# Alzheimer's Disease: Drug Discovery

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Edited by
XUDONG HUANG, PhD

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**Alzheimer's Disease: Drug Discovery**
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# FOREWORD

Alzheimer's disease (AD) is the most common cause of age-related dementia, accounting for up to 70% of all dementia cases. There are 50 million individuals living with AD worldwide and its global prevalence is expected to grow due to predicted expansion of the older population. AD presents with irreversible cognitive decline, which commences as insidious short-term memory dysfunction and gradually spreads to other cognitive domains, rendering patients mute and non-ambulatory after 10-15 years of progressive course. AD is a genetically complex disease. The majority of AD cases are sporadic and their risk is predominantly controlled by the *APOE* genotype. The *APOE ε4* allele increases AD risk in allele-dose dependent fashion while the *ε2* allele has a risk-mitigating effect. Early-onset familial AD (FAD) accounts for 2-5% of all AD cases and is caused by mutations of the amyloid precursor protein (APP) or presenilin 1 (PS1) or 2 (PS2) genes, which are inherited in autosomal dominant fashion. Neuropathological hallmarks of AD include accumulation of insoluble β-amyloid (Aβ) peptides in the form of parenchymal plaques and vascular deposits, intraneuronal neurofibrillary tangles (NFTs) composed of misfolded and hyperphosphorylated microtubule-associated τ protein, activated astrocytes and microglia and widespread loss of synapses and nerve cell bodies. Unfortunately, there are neither effective preventive measures nor efficacious treatments available for this devastating disease.

Identification of Aβ peptides as the main constituents of Aβ plaques and vascular deposits by the late Dr. George Glenner in 1984 and later by Drs. Konrad Beyreuther and Colin Masters in 1985 has led to the development of the Alzheimer's Aβ cascade hypothesis, which proposes that the accumulation of Aβ is the primary culprit of AD pathogenesis and is thus a critical therapeutic target. Building on this premise, development of Aβ-directed immunotherapeutics and inhibitors of APP proteases, β-site cleaving enzyme 1 (BACE1) and γ-secretase complex (γ-SC), whose synergistic action generates Aβ has been pursued over the past 20 years. However, a series of setbacks these approaches have encountered during clinical trials testing hampered progress toward their successful clinical development. At the time this book is set for print, Aducanumab an Aβ-directed monoclonal antibody remains under evaluation by regulatory agencies for an approval as a possible first AD modifying treatment, while two similar antibodies remain in advanced phase III testing. Unfortunately, clinical efficacy of anti-Aβ antibodies remains limited to moderating disease progression, while also causing side-effects in the form of amyloid related imaging abnormalities (ARIA). Such a situation undoubtedly calls for the diversification of AD druggable targets and a search for poly-targets or drug combination therapeutic strategies.

Contributed by an assembly of clinicians and translational and basic AD research scientists, this book intends to provide a brief overview of AD drug discovery field. It covers the underlying AD pathogenic mechanisms and provides a review of Aβ amyloid- and τ protein-targeted immunotherapies, and peptide inhibitors for anti-Aβ amyloidosis. In addition, it also examines therapeutic

potentials of various AD drug targets such as brain metal homeostasis, protein kinase C, the blood-brain barrier, epigenetic therapies, as well as discusses chimeric conjugates, multifunctional ligands, and natural products as interventional approaches. I am convinced that this book will be valuable to healthcare professionals caring for AD patients, AD researchers and interested readers as it will provoke thoughts about identifying novel and more efficacious therapeutic agents for AD and related dementias.

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# PREFACE

In 1906, German psychiatrist Alois Alzheimer first reported the histological features of unique plaques and neurofibrillary tangles in the post-mortem brain of a 50-year-old female patient, whom he had followed up for 5 years from admission until her death, for progressive sleep disturbance, memory dysfunction and behavioral changes such as paranoia, aggression, delusion, and confusion. The disease is now recognized as Alzheimer's disease (AD), the most common form of senile dementia. The plaques are now known as amyloid beta (Aβ) plaques, and the neurofibrillary tangles are known as microtubule-associated hyperphosphorylated protein τ in the paired helical filaments.

More than 200 clinical research programs and clinical trials targeting Aβ and protein τ, either directly or indirectly, as a therapeutic strategy for AD have failed thus far. A growing database of the etiopathological, genetic, and biochemical features of AD indicate that it is a heterogeneous, polygenic, multifactorial, and complex disease. Hence, more rational therapeutic strategies for AD should be druggable target diversification, multi-targeting, or drug combinations. While current drug discovery programs for AD continue to focus on anti-Aβ and anti-τ strategies, a deeper understanding of the disease in recent years has opened drug discovery avenues involving neuroinflammation, metabolic derangements, stem cells, gene therapy and alternative therapies. Despite the high attrition rate in AD drug discovery and development, and serial failures of AD drug trials, we remain hopeful for effective AD therapeutics to come in the near future.

This book takes a snapshot of current AD drug discovery approaches to satisfy interested readers' curiosity for diversity and complexity of AD drug discovery. Chapter 1 provides an overview of the underlying pathogenic mechanisms of AD. Chapters 2-4 summarize Aβ immunotherapy, Aβ-targeted inhibitory peptides, and τ protein immunotherapy, respectively. Chapter 5 reviews AD therapeutic strategy targeting brain metal homeostasis, while Chapter 6 examines atypical protein kinase C as a potential AD drug target. Chapters 7-8 discuss blood-brain barrier (BBB) models in AD drug delivery and the BBB degradation-related protein- secreted protein acidic and rich in cysteine (SPARC) as a potential AD druggable target, respectively. Chapter 9 examines the therapeutic potential of epigenetic therapies for AD. Chapters 10 and 11 discuss the search for effective AD therapies using chimeric conjugate and multifunctional ligand approaches, and Chapter 12 examines natural products as potential interventions for neurocognitive disorders such as AD.

I am grateful for all the authors' intellectual contributions and diligence toward the fruition of this book. The 12 chapters cover diverse AD therapeutic approaches, but by no means do they completely reflect the dynamic and challenging field of

AD drug discovery. I hope this book will encourage interested readers to dive into this field and appreciate both the challenges and excitement of developing effective therapeutics for AD.

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# 1

# Alzheimer's Disease: Etiology, Neuropathology and Pathogenesis

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**Abstract:** Alzheimer's disease is the most common form of dementia and the most common neurodegenerative disease. It manifests as a decline in short-term memory and cognition that impairs daily behavior. Most cases of Alzheimer's disease are sporadic, but a small minority of inherited forms allow gene identification which, together with neuropathology, yields important clues about the wider causes. Environmental and metabolic risk factors, including inflammation and vascular impairment, play a role in disease onset and progression. While neuronal atrophy and a loss of synapses occur throughout the cerebral cortex, we lack a full understanding of how this arises. The known hallmarks of Alzheimer's disease include amyloid-β plaques and neurofibrillary tau tangles and while extensive research has been carried out throughout the past few decades, the exact role of these protein aggregates in the disease remains elusive. In this chapter, we discuss mechanisms that have been implicated, including inflammation, mitochondrial dysfunction, oxidative stress and changes in protein clearance.

**Keywords:** amyloid-β plaques; etiology of Alzheimer's disease; dementia; neurodegeneration in Alzheimer's disease; neurofibrillary tau tangles

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## INTRODUCTION

Around 50 million people worldwide suffer from dementia (1). About two thirds have Alzheimer's disease (AD) (2), an irreversible neurodegenerative disorder involving a decline in memory and executive function, and personality change (3). It is named after Alois Alzheimer who first characterized AD in 1906 (4). AD results in synapse loss and neuronal atrophy predominately throughout the hippocampus and cerebral cortex. It is characterized by amyloid plaques and neurofibrillary tau tangles (NFTs), aggregates of misfolded proteins, throughout the brain. Both genetics and environmental factors are believed to play a role in AD. While there are a small number of cases due to dominant genetic mutations (5–7), a majority of AD cases are sporadic and have no single genetic cause. Environmental and metabolic risk factors such as diabetes, cerebrovascular disease, poor diet, head injury and stress are linked to increased dementia risk. The leading hypothesis as to how AD begins and progresses, the amyloid hypothesis, though quite widely accepted, leaves many questions. In particular it remains unclear "what is the best drug target?" and "what lies upstream of the rise in amyloid-β (Aβ) in sporadic cases?." We still lack a fundamental understanding of how AD comes to fruition, and therapies to help individuals fight the disease. AD is a chronic disease manifesting as loss of memory, language, cognition and problem-solving skills, changes in behavior and ultimately death. While the primary signs are memory loss and executive dysfunction, they are often preceded by changes in language and vision (8). Additionally, not all types of memory are equally affected. People with AD have severely impaired episodic, semantic and working memory, yet long-term memory, such as procedural memory, tends to remain intact (9, 10). Clinically, AD is classified into seven stages (Table 1) (11). Patients often die 3–10 years after onset of symptoms (12) with complications arising from immobility, such as pneumonia or blood clots (13, 14).

**TABLE 1** **The seven clinical stages of Alzheimer's disease (Global Deterioration Scale) (11)**

| | Symptoms and characteristics |
|---|---|
| **Stage 1** | Persons appear cognitively normal, but pathological changes are happening in the brain. |
| **Stage 2** | Prodromal stage: mild memory loss, but generally this is indistinguishable from normal forgetfulness. |
| **Stage 3** | Progression into mild cognitive impairment (MCI). Individuals may get lost or have difficulty in finding correct wording. |
| **Stage 4** | Moderate dementia; poor short-term memory. Individuals forget some of their personal history. |
| **Stage 5** | Cognition continues to decline and at this point individuals need help in their daily lives. They suffer from confusion and forget many personal details. |
| **Stage 6** | Severe dementia. Requiring constant supervision and care. Patients fail to recognize many of their family and friends and have personality changes. |
| **Stage 7** | Individuals are nearing death. They show motor symptoms, have difficulty communicating, are incontinent and require assistance in feeding. |

## ETIOLOGY

Both genetic and environmental risk factors play a role in the manifestation of AD. The greatest risk factor is age. At age 65, the likelihood of having AD is about 3%, rising to over 30% by age 85 (15). The incidence of AD under the age of 65 is less certain, but estimates suggest that this age group accounts for around 3% of AD cases (15). Although overall numbers are increasing with the ageing population, age-specific incidence appears to be falling in several countries (16–18).

AD can be classified by when the disease manifests, and whether it is inherited. Early-onset Alzheimer's disease (EOAD) occurs before age 65, whereas late-onset Alzheimer's disease (LOAD) accounts for over 95% of cases (19) and manifests beyond age 65. Familial AD shows Mendelian (usually dominant) inheritance, while sporadic AD shows no simple familial link (20). Nearly all EOAD are familial as these cases are due to mutations in *APP*, *PSEN1* or *PSEN2*, and a vast majority of LOAD are sporadic. Genome wide association studies (GWAS) and sequencing have now provided more than 20 risk loci in total that contribute to sporadic cases (21), but often there is no identifiable genetic cause.

### Aβ precursor protein

Aβ precursor protein (APP) was the first gene shown to have autosomal dominant mutations causing AD. As the precursor of the aggregated peptide in amyloid plaques, its discovery in 1991 by John Hardy and colleagues (5) led to the "amyloid hypothesis," which states that the toxic build-up of Aβ starts a cascade of events, leading to neuronal death and disease (22, 23). There are now over 50 known *APP* mutations, accounting for approximately 10% of familial cases. Widely studied ones include the London (V717I) (24), Swedish (KM670/671NL) (25), Indiana (V717F) (26) and Artic (E693G) (27) mutations, and most cluster around cleavage sites for β and γ-secretase (28). Research suggests that many of these mutations increase Aβ production, or the Aβ 42:40 ratio, leading to increased amyloid accumulation. In very rare instances, *APP* duplication or promoter mutations can cause AD (29, 30). Interestingly, studies have also found that there is an *APP* mutation (Icelandic—A673T) which lowers Aβ and protects against AD (31).

### Presenilins

Presenilin 1 (*PSEN1*) and Presenilin 2 (*PSEN2*) encode the catalytic components of γ-secretase, an enzyme complex involved in APP processing (32). Presenilin mutations cause autosomal dominant AD, with *PSEN1* variants being the most commonly known Mendelian genetic cause, estimated to account for around 30–50% of familial EOAD cases (33, 34). Research shows that *PSEN1* and *PSEN2* mutations alter Aβ production, similar to *APP* mutations (35) but paradoxically tend to confer loss of function, raising questions as to how this fits the amyloid hypothesis (36, 37).

### Other genetic risk factors

Other genes known to have variants associated with AD risk include *TREM2* (38), *APOE* (39), *CLU* (40–42), *SORL1* (43), *BIN1* (42) and *PICALM* (40, 42). APOE

(apolipoprotein E) is a protein involved in fat metabolism, and its E4 allele is the most common genetic risk factor for AD with an allele frequency of ~13.7% (44, 45). Heterozygosity for this allele increases the risk 3-fold (39). Although rarer, the variant *TREM2*$^{R47H}$ (triggering receptor expressed on myeloid cells 2) has a similar effect size (46). TREM2 is a receptor expressed on multiple cell types of the immune response, and its association supports a role for inflammation in AD pathogenesis.

## Down syndrome

By age 65, up to 80% of Down syndrome (DS) individuals develop dementia (47). As with other instances of EOAD, amyloid and tau pathology begin much earlier than in LOAD, even at <40 years of age (48–50). DS results from the trisomy of chromosome 21, where the *APP* gene is located, and having three copies of this gene is sufficient to increase Aβ levels. However, the increased risk of developing the disease may also be due in part to triplication of other genes on chromosome 21 (47, 51, 52).

## Inflammation

Sporadic AD often results from a combination of genetic and environmental risk factors, with cerebral hypoperfusion (53) and inflammation (54) being among the most common. Inflammation due to trauma, sepsis and infection has been linked to both short- and long-term cognitive impairment (55–57). Traumatic brain injury, and even bone fractures in the elderly, are implicated in dementia risk (58, 59). Higher levels of inflammatory markers such as interleukin 6 (IL-6) associate with greater risk of AD and vascular dementia (60). AD patients often have higher levels of certain inflammatory markers and activated microglia and astrocytes in the brain, which tend to surround plaques and tangles (61, 62). Finally, higher levels of these markers are associated with faster cognitive decline (63).

## Cerebral, cardiovascular disease and diabetes

There is a strong link between vascular disease and dementia. Cardiovascular disease, including high blood pressure and heart attack, and cerebrovascular disease such as ischemia are associated with increased risk of AD (64). Metabolic and lifestyle risk factors for developing vascular diseases, including poor diet, obesity, high cholesterol and sedentary lifestyle, are also risk factors for dementia (65, 66). Poor diet and high cholesterol can produce metabolic changes both systemically and in the brain, and alter oxygen levels (67). Additionally, type 2 diabetes approximately doubles the risk for dementia (68–70).

## Other environmental risk factors

The list of environmental and metabolic risk factors discussed here is not intended to be comprehensive, especially as the nature of epidemiology in populations with diverse genetics and lifestyle means that important mechanisms will not always

generate conclusive evidence. Other risk factors implicated include pollution, stress and heavy metal exposure (71–76). Many of these risk factors share some common characteristics with one another which can thus make it difficult to determine how their presence affects the brain. Some may act through similar mechanisms, such as inflammation or oxidative stress, which will be discussed later in this chapter.

## NEUROPATHOLOGY

AD is characterised by synapse loss, followed by the atrophy of neurons throughout the cerebral cortex, with the medial temporal lobe being the most severely affected (77–79). Pathology appears to start within the hippocampus and entorhinal regions and spreads subsequently throughout the fronto-temporal cortices. It reaches as far as the striatum and thalamus, usually with sparing of the cerebellum (80–83). On a macroscale level, MRI scans show shrinkage of these regions (84). In particular, pyramidal cells of the CA1 of the hippocampus are vulnerable to morphological changes and cell death, consistent with the main symptom of memory loss (85, 86). The appearance of Aβ plaques and NFTs precedes clinical symptoms suggesting that by symptom onset, there have been years of pathological changes making early intervention difficult.

### Aβ plaques

Senile plaques are primarily made of a variety of 36–43 residue-long amyloid peptides that undergo fibrilization to form Aβ sheets that are resistant to degradation (87). They often co-localize with neuronal debris and activated microglia and astrocytes (88), and first appear in the frontal, temporal and occipital lobes of the neocortex. They spread throughout neocortical areas as well as the hippocampal formation and entorhinal region, and eventually spread further throughout the cerebral cortex to the striatum and thalamus (83) (Figure 1). Amyloid pathology appears to precede that of tau, with NFTs only being found in regions where amyloid was already present. Numerous studies have shown that cognitively unimpaired elderly individuals can also have significant Aβ deposition (89–91), while on the contrary, others have reported a correlation of deposition to cognitive decline (92) and dementia severity (93). A recent study has more specifically shown that differences in Aβ oligomer concentration may be a better correlate of disease (94, 95). It is likely that differences in methodology are responsible for the varying conclusions from these studies. It has also been suggested that cognitively normal persons with high plaque levels may have "prodromal" disease, with Aβ pathology that precedes cognitive changes (96, 97).

### Neuronal fibrillary tau tangles

NFTs are intraneuronal aggregates of hyperphosphorylated tau protein, encoded by the microtubule associated protein tau (*MAPT*) gene (98) (Figure 1). NFTs are

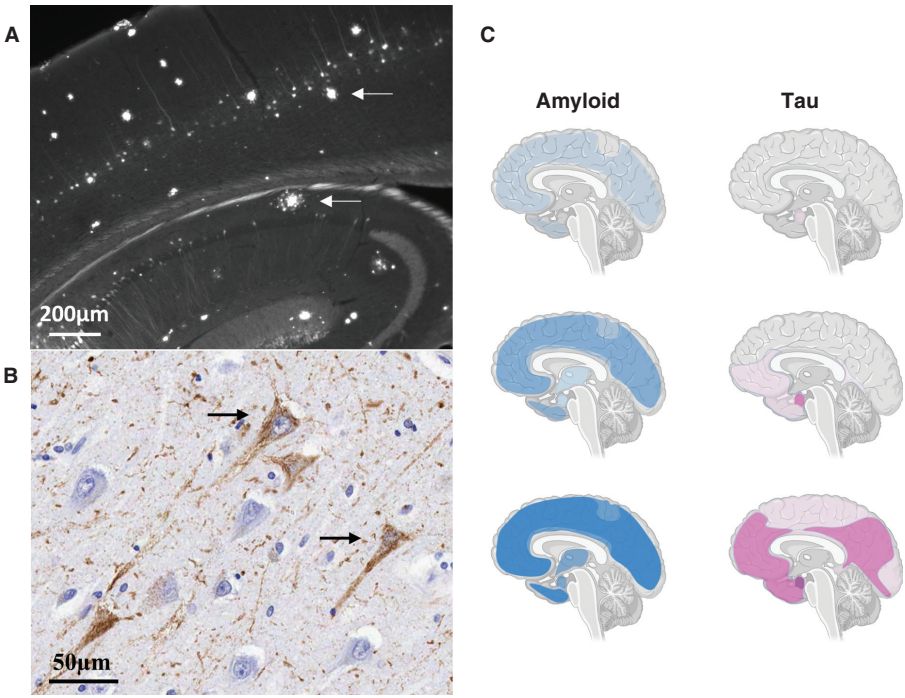


**Figure 1. Amyloid and tau pathology.** (A) Thioflavin S staining of Aβ plaques in the cortex of a CRND8 APP transgenic mouse. (B) AT8 staining of neurofibrillary tau tangles (NFTs) within an aged human CA1 region of the hippocampus. (C) The spread of amyloid and tau pathology throughout the brain during AD, adapted from Braak and Braak 1991 (83).

composed of paired helical fragments (PHFs) of tau fibrils approximately 20 nm in diameter (Figure 2). Like plaques, they spread throughout the brain as disease progresses, beginning near the entorhinal cortex. Braak staging is commonly used as a means of defining the progression of disease as determined by tau pathology. In stages I–II, tangles appear in the trans-entorhinal region; in stages III–IV, tangles have spread to the limbic system and start to show in the neocortex; in stages V–VI, pathology is present throughout the neocortex (83) (Figure 1). In addition to AD, several other neurodegenerative diseases are classified as tauopathies due to the presence of NFTs; these include Parkinson's disease, progressive supranuclear palsy, corticobasal degeneration and frontotemporal dementia (FTD) (99). While aggregates of amyloid and tau have both been associated with neuronal loss and toxicity, they have a poor correlation with cognitive decline as AD progresses. On the contrary, the loss of synapses is one of the strongest correlates to cognitive decline in AD (100). Familial cases and PET imaging have allowed us to identify changes in both Aβ and tau prior to changes in brain structure and symptom onset (101). A combination of psychological and cognitive testing, scans and CSF and blood tests (to rule out other neurological disorders) are required to obtain the diagnosis of AD. Ultimately though, definitive confirmation of the disease requires post-mortem histopathology.

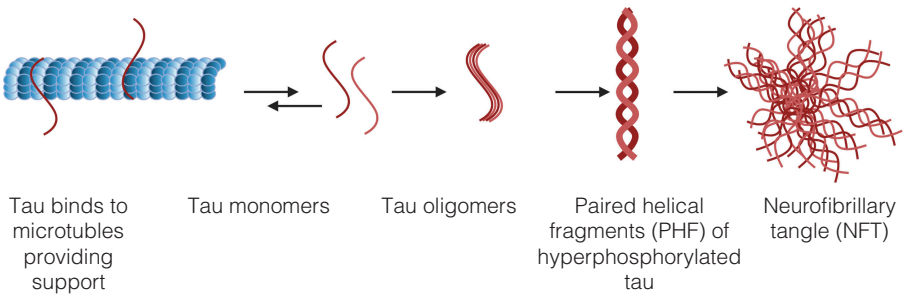


**Figure 2. Microtubule-associated protein Tau (MAPT) aggregation results in the accumulation of neurofibrillary tau tangles (NFTs).** Tau is believed to play a role in the stabilization of microtubules. Hyperphosphorylated tau polymerization leads to the creation of insoluble paired helical fragments (PHFs), which further aggregate into NFTs.

## PATHOGENESIS

The mechanism of AD pathology and neuronal loss remains elusive. The roles of both Aβ and tau have been extensively researched in the past few decades, yet we are still unsure of their role in disease. A variety of mechanisms have been proposed to explain what occurs in the pathogenesis of AD. It is possible that different combinations of risk factors in different patients activate the disease in different ways, and that these converge on a common pathway of degeneration.

### Aβ and APP

The amyloid hypothesis remains the dominant hypothesis in AD research due to the causal mutations found in both *APP* and presenilin genes. APP is processed via either the amyloidogenic or non-amyloidogenic pathway. For Aβ, APP is sequentially cleaved by the β- and γ-secretases, releasing the peptide into the cytosol (Figure 3). Functions of APP and Aβ are largely unknown, but they are thought to play a role in signal transduction for neuronal development, growth and survival (102, 103). While genetic mutations may explain Aβ accumulation in EOAD, it is still unclear how this occurs in LOAD. Aβ accumulation has been proposed to cause neuronal death via a number of mechanisms, including excitotoxicity, synaptic disruption, oxidative stress and mitochondrial dysfunction. Excitotoxicity can occur when NMDA receptors are continually activated, either by Aβ directly or by a downstream mechanism. In conjunction with synapse loss, both AD patients and animal models show reductions in the synaptic proteins synaptophysin and PSD-95 (104–108). Aβ oligomers accumulating in an AD brain (109) may be even more toxic than fibrils or plaques. Soluble oligomers appear to amass in a different manner compared to plaques and appear early in pathogenesis (110). Oligomers can disrupt cognitive function (111) and inhibit long-term potentiation (LTP) (112) *in vivo*, and can be neurotoxic (113) *in vitro*. Interestingly, oligomers tend to cluster near synapses (114) and can induce synapse loss and dysfunction (115). It has also been suggested that changes in another APP

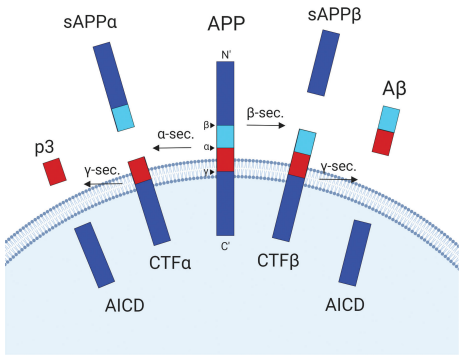


**Figure 3. Post-translational processing of Aβ precursor protein (APP) is thought to occur at the cell surface or within endosomes.** It includes cleavage by either α- then γ-secretase (non-amyloidogenic), or β- then γ-secretase (amyloidogenic pathway).

processing product could be a contributor to AD (103). Though many APP mouse models present with aspects of AD pathology, most fail to fully recapitulate the neurodegeneration seen in the human AD brain. While this most likely reflects inter-species differences, it also raises questions about the relative importance of APP/Aβ in driving dementia (116).

## NFTs and Tau

While no *MAPT* mutations are associated with AD, causal mutations in tau have been found for other neurodegenerative diseases such as FTD, suggesting that tau dysfunction and aggregation can be neurotoxic. Tau's major role is thought to be that of a cytoskeletal protein, interacting with tubulin to help assemble and stabilize microtubules (117). In humans there are six isoforms of tau generated by alternative splicing of exons 2, 3 and 10. The incorporation of exon 10 leads to four microtubule-binding repeats (4R tau) instead of three (3R tau), altering how tightly the protein binds to microtubules and its propensity to aggregate (118). Healthy adult humans express similar amounts of 3R and 4R tau. Research has shown that the ratio between the two may impact disease, with higher 4R isoforms leading to greater degeneration. In AD, there is a higher ratio of 4R to 3R, and reported downstream consequences include transcriptional alterations in the Wnt signaling pathway (119) and altered axonal transport (120). Prior to NFT formation, tau becomes hyperphosphorylated, and tau phosphorylation not only plays a large role in regulating tau function, but could be the key change resulting in the accumulation, and potential toxicity, of this protein. In fact, multiple tauopathy mutations cause tau to be more readily phosphorylated (117).

Mutant tau mouse models have shown that mutations in this gene can result in severe neurological phenotypes (121, 122). Tau has been hypothesized to induce neurotoxicity via loss of function, gain of function and/or mis-localization. Loss of function of tau occurs when tau is no longer able to stabilize microtubes having an impact on neuronal cytoskeleton, and similarly could lead to deficiencies in axonal transport (123, 124). Higher levels of tau have also been shown to

inhibit vesicle and organelle trafficking, including those carrying APP, and increase levels of oxidative stress (125), as well as have an effect on axonal transport (126). The mis-localization of tau to dendritic spines has been shown to effect cognition and synapses *in vivo* (127, 128). As with APP, it remains unclear as to exactly how tau influences disease progression, but interestingly, Aβ induced toxicity and impairment in LTP has been found to be a requirement for the presence of endogenous tau (129, 130). It has also been suggested that tau and Aβ work together to result in transcriptional deficits (131) and synaptic changes (132) in AD.

## Mitochondrial dysfunction and oxidative stress

One of the many processes that is compromised in AD is mitochondrial function. Alterations in mitochondrial morphology, number and transport, reduced cytochrome oxidase activity, deficiencies in metabolic proteins, changes in mitochondrial membrane potential and an increase in oxidative stress have been observed in AD (133, 134). Neurons are highly dependent on mitochondria, and mitochondria accumulate at synapses, helping to power their high metabolic demand. The high level of ROS production which occurs at synapses, in conjunction with insufficient antioxidants, can lead to oxidative stress (134). In addition, the brain is composed of high levels of cholesterols, which are also very vulnerable to oxidative damage (135). Thus, the high energy demands of the brain and its high lipid concentration naturally put it at risk for oxidative damage. Rather than aging driving amyloid pathology, as in the case of the amyloid hypothesis, the mitochondrial cascade hypothesis proposes that genetic and environmental factors determine the rate of mitochondrial decline, which in turn determines the rate of aging and subsequently AD (133). In terms of EOAD, APP or Aβ induces mitochondrial deficits, inducing an increase in the rate of aging, thus making some people susceptible to AD. This has been suggested as a potential link between EOAD and LOAD pathogenesis (136). Supporting this hypothesis, Thy-1-APP mice show reduced mitochondrial membrane potential and ATP synthesis and increased ROS production (137). Similarly, transgenic APP mice have shown an increase in Aβ within synaptic mitochondria, leading to dysfunction and oxidative stress prior to plaque accumulation (138). Paradoxically, oxidative stress, a by-product of mitochondrial deficiency, has been known to affect β-secretase activity (139), which in turn could alter Aβ production.

## Insulin

Insulin resistance and a decrease in insulin receptors have been observed in the AD brain (140). Late stages of diabetes also result in insulin resistance in the brain. As cells are heavily dependent upon glucose metabolism for energy production, this can lead to energy deficiencies, potentially leading to oxidative stress. It has also been shown that insulin plays a role in neurotransmission (141) and can be neuroprotective during insults such as ischemia (142). Additionally, it has been reported that insulin and metabolic inhibitors result in increased levels of β-secretase in both wild-type and Tg2576 mice (an APP transgenic model). In Tg2576 mice, this also resulted in an increase in Aβ levels (143). Yet, as others report a protective role of insulin, it is likely that there is a certain level of this hormone which allows the brain to function optimally.

## Hypoglycemia and vascular dysfunction

In addition to insulin resistance, the link between diabetes and AD could be due to changes in metabolic proteins, glucose receptors/transporters or even hypoglycemia due to over-medication. Glucose metabolism decreases in the normal aging brain (144) and even further in the AD brain (145). It has also been reported that there is a decline in the expression of glucose transporter at the blood brain barrier (BBB) in both AD patients and animal models of AD (146, 147), as well as in aged wild-type mice (147, 148). In addition, insulin-induced hypoglycemia has also been shown to cause neuronal death *in vitro* and *in vivo* (149). Glucose deprivation can elevate tau levels *in vitro* (150), and hypoglycemia has also been linked to increases in oxidative stress (151). Hypoglycemia could also be the link between cardiovascular and cerebral-vascular diseases and dementia, but whether it be hypoglycemia, hypoxia, a change in another blood component or a combination of these which increases one's risk of disease is still unknown. Finally, abnormal angiogenesis and alterations of vasculature, including changes in blood flow, have been shown in AD patients and animal models of the disease (152–154).

## Inflammation

The role of inflammation is a more recent topic of interest in the AD field. As discussed previously, people with inflammation are more likely to develop dementia, and dementia patients with higher levels of inflammatory markers tend to deteriorate more rapidly. Studies in animal models have shown that inflammation can result in cognitive impairment (155), as well as neuronal damage and synaptic loss *in vivo* and *in vitro* (156–159). Although inflammation and the activation of microglia are thought to play a neuroprotective role in acute circumstances, in the long term, this may lead to neurotoxicity, and an increase in Aβ load (155, 160, 161). Aβ itself is thought to activate microglia, attracting them to plaques and enhancing phagocytosis (162–164). Potentially, microglial response to Aβ is protective, but after chronic activation, the microglia begin to play a detrimental role, resulting in a feed-forward loop of degradation (54). Similarly, it has been shown that increased ROS levels increase inflammatory markers, and that immune cells influence the production of ROS (165–168), demonstrating the complex interplay between Aβ, oxidative stress and inflammation.

Tau pathology also appears to be influenced by (169, 170), and have an effect upon (171, 172), inflammation. Research looking at the ability of microglia to phagocytose tau aggregates is conflicting, potentially due to microglia playing an initial role in clearance, but losing their ability to maintain this over extended periods (173). And finally, it has been reported that altering expression of TREM2, which plays a role in inflammation, may have an effect on Aβ levels and plaque-associated macrophages (174).

## Ubiquitin-proteasome system

The ubiquitin-proteasome system (UPS) is involved in the degradation of misfolded and excess proteins. It is particularly important for synapse function, where there is high protein turnover (175). Proteins to be degraded go through an

enzymatic process where they are labelled with a polyubiquitin chain which is recognized by the proteasome (176), and subsequently broken down. The proteasome targets monomeric proteins, so is not thought to break down plaques or tangles, but both have been shown to potentially inhibit proteasome activity (177). This could lead to a toxic build-up of excess and misfolded proteins in the brain, and more specifically synapses.

### Autophagy lysosome pathway

Autophagy and lysosomal dysfunction are also proposed mechanisms of AD pathogenesis. Autophagy is involved in tau clearance (178), and plays a role in both the generation and clearance of Aβ. APP amyloidogenic processing involves trafficking through the endo-lysosomal pathway (179). Several genes implicated in AD including *BIN1*, *SORL1* and *PICALM* are involved in endosomal recycling, and studies have reported that each may directly play a role in APP endosomal processing (95, 180, 181).

### Cholinergic hypothesis

The cholinergic hypothesis was one of the first proposed theories on the manifestation of AD (182, 183). This came to fruition due to abnormal levels of acetylcholine in the AD brain. Cholinergic neurons of the basal forebrain are one of the earliest affected by AD and there is a decrease in choline acetyltransferase (ChAT) transcription and activity in remaining neurons. Studies have also shown a relationship between acetylcholinesterase (AChE) and Aβ accumulation (182). However, as the AD field has moved forward there has been difficulty in linking acetylcholine with other AD pathologies. Indeed, pyramidal neurons are lost in greatest numbers in regions with plaques and tangles and these are, for the most part, glutamatergic neurons (184).

## CONCLUSION

Although we have amassed a vast amount of knowledge in the search for a central, unifying mechanism behind dementia and AD, we are still lacking suitable therapies to help slow down the progression of disease. The amyloid hypothesis remains the dominant theory, yet drugs aimed at lowering Aβ levels have been largely unsuccessful. The possibility of NFT and plaque-load being correlative rather than causative with disease progression is entirely possible. There is much overlap between many of the risk factors, both genetic and environmental, and the known pathogenesis, highlighting the complexity of dementia. Similarly, we lack a firm understanding of how familial EOAD and sporadic LOAD ultimately produce the same neurodegenerative outcome. By enhancing our understanding of AD etiology, pathology and pathogenesis, we hope to one day find an effective therapy.

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# Immunotherapy Targeting Amyloid-β Peptides in Alzheimer's Disease

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**Abstract**: Neurodegenerative diseases, in particular Alzheimer's disease, represent significant unmet medical needs due to a lack of effective therapeutic treatment options and cause a substantial burden for health care systems. Accumulation of β-amyloid peptides within the brain is believed to be an initial trigger of the disease process. In the last 20 years, immunotherapy has emerged as a promising target-directed strategy to develop efficient treatment options with disease-modifying potential. Unfortunately, either active vaccination against β-amyloid or its fragments, as well as passive immunization using monoclonal antibodies, have largely failed to show a clinical benefit in a variety of clinical trials. This chapter addresses progress and developments with regard to active and passive immunization against Aβ and summarizes the current state of clinical trials.

**Keywords:** Alzheimer's disease; amyloid; immunization; immunotherapy; monoclonal antibodies

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## INTRODUCTION

With an estimated 50 million people affected worldwide, Alzheimer's disease (AD) is the most frequent cause of dementia, accounting for about 60–80% of all cases (1). The incidence is expected to increase in the next decades, due to the rapid increase of age in the population of the developing nations, possibly reaching 152 million cases by 2050 (1). Despite numerous and continuous efforts to find an effective cure, no drug has been approved for AD in the last 17 years (2). Additionally, the currently available therapies, comprising cholinesterase inhibitors and N-methyl-D-aspartate receptor agonist, do not modify the underlying pathophysiology of the disease and offer only modest, symptomatic and transient effects (3, 4). The amyloid cascade hypothesis is still widely considered the main theory for the pathology of AD (5), supported by the discovery of genetic autosomal dominant mutations in the amyloid precursor protein (*APP*), presenilin 1 (*PSEN1*) or presenilin 2 (*PSEN2*) genes in patients with early onset AD (EOAD), resulting in an enhanced formation and accumulation of amyloid-β (Aβ) peptides in plaques (6). Aβ accumulation in the brain, which starts 15–20 years before the manifestation of clinical symptoms, is believed to be the starting point for the progression of AD, driving tau phosphorylation and leading to synaptic and neuronal loss, which ultimately translates to cognitive impairment. The cascade hypothesis has been revised and modified due to, among other reasons, lack of correlation between fibrillary Aβ aggregates and AD severity (7). The focus shifted to intraneuronal Aβ accumulations as a site of Aβ toxicity (8) or oligomeric forms of Aβ, which are considered the toxic and pathogenic driving force in AD (9). The cascade hypothesis is the rationale for the development of passive and active anti-Aβ immunotherapy strategies, targeting both fibrillary aggregates and soluble forms of Aβ. Reducing Aβ burden by employing monoclonal antibodies (mAb) appears a straightforward and appealing strategy to slow or prevent the progression of the disease. Numerous antibodies have been tested so far and are currently under investigation in clinical trials; however, the outcomes of the past two decades have been disappointing. Though some antibodies, such as bapineuzumab and aducanumab, appeared to clear parenchymal amyloid (10, 11), failure to meet the primary endpoints or the occurrence of adverse side effects such as vasogenic edema and/or microbleeding (12) caused the termination of the ongoing trials for most of the tested mAb. The reasons for the disappointing outcomes could also be imputable to factors independent from the actual mode of action of the tested mAbs. An inaccurate selection of trial patients, leading to huge variations in cognitive and clinical decline during the trial period, as well as a late intervention and insensitive efficacy measures are potentially confounding factors. Proper target engagement (e.g. soluble, monomeric, dimeric, oligomeric, fibrillary Aβ) is also a critical aspect that needs to be addressed.

## Aβ GENERATION AND AMYLOID CASCADE HYPOTHESIS

The vast majority of AD cases are of sporadic origin, occurring beyond 65 years of age with an unknown cause. While mutations in *APP* or the *PSEN* genes have

been linked to early-onset autosomal dominant forms of familial AD (FAD) with an early disease onset (13, 14), so far, only genetic risk loci have been identified as potentially involved in APP processing or β-amyloid peptide generation in sporadic cases (15). APP is a single-pass transmembrane protein, and Aβ peptides are generated via a series of consecutive proteolytical cleavage steps from this larger precursor protein (16). The generation of Aβ peptides from its precursor APP is linked to the so-called amyloidogenic processing pathway, which is initiated by β-secretase cleavage. This cleavage is predominantly carried out by an aspartic protease named β-site APP cleaving enzyme (BACE1) (17), resulting in the release of a soluble APP fragment (sAPP-β) and a slightly longer APP C-terminal fragment of 99 amino acids (CTF-β). Further cleavage by γ-secretase, a protein complex consisting of PSEN1/2 among others (18), releases Aβ peptides. This complex is able to cut APP at slightly different positions, mainly resulting in the production of ~90% of $A\beta_{1-40}$ and less than 10% of $A\beta_{1-42}$ under basal conditions (19), but also shorter as well as slightly elongated Aβ peptides ($A\beta_{37} - A\beta_{43}$) (20). Processing by BACE1 and γ-secretase generates full-length Aβ peptides starting with an aspartic acid residue at position 1 (mainly $A\beta_{1-40}$ and $A\beta_{1-42}$). While most research efforts have concentrated on the full-length peptide species $A\beta_{1-40}$ and $A\beta_{1-42}$, there is accumulating evidence that a variety of other N- and C-terminally modified Aβ peptides may play an important role in the disease process (21–23).

The accumulation of Aβ peptides is regarded as one of the central processes underlying the neuropathological changes in AD. Almost 30 years ago, the amyloid cascade hypothesis was formulated, theorizing that Aβ accumulation is the initial event triggering further pathological alterations such as tau phosphorylation and neurofibrillary tangle formation, neuron and synapse loss, as well as cognitive impairment (5). While research efforts initially focused on fibrillar Aβ deposits in the form of extracellular plaques, the significance of soluble Aβ species (24, 25), mainly in the form of oligomers, became more and more recognized. They may directly injure synapses and neurites of brain neurons (26, 27), in addition to activating microglia and astrocytes (9). These metastable oligomeric forms likely exist in an equilibrium with amyloid plaques and consist of cross-β-sheet Aβ peptide units of variable size, including protofibrillar intermediates (28, 29) (Figure 1).

## MECHANISM AND PRINCIPLES OF (AMYLOID-β) IMMUNOTHERAPY

Immunotherapy focuses on the generation (in case of active) or use (in case of passive) of antibodies targeting a specific antigen, Aβ in this specific context, counteracting the disease by activation of the immune system. In active immunizations, a vaccine containing the Aβ-antigen is administered usually intramuscularly. Depending on the type of antigen used, a humoral response with B-cell and helper T-cell ($T_H$) involvement, cytokine secretion and production of polyclonal antibodies, and/or a cell-mediated immunity response with the activation of phagocytes (antigen-specific cytotoxic T-lymphocytes) is induced. T-cell populations can be further divided into cytotoxic T-cells, which kill target cells by

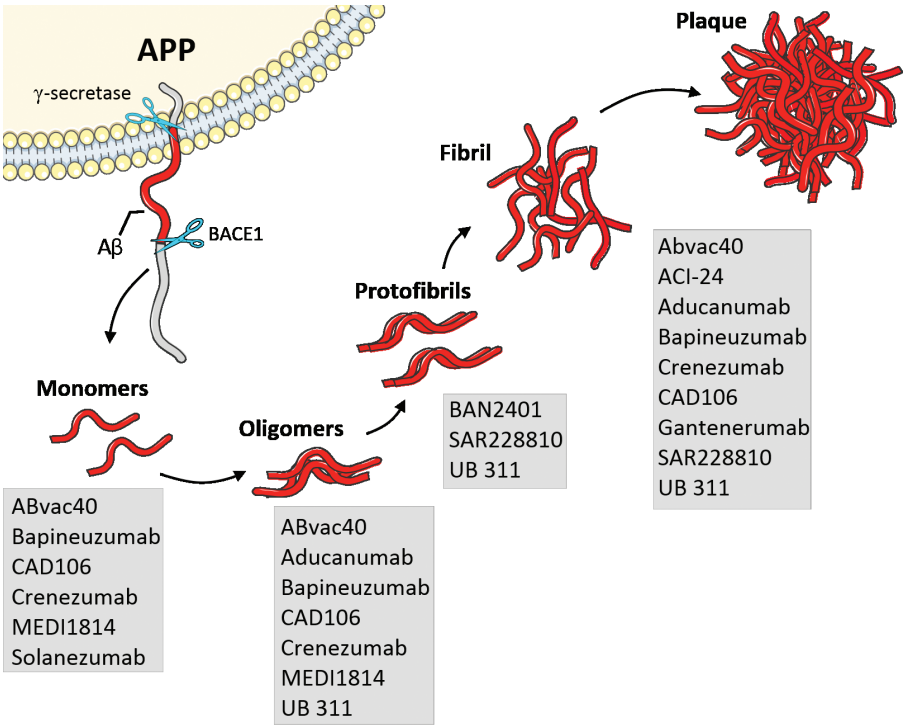


**Figure 1. Proposed targets of anti-amyloid-β (Aβ) drugs used in active and passive immunization approaches (modified from (2)).**

inducing apoptosis, macrophage-activating proinflammatory Th1 cells, and Th2 cells that stimulate B-cells into antibody-producing cells (30).

In passive immunization, monoclonal antibodies (mAb) against specific Aβ forms are administered by intravenous infusions or subcutaneous injection. In both cases, the antibodies are at first peripherally located and are required to pass the blood–brain barrier (BBB), greatly restricting the transport of antibodies, in order to reach the brain parenchyma. The access route for immunoglobulins has not been clearly identified yet, but could comprise passive diffusion, the lymphatic system, and perivascular spaces. The absence of active transport systems for antibodies, the presence of receptors (such as the neonatal Fc receptor) acting as a pump to remove antibodies in the central nervous system (CNS), as well as other not yet understood clearance mechanisms, are reasons why only a small fraction of antibodies (approximately 0.1%) introduced into the peripheral circulation can be detected in the brain or cerebrospinal fluid (CSF) (31). The presence of a large number of antibodies in the periphery could also act as a driving force for the efflux of Aβ out of the CNS, likely by changing the dynamic equilibrium between Aβ in the blood and the brain. Antibodies might therefore act as a peripheral Aβ "sink," creating a concentration gradient that attracts monomeric Aβ out of the CNS via passive diffusion mechanisms (32). In the brain parenchyma there

are several mechanisms, that are not mutually exclusive, by which the humoral response could exert its effects (33), and the Aβ epitope against which the antibody is directed (monomeric, oligomeric, fibrillary Aβ) may lead to a preferred mechanism over another. The antibodies could directly be responsible for the disassembly of Aβ deposits in the brain (34) or prevention of reassembly and inhibition of toxicity, as shown by *in-vitro* experiments (35, 36). Direct binding to Aβ oligomers, thus neutralizing their toxicity, is also a putative mechanism (37). The clearance of Aβ could also be enhanced by the antibodies through microglial activation, leading to Fc-mediated (32) or Fc-independent phagocytosis (38) (Figure 2). Peripherally, large immunoglobulin IgM, which is able to cross the BBB

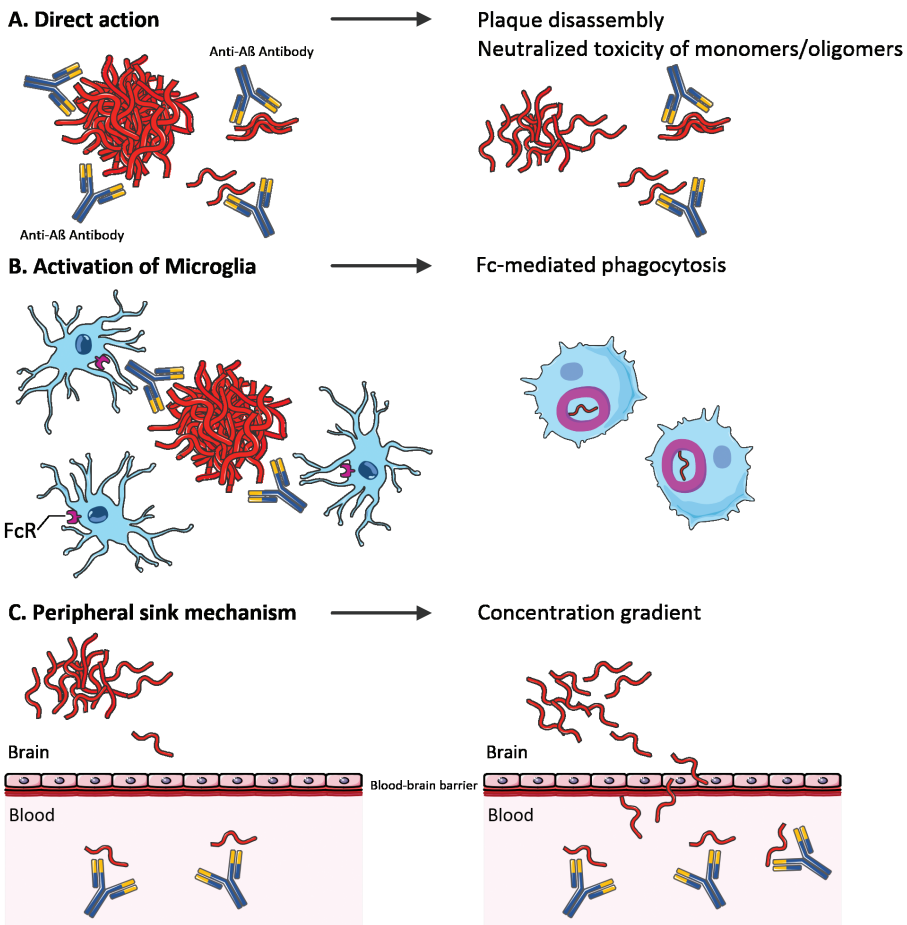


**Figure 2. Proposed mechanisms of anti-amyloid-β (Aβ) antibodies.** Antibodies might either directly target Aβ assemblies, leading to a neutralization of Aβ toxicity (A), or activate microglia, resulting in Fc-receptor (FcR) mediated phagocytosis (B). Alternatively, antibodies might not enter the brain but create a concentration gradient between the brain and the blood, leading to Aβ removal via a peripheral sink mechanism (C) (modified from (121)).

to a lesser extent compared to IgG but is likely involved in the already-mentioned peripheral-sink effect, is believed to directly hydrolyze Aβ (39). The specific advantages and disadvantages of active and passive immunization are described in the next paragraphs.

## ACTIVE AMYLOID-β-DIRECTED IMMUNOTHERAPIES

In active immunization, immunity is achieved following exposure to an Aβ antigen that causes the generation of antibodies in the recipient. It engages the cellular and humoral immune system, including T and B cells. Typically, an active vaccine is comprised of an antigen (alone or conjugated to a non-self T helper cell epitope) combined with an immune boosting adjuvant to ensure high antibody production. An advantage of active immunization is that with few vaccinations the patient should be able to produce a prolonged antibody response. The variability of the induced response across patients is, on the other hand, a problematic aspect, especially when dealing with elderly individuals. Adverse side effects may occur after active immunization: when a T-cell response is induced, the risk of an abnormal immune response increases. With age, the competency of the immune system reduces and the probability of developing autoimmune responses is enhanced. Additionally, vaccines lead to the formation of polyclonal antibodies, which can recognize multiple and possibly overlapping epitopes on the target protein. Polyclonal antibodies may be problematic in case the goal is the recognition of a specific form of the antigen.

The first effort to explore active immunization as a possible therapy for AD was made in 2001 with a vaccine called AN1792, consisting of synthetic full-length $A\beta_{1–42}$ peptide with QS-21 adjuvant. Despite initial positive findings in an APP-overexpressing mouse model (40), the phase II clinical trial in individuals with mild-to-moderate AD was interrupted, as 6% of the treated patients developed a T-cell-mediated meningoencephalitis (41). Additionally, only 20% of patients produced antibodies above the preset therapeutic cut-off titration level and clinical outcomes were no better than those of the placebo-treated control subjects (42). Despite the cessation of the trial, several follow-up studies were carried out as post-mortem brain samples from trial participants became available. Neuropathological analyses from AN1792 recipients in general showed a lower mean Aβ load compared to an age-matched unimmunized control group. The degree of plaque removal varied among immunized patients along with mean antibody response, and no evidence of improved survival or delay in the development of severe dementia was observed (43). It was further reported that immunized patients showed several-fold increases in $A\beta_{42}$-containing blood vessels in the cerebral cortex and leptomeninges, as well as a higher density of micro-hemorrhages. However, no major cerebral amyloid angiopathy (CAA)-related intracerebral hemorrhages were noted and, interestingly, two of the longest survivors showed a virtually complete absence of both plaques and CAA (44). Further studies revealed that active immunotherapy with AN1792 was associated with wall splitting in leptomeningeal vessels (45) and an accelerated loss of damaged degenerating neurons, an observation consistent with imaging data indicating an increased rate of cerebral atrophy among immunized AD individuals (46). A recent study reporting on post-mortem data from two AD patients who died

14 years after immunization revealed that these patients remained virtually plaque-free, however, an extensive overall distribution of neurofibrillary tangles (Braak stage V/VI) was observed (47).

In order to control the immune response by eliciting a strong antibody production but avoiding inflammatory T-cell activation, second-generation vaccines were designed to target more specific epitopes (48). One of these second-generation Aβ vaccines, ACC-001 (vanutide cridificar), studied by Janssen Immunotherapy and Pfizer, was discontinued in phase II clinical trials, as the primary efficacy-biomarker endpoints were found not statistically significant in the considered dosage groups (49, 50). The vaccine was composed of $A\beta_{1-7}$ with QS-21 adjuvant, designed to avoid the autoimmune meningoencephalitis caused by Th1 lymphocyte activation seen with AN1792, attributed to Aβ residues 15–42. CAD106 (Amilomotide) was another second-generation Aβ vaccine that reached phase II clinical trials involving patients with mild AD. CAD106 is composed of multiple copies of $A\beta_{1-6}$ peptide, coupled to a Qβ virus-like particle. Phase II trials in the United States and Europe ended in 2010 and 2011, supporting the favorable safety profile found in phase I trials and reporting prolonged antibody titers in responders (51). In a phase IIb trial, 120 patients suffering from mild AD received up to 7 intramuscular injections of CAD106 or placebo over 60 weeks. The vaccine was generally well tolerated and elicited an Aβ-specific immune response with an acceptable safety profile and preliminary evidence of target engagement by amyloid positron emission tomography (PET) (52). Despite a phase II/III trial began in November 2015, set to run until 2023, in September 2019 Novartis noted in its quarterly financial report that it had "retired" the CAD106 program. Several other candidates have been investigated and reached different stages of clinical development (Table 1).

## ABvac40

ABvac40 is an investigational vaccine targeting the C-terminus of $A\beta_{40}$. The agent comprises multiple repeats of a short C-terminal fragment of the Aβ peptide ($A\beta_{33-40}$), conjugated to the keyhole limpet cyanine (KHL) carrier protein and formulated with the adjuvant alum hydroxide. The phase I clinical trials demonstrated a favorable safety and tolerability profile with no incidence of vasogenic edema nor microhemorrhage (53). A phase II clinical trial by Araclon Biotech S.L. is ongoing in several European countries to confirm the results and explore the clinical efficacy of ABvac40 in patient with amnestic MCI and very mild AD (Clinical Trial: NCT03461276) and is due to be completed in February 2022.

## ACI-24

ACI-24 is a liposome vaccine that is designed to elicit an antibody response against aggregated Aβ peptides. ACI-24 is based on the truncated $A\beta_{1-15}$ sequence, thus avoiding the T-cell epitopes. At each end of the peptide, a palmitoylated lysine residue was attached, enabling anchoring the peptide in the lipid bilayer of a liposome adjuvant thus adopting an aggregated β-sheet structure and forming a conformational epitope. After promising preclinical results (54), a phase I/II trial to assess safety, tolerability, immunogenicity as well as efficacy of the vaccine in patients with mild-to-moderate AD began in 2009 in Denmark, Finland and

**TABLE 1** **Principal active amyloid-β-directed immunotherapy vaccines**

| Drug | Main ref. | Peptide | Adjuvant | Subjects | Phase | Clinical trial # | Outcome |
|---|---|---|---|---|---|---|---|
| ABvac40 | 53 | $A\beta_{33-40}$ (multiple copies) conjugated to KHL | Alum hydroxide | a-MCI or very mild AD | Phase II | NCT03461276 | Ongoing trial |
| ACC-001 | 49 | $A\beta_{1-7}$ | QS-21 | Mild-to-moderate AD | Phase II | NCT00479557 NCT00498602 | Failed |
| ACI-24 | 54 | $A\beta_{1-15}$ with palmitoylated lysine residues | Liposome adjuvant | AD in Down Syndrome | Phase II | NCT04373616 | Trial scheduled for late 2020 |
| Affitope AD02 | 55, 56 | $A\beta_{1-6}$ conjugated to KHL | Aluminum | Early AD | Phase II | NCT01117818 | Failed |
| AN1792 | 42 | $A\beta_{1-42}$ | QS-21 | Mild-to-moderate AD | Phase II | NCT00021723 | Failed |
| CAD106 | 51, 52 | $A\beta_{1-6}$ (multiple copies) conjugated to Qβ | – | Mild AD | Phase II | NCT02565511 | Failed |
| UB 311 | 57 | two $A\beta_{1-14}$–targeting peptides | Alum-containing Th2-biased delivery system | Mild AD | Phase II | NCT02551809 | Completed |

The list is presented in alphabetical order. The table shows the status of studies on 31 July 2020, as reported in ClinicalTrials.gov.
Aβ, amyloid-β; AD, Alzheimer disease; a-MCI, amnesic mild cognitive impairment.

Sweden. In 2016, ACI-24 became the first anti-Aβ vaccine to be evaluated for the treatment of Alzheimer's disease in Down's syndrome, and in late 2020 a double-blind, randomized, placebo-controlled phase II trial to assess the safety, tolerability and target engagement in adults with Down syndrome is scheduled to start (Clinical Trial: NCT04373616).

## Affitope AD02

Affitope AD02 consists of a synthetic peptide of six amino acids mimicking the N-terminus of Aβ, lacking the most common T-cell epitopes, but including the B cell epitope. This peptide induced an anti-Aβ antibody response when conjugated to Keyhole Limpet Hemocyanin and adjuvanted with aluminum (55). In 2009, a phase I study was conducted in Austria by AFFiRiS AG and showed a favorable safety and tolerability profile 1 year after treatment. A phase II trial of AD02 was conducted in Europe between 2010 and 2013 in patients with early AD, but no significant treatment effects were seen with AD02. Surprisingly, the placebo group receiving a dose of the immunomodulator aluminum oxihydroxide which was part of the formulation, then called AD04, showed a significantly reduced cognitive decline correlating with a reduced hippocampal shrinkage (56). The company declared to be interested in further investigating the potential therapeutic effects of AD04; however no further data have been disclosed yet and no further activities with regard to AD are listed on the company website.

## UB 311

UB 311 is a synthetic peptide vaccine developed by United Neuroscience, coupling a helper T-cell epitope to the $A\beta_{1–14}$ sequence. The approach aims to stimulate a T helper type 2 regulatory immune response over a T helper type 1 proinflammatory response (57). In a transgenic AD mouse model (hAPP751), UB-311 reduced levels of $A\beta_{1–42}$ oligomers and protofibrils, as well as extracellular amyloid plaque load (57). In a first-in-human clinical trial in patients with mild-to-moderate AD, each participant received three immunizations (300 μg/dose) by intramuscular injection. The vaccine was well tolerated and showed encouraging improvement in ADAS-Cog scores in the subgroup of mild AD patients (57). As a result, a phase-II clinical trial started in Taiwan in October 2015 enrolling people with a clinical diagnosis of mild AD, which was followed by a safety extension in 2018. A press release from United Neuroscience at the beginning of 2019 reported a favorable safety profile and promising, yet not statistically significant, changes in the secondary endpoints (amyloid PET burden, CDR-SB, ADCS-ADL, ADAS-Cog and MMSE [Mini-Mental State Examination]) (58). United Biomedical, as of July 2020, lists UB 311 as investigational vaccine but no current clinical trials are registered.

## Passive Immunotherapy with Monoclonal Antibodies

In passive immunization, externally produced antibodies are administered through intravenous infusions or subcutaneous injections. They can be humanized versions of murine mAb evaluated in previous preclinical trials (such as Bapineuzumab) or fully human mAbs (like Gantenerumab). In the first group,

murine mAbs are modified so that a large part of their protein sequences is similar to naturally produced human antibody variants, in order to reduce the immunogenicity that (foreign) murine antibodies would cause. Fully human mAbs are produced for example with transgenic mice that have been genetically engineered with the human immunoglobulin locus, while in contrast, humanized mAbs are initially generated in wild type mice with the endogenous murine immunoglobulin locus (59). Avoiding some of the side effects that the humanized murine mAb still possess, fully human mAb are considered safer and more effective (60).

The passive immunization strategy allows for a precise titration of the administered antibodies and a possible rapid clearance in case adverse effects develop, but has the disadvantage that repeated infusions/injections over time are required to maintain a constant amount of therapeutic antibodies. Passive immunization might allow for targeting specific conformations of the Aβ peptide, presumably leading to the specific removal of distinct Aβ assemblies such as monomers, oligomers, or fibrils (61). The employment of mAbs against Aβ has been associated with the risk of developing amyloid-related imaging abnormalities (ARIAs) as severe adverse effects. These abnormalities seen in neuroimaging of AD patients comprise "vasogenic edema" and/or sulcal effusion (ARIA-E) and hemosiderin deposits (ARIA-H) including microhemorrhage and cortical superficial siderosis (62), and are believed to be the consequence of the removal of vascular amyloid leading to increased vascular permeability. The development of ARIAs after mAb treatment appears to be compound-dependent and dose-related. ARIAs represent a core safety issue in immunotherapy trials and challenged the progress of mAbs as a treatment for AD. Magnetic resonance imaging (MRI) imaging is used to detect active stages of ARIAs in clinical trials, but is not appropriate for predicting the risk of developing ARIAs during treatment (63). Currently, efforts to discover and use specific biomarkers for ARIAs in clinical trials are being made to better manage these severe side effects and reduce the delay caused by this side effect often seen in clinical trials (64).

Amyloid clearance in immunotherapy is largely correlated with IgG Fcγ Receptor (FcγR)-mediated activation of microglia and antibody-mediated phagocytosis, however, these same effects are probably responsible for an increased inflammatory response and vascular side effects (ARIAs) observed in a variety of studies (65). FcγRs are activated by human IgG1 and mouse IgG2a with higher affinity compared to other IgG subclasses. Using a different class of immunoglobulin G (e.g. IgG4) could help prevent an excessive microglial activation, reducing the risk of vascular damage (66). Modification of the effector function, such as de-glycosylation, by antibody engineering, was also used as a strategy to reduce the incidence of adverse ARIAs (67). Even though the role of the antibody effector function in the development of vascular side effects is clear, the engaged epitope is also crucial. A comparative study of murine versions of therapeutic Aβ antibody candidates with a constant IgG2a region showed strong differences in their plaque-removing potential, demonstrating that the ability of an antibody to remove plaques and activate inflammation is critically dependent on its epitope and affinity (68). A variety of antibodies have been evaluated in passive immunotherapy approaches and reached different stages of clinical trials (Table 2).

## TABLE 2 Principal passive amyloid-β-directed immunotherapy drugs

| Drug | Main ref. | Target | Antibody - subtype | Subjects | Phase | Clinical trial # | Outcome |
|---|---|---|---|---|---|---|---|
| Aducanumab | 122 | $A\beta_{3-7}$ | Humanized mAb - IgG1 | Early AD | Phase III (ENGANGE and EMERGE) | NCT02477800<br>NCT02484547 | Terminated |
| BAN2401 | 115 | Soluble Aβ protofibrils | Humanized mAb - IgG1 | Early AD<br>Preclinical AD | Phase III<br>Phase III | NCT03887455<br>NCT04468659 | Ongoing Trial<br>Ongoing Trial |
| Bapineuzumab | 71 | $A\beta_{1-5}$ (fibrillary and soluble Aβ) | Fully human mAb - IgG1 | Mild-to-moderate AD | Phase III | NCT00676143<br>NCT00667810<br>NCT00575055<br>NCT00574132 | Failed |
| Crenezumab | 81<br>82 | $A\beta_{13-24}$ (pentameric Aβ oligomers and fibrils) | Humanized mAb - IgG4 | Prodromal-to-mild AD<br>Preclinical AD with PSEN1 E280A mutation | Phase III (CREAD 1 and 2)<br>Phase II | NCT02670083<br>NCT03114657<br>NCT01998841<br>NCT03977584 | Failed<br><br>Ongoing Trials |
| Gantenerumab | 85, 86 | Aβ fibrils | Humanized mAb - IgG1 | Early AD | Phase III (GRADUATE 1 and 2) | NCT03443973<br>NCT03444870 | Ongoing Trials |
| MEDI1814 | 129 | $A\beta_{42}$ C-terminus (Aβ monomers and low-n oligomers) | Fully human mAb - IgG1λ | Mild-to-moderate AD | Phase I | NCT02036645 | Completed |
| Ponezumab | 95 | $A\beta_{40}$ C-terminus (Aβ monomers) | Humanized mAb - IgG2Δa | Mild-to-moderate AD | Phase II | NCT00722046<br>NCT00945672 | Failed |
| SAR228810 | 130 | Protofibrillary and fibrillary Aβ | Humanized mAb - IgG4 | AD | Phase I | NCT01485302 | Completed |
| Solanezumab | 105, 106 | $A\beta_{16-26}$ (Aβ monomers) | Humanized mAb - IgG1 | Mild-to-moderate AD<br>Individuals with EOAD-associated mutations | Phase III (EXPEDITION 1,2 and 3)<br>Phase II/III | NCT00905372<br>NCT00904683<br>NCT01900665<br>NCT01760005 | Failed<br><br>Ongoing Trial |

The list is presented in alphabetical order. The table shows the status of studies on 31 July 2020, as reported in ClinicalTrials.gov.
Aβ, amyloid-β; AD, Alzheimer disease; EOAD, early onset Alzheimer disease; mAb, monoclonal Aβ.

## Bapineuzumab

Bapineuzumab is a humanized form of the murine monoclonal antibody 3D6, directed specifically towards the N-terminus of the Aβ sequence starting at Asp1 (69, 70). This antibody of the IgG1 subclass binds fibrillary and soluble Aβ and activates microglial phagocytosis as well as cytokine production, aiming to reduce plaque formation and promote Aβ clearance (71). Preclinical studies and phase I–II clinical trials gave initial promising results. When 3D6 mAb was administered to 4-month-old PDAPP mice with i.p. injections of 10 mg/kg/week for 12 months, total Aβ deposition was reported to be almost completely reduced (72). Although the translatability of these preclinical studies was later questioned (73), bapineuzumab was tested in a phase I clinical trial where a single ascending dose was administered to patients with mild-to-moderate Alzheimer's disease in order to determine the safety, tolerability, and pharmacokinetics of the mAb (74). MRI abnormalities, consistent with vasogenic edema, were observed in 3 out of 10 patients receiving the higher dose of 5 mg/kg, but this resolved with time. MMSE scores improved at the lower doses (0.5 and 1.5 mg/kg) of bapineuzumab compared to the placebo, a finding not observed with the highest dose.

In a phase II clinical trial, patients with mild-to-moderate AD were randomly assigned to one of four dose cohorts (0.15, 0.5, 1.0, or 2.0 mg/kg) and received six infusions 13 weeks apart. The final assessments were performed at week 78 but no significant differences were found in co-primary efficacy endpoints, the ADAS-cog and Disability Assessment for Dementia (DAD). Exploratory analyses showed potential treatment differences on cognitive and functional endpoints. Differences based on APOE ε4 carrier status were also observed. ARIA-E was found in 12/124 treated patients, with a dose and APOE ε4 carrier-dependent incidence increase (71). Additional phase II studies reported a reduction in exploratory CSF biomarkers T-Tau and p-Tau, the latter being significantly different between treated and placebo groups (75). A reduced cortical $^{11}$C-Pittsburgh compound B (PiB) average uptake, visualized by PET, was also found after 78 weeks of treatment with bapineuzumab (76). The feasible and tolerable administration of bapineuzumab, together with evidence that the mAb could be disease modifying, led to the actualization of phase III clinical trials.

A four-trial phase III program was launched in North America and Europe. The first two double-blind, randomized, placebo-controlled 18-month phase III trials tested bapineuzumab in patients with mild-to-moderate Alzheimer's disease, divided into APOE ε4 carriers and non-carriers (71). Bapineuzumab was administered by intravenous infusion every 13 weeks for 78 weeks at a dose of 0.5 mg/kg in APOE ε4 carriers and at 0.5 mg/kg, 1 mg/kg, and 2 mg/kg doses in non-carriers, even though the highest dose was soon discontinued due to ARIA-E and ARIA-H development. No significant differences were found in the primary outcome measures (ADAS-cog11 and DAD) between groups. The APOE ε4 carriers group showed a modest reduction in PiB PET binding as well as a significant reduction of CSF p-Tau when compared to the placebo group. Consistent with the phase II data, a dose-related and APOE ε4 carriers-dependent increase in ARIA-E was observed. The failure to meet the primary endpoints led to the discontinuation of two additional phase III clinical trials and the further evaluation of bapineuzumab as treatment for AD.

## Crenezumab

Crenezumab is a humanized mAb designed on an IgG4 backbone targeting multiple species of Aβ. Its epitope is located in the central part (~ $A\beta_{13–24}$) of the peptide and it shows particular affinity for pentameric oligomeric and fibrillary 16mer assemblies of aggregated Aβ (77, 78). A recent study confirmed that it detects a variety of full-length and N-terminal truncated Aβ variants in post-mortem human AD brain samples (70). Limited preclinical data are currently published on the efficacy of chronic treatments with crenezumab. The murine version of the antibody (mC2) was tested in 18-month Tg2576 transgenic mice with a single intracerebral injection of 2 µg of antibody, which did not cause significant inflammatory changes (68). *In vivo* imaging of 10-month-old transgenic $hAPP^{(V717I)}$/PS1 mice showed decreased plaque volumes over a period of 3 weeks after an intraperitoneal injection of 60 mg/kg antibody (77). The same study reported the results of a phase I clinical trial, performed in patients with mild-to-moderate AD. No ARIAs were observed either with a single or multiple ascending dosage.

Crenezumab was further tested in phase II clinical trials in patients with mild-to-moderate AD. A total of 431 patients were enrolled in the ABBY study, receiving either a low subcutaneous dose (300 mg) or placebo every 2 weeks, or an intravenous high dose (15 mg/kg) or placebo every 4 weeks, for a total period of 68 weeks (79). The primary endpoints (changes in ADAS-Cog12 and CDR-SB scores), measured at week 73, were not met. Exploratory analyses pointed towards a reduction in decline on the ADAS-Cog12 in the high-dose group, and the patients with mild AD showed the greatest deviation from the placebo group. This difference became significant in the group with MMSE scores ranging from 22 to 26. These trends were also observed in a smaller phase II brain imaging study (BLAZE), enrolling 91 patients. Even though no significant differences were observed in the primary outcome measures, non-significant trends toward ADAS-Cog12 and CDR-SB score improvements were observed in the mild AD group receiving the higher dose of antibody (80). Throughout these studies, no ARIAs adverse effects were reported.

Two large phase III clinical trials, CREAD1 and CREAD2, started in 2016 and 2017 respectively, and enrolled patients with prodromal-to-mild AD. These double-blind, placebo-controlled global studies recruited overall more than 1500 patients, testing a 60 mg/kg dose by intravenous infusion every 4 weeks for a period of 100 weeks with the primary endpoint being changes in the CDR-SB score at 2 years (81). In January 2019 the company Roche announced the decision to discontinue both trials, based on preliminary analyses suggesting that the primary endpoint would unlikely be met. Crenezumab is, to date, being tested as a preventive treatment as part of the Alzheimer Prevention Initiative (API) in a randomized, double-blind, placebo-controlled phase II study by Genentech, estimated to end in 2022 (Clinical Trial: NCT01998841). The 5-year trial started in 2013 and recruited patients who carry the PSEN1 E280A autosomal-dominant mutation and are still in a preclinical phase of AD (82). In a subgroup of participants (carriers and non-carriers) the longitudinal tau burden will be evaluated with a tau positron emission tomography (PET) scan after IV injection of the probe [18F]GTP1 (Clinical Trial: NCT03977584).

## Gantenerumab

Gantenerumab is a recombinant human IgG1 antibody, designed to recognize a conformational epitope present on Aβ fibrils, in order to disassemble and degrade aggregated Aβ peptides via recruiting microglia and activating phagocytosis (83). Using peptide mapping, N-terminal as well as central portions of Aβ were recognized and no evidence of altered plasma Aβ was detected. In a preclinical study, gantenerumab bound cerebral Aβ and significantly reduced small amyloid-β plaques in APP/PS2 transgenic mice with chronic treatment (83). An initial randomized study of AD patients receiving either 60 mg or 200 mg intravenous gantenerumab or placebo, showed a ~16% or ~36% reduction in Pittsburgh Compound B retention in the 60 mg and 200 mg gantenerumab group respectively. However, two patients in the 200 mg group showed vasogenic edema and focal areas of inflammation on MRI scans at sites with the highest level of amyloid removal (84).

The Scarlet RoAD trial assessed the efficacy and safety of gantenerumab in prodromal AD patients. Participants enrolled in this 2-year randomized double-blind phase III study received 105 mg, 225 mg or placebo every 4 weeks subcutaneously. A dose- and APOE ε4 genotype-dependent increase of generally asymptomatic ARIAs was noticed and the study was terminated for futility when no differences in primary or secondary endpoints were observed (85). Of note, significant reductions in total and phosphorylated tau in the CSF, as well as a dose-dependent reduction in brain amyloid on PET scans were observed in an exploratory biomarker analysis (85). A 2-year PET sub-study evaluating the effect of up to 1200 mg of gantenerumab every 4 weeks in patients with prodromal-to-moderate AD, revealed a 3.5-times greater reduction in amyloid-PET signal than seen after 2 years at a dose of 225 mg, with 51% of patients having amyloid-β plaque levels below the positivity threshold (86).

A phase I randomized, open-label study including healthy volunteers aged 40–80 years, evaluated different subcutaneous injection regimens of gantenerumab, with regard to pharmacokinetic properties and tolerability. The results of this study suggest that subcutaneous injections at speeds of 5 and 15 s were well-tolerated and might enable at-home administrations by AD patients or their caregivers (87). Gantenerumab is currently under investigation in two large phase III trials (GRADUATE 1 and 2), which started enrolling patients with early AD in 2018 with the goal of more than 1500 patients in up to 350 study centers with a data read-out expected in 2022 (Clinical Trial: NCT03443973 and NCT03444870).

## Ponezumab

Ponezumab is a humanized monoclonal IgG2Δa anti-Aβ antibody reported to bind to the C-terminus of the most abundant $A\beta_{1–40}$ peptide. It contains two mutations that eliminate effector function and therefore potential cell toxicity depending on the antibody. Structural analyses revealed extensive contacts of ponezumab with the carboxyl moiety of $A\beta_{40}$ (88). Preclinical analyses using the murine antibody 2H6, similarly binding to the C-terminal of $A\beta_{1–40}$, demonstrated a robust reduction of amyloid deposits in aged Tg2576 (89). Intraperitoneal

injections of ponezumab increased plasma $A\beta_{1-x}$ and $A\beta_{x-40}$ levels in PS1xAPP mice in a concentration-dependent manner, while $A\beta_{x-42}$ plasma concentrations remained unchanged. This led to the suggestion that ponezumab removes brain Aβ via a peripheral sink mechanism (88). Another preclinical study in cynomolgus monkeys, sharing the same Aβ peptide sequence with humans, confirmed increased plasma $A\beta_{1-40}$ and $A\beta_{1-x}$ levels in treated animals versus controls (90).

An initial randomized, double-blind, single-dose-escalation study evaluated safety, pharmacokinetics and pharmacodynamics using doses of 0.1 mg/kg up to 10 mg/kg. The 2-h infusion was well-tolerated, and in individuals receiving the highest dose increases in CSF Aβ were observed, which is suggestive of altered central Aβ levels (91). A related study in a cohort of Japanese subjects yielded comparable results (92). A different administration protocol of a single 10-min intravenous infusion was evaluated and produced comparable effects on plasma Aβ species (93). Individuals aged 50 and older with a diagnosis of mild-to-moderate AD and a MMSE score of 16 to 26 were enrolled in a placebo-controlled, multiple dose study (0.1 mg/kg up to 8.5 mg/kg) of ponezumab. The treatment was administered as 10 2-h infusion every 2 months, and was generally well tolerated with an acceptable safety profile and robust plasma Aβ increases but no evidence of a dose response with regard to CSF biomarkers (94). Effects on peripheral and central Aβ were characterized in small Swedish cohorts suffering from mild-to-moderate AD. One cohort received ponezumab (10 mg/kg) or placebo quarterly over 1 year, whereas a second cohort started with an initial dose of 10 mg/kg or placebo, followed by monthly infusions of 7.5 mg/kg or placebo respectively. This phase II study again showed that ponezumab was generally safe and well tolerated, with dose-dependent increases in plasma Aβ. However, no apparent differences in brain amyloid burden assessed by PiB-PET were detected and changes in both cognitive and functional decline were observed during the course of the study without, however, differences between treatment arms (95). The potential effect of intravenous ponezumab was also investigated in patients with probable cerebral amyloid angiopathy (CAA) (96), a disease condition with amyloid deposition in the walls of leptomeningeal and intracortical blood vessels of the CNS (97, 98). In this study, again, ponezumab was safe and well tolerated; however, this antibody has been discontinued as prespecified efficacy criteria were not met in the majority of the trials.

## Solanezumab

Solanezumab is a humanized monoclonal IgG1 antibody (mouse version m266), targeting the mid-region of Aβ. Co-crystallization studies revealed that solanezumab accommodates a large Aβ epitope (residues 16–26), forming extensive contacts and hydrogen bonds with the antibody (99). As administration of solanezumab as well as its murine precursor m266 cause substantial dose-dependent increases in plasma antibody-bound Aβ levels (100–102), it has been suggested that this antibody primarily targets soluble monomeric forms of Aβ. On the contrary, neuropathological studies employing human brain samples indicated that a recombinant biosimilar antibody of solanezumab showed a strong binding affinity to amyloid plaques (103), calling its assumed selectivity for monomeric Aβ into question. In transgenic PDAPP mice, administration of m266 resulted in a rapid

reversal of memory deficits in the absence of amyloid plaque reductions (102); however, a more recent study in the J20 mouse model of AD reported no improvement of behavioral deficits and even a strongly increased mortality rate following m266 immunization (101).

Solanezumab has been investigated in several clinical trials in order to evaluate its disease-modifying potential. Following a phase II trial with 52 patients suffering from mild-to-moderate AD evaluating diverse dose regimens (104), two large phase III studies (EXPEDITION-1, EXPEDITION-2) were launched. These studies recruited 2,052 mild-to-moderate AD patients, who received monthly 400 mg infusions. However, both showed a lack of efficacy with regard to cognitive performance, the primary outcome measure of both studies (105). Pooled analyses of both studies suggested less functional and cognitive decline in the mild AD population; however, no significant differences in baseline-to-endpoint changes were found for a variety of secondary outcome measures such as activities of daily living (106). Following the review of the data obtained from the pooled mild AD population, a third phase III trial (EXPEDITION-3) was initiated. This trial enrolled 2129 patients with mild dementia and evidence of amyloid deposition, shown by either florbetapir PET or $A\beta_{1\text{-}42}$ measurements in CSF, and patients received 400 mg solanezumab or placebo every 4 weeks for 76 weeks. As a result, the secondary analyses of the previous EXPEDITION trials were not reproduced and solanezumab showed no benefit with regard to cognitive decline in patients with mild AD (107).

Solanezumab is being tested within the Dominantly Inherited Alzheimer Network (DIAN) trial in a phase II/III study as a potential disease-modifying treatment, together with gantenerumab, in individuals at risk for or with a mutation associated with EOAD. The trials are estimated to be completed by March 2021 (Clinical trial: NCT017660005).

## BAN2401

BAN2401 is the humanized version (IgG1) of the mouse monoclonal antibody mAb158, which has been shown to primarily bind to large soluble Aβ protofibrils (108). Selectivity for this type of aggregate has been described to be at least 1000-fold higher than for monomers and 10–15 times better than for Aβ fibrils (109, 110). Administration of mAb158 to plaque-bearing AD transgenic mice carrying both the Arctic and Swedish APP mutations (tg-ArcSwe) resulted in lowered Aβ protofibrils, albeit unchanged insoluble Aβ levels. When treatment was started prior to extracellular plaque onset, a prevention of amyloid deposition and a reduction in protofibril levels was observed (111). Interestingly, individual performance of young tg-ArcSwe mice in a spatial memory test (Morris water maze) was inversely correlated with protofibril but not total Aβ levels (112). This antibody, as well as its humanized version BAN2401, efficiently precipitated soluble Aβ aggregates from the human brain, and more than 50% reduction of protofibrils/oligomers was observed after long-term mAb158 treatment in the CSF of tg-ArcSwe mice (113). A radiolabeled version of mAb158 conjugated to a transferrin receptor antibody has been shown to effectively visualize Aβ in the brain of two AD mouse models, enabled via receptor-mediated transcytosis across the BBB (114).

Safety and tolerability of BAN2401 were investigated in an ascending dose study (0.1 mg/kg up to 10 mg/kg biweekly) for 4 months in mild-to-moderate AD cases. The treatment was well-tolerated across all doses and a slight elevation of plasma $A\beta_{1-40}$ was noted, albeit in the absence of measurable effects on CSF biomarkers (115). A subsequent placebo-controlled, double-blind, randomized phase IIb study enrolling 856 patients with mild cognitive impairment (MCI) caused by AD or mild AD-dementia evaluated several doses in a Bayesian adaptive design. A statistically significant reduction in amyloid PET standard uptake value ratio (SUVR) was observed after 18 months at the highest dose, together with a significant clinical benefit measured by ADCOMS at 6 and 12 months. The drug was well-tolerated with an incidence of ARIA-E of not more than 10% in any treatment arm and less than 15% in APOE ε4 carriers at the highest dose (116). Assessment of amyloid PET status in patients in an ongoing open-label extension (OLE) of BAN2401-G000-201 revealed that all amyloid-negative, BAN2401-treated individuals entering the OLE were also amyloid negative at OLE baseline, despite subjects being off treatment for 9–52 months (117).

A phase III trial for individuals with preclinical AD and elevated amyloid (AHEAD 3–45 study) is currently underway and participants are being recruited and is expected to be completed in October 2027 (Clinical Trial: NCT04468659).

## Aducanumab

Aducanumab (BIIB037) is a recombinant human IgG1 antibody that has been isolated from blood lymphocytes of a healthy donor population of elderly subjects with unusually slow cognitive decline and lack of symptoms of cognitive impairment. Preclinical studies in the Tg2576 mouse model employing chronic dosing of a murine IgG2a/k chimeric aducanumab analogue showed significant reductions of Aβ in both soluble and insoluble protein extracts, as well as significantly reduced Aβ deposits in both the cortex and hippocampus; however, no data on behavioral performance was provided (11). Structural and biochemical analyses revealed that aducanumab binds a linear Aβ epitope comprised of amino acids 3–7 in an extended conformation, discriminating between monomers and higher molecular weight peptide assemblies, based on a strong avidity for epitope-rich aggregates and very weak monomer affinity (118). The linear sequence recognized by aducanumab substantially overlaps with other Aβ antibodies (such as bapineuzumab or gantenerumab), while specific interactions such as critical contacts formed with Phe-4 and His-6, are different and the interaction of Aβ and aducanumab is quite shallow (118).

An initial phase I study investigated the safety, tolerability, and pharmacokinetics of a single ascending aducanumab dose (0.3–60 mg/kg) or placebo in mild-to-moderate AD patients. While doses up to 30 mg/kg were generally well-tolerated, all three patients receiving 60 mg/kg developed serious adverse events (SAEs) of symptomatic ARIA, which completely resolved after several weeks (119).

A subsequent phase Ib, 12-month, double-blind placebo-controlled, multiple ascending-dose (1 – 10 mg/kg) study (PRIME) enrolled 165 patients with a clinical diagnosis of prodromal or mild AD (11). Amyloid PET imaging using florbetapir was used as an adjunct tool to identify and select patients for enrollment (120). Of the 165 dosed patients, 40 discontinued treatment, mainly due to adverse events

or withdrawal of consent. Aducanumab reduced brain Aβ plaques as quantified by florbetapir PET in a dose- and time-dependent manner, with significantly reduced SUVR composite scores in the 3, 6 and 10 mg/kg dose groups after 54 weeks of treatment. A slowing of clinical progression as measured by both the MMSE as well as the CDR-SB was observed in patients receiving the highest dose after 1 year of treatment (11). Although this represents the first study reporting an effect of lowering the brain Aβ load coupled to beneficial effects on cognitive outcomes, the small sample size, a staggered parallel-group design and potential unblinding due to ARIA-E in the treatment groups receiving higher antibody doses, impede interpretation of the results. In addition, the clinical stage of dropouts might bear a potential interpretation bias. Similar discontinuation rates were reported among prodromal and mild AD patients in the placebo group; however, more mild than prodromal AD patients at baseline dropped out in the 10 mg/kg group, with a potential impact on the observed slower cognitive decline (121).

Two large 18-month, randomized, double-blind, placebo-controlled phase III trials (ENGAGE & EMERGE) evaluated aducanumab in patients with early AD and MCI due to AD with PET-confirmed amyloid pathology (122). The participants were randomized to receive a low dose (3 mg/kg for ApoE ε4 carriers, 6 mg/kg for non-carriers) or a high-dose of 6 or 10 mg/kg for 78 weeks. The protocol has been amended during the course of the study, allowing ApoE ε4 carriers to receive up to 10 mg/kg and increasing the sample size of each trial to 1650 to compensate for larger than expected standard deviation. A planned futility analysis indicated little chance of treatment efficacy and the trials were terminated in March 2019 (123). Later in 2019, analyses of a more complete data set from both studies were presented, with 29% of patients in EMERGE and 22% in ENGAGE receiving the full possible 14 doses of 10 mg/kg and final participant numbers of 982 and 1084 respectively (124). In EMERGE, the high dose aducanumab group showed a significant 23% reduction in decline on the CDR-SB and 27% reduction on the AD Assessment Scale-Cognitive Subscale 13 items (ADAS-Cog 13) compared to placebo; however, only a 2% reduction on CDR-SB and a 12% reduction in ADAS-Cog 13 were observed in the high-dose group in the ENGAGE sister trial (124). This was explained by the greater exposure to high-dose aducanumab in the EMERGE trial; however, other possibilities such as greater worsening in the placebo group are conceivable as well (125). On July 8, 2020 Biogen announced that it had completed the submission of a Biologics License Application (BLA) to the U.S. Food and Drug Administration (FDA) for the approval of aducanumab (126). On August 7, 2020, Biogen announced that the agency accepted this BLA granting priority review, which means that the time to review is cut down to 6 months. In case of successful approval, aducanumab will be the first approved biological capable of removing amyloid plaques.

## MEDI1814

MEDI1814 is a fully human monoclonal IgG1λ antibody targeting the C-terminus of $A\beta_{42}$, with a triple mutation in the Fc tail to reduce its effector function. It aims to bind and remove monomers and low n-oligomers from circulation, thus preventing further aggregation of the peptide (127). MEDI1814 showed a dose-dependent suppression of up to 90% of free $A\beta_{42}$ in the CSF of V717I transgenic mice, naıve rats and cynomolgus monkeys (128).

AstraZeneca started a clinical trial in the United States in 2014 testing single and multiple ascending dose in subjects with mild-to-moderate AD. Safety, tolerability, pharmacokinetics, and pharmacodynamics were analyzed and none of the participants on the drug showed signs of ARIAs. In addition, pharmacokinetics and pharmacodynamics data provided evidence of dose-dependent and selective $A\beta_{42}$ target engagement in the CNS (129).

### SAR228810

SAR228810 is a humanized version of murine IgG1 SAR255952 antibody with an engineered human IgG4 backbone with two amino-acid substitutions to reduce the Fc effector function-dependent risk of ARIAs. It binds specifically to soluble protofibrillar and fibrillar forms of Aβ and it is relatively inactive against Aβ monomers and small oligomeric aggregates (130). Co-application of SAR228810 and oligomeric $A\beta_{42}$ preparations significantly inhibited Aβ-induced neurotoxicity in primary neurons (131). Preclinical pharmacological studies of SAR255952 in APPSL mice showed that a chronic 4-month treatment dose-dependently prevented brain amyloid plaque formation. Even with high doses (up to 50 mg/kg/week intravenously), SAR255952 did not increase brain micro-hemorrhages in old mice. In immunotolerized APPSL mice, in which $CD4^+$ T lymphocytes have been transiently depleted, SAR228810 demonstrated the same efficacy as its murine precursor (130, 132).

A multi-center, double-blind, placebo-controlled phase I clinical trial testing escalating single and multiple doses by Sanofi has been completed and no further clinical trials are ongoing at the moment. SAR228810 has been administered by intravenous infusion or subcutaneous injection in patients with mild-to-moderate AD.

## CONCLUSION

A multitude of preclinical biochemical, histopathological and animal studies, as well as a large number of genetic, biomarker and clinical reports support the central role of Aβ in AD pathogenesis. While the amyloid cascade hypothesis, with all its modifications, is still considered relevant, the continuous failures of late stage clinical trials with immunotherapy approaches raise questions about considering the right target. There is increasing evidence that Aβ peptides might also play important physiological roles, as neurotrophic effects (133) or improved synaptic function after application of picomolar Aβ concentrations in mice depleted of endogenous Aβ have been described (134, 135). The observation that Aβ is elevated in the CSF after sleep deprivation in healthy adults (136), together with its increased brain levels in a variety of other neurologic disease conditions such as traumatic brain injury (137) or cerebrovascular lesions (138) may indicate, that Aβ production in the case of neuronal stress or damage might represent response rather than origin. While immunotherapy trials targeting Aβ have been regarded as the final proof of the validity of the amyloid cascade hypothesis, the aforementioned studies still paint a nebulous picture and alternative therapeutic strategies and approaches should be vigorously investigated.

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# Amyloid β-Targeted Inhibitory Peptides for Alzheimer's Disease: Current State and Future Perspectives

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**Abstract:** Alzheimer's disease is the most common irreversible neurodegenerative disorder. To date, there is no cure for Alzheimer's disease. While multiple pathological mechanisms have been proposed for the onset and progression of Alzheimer's disease, the hypothesis that attracted much attention is the amyloid hypothesis. The senile plaques that accumulate in the brain of Alzheimer's disease

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patients are predominantly composed of beta amyloid (Aβ). Aβ deposition in the brain is thought to occur years before the emergence of clinical symptoms. The overproduction, aggregation, and fibrillation of Aβ, combined with reduced clearance, eventually lead to amyloid plaque formation and subsequent neurotoxicity. Hence, inhibition of Aβ aggregation and the promotion of Aβ clearance have been actively explored as therapeutic strategies for Alzheimer's disease. This chapter provides an overview of the current knowledge on one such strategy, Aβ-targeted inhibitory peptides.

**Keywords:** Aβ aggregation in Alzheimer's disease; biopanning; inhibitory peptides for Alzheimer's disease; peptide–nanostructure conjugates; peptidomimetics

## INTRODUCTION

Alzheimer's disease is an age-dependent disorder that is the fifth leading cause of death in people aged 65 years and older. It is estimated that over 50 million people worldwide suffer from Alzheimer's disease, and this figure is set to increase to 152 million by 2050 with a financial burden of 1.1 trillion US dollars by 2050 (1–3). Several hypotheses, including the amyloid, cholinergic (4), and Tau protein hypotheses have been proposed to explain the pathophysiology and etiology of Alzheimer's disease (5). Because of the presence of Aβ in the brain tissue, cerebrospinal fluid, and plasma, the amyloid cascade hypothesis is the most widely accepted. The amyloid cascade hypothesis states that neurodegeneration in Alzheimer's disease is the result of amyloid plaque and neurofibrillary tangle formations (6, 7). The overproduction, clearance failure, aggregation, and fibrillation of Aβ eventually leads to amyloid plaque formation. These factors also contribute to neuroinflammation and cell death. Aβ deposition in the brain is likely to be the first pathological incident that occurs years before the emergence of clinical symptoms. Aβ is produced through the proteolytic cleavage of the amyloid precursor protein (APP), a transmembrane glycoprotein, which is made up of a cytoplasmic domain with 55 amino acids and a long extracellular domain with 590–680 amino acids (8). APP cleavage by the proteases β- and γ-secretases produce Aβ fragments of varying size depending on the cleavage site (9), of which $A\beta_{40}$ (about 90%) and $A\beta_{42}$ (about 5–10%) are the most prevalent (Figure 1). $A\beta_{42}$ is more toxic than $A\beta_{40}$. After production, the Aβ peptides aggregate to form amyloid deposits. There are different aggregation forms such as low molecular weight oligomers, protofibrils, as well as mature fibrils that eventually come together to form amyloid deposits in the brain parenchyma and cerebrovascular spaces (10, 11).

Therefore, inhibition of Aβ aggregation and the promotion of Aβ clearance have been investigated as therapeutic strategies for Alzheimer's disease. Some of these strategies include the use of metal chelators (12), peptides (13), organic molecules (14), and biomolecules (15, 16). Peptides are considered a better option than small molecule-based compounds because of their high affinity for Aβ and low toxicity (17). Although natural amino acid-based peptides are effective inhibitors of Aβ aggregation, they are prone to faster enzymatic degradation and show a tendency for self-assembly into fibrils during administration (15). To overcome these problems, modified peptides have been generated (18) with

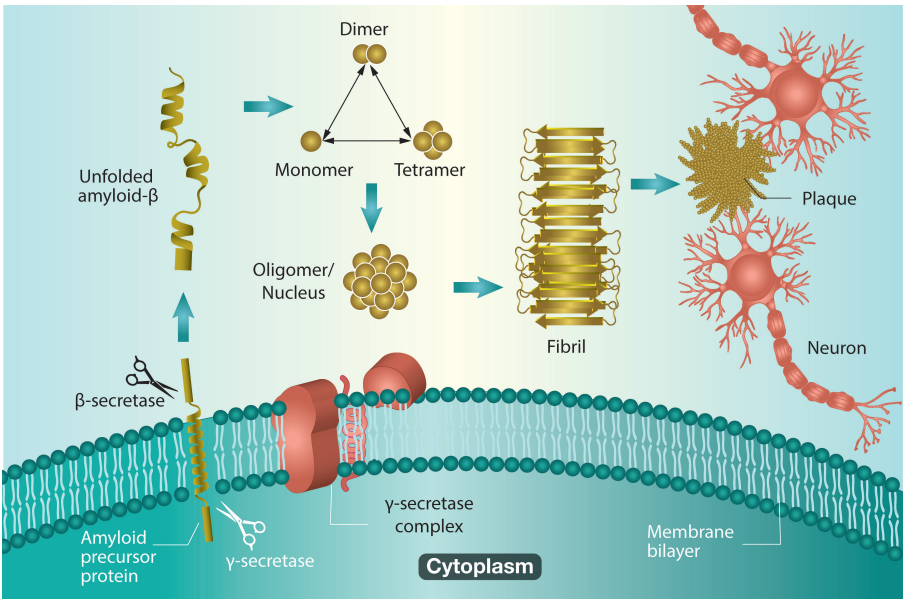


**Figure 1. Amyloid-β fibrillation and neuronal damage.** First, amyloid precursor protein is cleaved by β and γ secretases, respectively. A peptide fragment of 39-42 amino acid is formed depending on the site of cleavage. After cleavage, Aβ monomers start to self-assemble to form soluble toxic aggregates, and finally into insoluble fibrils, which subsequently cause synaptic dysfunction and neuronal death.

D-amino acids, retro-inverso cyclization, fluorination, as well as N-methylation of the ester bond (19). With this knowledge, peptides could be potential candidates for inhibiting Aβ conformational transitions, self-assembly, and toxicity against neurons, and promotion of the pathways of the nontoxic fibrillation and early diagnosis of Alzheimer's disease (20). This chapter provides an overview of the therapeutic potential peptides as Aβ aggregation inhibitors.

## PEPTIDIC INHIBITORS

Luhrs and co-workers first experimentally described the structure of the $A\beta_{42}$ fibril (Figure 2, right) (21). At least four specific structural sites for interaction have been identified on the Aβ fibril (22): (i), hydrophobic regions of Ala30–Val36, and Leu17–Ala21 residues from the C and N-terminal β-sheets respectively; (ii), hydrophilic part using electrostatic interactions between Asp23 and Lys28 residues; (iii), central cleft in the interior of the U-shaped turn; and (iv), Glu22 ladder between the side chains of the Glu22 residues of the adjacent β-strands (Figure 2, bottom right). The formation of the salt-bridge between Asp23 and Lys28 is an essential β-sheet conformation stabilizer. Moreover, it might stimulate the oligomerization of Aβ via stabilizing the Val24–Asn27 turn (23). The hydrophobic residue of Met35 in the C-terminus domain could support

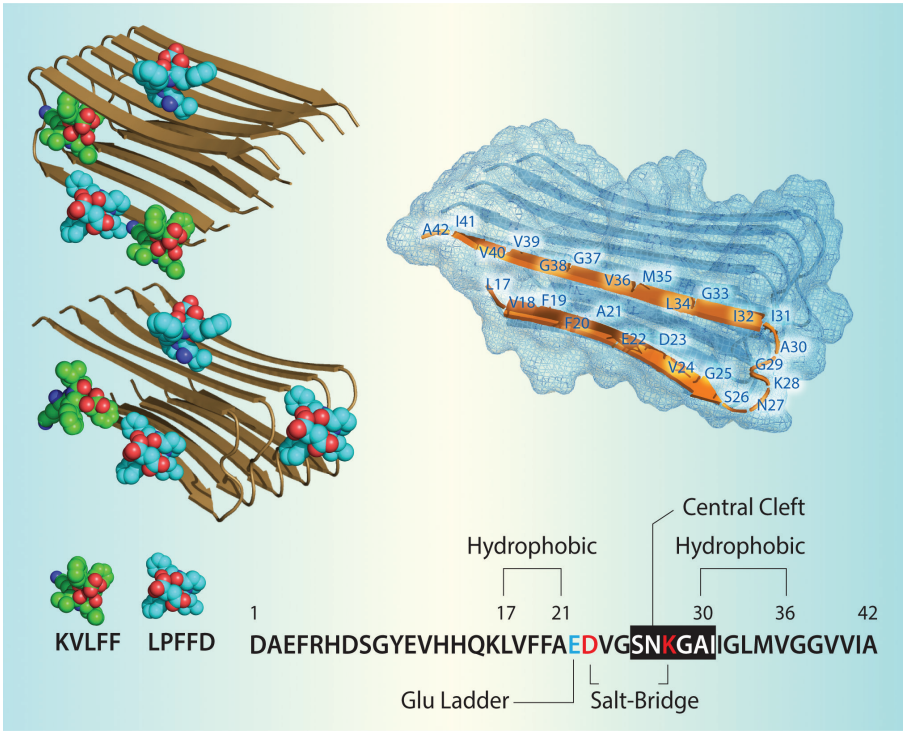


**Figure 2. The structure of Aβ.** Right, schematic of the $A\beta_{42}$ amyloid fibril. Left, binding sites of inhibitory peptides of LPFFD and KVLFF on $A\beta_{40}$ fibril.

fibril stability by hydrophobic interactions. The Met35 binding site can potentially inhibit protein-protein interactions and prevent amyloid fibril formation (24). These sites are probably critical regions in the initiation of Aβ nucleation, conformational transition promotion, and fibril formation. The residues $^{16}$KLVFFA$^{21}$ (Figure 2) of the central hydrophobic core (CHC) region is a critical nucleation site, or self-recognition sequence. The Ile41 and $Ala_{42}$ residues can modulate $A\beta_{42}$ oligomer formation (25) by interacting with the N and C-terminus of $A\beta_{42}$ (26). Figure 2, left, shows the binding sites of the most common inhibitory peptides, such as LPFFD and KVLFF, on the $A\beta_{40}$ fibril structure.

Peptide inhibitors are generally divided into Aβ-based peptide inhibitors and non-Aβ-based peptide inhibitors. A list of select Aβ inhibitory peptides are presented in Table 1.

## Aβ-based peptide inhibitors

These are based on the structure of the C-terminal fragments (CTFs) and the CHC sequences of the Aβ peptide. They bind to the Aβ peptide at specific sites and prevent its assembly into amyloid fibrils. Peptides consisting of D-enantiomeric amino acids exhibit greater stability against proteases and show a higher binding

## TABLE 1 A select list of inhibitory peptides against Aβ peptide aggregation

| No | Name/sequence of inhibitor peptide | Configuration | Type | Therapeutic findings | Ref. |
|---|---|---|---|---|---|
| 1 | RR (RYYAAFFARR) | L-enantiomer | Rational design based on hydrophobic core and salt bridge region | • Inhibition of $A\beta_{40}$ fibrillation and decrease of $A\beta_{40}$ induced toxicity (*in vitro*) | (27) |
| 2 | D-(PGKLVYA) and D(KKLVFFARRRRA) | D-enantiomer | CHC-derived sequence | • Inhibition of Aβ aggregation<br>• Increase life time in an animal model of expressing $A\beta_{42}$ | (28) |
| 3 | CP-2 (lLwHsK) | Cyclic d,l-α-peptide | Screening of the randomly library members (500) | • Nontoxic<br>• Stabilize small Aβ oligomers<br>• Disassemble formed Aβ fibrils | (29) |
| 4 | D3D3 (D-RPRTRLHTHRNRRPRvTRLHTHRNR) | D-enantiomer | Not derived from Aβ sequence | • More inhibitory effect against $A\beta_{42}$ oligomerization than D3 peptide<br>• Formation of nontoxic amorphous instead of toxic oligomers (*In vivo*) | (30) |
| 5 | RD2 (DPTLHTHNRRRR-$NH_2$) | D-enantiomer | • Not derived from Aβ sequence | • Good binding affinity to Aβ<br>• Reduce Aβ fibrillation formation<br>• High stability in mouse plasma<br>• High bioavailability (*In vivo*) | (31, 32) |
| 6 | AOEP2 (FDYKAEFMPWDT) | D-enantiomer | • A mimotope of the Aβ oligomer<br>• Selected by phage display | • Targeting of all forms of Aβ<br>• The considrable reduction of TNF-α | (33) |
| 7 | LPYFD-amide | L-enantiomer | CHC-derived sequence | • Neuroprotective<br>• Decrease tau aggregation (*In vivo*) | (34) |
| 8 | Ac-LPFFN-$NH_2$ | L-enantiomer | CHC-derived sequence | • Inhibit of $A\beta_{40}$ aggregation<br>• Stabilize the α-helical conformation of $A\beta_{40}$<br>• Prolong the fibril formation | (35) |
| 9 | D-4F peptide | D-enantiomer | Not derived from Aβ sequence | • Inhibit Aβ deposition<br>• Improve cognitive function | (36) |

*Table continued on following page*

**TABLE 1** **A select list of inhibitory peptides against Aβ peptide aggregation** (Continued)

| No | Name/sequence of inhibitor peptide | Configuration | Type | Therapeutic findings | Ref. |
|---|---|---|---|---|---|
| 10 | Diazirine-equipped cyclo-KLVF(b-Ph)F | cyclic | CHC-derived sequence | • Inhibit Aβ aggregation<br>• Prevent $A\beta_{42}$ induced toxicity | (37) |
| 11 | Attached flavin to an Aβ-binding peptide | cyclic | CHC-derived sequence | • Inhibition of Aβ aggregation<br>• Decrease of the aggregation potency and Aβ induced neurotoxicity | (38) |
| 12 | ZAb3 affibody | L-enantiomer | Selected by phage display technique | • Inhibit Aβ aggregation<br>• Disassemble preformed oligomers | (39) |
| 13 | H102 (HKQLPFFEED) | L-enantiomer | CHC-derived sequence | • Inhibit $A\beta_{42}$ fibrillization | (40) |
| 14 | Th-NT, Th-CT, and Th-SC | Trehalose conjugated peptides, L-enantiomer | CHC-derived sequence | • Inhibit fibril formation | (41) |
| 15 | Fc-KLVFF | Ferrocene- conjugated peptide, L-enantiomer | CHC-derived sequence | • Inhibit Aβ aggregation | (42) |
| 16 | N-methylated Aβ(32–37) | L-enantiomer | C-terminal fragment | • Inhibits Aβ aggregation<br>• Improve Drosophila longevity<br>• Increase locomotion | (43) |
| 17 | D-Trp-Aib | D-enantiomer | Rational design | • Inhibits Aβ oligomerization<br>• Decrease cerebral amyloid deposits in transgenic model<br>• Improves cognitive activity (*in vivo*) | (44) |
| 18 | GABA-FPLIAIMA | D-enantiomer | C-terminal fragment | • Inhibit and reduce the Aβ aggregation | (22) |
| 19 | Tyr(Allyl-RCM)-Xaa-Gly(Allyl-RCM) and 'Gly(Allyl-RCM)-Xaa-Tyr(Allyl-RCM) | cyclic | CHC-derived sequence | • Inhibit significantly the $A\beta_{42}$ aggregation | (45) |
| 20 | D(KLVFW)- aminobutyric acid (Aib) | D-enantiomer | CHC-derived sequence | • High specific interaction with $A\beta_{42}$ monomers and oligomers<br>• Remarkable inhibition of A $A\beta_{42}$ fibril formation<br>• No cytotoxic effects | (46) |

affinity for Aβ compared with their L-enantiomeric counterparts. Moreover, D-peptides inhibit Aβ aggregation in animal models (28). Retro-inverso peptides are a special class of modified peptides that contain D-amino acids and reversed NH and CO groups in the peptide bonds. These peptides could keep the same spatial position in the side chain of the residues and preserve the desirable 3D structure compared to unchanged L-peptides (47). They also displayed advantages in terms of Aβ aggregation inhibition, higher proteolytic stability, lower self-assembly, and better blood-brain-barrier (BBB) permeability when compared with L-peptides in an animal model (48, 49). Fluorinated hydrophobic valine or phenylalanine in the LVFFA-based peptides can considerably delay the formation of Aβ aggregation. Fluorinated amino acids can also inhibit Aβ aggregation (50). Modification of amide functional groups with a methyl group is another strategy in the development of new inhibitors. N-methylated amide groups could enhance the peptide's solubility in aqueous solutions and decrease Aβ-induced toxicity. Cyclic peptides have a higher inhibitory activity than acyclic derivatives (51). Because of their high enzymatic resistance, they are degraded slowly. Residues of lysine and glutamic acid have been known to be effective stabilizing and enhancing agents of Aβ fibrillation due to their ability to improve surface tension. In contrast, arginine residues have been reported as aggregation inhibitors or destabilizers (chaotropes) (52).

The $^{16}$KLVFF$^{20}$-based peptides play a crucial role in disrupting Aβ aggregation by binding to full-length Aβ peptides and preventing fibril formation (53, 54). Ac-LVFFARK-NH2 (LK7), designed by adding arginine and lysine to KLVFF, induced a dose-dependent inhibition on $Aβ_{42}$ fibrillation; however, it was cytotoxic due to high self-assembling properties (55). When conjugated with poly (lactic-co-glycolic acid) nanoparticles (NPs), the LK7-PLGA-NPs complex resulted in the elimination of the LK7 self-assembly feature while inhibiting $Aβ_{42}$ fibrillation (55). Binding β-cyclodextrin to LK7 (56) improved LK7 peptide solubility, inhibited its tendency to self-aggregate, improved its binding to Aβ, and inhibited Aβ aggregation. Head-to-tail cyclization of LK7 peptide also resulted in a decrease in self-assembly of the LK7, an increase in binding affinity to the $Aβ_{40}$ peptide, and proteolytic stability in serum. This derivative also can stabilize the $Aβ_{40}$ secondary structure and inhibit $Aβ_{40}$ –mediated cytotoxicity. Another derivative of LK7 peptide is Ac-LVFFARKHH-NH2 (LK7-HH), in which LK7 has been conjugated to the HH ligand as a chelator for reducing reactive oxygen species (ROS) production and capturing free and complexed ions of $Cu^{2+}$ (57). This chelator also improved the anti-aggregate effects of LK7 on Aβ peptide and reduced its self-aggregation properties.

Proline and aspartic acid were exchanged for valine and alanine, respectively, in KVLFFA (58, 59). The derived peptide, referred to as 5-mer iAβ5 with sequence LPFFD, inhibited Aβ aggregation, neurotoxicity, and reduced plaque load (58). Due to the lack of a proton on the secondary substituted nitrogen in the peptide bond of proline residue, it could inhibit the formation of the intramolecular hydrogen bonds into fibrils. Since these small peptides are prone to faster enzymatic degradation and have reduced BBB permeability *in vivo*, iAβ5p was modified by N-methylation between Pro and Phe residues to improve its stability (60). The results from *in vitro* and *in vivo* studies showed that it has the same inhibitory activity as the parental iAβ5 peptide against amyloid fibril formation and neurotoxicity but with improved protease resistance. Also, molecular dynamics

simulations show that this peptide has more durable binding and enhanced activity against $A\beta_{40}$ aggregation in comparison to the iAβ5 peptide. In a similar study, the RIVFF sequence was produced by residue mutations of lysine16 (K) to arginine (R) and leucine17 (L) to isoleucine (I) on the KLVFF segment (61). The results indicated that this peptide could self-aggregate into β-sheet structures by reducing the surface tension of water and at higher concentrations (>250 μM) enhanced the Aβ-induced cytotoxicity.

The peptide D-GRKKRRQRRR-GGGG-DVEFRH ($A\beta_{1-6}$ A2V-TAT) was investigated *in vivo* (62, 63). It was generated by modifying the N-terminal fragment of $^{1}$DAEFRH$^{6}$ through mutation of alanine in position 2 to valine and conjugating with the HIV protein transduction domain GRKKRRQRRR (TAT). The resulting peptide showed strong anti-amyloidogenic effects *in vitro* and Aβ aggregation inhibition in mouse models of Alzheimer's disease (64). The KLVFWAK motif was designed based on the $^{16}$KLVFFAE$^{22}$ sequence with mutations introduced at phenylalanine and glutamic acid residues to tryptophan and lysine respectively to enhance solubility and disrupt self-assembly via electrostatic repulsion. Results showed that the designed motif could only target the C-terminal region of Aβ oligomers. The designed motif exhibited a lower self-aggregation tendency in comparison to other KLVFF-related sequences. Moreover, it demonstrated a higher binding affinity to Aβ aggregates and fibrils than monomers (65).

RGKLVFFGR (OR1) and RGKLVFFGR-NH (OR2) are retro-inverso peptides (66), designed by the addition of arginine (R) and glycine (G) to the KLVFF sequence. They exhibit high solubility and stability against enzymes. However, only the OR2 peptide showed inhibitory effects on Aβ oligomer formation and cytotoxicity. OR2 was modified to HN-rGklvffGr-Ac (RI-OR2) by acetylation of the C-terminal residue (49). The result illustrated that the peptide has a high resistance to proteolysis, while maintaining the same inhibitory activity *in vivo*. In a follow-up study, the RI-OR2 peptide was attached to the TAT peptide to improve its permeability into cells and the BBB (48). The results showed the peptide was able to decrease Aβ aggregation, plaque levels, and oxidative damages as well as increase the number of young neurons in the brain.

$^{31}$IIGLMVGGVVIA$^{42}$ and $^{39}$VVIA$^{42}$ sequences were designed based on the C-terminal domain of $A\beta_{42}$ (67). The $^{39}$VVIA$^{42}$ sequences could interact with $A\beta_{42}$ monomers and smaller oligomers at several sites, specifically at the N-terminal domain. At micromolar concentrations, the VVIA-$NH_2$ peptide inhibited $A\beta_{42}$ aggregation, exhibited less toxicity, and protected synaptic activity. However, these effects were not observed for the acetylated Ac-VVIA sequence (68). The non-acetylated VVIA-$NH_2$ sequence particularly interacts with the C-terminal domain while the Ac-VVIA peptide has a dispersed binding distribution (68). The Ac-$^{32}$IGLMVG$^{37}$-$NH_2$ sequence, a hexapeptide from the C-terminal fragment, has been shown to have a moderate efficacy with less toxicity (69).

O-acyl isopeptide and NMe-b-Ala26 (70) were derived from the full-length Aβ sequence with modification of an ester bond at the Gly25-Ser26 moiety and an N-methyl amide-β-Ala26, respectively. O-acyl isopeptide inhibited $A\beta_{42}$ fibrillation at equimolar concentrations through an inhibitory mechanism distinct from any other peptidic inhibitors reported previously. Also, this derivative was more soluble than $A\beta_{42}$ peptides and rapidly decomposed to $A\beta_{42}$ monomers under physiological conditions through an O-to-N acyl rearrangement reaction whereas NMe-b-Ala26 showed higher chemical stability at physiological conditions.

### Non-Aβ-based peptide inhibitors

Carnosine, a natural imidazole-containing dipeptide is a metal ion chelator (71). It inhibits the fibrillation and toxicity of amyloidogenic species such as glycated α-Crystallin, Aβ peptide, and prions. This peptide also inhibits the intramolecular salt bridge formation, which is vital to the stability and elongation of fibrils (71). Peptide D1, QSHYRHISPAQV (72), is another non-Aβ peptide that reduces Aβ aggregation and Aβ-associated cytotoxicity at high concentrations. N-methylated proprietary peptides such as D-$NH_2$ (SEN304) and SEN1576 can inhibit Aβ-associated toxicity *in vivo* (73). Furthermore, SEN304 is a more potent inhibitor than customized versions of the KLVFF peptide. These peptides could interfere with the nucleation of Aβ, convert them into non-toxic forms, and eliminate toxic oligomers.

## PEPTIDE LIBRARY SCREENING

There are many screening approaches to identify target-specific ligands (74, 75). Phage display is one such efficient high-throughput screening method that allows the screening of a wide variety of peptide libraries to identify specific peptide sequences against the desired target (76, 77). Wang et al. synthesized a linear peptide with sequence PYRWQLWWHNWS selected based on the screening of a randomized 12-mer peptide library against the target $Aβ_{1–10}$ sequence (78). After screening, specific phages were selected and their binding affinity to $Aβ_{1–10}$ was evaluated by real-time biomolecular interaction analysis. This peptide could specifically bind to $Aβ_{1–10}$, inhibit the aggregation of Aβ into plaques, and reduce $Aβ_{1–42}$ induced-apoptosis. Furthermore, it illustrated a protective effect against $Aβ_{1–42}$-induced memory and learning impairments in animal models (59).

Larbanoix et al. utilized the phage display method to discover a linear hexapeptide against $Aβ_{1–42}$ aggregation (79). Two of the selected clones, Pep1: LIAIMA and Pep2: IFALMG, corresponding to fragment $^{31}$IIGLMV$^{36}$ from $Aβ_{1–42}$ peptide, demonstrated the highest binding affinities to $Aβ_{1–42}$ with Kd values in the micromolar range. Their specific interactions with $Aβ_{1–42}$ plaques were identified by immunohistochemistry on harvested brain tissue from an animal model of Alzheimer's disease. The peptides did not induce any toxicity in neurons *in vitro*. Moreover, the thioflavin T aggregation assay indicated that the designed peptides could suppress the amyloid fibril formation.

In 2010, a random heptapeptide library (XX–P–XXXX) on T7 phage was reported by Kawasaki et al. (80). The library was designed based on the LPFFD sequence XX-P-XXXX, where P is proline, and X is any amino acid. After the fifth-round of biopanning against $Aβ_{1–42}$ soluble oligomers, eight new peptides containing arginine residues were obtained. The peptide with the strongest affinity to Aβ (RGPRGRV) suppressed the formation of 37–48 kDa oligomers and maintained the monomeric form of $Aβ_{1–42}$ for up to 24 h. In follow-up studies, to assess the effect of the peptide length on the inhibition of soluble oligomers formation, random libraries containing 3-residue and 4-residue peptides were prepared by phage display and evaluated. The results demonstrated that the 3-residue peptides could not significantly inhibit oligomers formation because of their

smaller size. In contrast, the 4-residue peptide with the RFRK sequence inhibited the soluble oligomer formation like the heptapeptide (RGPRGRV). It also showed a slight decrease on Aβ fibrillation (81), similar to the inhibitory activity of the N-Methylated Peptide (SEN304), against $A\beta_{42}$ aggregation (25, 82, 83). Tsuji-Ueno et al. utilized the all-steps-all-combinations (ASAC) method to explore $A\beta_{42}$-binding peptide aptamers. The identified peptides from the primary and secondary libraries showed a weak binding affinity to $A\beta_{42}$ (Kd values in the μ m range) (84). To further improve the peptide aptamers, Gautam et al. applied the mRNA display technique and paired-peptide library method. The library was assembled by a random shuffling method on selected peptide blocks taken from the formed primary and secondary peptide libraries by Tsuji-Ueno et al. (84). They reported two peptides with high binding affinity to $A\beta_{42}$ (Kd in the nM range) which significantly inhibited the $A\beta_{42}$ aggregation (85). The improved peptide aptamers, P84 (CGILDPIPWGGSGGSCGILDPIPW) and P131 (GCPCIGIIGGSGGSDCSSDLTPS), where GGSGGS is the linker sequence, demonstrated a higher binding affinity for the $A\beta_{42}$ peptide (Kd values in the nanomolar range) compared to the primary and secondary $A\beta_{42}$-binding peptides (86). The results showed that both peptides could inhibit the $A\beta_{42}$ aggregation and result in the reduction of the cytotoxic effects of $A\beta_{42}$ fibrils and $A\beta_{42}$ oligomers in PC12 cells; P84 showed better efficacy than P131 on the cell line.

Groen and co-workers employed mirror-image phage display to identify selective and high-affinity D-peptide ligands for $A\beta_{1–42}$. The D-enantiomer $A\beta_{1–42}$ was used as a target for selection from a randomized 12- amino acid peptide library with more than 1 billion different peptides. After six rounds of biopanning, they identified a specific D-enantiomeric peptide, RPRTRLHTHRNR, called D3 (73). The D3 ligand inhibited Aβ aggregation, and dissolved pre-formed Aβ fibrils. Additionally, D3 ligand could disaggregate pre-existing amyloid plaques in the brain and result in an increase in the amount of Aβ monomeric form, which has high clearance from the brain (87). FITC fluorescence data demonstrated that Aβ–D3 clearance might have been associated with pericytes, which have a major role in the clearance of different $A\beta_{40/42}$ species (88, 89). Glial fibrillary acidic protein (GFAP) staining of astrocytes and CD11b staining of microglia in brain sections revealed that the D3 significantly decreased the amount of plaque-related inflammation markers (active astrocytes and microglia) around the Aβ plaques in comparison to the untreated animals. In addition to the anti-inflammatory properties, this peptide ligand could drastically reduce the Aβ plaque load in brain tissue of transgenic APP-PSD mice after a 30-day treatment with administration of 9 mg D3 per day per mouse. Computational simulation studies demonstrated strong electrostatic interactions between the arginine-rich D3 and negatively charged groups of Aβ nonamer; D3 binding to Aβ nonamer could change the topology of the Aβ oligomers by inducing a twist in them and consequently promote the formation of Aβ nonfibrillar aggregations (73, 90, 91).

Luo et al. applied peptoid chemistry, N-substituted glycine oligomers as a class of peptidomimetics, to develop and improve selective high-affinity ligands for $A\beta_{42}$ (92). They constructed an on-bead peptoid library of 38,416 unique peptoids. After screening for $A\beta_{42}$-selective peptoid ligands, the IAM1 ligand and its dimeric form were selected and further evaluated. IAM1 peptide showed about 10-fold more affinity for $A\beta_{42}$-binding than for $A\beta_{40}$, and inhibited $A\beta_{42}$ aggregation *in vitro*. The dimeric derivative $(IAM1)_2$ demonstrated a 7.4-fold higher

affinity for $A\beta_{42}$ (60 nM) than the monomeric form. Moreover, $(IAM1)_2$ demonstrated neuroprotective effects on primary hippocampal neurons against $A\beta_{42}$-induced toxicity.

Due to the considerable similarities between the self-assembly of cyclic d,l-α-peptides and amyloid structures, it is possible such peptides can bind to Aβ nontoxic forms and stabilize them (29). Richman et al. described the cyclic peptide CP-2, cyclo-[l–J–w– H–s–K]s (J denotes l-norleucine), by screening a 6-residue library of head-to-tail cyclic d,l-α-peptides consisting of residues Lys, Glu, Ser, Leu, Trp, and His using a one-bead-one-peptide combinatorial approach (29, 93). The selected peptide strongly interacted with $A\beta_{40}/A\beta_{42}$ and prevented their assembly, entirely disassembled $A\beta_{40}$ fibrils, and protected PC12 cells against $A\beta_{40}/A\beta_{42}$-induced toxicity, without having any toxic effects of its own. NMR spectroscopy revealed that the CP-2 peptide, in a self-assembled form, interacted with monomeric and low-oligomeric structures of $A\beta_{40}$ and induced weak α-helix structures during the initial stage of $A\beta_{40}$ aggregation and subsequently promoted the conformational transition shift from a more toxic antiparallel β-sheet conformation to the less toxic parallel β-sheet.

In another study, Acerra et al. utilized an intracellular protein-fragment complementation assay (PCA) methodology for the screening of selective high-affinity peptides to Aβ (94). The $A\beta_{25–35}$ sequence, known to self-assemble into toxic fibrils (95), was inserted into one half of the murine dihydrofolate reductase enzyme as a target, and the Aβ29–35 sequence-based peptide was inserted on the other half of the enzyme (96). After the screening of primary and secondary libraries, two new targeting peptides L2P1, FSKATSN, and L2P2, PVKATTA were selected. These peptides shared no homology with the starting template $A\beta_{29–35}$. The results showed that all selected peptides could bind $A\beta_{42}$, inhibit fibril formation, and disaggregate pre-formed fibrils. To further improve the metabolic stability of selected peptides from primary and secondary libraries, their retro-inverso (RI) analogs were evaluated (86). All RI peptide ligands, such as KAR-R1, L2P1a-RI, L2P1b-RI, L2P2a-RI, and L2P2b-RI, inhibited Aβ fibrillation and disaggregated pre-formed fibrils, and reduced $A\beta_{42}$-induced toxicity in PC-12 cells.

## THE CURRENT STATE AND FUTURE DIRECTIONS OF Aβ INHIBITORY PEPTIDES IN ALZHEIMER'S DISEASE

A wide range of peptide-based inhibitors has been evaluated in cellular and animal models as new therapeutic compounds for inhibition of Aβ aggregation. While experimental studies generated promising results, only a few of these inhibitory peptides have been successful enough to enter clinical trials. NAP or Davunetide peptide with NAPVSIPQ sequence, derived from the activity-dependent neuroprotective protein (ADNP), was reported in 2003 by Gozes et al. (97). NAP was able to inhibit Aβ aggregation, disassemble pre-formed fibrils, and protect the neuronal cells from Aβ induced toxicity. Though NAP demonstrated benefits in phase II clinical trials for mild cognitive impairment, it failed in a phase III trial (98–100). PPI-1019 peptide (APAN), with a sequence of D-(H-[(Me-L)-VFFL]$NH_2$), is an N-methylated peptide which is derived from the D-enantiomeric

Cholyl-LVFFA-$NH_2$ that could inhibit Aβ aggregation and the induce toxicity in experimental studies (101). The phases I and II clinical trials of APAN was completed in patients with mild-moderate Alzheimer's disease in 2005 (NCT00100282, NCT00100334), but the outcome of this study is still unknown (https://clinicaltrials.gov/, last assessed 28 October 2020). Other reported inhibitory peptides including, D3 (102), D-Trp-Aib–OH (44), D-4F (36), TAT-R1-OR2 (48), NL-RI-OR2-TAT90 (103), and R1-OR2 (49) have shown considerable efficacy in preclinical trials, but they have not yet entered clinical trials.

The reality is that, to date, there is no cure for Alzheimer's disease. Only optimism remains. Therefore, it is necessary to discover potential peptides for testing in clinical trials. The current inhibitory peptides have certain limitations such as poor BBB permeability and high cytotoxicity. To overcome these problems and further improve the inhibitory activity, a number of studies have focused on peptide–nanostructure conjugates (PNCs) approach that provides an opportunity to increase the capabilities of both these classes of materials (55, 104, 105). Nanostructures could be considered as a potential vehicle to overcome poor BBB permeability and bring hope for neurodegenerative diseases therapy due to their size and various surface modifications (106). As an example, multivalent inhibitors can be developed against Aβ aggregation by decorating gold nanoparticles with VVIA and LPFFD (107). The PNCs approach gives a fascinating insight into the fields of diagnosis and treatment, and provides new opportunities for the design of high-performance peptides (108, 109).

## CONCLUSION

Despite a better understanding of the pathogenic mechanisms of Alzheimer's disease, finding efficient therapeutic compounds to prevent or halt the progression of Alzheimer's disease continues to be a challenge. Aβ aggregation inhibition-based approaches are being developed with the aim to stop disease progression. While the reported inhibitory peptides have considerable advantages over other compounds, and experimental evidence has been encouraging, bench-to-bedside has not yet become a reality. Therefore, adequate knowledge of binding interactions of these peptides with their biological targets, the ligand-target complex, is required to design more accurate therapeutic biomolecules. Peptide inhibitors have unique properties, particularly, high selectivity, low accumulation in tissues, low side-effects and toxicity, and different chemical and biological synthesis routes when compared with other compounds. As researchers continue to focus on rational design, characterization, optimization, and interaction between the inhibitor and the Aβ peptide complex, more peptide inhibitors are expected to succeed in clinical trials.

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# Tau Protein-Targeted Therapies in Alzheimer's Disease: Current State and Future Perspectives

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**Abstract:** Drugs available on the market for the treatment of Alzheimer's disease show only low symptomatic efficacy and phase 3 clinical trials against amyloid have been negative over the past 20 years. As dysfunctional tau protein is more closely correlated with dementia than amyloid, targeting tau protein may be more effective in improving cognitive function in cases of Alzheimer's disease. It should be emphasized that the development of tau protein therapy is in many ways more complicated than the development of anti-amyloid therapy. Several antibodies to the tau protein and two vaccines are currently undergoing clinical trials. Relatively speaking, tau protein therapy for Alzheimer's disease is still in its infancy. The purpose of this chapter is to draw the readers' attention to the various uncertainties and barriers to the success of tau protein therapy in treating Alzheimer's disease, and to show how future research and clinical trials can avoid previous limitations or mistakes.

**Keywords:** Alzheimer's disease; immunotherapy; neurofibrillary tangles; tau protein; vaccine for Alzheimer's disease

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## INTRODUCTION

Alzheimer's disease is an age-related disorder characterized clinically by gradual memory loss and cognitive impairment. The disease affects more than 47 million people worldwide, and this number is expected to reach over 131 million by 2050. Alzheimer's disease is considered the most common cause of dementia (1, 2), covering about 60–80% of dementia cases worldwide (3). Available data indicate that the incidence of sporadic Alzheimer's disease is common and reaches 10–50% persons over the age of 65 (4). Factors that may be involved in the development of the disease include lifestyle habits such as diet, exercise, education, cognition and aging, immunosenescence, chronic infections and inflammation, latent infections, vascular factors, sleep problems and more (5–9). It has been suggested that intestinal microorganisms and ischemic episodes may also be involved in the development of this disease (10–13). Heritability of this form of dementia is high and estimated at 70–79% based on twin-studies. However, most evidence points to a heterogeneous etiology, with the disease resulting from a combination of many genetic, environmental, vascular and other currently unknown factors (5, 6, 10, 14–16). One particular genetic factor, the epsilon allele in the apolipoprotein E gene, has been identified as being associated with an increased risk of sporadic Alzheimer's disease, but a large percentage of the genetic risk remains unidentified. Alzheimer's disease is the leading cause of acquired disability in the world, affecting 1 in 2 in women and 1 in 3 in men (17). Alzheimer's disease is described as one of the unsolved problems of modern medicine (18), one which has a significant impact on the global economy, society and the families of the sick (19). In addition to significant personal costs, the total estimated worldwide financial burden due to dementia in 2010 was USD 604 billion (19). This is a serious public health problem that can grow to epidemic proportions over the next few decades if the disease cannot be prevented or slowed down (18).

Neuropathologically, Alzheimer's disease is characterized by the accumulation of amyloid in the form of plaques in the extracellular space and tau protein dysfunction in the form of neurofibrillary tangles present in the intracellular space, which are important in the final *post-mortem* diagnosis. The most neurotoxic forms of amyloid and tau protein are believed to be oligomeric forms that spread extracellularly as soluble oligomers through a prion-like mechanism (20, 21). The causes or mechanisms of amyloid plaques and neurofibrillary tangles formation are not yet well understood, but they are generally considered to be the result of a process of misfolding of proteins that leads to the development of pathological phenomena. Alzheimer's disease develops due to a combination of different neuropathological processes in the brain that cause massive neuronal death and loss of synapses. The resulting atrophy causes patients' brains to weigh about a third less than age-matched non-demented people (3). Today we know that the onset of Alzheimer's disease begins between 15 (in familial cases) and 20–30 years (in sporadic cases) before any clinical symptoms appear (2, 8, 22). By the time the clinical phenotype is recognized, significant neuronal and synaptic degeneration and massive neuroinflammatory changes have already occurred. Though the cause of Alzheimer's disease is not completely certain, it has been proven by

diagnostic methods based on the analysis of the patient's brain images that the accumulation of amyloid in the brain precedes the appearance of clinical symptoms and indicates a number of pathological factors that are ultimately not defined.

The amyloid hypothesis of Alzheimer's disease suggests that increased amyloid aggregation causes Alzheimer's disease by triggering toxic events leading to progressive neurodegeneration. However, no drug candidate targeting amyloid has yet led to effective treatment (3, 23). It is currently speculated that treatment requires early targeting of amyloid when the changes remain reversible, and clinical trials should focus on assessing amyloid compounds in pro-dromal Alzheimer's disease. There is no prophylactic or causal therapy for the disease, and the lack of knowledge about etiology and when or why the disease really begins, significantly complicates the work of physicians (24). Currently available treatments for Alzheimer's disease are only aimed at mitigating clinical symptoms and delaying cognitive decline. The development of a therapy for Alzheimer's disease has resulted in only a few approved drugs that provide temporary symptomatic relief in some patients. None of these clinically used drugs stop or slow the progression of the disease. Currently, the only drugs that have an impact, albeit modest and transient, on the main symptoms in patients with mild to moderate dementia of Alzheimer's disease are acetylcholinesterase inhibitors and N-methyl-D-aspartate (NMDA) receptor antagonists. For therapeutic measures to have a significant effect on the delay or actual prevention of Alzheimer's disease, it is likely that patients will need to be diagnosed at the stage of preclinical Alzheimer's disease (i.e., presence of Alzheimer's disease neuropathology, but without clear clinical symptoms) or during early signs of Alzheimer's disease that could be treated with disease modifying agents. Treatments that target the etiological mechanisms of Alzheimer's disease are urgently needed.

That treatments targeting amyloid plaques have proven ineffective draws attention towards another pathologic hallmark of Alzheimer's disease, neurofibrillary tangles arising from hyperphosphorylation of the tau protein. Experimental evidence indicates that the symptoms of Alzheimer's disease appear in the presence of both accumulating amyloid and dysfunctional tau protein (3), and research has shown a strong correlation between the accumulation of neurofibrillary tangles and the deterioration of cognitive functions (3, 25, 26). Thus, it is believed that tau protein dysfunction cannot be ignored as an etiological factor of Alzheimer's disease. Compounds that prevent tau protein hyperphosphorylation may therefore affect disease progression; however, the failure of previous trials to treat tauopathy in progressive supranuclear palsy (26) gives a strong warning of a possible failure. Nonetheless, the importance of tau protein as a potential independent cause of Alzheimer's disease, and therefore a potential target for treatment, serves as the basis for ongoing clinical trials against the protein. Despite the lack of an in-depth understanding of the role of tau protein in the pathology of Alzheimer's disease, efforts are underway to develop new therapies targeting tau protein in Alzheimer's disease. This chapter presents some of the proposed therapeutic compounds in preclinical and clinical studies that may affect the development of the next generation of anti-Alzheimer's disease drugs.

## SUBSTANCES PREVENTING TAU PROTEIN POST-TRANSLATIONAL CHANGES

The strong correlation between tau protein phosphorylation and its influence on the development of pathological processes gave rise to the search for tau protein kinases inhibitors as potentially effective therapeutic agents in Alzheimer's disease. The most advanced strategy for inhibiting protein kinase in the clinic is currently directed at glycogen synthase kinase-3 (GSK-3) (27). To this end, pilot studies were performed to determine the clinical effects of lithium in patients with Alzheimer's disease; however, the results of this study were inconsistent, perhaps due to the small number of patients, low susceptibility and narrow therapeutic range of lithium (28).

Tideglusib (NP031112, NP-12) is a GSK-3 inhibitor that, in preclinical studies, reduced neuronal loss, gliosis and tau protein phosphorylation, and improved spatial memory deficit in transgenic mice (29). Further investigation with human trials included a pilot study conducted on 30 patients affected with Alzheimer's disease who were treated for 5 months with Tideglusib in a placebo-controlled phase IIa increasing dose clinical trial (NCT00948259). The results showed overall positive, but not significant, trends in their cognitive health, though the study affirmed the safety of the drug (30). Another 6-month phase IIb study on 308 patients affected with Alzheimer's disease was conducted at 55 centers in Europe (NCT01350362). Tideglusib proved to be safe in the study, but those treated with the drug did not show significant clinical benefit (31).

Another protein kinase that is increasingly being considered as a potential therapeutic target is Fyn tyrosine kinase, which phosphorylates tau protein at the N-terminal domain, and also plays a role in the amyloid signaling pathway (32). Saracatinib (AZD0530) is a Fyn inhibitor that improves memory deficiencies in transgenic mice and is considered safe and well tolerated based on a phase I clinical trial (NCT01864655) (33). A multicenter phase IIa study in 159 Alzheimer's disease patients treated with Saracatinib is still ongoing (NCT02167256).

The tau protein is also modified post-translation by lysine acetylation, which leads to impaired protein activity and triggers pathological aggregation. This suggests that acetyltransferase inhibitors may be a potential therapeutic strategy for Alzheimer's disease (34). A phase 1 clinical trial of salsalate has recently started to assess its safety and tolerability in patients with Alzheimer's disease (NCT03277573). Patients will be randomly assigned to receive salsalate or placebo twice a day for 1 year. At present, the results of this study have not been published.

Phase I studies on substance AZP2006 are coming to an end for Alzheimer's disease cases in France, but no detailed results are available as yet. The oral substance has been proposed to block phosphorylation of the tau protein thereby preventing the tau protein from folding incorrectly. In addition, it appears to stimulate macrophages, inducing the removal and elimination of an incorrectly folded tau protein.

Nilotinib is a c-Abl tyrosine kinase inhibitor, and the rationale for the use of nilotinib in cases of Alzheimer's disease is based on the clearance of tau protein and amyloid accumulated in the brain in neurodegenerative processes. Although the exact molecular mechanism is uncertain, nilotinib appears to cross the

blood–brain barrier and trigger autophagy in neurons to remove both tau protein and amyloid. The properties of nilotinib mentioned formed the basis of a randomized, double-blind, placebo-controlled, phase II trial to assess the effect of nilotinib on safety and clinical outcomes in patients diagnosed with Alzheimer's disease (NCT02947893).

## SUBSTANCES THAT PREVENT MICROTUBULE DESTABILIZATION BY AMYLOID

The observation that amyloid oligomers destabilize microtubules and interfere with rapid axonal transport by activating calcineurin in tau protein-deficient mice has led to the conclusion that microtubule destabilization may be a key process during neurodegeneration (35). Epothilone D (BMS-241027), a small molecule stabilizer of microtubules that can pass through the blood–brain barrier, was able to increase the density of microtubules in axons, and improved cognitive function in a mouse transgenic tauopathy model; only insignificant changes in tau protein pathology were noted in the study (36). The substance was also tested in a double-blind, randomized, placebo-controlled, multicenter phase I clinical trial (NCT01492374) intended to assess its safety and tolerability in patients with Alzheimer's disease, but no results have been published and use of the drug in Alzheimer's disease has been suspended.

Recently, a small molecule called abeotaxane (TPI-287) was tested in a phase I study to assess safety and tolerability in patients with Alzheimer's disease (NCT02133846). Abeotaxane was administered intravenously for 9 weeks once per 3 weeks, with the option of extending the open label to 3 months. Ultimately, treatment was not well tolerated by people with Alzheimer's disease, and exploratory cognitive endpoints showed no significant improvement.

## SUBSTANCES THAT PREVENT TAU PROTEIN AGGREGATION

Methylthioninium chloride easily crosses the blood–brain barrier and prevents tau protein aggregation *in vitro*, as well as in cells and animals models (37). A double-blind clinical trial in which single-site, 6-month methylthioninium monotherapy was conducted on patients with Alzheimer's disease (NCT00515333) showed signs of benefit in moderate cases of the disease (38). Leuco-methylthioninium bis, a stable, reduced form of the methylthioninium moiety, acts as a selective inhibitor of tau protein aggregation both *in vitro* and in transgenic mouse models. The primary analysis using leuco-methylthioninium bis derivative of methylthioninium chloride in the treatment of Alzheimer's disease was negative and the results did not suggest therapeutic benefits for Alzheimer's disease (39). Recently, a second phase-III study in patients with Alzheimer's disease treated orally, twice daily, (NCT01689233) showed no effect on primary endpoints (40). The authors' explanations of the effectiveness after the secondary analysis of the post-hoc subgroup raised many doubts, mainly regarding the methodology used and the interpretation of the results. Although these studies

did not produce positive results, they are nevertheless an important step in the development of anti-tau protein drugs.

Since positron emission tomography (PET) tau protein imaging is currently used in conjunction with amyloid PET imaging in an increasing number of Alzheimer's disease clinical trials, our knowledge of the ideal stage of the disease for testing anti-amyloid, anti-tau protein or a combination of both will undoubtedly be improved. There is growing optimism that we have the right tools to evaluate compounds that can stop and even prevent Alzheimer's disease.

## IMMUNOTHERAPY AGAINST TAU PROTEIN

The first mention of data on possible immunotherapy in Alzheimer's disease appeared during research into the possibility that human β-amyloid peptide 1–42 may be able to cross the blood–brain barrier (41, 42). Despite the disappointing data from several advanced clinical studies on anti-amyloid immunotherapy in the treatment of Alzheimer's disease (23), immunotherapy in neurodegenerative diseases is very actively sought as a promising approach for the removal of pathological proteins, particularly in Alzheimer's disease. Recently tested anti-tau protein immunotherapy strategies in animal models have shown that immunotherapy may be clinically viable to remove toxic protein species in tauopathies such as Alzheimer's disease (43). Anti-tau protein immunotherapy strategies involve the removal of pathological species of tau protein with antibodies, which may ultimately improve neuronal function (26, 43). Thus, choosing the right epitope is crucial for obtaining effective immunotherapy (26). Because hyperphosphorylation is thought to be the cause of tau protein aggregation and the development of neurofibrillary tangles, many phospho-epitopes have been tested in animal models with final positive effects (13, 26, 27, 29, 32, 33, 35–37, 44). It should be noted that the tau protein undergoes modifications other than phosphorylation during the transformation from soluble protein to insoluble aggregates and deposits (13, 27, 44). As mentioned above, the cascade of events leading to the development of neurofibrillary tangles may include post-translational modifications such as phosphorylation, glycosylation, truncation and ubiquitination (13, 27, 29, 34, 44). This gave rise to a series of active and passive immunotherapy programs in the treatment of patients associated with Alzheimer's disease (26). Some of the clinical studies of tau protein-targeted immunotherapy described below target different protein domains rather than specific phospho-epitopes (26).

At present, studies of anti-tau protein immunotherapies in clinical trials are in their early stages. AADvac-1 is an active immunotherapy (vaccine) based on a synthetic tau protein peptide containing residues 294–305 derived from a fragment of a misfolded tau protein. Phase I clinical trials of the therapy have been recently completed (NCT01850238). In this first-ever, randomized, double-blind, placebo-controlled study, 30 Alzheimer's disease patients aged 50–85 received subcutaneous injections of AADvac-1 for 3 months; generally mild, if any side effects were reported, and high tau protein titers indicated effective immunogenicity (45). The study was then extended to patients who completed the AADvac-1 phase 1 study to administer additional immunization doses that they received for the next 18 months (NCT02031198) (46). The recruitment of 185 Alzheimer's disease patients in a 24-month, randomized, placebo-controlled, double-blind, parallel

**TABLE 1 Clinical trials of tau protein-targeted immunotherapies (21, 26, 45–55)**

| Compound | Isotype | Therapy type | Patients | Trial phase |
|---|---|---|---|---|
| AADvac1 | IgG1 | Active | Alzheimer's disease | 1/2 |
| ACI-35 | n.a. | Active | Alzheimer's disease | 1 |
| BIB076 | IgG1 | Passive | Alzheimer's disease | 1 |
| BIB092 | IgG4 | Passive | Alzheimer's disease | 2 |
| LY3303560 | n.a. | Passive | Alzheimer's disease | 2 |
| RO7105705 | IgG4 | Passive | Alzheimer's disease | 2 |
| JNJ-63733657 | n.a. | Passive | Alzheimer's disease | 2 |
| LuAF87908 | n.a. | Passive | Alzheimer's disease | 1 |
| ABBV-8E12 | IgG4 | Passive | Alzheimer's disease | 2 |
| UCB0107 | IgG4 | Passive | Alzheimer's disease | 2 |
| IVIg | pIgG | Passive | Alzheimer's disease | 2/3 |

n.a, not available.

group study, multicenter clinical safety and phase II efficacy study for AADvac-1 began in 2016 with an option to end by 2019 (NCT02579252) (Table 1).

So far only limited data from clinical trials and only for the AADvac1 vaccine have been presented (45, 46). Administration of the AADvac1 vaccine induced an IgG response against tau protein in 29 of 30 patients and a response to the shortened form of tau protein (151-391/4R) was noted in 25 of 28 patients (45). Although the phase 1 trial was not designed as an efficacy study, inter-individual differences in AADvac1-induced antibody titers enabled an assessment of the relationship between antibody response potency and disease progression. Patients with higher antibody titers were characterized by slower cognitive performance and lower hippocampal atrophy (46). Adverse reactions were generally mild, with the most common being local injection-site reactions (45, 46).

ACI-35 (vaccine) is a synthetic peptide comprising the tau protein sequence of human protein 393–408, phosphorylated at S396 and S404. In animal models, its administration has been shown to reduce both the quantity of aggregates of phosphorylated tau protein and the total pathological protein, as well as improve some cognitive functions. The vaccine triggers a specific antibody against the tau protein and an immune response that is independent of T cells. There are ongoing multicenter, double-blind, randomized, placebo-controlled phase I studies to assess the safety, tolerability and immunogenicity of ACI-35 in patients with mild to moderate Alzheimer's disease (ISRCTN13033912) (Table 1).

Data for AADvac1 and the fact that no dangerous adverse effects were reported for ACI-35 indicate that active immunization may be a safe way to counteract tau protein pathology. The safety aspect will become more and more important as active tau protein-targeted immunotherapy switches from the treatment of neurodegeneration towards its prevention (46).

Intravenous immunoglobulin (IVIg) derived from human plasma, consisting of polyclonal serum IgG obtained from blood donors, is effective in anti-inflammatory and immunomodulating therapy in cases of neurological diseases (50).

A randomized, double-blind, placebo-controlled phase III study (NCT00818662) involving 390 patients with Alzheimer's disease found no improvement in cognitive ability and function after IVIg infusions every 2 weeks for 18 months (Table 1) (47). Two further trials, one phase II (NCT01300728) and one phase III (NCT01561053) for the treatment of Alzheimer's disease are underway. Interestingly, it was found that tau protein-specific antibodies are present in the IVIg Flebogamma® product that recognizes a recombinant tau protein fragment containing residues 155–421, as well as tau protein aggregates from the brains of Alzheimer's disease patients (51).

In addition to active immunotherapy, strategies for passive immunotherapy using various antibodies to the tau protein are also under investigation (Table 1). It has been shown that this approach can improve behavioral and cognitive impairment in mouse models (52). In the past few years, three passive immunotherapy programs based on tau protein antibodies have been opened in clinical trials, mainly for the treatment of Alzheimer's disease as outlined in Table 1. BIIB092 is a humanized IgG4 monoclonal antibody against the extracellular N-terminal of a tau protein isolated from pluripotent stem cells of a patient with familial Alzheimer's disease (Table 1) (53).

ABBV-8E12 is a humanized monoclonal antibody against tau protein for the treatment of Alzheimer's disease in clinical settings. Currently, recruitment for a phase II clinical trial is done to assess the effects of ABBV-8E12 in patients with Alzheimer's disease (NCT02880956) (Table 1). A multicenter, randomized, double-blind, placebo-controlled study to assess the efficacy and safety of ABBV-8E12 in 400 patients with Alzheimer's disease should be completed by 2020 (54). A final approach to passive immunotherapy in clinical trials is R07105705, an antibody against tau protein, whose features regarding its nature are very limited and its preclinical efficacy unknown (55). In 2016, patients with Alzheimer's disease were recruited for the first stage of the study (NCT02820896). Although no results have been published, recruitment was initiated at the end of 2017 to an 18-month, randomized, double-blind, placebo-controlled phase II study to evaluate the efficacy and safety of RO7105705 in 360 patients with Alzheimer's disease (NCT03289143) (Table 1). Patients who complete the double-blind therapy will be invited to an optional 24-month extension period.

In summary, many active and passive tau protein-targeted immunotherapies are already in the clinical trial stage for the treatment of Alzheimer's disease (Table 1). Due to the fact that new strategies for tau protein-directed immunotherapy are still being developed, a number of key questions need to be answered, in particular regarding the choice of the immunogen, the species of tau protein to be targeted, as well as mechanism of action and safety (56–58).

## FUTURE PERSPECTIVES

Per the studies and data noted in this chapter, the status of tau protein targeting therapy in the treatment of Alzheimer's disease is unclear due to lack of evidence or mutually exclusive observations. The small number of studies, and variable non-uniform measures of results suggest the field to be in its infancy and limit the possibility of making generalized conclusions. Although Alzheimer's disease is a real challenge for the pharmaceutical industry, there has been no clear progress in

treatment options in the past few years. Current drugs on the market show only low effectiveness, and clinical studies from the last 20 years have ultimately proven to be negative in phase 3. This was perhaps due to the hegemony of the amyloid hypothesis and focus of therapeutic strategies on amyloid, while the focus on tau protein, the main component of neurofibrillary tangles which correlates better with the degree of dementia than amyloid (25) has only emerged in recent years. A limited number of studies provide evidence of low or very low quality. Some studies have shown side effects, although the study times were short and the long-term risk of side effects was not determined.

To date, several drug trials targeting the tau protein have failed in clinical trials. Although there are various causes for these failures, the following points can help improve the results of future attempts. Firstly, the tau protein should be ideally targeted intracellularly, since most pathologies of the tau protein affect neurons inside. Secondly, as previous anti-amyloid immunotherapy attempts have taught us, it is important to continue to develop second- and third-generation methods in the field of tau protein immunotherapy. Smaller antibodies that are fragments of whole antibodies should have better access to the inside of both the brain in general and the neurons themselves, while also enabling them to bind to different epitopes of the tau protein than whole antibodies, providing greater and more effective therapeutic benefits. Thus, due to their smaller size, they will also be better suited for gene therapy than whole antibodies. We will be looking forward to future preclinical studies examining antibody fragments as a novel therapeutic approach. Thirdly, in recent years, a major focus has been on the implementation of drug-screening models that have focused on preventing seeding or spreading aggregation. Much less attention has been paid to the identification of compounds that inhibit the neurotoxicity of these aggregates, which is not necessarily associated with their seeding or spreading tendency. Ideally, all these markers should be readings in a unified test or model. Fourthly, the variety of conformer or strain of aggregates complicates the development of drugs for small molecule aggregation inhibitors but will probably not pose a problem in antibody-based therapy. Fifthly, other more general goals related to neurodegeneration should still be pursued, but in many ways, they are more difficult to solve than the removal of amyloid and tau protein, which are the hallmarks of Alzheimer's disease. Sixthly, shifting the time of therapeutic intervention to the very early stages of Alzheimer's disease should be a feature of long-term clinical studies. Lastly, targeting the tau protein is likely to provide better therapeutic benefits at a later stage in the development of the disease because tau protein dysfunction is more closely correlated with performance on cognitive tests than amyloid (25). Based on the data discussed in this chapter regarding the presence and abundance of intra- and extracellular pools of tau protein and its epitopes, and the finding that antibodies can have disease-specific efficacy and separate efficacy against tau protein toxicity, are the right direction for future preclinical and clinical investigations.

Future randomized clinical trials are needed to demonstrate the effectiveness of test substances and provide necessary data on several unresolved practical problems, that is, how and how long these substances can be administered to patients with Alzheimer's disease. What part of the tau protein is affected by immunotherapy and other test substances? How? What effect do treatment attempts have on the physiological and neuropathophysiological function of the tau protein? What human data was obtained to confirm this? These questions can

only be answered by undertaking large, multi-center clinical trials. At the moment, information on the use of anti-tau protein substances as drugs in the treatment of Alzheimer's disease seems interesting because of the possible effect on the accumulation of tau protein. The few clinical trials available to date have no genuine control and group randomization. Evidence has shown the causative factors and indicates the need for further research to show that anti-tau protein substances have a positive effect on patients with Alzheimer's disease. Though there is uncertainty regarding the potential of tau protein-targeting therapies in the treatment of Alzheimer's disease, and indeed the reasoning behind this skepticism is important to consider, it is still worthwhile to investigate. Though published studies on the subject are often flawed, and the findings should be taken with caution, the promising results should not be completely disregarded.

Another issue is the study design for substances directed against tau protein. Current ethical standards for clinical trials require that substances under investigation must be compared to the current treatment standard; this makes it difficult to conduct a true randomized, placebo-controlled study that is independent of potentially confounding treatments. This necessitates that the question of what standards of treatment serve as the best basis of comparison be answered prior to further study. Based on comprehensive pre-clinical results, as well as the initial clinical data, it is clear that the next step must be to test these substances in well-designed and controlled clinical trials. However, further double-blind studies are also needed to determine the efficacy of these substances in treatment. In conclusion, future clinical trials should focus on the proper selection of patients. Accurate and definitive explanation of the therapeutic properties of substances against tau protein can give hope for a long-lasting therapeutic effect. Based on the results of verified clinical trials, it may seem that the clinical effectiveness of substances that target tau protein is promisingly high, but this is not to be considered certain. We must be patient and wait at least a few years for thorough confirmation. No substances against the tau protein have been approved for use in the clinic. We hope that the evidence from ongoing clinical trials will help us better understand the therapeutic efficacy of substances against the tau protein and put them at the forefront of new therapies that patients and their doctors are eagerly awaiting. While some open questions and challenges remain, the data presented here encourages and demonstrates the potential of tau protein-based therapeutic strategies in the future treatment of Alzheimer's disease. The complexity of abnormal tau protein folding, aggregation and propagation of neuropathology, as well as the immune response during aging and neurodegeneration should be considered so that we can design and develop safe and effective treatment in a better way. Lessons must be learned from the disappointing experience accumulated over the past 20 years to develop disease-modifying therapies so that we can continue to progress, with caution, translating results from preclinical models into the development of drugs at the clinic. The growing interest in the tau protein will certainly lead to a deeper understanding of its function and will give new insight into the precise mechanisms and nature of tau protein species responsible for neuronal dysfunction and its causal role in the development of Alzheimer's disease. Hopefully, this will lead to an extension of the range of potentially useful therapeutic tools for treating such a devastating condition as Alzheimer's disease.

## CONCLUSION

Although both amyloid and tau protein are very important, their relationship in causing Alzheimer's disease remains unknown. Treatment directed to amyloid and tau protein may be individually effective, but the convergent progression of amyloid and tau protein pathology suggests that combination therapy may eventually be required, especially in late stages when both are abundant. While ongoing works focused on single-goal therapies, the approach to double-targeting amyloid and tau protein is more likely to lead to a breakthrough (3). Referring to the above observations, it should be stated that Alzheimer's disease is an age-related neurodegenerative disease whose various neuropathological and therapeutic aspects are still being investigated and are not fully explained; pending success in the development of an effective treatment for Alzheimer's disease, it may be best to focus on preventive measures (59).

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5

# Targeting Metal Homeostasis as a Therapeutic Strategy for Alzheimer's Disease

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**Abstract:** Trace metals play an important role in the pathophysiology of amyloid precursor protein, amyloid beta, and tau, the key molecules involved in Alzheimer's disease. Altering trace metal concentrations in the brain has been explored as a therapeutic strategy for Alzheimer's disease. It is not only the accumulation of metals that drives amyloid beta aggregation, but also the lack of sufficient trace metals for other biological processes created through sequestration by amyloid beta that affects brain health and function. Thus, balancing metal levels to achieve therapeutic effects is an intricate process. This chapter summarizes the role of trace metals in Alzheimer's disease and highlights the preclinical and clinical studies targeting metal homeostasis in animal models and humans. It further discusses recent developments in pharmacological approaches targeting metals in Alzheimer's disease and provides an outlook on possible future treatments based on current translational research, for example, nanomedicine.

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## INTRODUCTION

Over the last decade, the literature covering the roles of metals in biological systems has increased considerably. Metallobiology has become a useful tool in investigating the connection between metals, metalloproteins, and neurodegenerative diseases and other disorders of the central nervous system (CNS). The primary risk factor for Alzheimer's Disease (AD) is aging (1, 2). However, evidence shows that dysregulation of trace metals such as copper (Cu), zinc (Zn), iron (Fe), aluminum (Al), manganese (Mn), and other elements such as lead (Pb) and cadmium (Cd) is also associated with AD (3). Fe, Zn, Cu, and Mn are essential for various biochemical and physiological processes in humans, playing roles in structural and regulatory functions in proteins, cellular signaling pathways, and oxygen transport. In the human body, the distribution of metals is different for each organ. For example, in the human brain, the most abundant trace metal is iron, mostly found as heme iron (iron bound to hemoglobin in red blood cells). Iron is essential not only for the transport of oxygen but also as a cofactor for a series of enzymes that play a role in the synthesis of neurotransmitters such as tryptophan hydroxylase and tyrosine hydroxylase (4, 5). Additionally, iron has several functions in the CNS including the regulation of synaptic plasticity (6), the myelination of neuronal axons (7), and the regulation of the neuronal energy status (8). Zinc is the second most copious trace element in the human brain (9). Like iron, zinc is a modulator of synaptic plasticity. In addition to this, it can regulate neurogenesis, neuronal migration, and differentiation, and plays a role in neurotransmission (10–12). Zinc is a fundamental structural and catalytic component of proteins, acting as a cofactor for over 300 enzymes. It also gives stability to various transcription factors. Finally, zinc has neuroprotective functions, and can protect against oxidative stress (13, 14). Copper is the third most prevalent trace metal in the brain. Like zinc, copper acts as a cofactor for a series of enzymes and plays a vital role in the biosynthesis of neurotransmitters (15). Manganese is a cofactor for several enzymes, including glutamine synthetase, pyruvate carboxylase, arginase, MnSOD, and protein serine/threonine phosphatase-1. Mn is also involved in a series of Mn-sensitive pathways, such as the ATM-p53 pathway (16).

Thus, trace metals are involved in enzyme activation, catalysis, regulation of gene expression, and protection against reactive oxygen species (ROS). Therefore, they are fundamental to appropriate brain growth, development, and function. The homeostasis of these charged elements is tightly regulated at the blood–brain barrier (BBB); metal ions are actively transported across the BBB by several transport proteins. Any disruption of metal balance, which can be caused by impairments in the processes of absorption, distribution, and excretion, or mutations in metal transport or metal-binding proteins, or by the competition between metals for the binding sites (e.g., Zn and Cu), can lead to an imbalance of essential trace metals or increased levels of non-essential trace elements (17, 18).

## METALS AND THE AMYLOID PRECURSOR PROTEIN

In AD, metal imbalances occur in the brain (3, 19) as a result of the interactions between the metals and the critical proteins amyloid precursor protein (APP) and its products, amyloid beta (Aβ), and tau (Figure 1). APP, which is mainly expressed

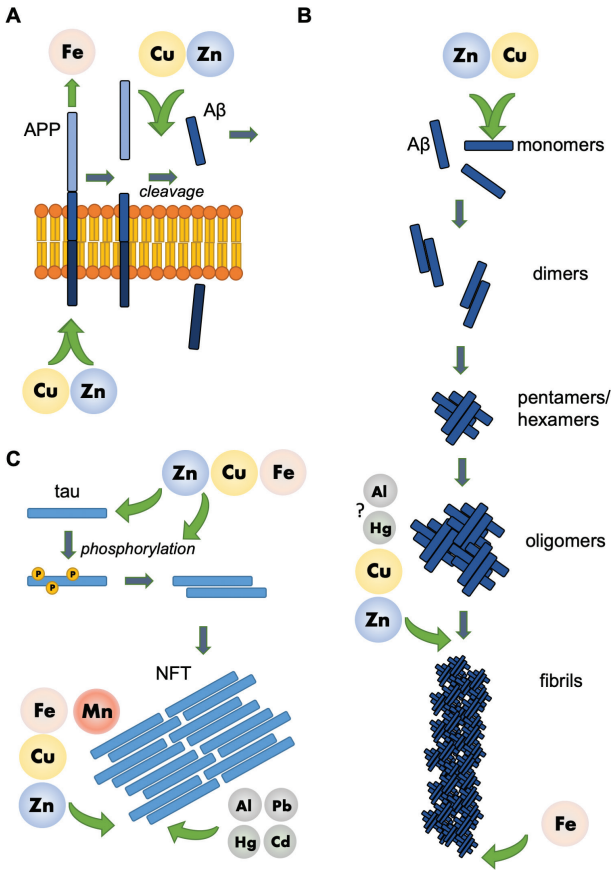


**Figure 1. Trace metals interactions with APP, Aβ, and tau.** (A) APP can be considered to act as a metalloprotein. It has two putative metal-binding sides with which it binds Cu and Zn ions. Fe is involved in the regulation of APP translation, and APP may mediate Fe export of neurons. Aβ is derived from APP cleavage through β- and γ-secretases. Cu and Zn indirectly influence Aβ generation by interacting with all three secretases (α, β, and γ) involved in the cleavage of APP. (B) Aβ directly binds Zn and Cu ions after enzymatic cleavage. Aggregation of Aβ into insoluble fibrils is mediated by the interaction of Aβ with Zn and Cu. Chelation of Zn and Cu results in the enrichment of these ions in amyloid plaques. Aβ may also bind Fe, and changes in Fe metabolism occur after the development plaques. Al has been detected in Aβ plaques suggesting an interaction of Al with Aβ, and Al and Hg promote the accumulation of Aβ aggregates *in vitro*. (C) NFTs include trace metals such as Zn, Cu, Fe, and Mn, but also Pb, Cd, Hg, and Al have been detected. Trace metals may promote tau hyperphosphorylation and induce tau aggregation. A direct interaction between Zn and tau has been reported. Similarly, a selective binding between tau and Cu was found. Fe, as Zn and Cu, interacts with some isoforms of tau. Al, Cd, Pb, and Hg may not directly bind tau, but influence NFT formation through secondary processes.

in the human brain, is involved in neuronal cell migration and neurite outgrowth (20). Copper binds to APP between residues 142 and 166 (21), playing essential roles in the structural stability of the protein, including folding stability and homodimerization, and expression levels (22). Zinc can also interact with APP using the site located between amino acid positions 170 and 188, regulating the homodimerization of APP (23, 24). In addition, there is bidirectional regulation between iron and APP: iron can directly control APP translation, and APP levels control the export of iron in neurons (25). The Aβ protein is derived from APP in the process of sequential proteolysis realized by β and γ- secretases. Insoluble amyloid fibrils of Aβ are associated with metals like Zn, Cu, and Fe and represent a pathological feature of AD (26). Zinc plays a role in the stabilization of amyloid fibrils (27), binding to Aβ at histidine (His)13 and His6 sites (28). High zinc concentrations have been found at the level of senile plaques in postmortem tissues of patients affected by AD and in plaques of genetic AD mouse models, underlining the critical role of zinc in this process (26). Copper is considered to play a significant role in the neurotoxicity of Aβ. The monomeric Aβ exhibits three high-affinity His Cu-binding sites: His6, His13, and His14. They are known to form a tetragonal complex with copper ions along with the Nterminal amino group and aspartate (29). In addition to this, copper is involved in the formation of β-sheet structures, the precursors of the toxic aggregates of the fibrillary form of Aβ. Several studies have shown that, in transgenic AD mouse models, Cu chelators could inhibit the accumulation of Aβ (30, 31). Iron also interacts with Aβ at the binding sites of Asp1, Glu3, and the three His residues: His6, His13, and His14. The consequence of this interaction is the release of free radicals via Fenton chemistry (32). Other metals such as Al and Hg have been identified in Aβ plaques. While the role of Al in the AD pathology is still not clear, Hg promotes the accumulation of Aβ deposits *in vitro*. Cd and Mn are also involved in increasing the Aβ accumulation (33).

Another pathological hallmark of AD is hyperphosphorylation of the protein tau that leads to the formation of abnormal intracellular structures known as neurofibrillary tangles (NFTs). Zn, Cu, Fe, Mg, Mn, Pb, Cd, Hg, and Al have been found in these structures. Zinc interacts with tau using the serine (Ser) and proline (Pro) binding sites, using two cysteine (Cys) residues (C291 and C322), or threonine (Thr) and Pro sites (34). Zinc also interacts indirectly with tau, through the activation of kinase and phosphatase pathways (35). The importance of copper in the hyperphosphorylation of protein tau via the activation of the cyclin-dependent kinase (CDK)5/p25 complex is still controversial, although high levels of Cu have been reported in amyloid plaques and NFTs (36, 37). Iron ($Fe^{3+}$) is also reported to interact with NFTs through His residues (38). Like Cu, Fe can also indirectly contribute to hyperphosphorylation of tau, by activating the CDK5/p25 complex and GSK-3β and MAP kinases (39).

The abundance of Aβ and the formation of NFTs lead to an imbalance in trace metals that interact with tau and Aβ. It is both the interaction with the hallmark proteins of AD and the loss of trace metals in their physiological processes due to excessive binding by Aβ and tau that contributes to the pathogenesis of AD. For example, in postmortem AD brains, increased Mn concentration, abnormal tau aggregation, and consequent hyperphosphorylation of tau caused by GSK-3β kinase, have been observed (40). The presence of toxic trace metals has also been reported in AD. For example, Cd is involved in the activation of GSK-3β kinase,

inducing hyperphosphorylation of tau (41). Pb plays a role in the modulation of tau by regulating the activity of (CDK)5/p25 complex and GSK-3β kinase (42). To modify Aβ aggregation, plaques, and the formation of NFTs, and to return essential trace metals to their effector proteins, balancing levels of trace metals is necessary and has been pursued as therapeutic strategy with some success in the past (43).

## NUTRITIONAL INTERVENTIONS

The essential metals (Na, K, Ca, Mg) and essential trace metals (Fe, Zn, Cu, Mn, Co, Mo) are obtained almost exclusively from the diet. Trace metal deficiencies may affect the elderly (44, 45) and alter immune function (46). While some meta-analyses of studies analyzing the nutrient status of patients with AD found no significant differences in Fe, Zn, and Cu status (47, 48), more recent meta-analyses revealed significantly lower brain Cu (49) and serum Mn levels (50). However, subclinical trace metal deficiencies are hard to diagnose (51) and may not be detected in many individuals. Considering this, nutritional supplementation studies may provide additional insights. A systematic review found that the amount of caloric intake and type of diet may influence AD onset and progression (52). For example, reports show that a Mediterranean diet, which is naturally rich in metals, such as Mg, K, Ca, and Zn, (53), can have a neuroprotective effect that may be relevant to AD (54). In mouse models of AD, zinc supplementation prevented cognitive deficits (55). In humans, despite case-control and postmortem studies showing decreased systemic Zn levels in AD patients (47, 56), studies investigating the effects of zinc supplementation provided inconclusive results. Zinc supplementation also reduces copper uptake due to an antagonistic relationship between the two metals (57). A diet low in Cu has been suggested as beneficial for AD (58–60), but a recent study does not support this hypothesis (61).

Trace metal homeostasis in AD is affected at cellular and sub-cellular levels in the brain and, thus, therapeutic strategies involving their manipulation may require more targeted approaches than dietary supplementation/restriction. However, trace metal supplementation in AD is still understudied. In addition to their direct effects on physiological processes in the brain, they may exert indirect effects through the microbiota composition of the gut (62). Regardless, maintaining adequate trace metal status will affect general health positively and may modify AD phenotypes, for example, by lowering pro-inflammatory responses and oxidative stress (63).

## TARGETED INTERVENTIONS: DELIVERY OF METAL-ATTENUATING COMPOUNDS

Altered homeostasis of certain essential and non-essential trace metals can be the cause, as well as the consequence of AD pathology. Abnormal trace metal levels have been associated with neuroinflammation, mitochondrial dysfunction, oxidative stress, and protein aggregation (19). The AD hallmark proteins Aβ and tau are metal-binding proteins. Therefore, metal chaperones

and/or metal chelators may help modulate and counteract AD progression. Metal chaperones or metallochaperones are a class of compounds that shuttle metal ions to specific intracellular targets. In contrast, metal chelators or buffers function to exclude or deplete metals from discrete cellular compartments to limit biological or pathological interactions of essential metal ions. Cumulatively, these processes serve to maintain tight regulatory control over cellular metal ion homeostasis such that the intracellular concentration of freely available metal ions (like Cu and Zn) is close to zero, and sufficient metals are available for crucial metal-binding proteins. Among the metal-focused interventions (Table 1), metal protein attenuating compounds

**TABLE 1 Metal interventions in preclinical and clinical trials**

| Drug | Targeted metal | Species | Effect of the drug | Reference |
|---|---|---|---|---|
| Clioquinol (CQ) | Fe, Cu, Zn | Mouse: Tg2576 | Reduction of sedimentable Aβ (50%), plaques surface area and Aβ serum levels and increase in Aβ soluble fraction | (30) |
| Clioquinol (CQ) | Fe, Cu, Zn | Human: severe AD patients | Prevention of cognitive deterioration and reduction of Aβ plasma levels | (64) |
| DP-109 | Fe, Cu, Zn | Mouse: Tg2576 | Reduction of Aβ plaques and CAA | (66) |
| PBT2 | Fe, Cu, Zn | Mouse: APPswe/PS1 dE9 and Tg2576 | Decrease in soluble and insoluble Aβ levels and plaques burden and improvement in learning and memory in the MWM | (67) |
| PBT2 | Fe, Cu, Zn | Human: early/mild AD patients | Reduction of Aβ plasma and CSF levels and improvement of executive tasks | (70, 71) |
| DFO | Al, Fe | Human: AD patients | Reduction of mental deterioration | (81) |
| Ferelex-G | Al, Fe | Human: postmortem AD brain | High Al/Fe removal bound to hyperphosphorylated tau | (79) |
| DFO | Al, Fe | Mouse: APP/PS1 | Reduction of Aβ, improvement in learning and memory retention | (82) |
| TETA | Cu | Mouse: APP/PS1 and Tg2576 | Reduction of Aβ deposition in APP/PS1 but not in Tg2576 mice | (73) |
| PDTC | Cu | Mouse: APP/PS1 | Improvement of spatial learning but no reduction of Aβ levels | (75) |
| Zn7MT3 | Zn | Mouse: Tg2576 | Reduction of behavioral deficits, Aβ aggregation, oxidative damage and neurodegeneration | (83) |
| XH1 | Fe, Cu, Zn | Mouse: APP/PS1 | Reduction of Aβ load | (87) |

AD, Alzheimer's disease; CAA, cerebral amyloid angiopathy; CSF, cerebrospinal fluid; MWM, Morris water maze.

(MPACs) have been employed in both preclinical animal studies and human clinical trials. MPACs are metal chelators with a moderate affinity for metal ions and can cross the BBB. They correct abnormal metal interactions and have subtle effects on metal homeostasis. Therefore, they may limit metal binding to plaques, inhibit Zn and Cu-induced oligomerization of Aβ, and consequently reduce Aβ aggregation and toxicity (64).

Clioquinol (CQ, 5-chloro-7-iodo-8-hydroxyquinoline, MW = 305.5) is a hydrophobic 8-hydroxy (OH) quinoline derivative and an archetype of MAPCs. CQ freely crosses the BBB and is a moderate chelator of Fe, Cu, and Zn. Additionally, with its ionophore activity, it works as a metal chaperon relocating metal ions into cells (65). Originally developed as an oral antiparasitic agent, CQ treatment significantly prevents the cognitive decline of patients with moderately severe AD, as demonstrated in a phase II clinical trial (64). The slowed cognitive decline was also accompanied by a transient decrease in $A\beta_{1-42}$ plasma levels. These results indicate the potential of CQ and related compounds as treatments for AD and have sparked further interest in MPACs. Also, in animal models of AD such as Tg2576 mice, chronic treatment with CQ can decrease Aβ loads without adverse effects (30). Treated mice showed significant improvements in general behavior and neurotoxicity, in accordance with the results obtained in severely affected AD patients (64).

DP-109 is a diester derivative of BAPTA (1,2-bis(2-aminophenyloxy)ethane-N,N,N′,N′-tetra acetic acid), a widely-used calcium chelator, that is able to cross the BBB and is designed to selectively chelate transition metals such as Zn, Cu, and Fe within membrane compartments (increased efficacy for divalent metals). Like CQ, prolonged administration of DP-109 to female Tg2576 mice markedly reduced the amyloid plaque load and the degree of cerebral amyloid angiopathy (CAA) (66).

PBT2 belongs to the second-generation MPACs and is a highly soluble derivative of 8-OH quinoline that lacks iodine. With increased BBB permeability and ionophore activity compared to CQ, PBT2 reduced Aβ levels and improved cognitive abilities in Tg2576 and APP/PS1 AD mouse models (67, 68). In these mice, both acute and chronic dosing resulted in a significant decrease in Aβ plaque burden and interstitial fluid Aβ levels, a significant increase in synaptophysin levels, and restoration of hippocampal dendritic spine density (69). In a 12-week phase-II clinical trial of early AD patients, PBT2 was also found to lower cerebrospinal fluid $A\beta_{42}$ concentrations and reverse frontal lobe functional deficits significantly, reducing executive dysfunction; however, no significant effects on cognition and memory were reported (70, 71).

The ionophore activity (the selective Aβ-metal-ion binding) of these compounds seems more promising than metal-chelation (metal-depletion) in counteracting AD pathology. While CQ and PBT2, possessing both features, are effective in reducing sedimented Aβ, triethylenetetramine (TETA), a classic hydrophilic Cu chelator, is not (72). TETA, a well-characterized anti-diabetic molecule, acts as a highly selective divalent copper (Cu(II)) chelator that prevents or reverses diabetic copper overload, thereby suppressing oxidative stress. Recent data showed that TETA disuccinate or trientine treatment also reduces Aβ production and deposition in APP/PS1 mice but not in Tg2576 mice in the same experimental paradigm in which CQ was effective (73). Given the

hybrid action of many of these compounds, the original proposed name shifted from MAPCs to "ionophores" and finally to "metallochaperones" (74). Other molecules with metal ion chaperone properties, like pyrrolidine dithiocarbamate (PDTC) and copper-bis(thiosemicarbazonoto) complexes, have also proved to ameliorate cognitive functions in AD mouse models, though they did not affect Aβ (75, 76). On the contrary, D-penicillamine chelates Cu by forming a stable complex with thiol groups and allowing renal copper excretion. A clinical pilot study indicates that D-penicillamine can reduce oxidative stress, but not the clinical progression of AD (77).

While Zn, Fe, and Cu mainly bind to Aβ (78), Al and Fe accumulate and bind to hyperphosphorylated tau in neurons with NTFs (79). As Fe binds to both misfolded proteins, Fe is a potential target for chelation therapy. A 2-year long, single-blind clinical study showed that sustained intramuscular administration of desferrioxamine mesylate (DFO), an Al/Fe metal chelator, slows the clinical progression of AD-associated dementia (80). Unfortunately, its hydrophilic nature and large molecular size limit its absorption across the gastrointestinal tract and prevent it from penetrating the BBB (80), inducing undesired side effects such as systemic metal depletion. All these features have prevented its final use in the clinics (81). More recent evidence showed that intranasal administration of DFO is accompanied by a reduction in Aβ burden/load as well as improved learning and memory abilities in APP/PS1 mice (82) confirming, again, the beneficial effects of this drug on AD pathology.

Other Fe chelators have been developed in the last 20 years. Feralex-G, for instance, showed enhanced chelating activity for Al/Fe associated with hyperphosphorylated tau compared to DFO (79).

Chelators such as TETA, penicillamine, and DFO are routinely used in the treatment of metal overload disorders, such as Wilson's disease. However, these molecules are hydrophilic and exert their effects by systemic depletion of metals and are poorly absorbed through the BBB with limited bioavailability. The efficacy of chelation therapy is still a matter of debate (74). Several metal-binding proteins are currently being tested as AD treatment. Metallothionein 3 (MT-3), a key regulator of metal homeostasis in the brain, has been found to decrease in AD patients. Intraventricular injection of Zn-loaded MT-3 (Zn/MT3) attenuates behavioral deficits, Aβ aggregation, oxidative damage, and neurodegeneration in the Tg2576 AD mouse model. This drug allows the exchange of Aβ Cu with Zn making Aβ ROS inert (83).

Finally, another group of molecules has been characterized for their ability to modulate APP expression, leading to a novel therapeutic approach aimed at reducing the amyloidogenic process (84). A common feature of these molecules, to which the previously mentioned DFO belongs, is their ability to recognize an IRE stem-loop upstream to the start codon of the APP transcript at the APP-5'UTR region (84, 85). Like tetrathiomolybdate (TM) (a Cu chelator) and dimercaptopropanol (a Pb and Hg chelator), DFO reduces Aβ secretion and restrains the expression of APP holo-protein *in vitro* (84, 86). Similarly, XH1, a bifunctional compound capable of both APP binding and metal-chelation, was able to lower APP expression in the SH-SY5Y cell line and reduce Aβ burden in APP/PS1 mice (87). Several other chelators with multifunctional properties with potential applications in AD treatment exist (88). However, so far, they have mainly been tested *in vitro*, and the beneficial effects they exert cannot all be attributed to their interaction with metals.

**TABLE 2 Novel approaches in AD using DDSs**

| Delivery system | Function | Effect of the drug | Reference |
|---|---|---|---|
| G7-Zn-NP | Zinc delivery | Plaque size reduction and decrease in pro-inflammatory cytokine release *in vivo* | (92) |
| ZnONP | Zinc oxide delivery | Dose-dependent inhibition of fibrillar amyloid growth and β-sheet formation *in vitro* | (93) |
| CQ-MSNP | CQ metal chelator | Inhibition of $Cu2^{+}$-induced Aβ40 aggregation *in vitro* | (94) |
| Nano-N2PY | Iron chelator | Inhibition of Aβ aggregation in human cortex *in vitro* | (95) |

DDSs, drug delivery systems; AD, Alzheimer's disease.

## TARGETED INTERVENTIONS: DELIVERY OF TRACE METALS

Although many studies support the efficacy of chelation therapy *in vitro*, the translation of metal chelators into clinical studies is currently limited due to their inability to cross the BBB easily: they are hydrophilic, and many are neurotoxic at high concentrations. The use of innovative drug delivery systems (DDSs) such as nanoparticles designed to overcome the BBB could be considered as a strategic approach in the prevention and treatment of neurological and neurodegenerative diseases such as AD (89–91). Several sustained-release nanocarriers have been studied for the treatment of AD; in particular, polymeric nanoparticles (NPs) allow a significant increase in bioavailability and effectiveness of the administered drugs. Although the efficacy and advantage of these systems have been supported by several experimental studies (Table 2), the translational efficacy is yet to be proven.

## CONCLUSION

The role of trace metals in AD is complicated as they are involved in both pathological and physiological processes in the brain. In addition, trace metals such as zinc, which binds to more than 10% of proteins encoded in the human genome (96), regulate a plethora of processes whose effects are neither fully understood nor assessable in preclinical and clinical studies. Furthermore, levels of one trace metal affect the levels of other metals (19). Therefore, any therapeutic strategy targeting trace metal levels in AD needs to be specific in terms of drug delivery and consider its effect on the concentration of a particular metal. Preclinical and clinical studies attempting to manipulate trace metals, although not entirely successful, were among the most promising approaches for the treatment for AD. Metal chelation and chaperoning successfully reduced Aβ

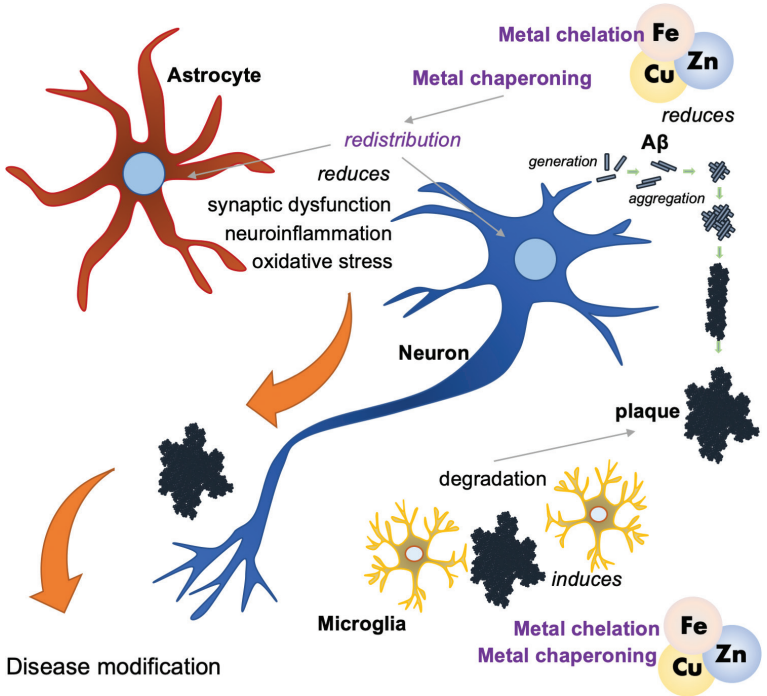


**Figure 2. Targeting trace metals as therapeutic strategy in AD.** Metal chelators and ionophores can prevent metal binding to Aβ, inhibit oligomerization of Aβ, and consequently reduce its aggregation and toxicity. Altering metal homeostasis by depletion, delivery, and chaperoning was reported to decrease Aβ plaque burden and interstitial fluid Aβ levels, positively affect synaptic spine density, and suppress oxidative stress. Zinc delivery further reduces inflammation, while iron chelation modifies tau phosphorylation, and APP expression.

generation and aggregation, synaptic dysfunction, neuroinflammation, oxidative stress, and induced degradation of senile plaques (Figure 2). In addition, effects on cognitive abilities were observed in some studies. To further develop treatment strategies targeting metal homeostasis, it is necessary to clearly understand the role of trace metals in AD. It is important to explore the role of trace metals, such as zinc, in physiological processes that could be exploited as therapeutic targets apart from Aβ aggregates, such as synaptic plasticity mediated by zinc-dependent SHANK proteins (97) or reducing excitotoxicity and Aβ burden by zinc-binding S100B proteins (98–101). A re-distribution and balancing of metals may be necessary between neurons, astrocytes, and microglial cells to optimize effects on synapses and inflammatory processes. This could be achieved by targeted delivery of trace metals to Aβ aggregates and NFTs. To this end, novel compounds, combinations of novel metal chelators and chaperones, and novel delivery platforms will be needed.

**Conflict of Interest:** The authors declare no potential conflict of interest with respect to research, authorship and/or publication of this chapter.

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# 6

# Atypical Protein Kinase C Hyperactivity in Insulin-Resistant and Insulin-Sensitive Forms of Alzheimer's Disease: A Potential Therapeutic Target

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**Abstract:** Alzheimer's disease (AD) is commonly, not always, associated with insulin-resistant, hyperinsulinemic, and obesity/type-2-diabetic (O/T2D) states. Partial deficiencies of brain insulin receptor (IR) indeed occur in both O/T2D-AD and human AD, but these deficiencies can be bypassed by hyperinsulinemia, which activates atypical protein kinase C (aPKC) and β-secretase, increases Aβ-peptide and phospho-thr-231-tau levels, and induces memory impairments; importantly, these aberrations are reversed by reduction of liver/aPKC-dependent hyperinsulinemia or direct blockade of brain aPKC. New evidence shows that aPKC acts via nuclear factor kappa-B to increase β-secretase mRNA/protein levels in brain, where β-secretase acts on both β-amyloid precursor protein to increase AD risk and IR to limit beneficial (aPKC independent) insulin effects, particularly in normo/hypoinsulinemic AD, and liver, where β-secretase acts on IR to initiate or abet development of insulin resistance and compensatory hyperinsulinemia

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that originates from diet-induced hepatic aPKC activation. Fortunately, agents that inhibit PKC-λ/ι in brain, liver, or both effectively reduce β-secretase levels and adverse actions therein, and moreover, prevent/reverse O/T2D and AD development in mouse models. This chapter summarizes work implicating the critical role of atypical PKC in the development of liver-dependent hyperinsulinemia as a risk factor in O/T2D-associated AD and β-secretase-mediated pathological alterations in brains of O/T2D-associated and O/T2D-independent AD.

**Keywords:** atypical protein kinase C; β amyloid precursor protein; β secretase; insulin-resistant Alzheimer's disease; insulin-sensitive Alzheimer's disease

## INTRODUCTION

In the United States, according to the Alzheimer Association, late onset (LO) Alzheimer's disease (AD) afflicts 1 in 5 women and 1 in 10 men over the age of 65, and 45% of people over the age of 85. Hyperinsulinemic forms of obesity, the associated metabolic syndrome (MetS) and type 2 diabetes mellitus (T2DM), which together afflict half of all adults in the United States, are thought to increase LO-AD risk. The extent and consequences of these interrelated disorders are enormous, in terms of morbidity, mortality, and cost. Although pathogenetic mechanisms of AD are still unsettled, both early-onset, inherited forms of AD and sporadic LO-AD are thought to be dependent on pathological increases of Aβ-plaques that arise from Aβ-peptides, whose production is initiated by the aspartyl peptidase, β-site β-amyloid precursor protein (βAPP) cleaving enzyme-1 (BACE1), acting on specific amino acids of βAPP (1–4). Indeed, BACE1 levels are increased in many or most, but not necessarily all, AD patients (5). In either case, BACE1 action is thought to be essential for the development of classical AD pathology, and this dependency spurred the widely heralded development of BACE1 inhibitors for AD treatment. Unfortunately, however, in human clinical trials, BACE1 inhibitors apparently produced cognitive/memory impairments, and this may reflect requirements for uncertain actions of BACE1 in cognitive/memory functions, or development of compensatory increases in BACE1-containing vesicles (6) that may be dysfunctional owing to inhibitor binding, with impairment in vesicle trafficking needed for cognitive/memory functions.

As to whether BACE1 is needed for normal cognition and memory, BACE1 knockout (KO) does not produce significant aberrations in mice (7, 8), but it is uncertain if humans have greater dependence on BACE1. It is also uncertain if vesicle trafficking needed for memory function is disrupted by BACE1 inhibitors. However, there is another approach that would avoid the use of BACE1 inhibitors in AD, that is, development of therapeutic measures to partially diminish BACE1 content to levels that sufficiently reduce Aβ-peptide production while maintaining essential BACE1 functions. In this regard, we recently found that the atypical protein kinase C (aPKC) signaling factor, PKC-λ/ι, is critically needed for the production of BACE1 mRNA and protein (presumably BACE1 transcription), not only in the brain, where BACE1 acts upon βAPP to generate Aβ-peptides in AD, but also in liver, as discussed later in greater detail. BACE1 diminishes insulin receptor (IR) levels (9) and thereby contributes to the development of hepatic and

systemic insulin resistance that, in turn, as alluded to, leads to hyperinsulinemia, which we postulate, increases AD risk in states of obesity, MetS and T2DM. Moreover, we have developed chemical agents that, when administered to mice orally or parenterally (subcutaneously [SC] or intravenously [IV]), inhibit both liver and brain PKC-λ/ι, and thereby reduce BACE1 levels in both organs; but, when given nasally, it inhibits brain, but not liver, PKC-λ/ι, and thereby reduce brain, but not liver, BACE1 levels. Furthermore, in very recent studies, we found that oral and nasal treatment of transgenic (Tg) AD mice with an inhibitor of PKC-λ/ι over several months diminished brain aPKC activity and BACE1 levels and, most importantly, largely prevented the development of AD pathology and memory impairment.

## THE ESSENTIAL ROLE OF BACE1 IN THE PATHOGENESIS OF AD

It seems abundantly clear that the aspartyl protease, BACE1, acts upon βAPP in the brain to initiate the production of $A\beta_{1–40/42}$ peptides that polymerize to form the characteristic Aβ-plaques of AD (1–4). Indeed, genetic KO of BACE1 blocks $A\beta_{1–40/42}$ production and Aβ-plaque formation in Tg AD mice carrying human mutations that increase susceptibility to actions of BACE1 (e.g., $APP_{Swede}$) and γ-secretase (e.g., presenilin-1 [PS1]) (7, 8). This BACE1-KO-induced protection against the development of AD pathology in Tg AD mice is accompanied by the improvement of cognition/memory losses that occur in unprotected wild-type (WT) Tg AD mice, suggesting that (i) BACE1-dependent pathology is required and may account for cognitive/memory losses in Tg AD mice (and presumably other AD states) and (ii) BACE1 itself is not required for cognitive/memory functions, at least in mice. Moreover, delayed conditional KO of brain BACE1 in Tg AD mice, in which AD pathology and cognitive/memory losses are already well established, was found to reverse pathology and cognitive/memory losses therein (7), suggesting reversibility following BACE1 depletion, at least in Tg mouse AD models.

Although elevations of brain BACE1 levels have been observed in some series of human AD patients (5), this is not always the case, and increases in BACE1-dependent Aβ-peptide production may also be due to enhanced trafficking of BACE1-containing vesicles (10) and interactions with βAPP-containing vesicles in the Trans Golgi Network (TGN), where BACE1 is activated by increases in intravesicular acidity and perhaps other factors, for example, increased phosphorylation of ser-498 in the cytosolic, C-terminal tail of BACE1. Also, changes in other factors, for example, mutations in βAPP, PS1, or γ- or α-secretase(s), or altered clearance of Aβ-peptides, may serve to increase Aβ-peptide levels. Regardless of the initial or later causes, BACE1 action is critically needed for Aβ-peptide production and Aβ-plaque formation in AD, and BACE1 is an attractive target for the treatment of AD and minimal cognitive impairment (MCI).

Unfortunately, as alluded to, the use of chemical agents that directly inhibit BACE1 in humans with AD or MCI resulted in impairments in cognition/memory, despite apparent reduction of Aβ production. This raises the possibility that increases in BACE1 levels, activity and/or action, do not explain cognition/

memory losses in human AD/MCI, or losses of cognition/memory reflect a requirement for BACE1 itself, or there are direct or indirect untoward effects of BACE1 inhibitors. With respect to the latter possibility, as discussed, BACE1 levels increase with BACE1 inhibitor treatment (6), and increases in vesicles containing chemically inhibited forms of BACE1 may interfere with the vesicular trafficking needed for neurite formation, cognition, and memory induction. Whether decreases in BACE1 levels that are elicited by inhibition of regulatory factors upstream of BACE1 transcription, in particular for the present discussion, PKC-λ/ι, or downstream factors linking PKC-λ/ι to BACE1, will adversely affect cognition/memory is uncertain, but this approach seems worthy of consideration, given the present dearth of effective treatments for MCI and AD.

## SYSTEMIC VERSUS BRAIN INSULIN RESISTANCE IN DIABETES-ASSOCIATED AND NONDIABETIC AD

LO-AD and the insulin-resistant states of T2DM and its forerunners, that is, obesity and MetS, have frequently been found to coexist, for example, in a particularly important study, 80% of all AD patients seen at the Mayo Clinic were found to have, in half the cases, overt T2DM (fasting blood sugar [FBS] over 125 mg/dL) or, in the other half, pre-T2DM (FBS 110–125 mg/dL) (11). In view of this and other reports, it has been speculated that the systemic insulin resistance, which is generally, if not usually, seen in T2DM, pre-T2DM, obesity, and the MetS, serves as a risk factor for LO-AD. It has further been speculated or tacitly assumed that, as in peripheral tissues, most notably, muscle, where resistance to insulin is particularly severe, the brain is similarly insulin resistant, that is, essentially unresponsive to insulin, and therefore hypoinsulinized in the aforesaid insulin-resistant states. Accordingly, it is proposed that this hypoinsulinization *as such* provides an explanation for increases in AD risk in states of systemic insulin resistance.

In keeping with the idea that insulin action in brain is reduced in AD, brain IR activity and/or number is reportedly mild-moderately reduced in the brains of humans with *nondiabetic* AD (12); accordingly, the concept of brain hypoinsulinization has been reemphasized and expanded, and it has been proposed that the brain itself is insulin resistant and, moreover, hypoinsulinized, in both nondiabetic AD and obesity/MetS/T2DM-associated AD.

However, in view of our more recent findings, as discussed later, this proposal appears to be overstated and in need of modification. Thus, it is indeed reasonable to postulate that deficiencies of the brain IR would of necessity impair insulin effects in the brains of AD patients that have normal or low circulating levels of blood/plasma insulin, as in non-diabetic, normo-insulinemic AD, or in late forms of T2D, if and when insulin levels fully diminish to normal or subnormal levels. On the other hand, the same cannot be said in conditions of hyperinsulinemia, wherein elevated levels of blood insulin can bypass partial defects in the IR in many or most tissues by using "spare" IRs, that is, IRs in excess of those needed to produce maximal downstream effects.

In this respect, note that a more severe insulin resistance that exists in T2D muscle is largely a result of down-regulation of IRS-1 and its ability to activate phosphatidylinositol (PI) 3-kinase (3K), which is needed to produce

PI-3,4,5-$(PO_4)_3$ ($PIP_3$), which activates signaling factors required for insulin-stimulated glucose transport in muscle, that is, Akt and aPKC. Also note that this defect in muscle IRS-1/PI3K is a "post-IR" impairment that cannot be bypassed by hyperinsulinemia and should not be confused with IR defects that can be bypassed via "spare" IRs, which, as will become evident in this chapter, are present and operative in both liver and brain (13–17).

And, in marked contrast to muscle, insulin action in *liver* and activation of hepatic Akt and aPKC in obesity, the MetS and *early* T2DM are excessive in many respects owing to hyperinsulin-emia-induced activation of "spare" hepatic IRs. Indeed, note that, unlike the post-IR defect in muscle IRS-1/PI3K which occurs early in high-fat-fed (HFF) mice (18), the post-IR impairments of *hepatic* IRS-1/PI3K and subsequent Akt activation by insulin occur only later as T2DM progresses, that is, only after 2–3 months of high-fat feeding in the classical mouse model of diet-dependent obesity/MetS/T2DM (15). Also note that the activation of hepatic IRS-2/PI3K and subsequent aPKC activation by insulin remains intact even in late T2DM when IRS-1/PI3K/Akt activation is impaired: in short, hepatic aPKC remains activated by hyperinsulinemia and continues to promote increases in hepatic production of glucose and lipids throughout the course of obesity, the MetS and T2DM. Moreover, as stated, the post-IR defect in muscle described earlier occurs very early in initial stages of the development of diet-induced obesity/MetS/T2DM in HFF mice (18), viz, and several months before impairments in hepatic IRS-1/PI3K and Akt develop. And this muscle defect in glucose metabolism undoubtedly contributes importantly to early elevations of blood insulin to levels (18) that are sufficient to bypass IR defects and strongly/maximally activate key processes in other tissues, most notably for the present discussion, liver and brain. In keeping with mouse studies, note that defective glucose transport in muscle occurs early in human obesity, well before the development of pre-T2D and overt T2DM.

## INSULIN RECEPTOR DOWNREGULATION IN OBESITY/T2DM AND AD

The downregulation of the brain IR that is seen in nondiabetic AD (12) and diabetes-associated AD (see below) may well be at least partly due to increases in BACE1-dependent degradation of the IR, as this mechanism has recently been shown to clearly be operative in the liver in various murine and human forms of obesity and T2DM (9). Indeed, in initial studies, we too have found that there are decreases of IR levels in both brain and livers of hyperinsulinemic, HFF, and obese/MetS/T2DM mice, and these decreases in IR levels in both brain and liver are associated with, and apparently dependent on, increases in PKC-λ/ι activity and aPKC-dependent increases in BACE1 levels and proteolytic activity, as aberrant elevations in BACE1 levels and subsequent deficiencies in IR levels are abrogated by the inhibition of aPKC.

In addition to the above-described aPKC/BACE1-dependent degradative mechanism for the decreases in IR levels, the activity of the IR may also be diminished by inhibitory phosphorylation of the IR that is provoked via negative feedback mechanisms that occur during actions of a wide variety of factors, many of which are likely to be increased and operative in damaged AD tissues.

These factors include lipids, such as ceramide and phosphatidic acid (PA), both of which directly activate aPKCs; cellular stress, inflammatory, and oxidative factors that activate phospholipases C and D (PLC and PLD) that produce diacylglycerol (DAG) and PA, respectively (note that DAG and PA can interconvert); DAG-sensitive conventional (c) and novel (n) PKCs (α,β,γ,δ,ε,θ) that phosphorylate and diminish activity of the IR and/or IRS-1 which activates PI3K. Also note that negative feedback on the IR and IRS-1 can be mediated by phosphorylation provoked by a variety of signaling factors, including Akt, aPKC, mammalian target of rapamycin (mTOR), and ERK, all of which are activated by insulin and various polypeptides that operate via PI3K. In this regard, note that aPKC, and Akt and ERK are all reportedly hyperactivated in brains of humans with nondiabetic AD (12), and any of these downstream factors may negatively feedback on the IR.

Finally, note that the *partial* resistance at the IR level in nondiabetic AD brain is in fact readily overcome by elevated insulin levels (12), which may be acting via "spare" IRs, that is, present in excess of that needed for maximal activation of downstream factors; alternatively or additionally, IGF-1 receptors may also be activated by elevated insulin levels and may contribute to the activation of IRS-1/PI3K, Akt, and aPKC. And, in this regard, note that, despite decreases in IR number that we recently observed in brains of obese/MetS/T2D HFF mice, the IR is nevertheless *maximally* activated by hyperinsulinemia in HFF mice (13), apparently by the activation of "spare" IRs. Further note that the treatment of HFF mice with inhibitors of brain aPKC (discussed later) diminished brain BACE levels and simultaneously increased brain IR levels.

## HYPERINSULINEMIA IN INSULIN-RESISTANT FORMS OF OBESITY AND T2DM AS A RISK FACTOR FOR AD

With the prevailing assumption that the brain itself is insulin resistant and more importantly *hypo-insulinized* in AD, we were surprised to find (14), in whole brain and individual neurons of the anterior cortex and hippocampus of *systemically* insulin-resistant, hyperinsulinemic HFF mice, ob/ob mice, and, very importantly, monkeys with long-standing, diet-dependent obesity/T2D, that insulin signaling to both Akt and aPKC, and to all examined Akt substrates, viz, glycogen synthase kinase-2β, mTOR, and forkhead homeobox factors (FoxO1, FoxO3a, and FoxO4), is uniformly increased (Figure 1). Indeed, increased Akt/aPKC signaling in obese/T2D HFF and ob/ob mice appears to be maximal, as insulin treatment (which rapidly, over 15 min, maximally increases brain Akt and aPKC activities in normal mice) did not elicit further increases in these activities in hyperinsulinemic HFF and ob/ob mice (13, 14).

As alluded to, we moreover found that the brain IR itself, despite a reduction in total levels, is nevertheless maximally activated (as per total phosphotyrosine content of the IR-β subunit) by the hyperinsulinemia that exists in HFF obese/T2D mice (13). It therefore seems clear that any IR deficiency that is present in the brains of HFF obese/T2D mice is readily bypassed by the activation of "spare" IRs by hyperinsulinemia in HFF mice (13).

Finally, note that the hyperinsulinemia as such in insulin-resistant mice appears to be largely/fully responsible for increases in brain IR and Akt and aPKC signaling, as

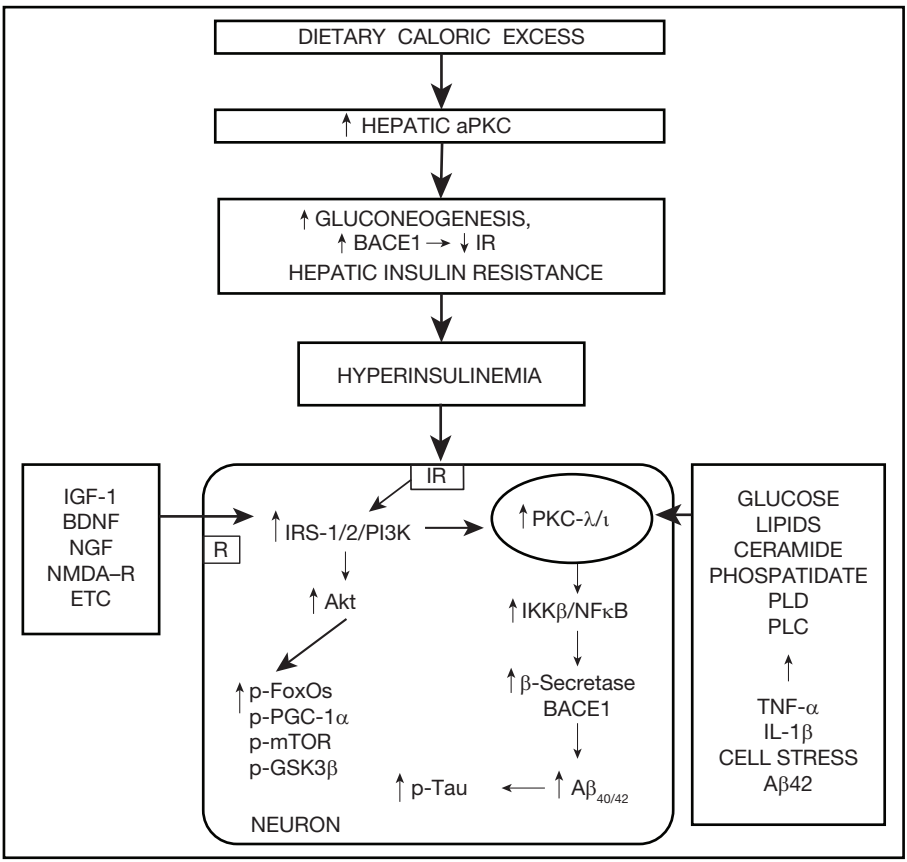


**Figure 1. Atypical PKC (aPKC)-dependent risk factors in Alzheimer's disease.** Activity of neuronal aPKC (encircled) can be excessively activated by (i) diet/liver/aPKC-dependent hyperinsulinemia acting on insulin receptors (IRs) and IR substrates (IRS-1/2), (ii) other factors (left box) acting on noninsulin receptors (R) that similarly activate phosphatidylinositol 3-kinase (PI3K) by IRS-1/2 and/or other factors, and (iii) various agents and metabolites (right box) that operate directly or indirectly via phospholipases D and C to increase levels of ceramide and phosphatidic acid, which directly activate aPKC. In turn, aPKC acts via inhibitor of kappa-B kinase-beta (IKKβ) to increase the activity of nuclear factor kappa-beta (NFκB), which acts directly to increase β-secretase (aka, BACE1) transcription, and thus increase levels of Aβ-peptides and phospho-tau, that is, precursors of Alzheimer plaques and tangles. Note that, although not shown, NFκB also increases transcription and levels of proinflammatory cytokines, tumor necrosis factor-alpha (TNF-α) and interleukin 1-β (IL-1β), and perhaps other cellular stress/oxidant/inflammatory factors, which, along with increases in Aβ42 peptides, act, as depicted (right box), to increase aPKC activity, perhaps creating multiple vicious cycles.

the correction of hyperinsulinemia (elicited by improvement in hepatic abnormalities following treatment with aPKC inhibitor, aurothiomalate [ATM] (14), in doses that selectively act in liver [but not in brain] to fully ameliorate obesity/MetS/T2DM and correct hyperinsulinemia (16)) was attended by (i) return of all brain insulin signaling aberrations, including increases in activities of Akt, Akt substrate, and aPKC, to their normal resting/basal levels and (ii) restored ability of insulin to acutely and fully activate both Akt and aPKC in the brain (14). Obviously, unlike aPKC inhibitors

1H-**i**midazole-4-**c**arboxamide, 5-**a**mino-1-[2,3-dihydroxy-4-[(**p**hosphono-oxy) methyl]cyclo**p**entyl-[1R-(1a,2b,3b,4a)] (ICAPP), 1H-**i**midazole-4-**c**arboxamide, 5-**a**mino-1-[2,3-dihydroxy-4-[(hydroxyl)methyl]cyclo**p**entyl-[1R-(1a,2b, 3b,4a)] (ICAP), and 2-**a**cetyl-**c**yclo**p**entane-1,3-**d**iketone (ACPD) (see later), ATM did not cross the blood–brain barrier (BBB) in these studies.

Accordingly, as depicted in Figure 1, we postulate that persistent hyperactivation of brain Akt in insulin-resistant states and phosphorylation/inhibition of activities of all brain FoxOs (1/3a/4/6) and PGC-1α (14) may be problematic over time, as their transcriptional actions are needed to maintain cognitive functions and neuronal integrity (see discussion in (14)). On the other hand, Akt may also have more acute beneficial effects on memory and overall brain metabolism and may also function as an antiapoptotic agent. In any case, the hyperactivation of brain aPKC, and subsequent increases in BACE1, $A\beta_{1-40/42}$, and phospho-tau (p-tau) (13, 14) most likely have detrimental effects.

To summarize, systemic insulin resistance that originates in the liver due to diet-dependent caloric excess (15) or in the brain appetite/energy center as in leptin-deficient ob/ob mice (17), or in muscle that is defective in glucose transport (16) is accompanied by key abnormalities in liver that includes the hyperactivation of hepatic aPKC, aPKC-dependent increases in gluconeogenesis and lipogenesis, and development of hyperinsulinemia. In turn, hyperinsulinemia leads to chronic *hyperinsulinization* of the brain, which, by hyper-activating brain aPKC, increases levels of the factors that produce pathological plaques and tangles in AD, that is, BACE1 expression, BACE1 activity, and BACE1-dependent increases in Aβ-peptides and p-tau. Obviously, this hypothesis is at odds with postulates that the brain itself is uniformly insulin resistant, unresponsive to insulin, and hypoinsulinized in hyperinsulinemic states of obesity. MetS and T2DM, and brain insulin resistance *per se* increase AD pathology in these states (19–22). In any case, liver involvement plays a critical role in causing systemic insulin resistance, and liver abnormalities are dependent on excessive activation of *hepatic* aPKC (15–18). This dependence on hepatic aPKC explains how treatment with inhibitors of hepatic aPKC and correction of hyperinsulinemia can reduce brain aPKC activity and thereby improve BACE1, $A\beta_{1-40/42}$, p-tau, and memory alterations (13, 14).

On the other hand, in AD that is not associated with hyperinsulinemia, for example, in lean nondiabetic subjects, there may well be an impairment in brain IR activity or a deficiency of brain IR, which limits insulin action in the brain, that is, in response to normal or low blood insulin levels. As discussed, this, of course, may occur in nondiabetic, normo-insulinemic AD and possibly in later stages of T2D-related AD if and when insulin levels are reduced to normal or subnormal levels. These differences in ambient insulin levels may explain how some, but not all, studies suggest that nasal insulin treatment, as well as subsequent brain insulinization, has beneficial effects in AD (19–22).

## EFFECTS OF DAG-SENSITIVE PKCs (α,δ,ε) VERSUS aPKC-λ/ι ON BACE1 AND AD

In addition to $PIP_3$-activated aPKCs, DAG-activated conventional (c) cPKCs and novel (n) nPKCs can influence AD pathology in a variety of ways. For example,

the activations of both DAG-sensitive cPKC-α and nPKC-ε diminish the amyloidogenic pathway and limit $A\beta_{1–40/42}$ production by activating α-secretase, which cleaves βAPP between sites cleaved by β-secretase and γ-secretase, thus producing shortened, nonamyloidogenic peptides (1, 2, 23, 24). In this regard, note that insulin activates all DAG-sensitive PKCs in muscle, liver, and adipose tissues, but whether this occurs in brain is uncertain and is in need of study.

Additionally, the activation of PKC-α may diminish $A\beta_{1–40/42}$ production indirectly by restraining PKC-ε-dependent βAPP expression, as it has been reported that phorbol-ester-induced *deficiency* of PKC-α is accompanied by increases in PKC-ε, βAPP expression, βAPP accumulation in the TGN, and subsequent increases in BACE1-dependent $A\beta_{1–40/42}$ production (25).

On the other hand, some LO-AD patients have gain-of-function mutations in PKC-α that diminishes cognitive/memory functions by altering synaptic activity (26). However, in the absence of this mutation, it is unknown if there are similar memory problems that result from PKC-α activation arising from (i) *de novo* synthesis of PA/DAG owing to increases in glucose and/or fatty acids, (ii) PLC-produced DAG, or (iii) increases in levels of blood insulin, which activates all c/nPKCs, including PKC-α, by a variety of mechanisms in muscle, liver, and adipose tissues (information on brain c/nPKCs during insulin action is lacking).

In marked contrast to the potentially beneficial effects on AD conferred by the activation of DAG-sensitive aPKC-α and nPKC-ε and subsequent activation of α-secretase, the effects of the DAG-sensitive nPKC, PKC-δ, are much different, in that PKC-δ overexpression increases, and PKC-δ knockdown decreases, BACE1 levels and $A\beta_{1–40/42}$ production in neuronal cells (27). Further, like the increases in activity or levels of PKC-λ/ι in brains of humans with AD (12), PKC-δ levels are increased and correlate with increases in BACE1 levels in brains of AD humans (27).

And, very interestingly, inhibition of PKC-δ by *rottlerin* simultaneously protects Tg/APP/PS1/AD mice from developing increases in BACE1 and Aβ-peptide/plaque production, and aberrations in cognition/memory functions (27). However, note that, like PKC-λ/ι, which activates NFκB in liver (15–17, 28) and at least certain other tissues, for example, lymphocytes (29) (and presumably in brain, as discussed further later), PKC-δ similarly activates brain/neuronal NFκB (27), which, in turn, increases the expression of proinflammatory cytokines, TNF-α, and IL-1β, which, in turn, activate NFκB by a *PKC-λ/ι-dependent* mechanism (30). And, as PKC-δ is also activated by NFκB (27), this suggests operation of a vicious cycle and raises the possibility that the activation of PKC-λ/ι by TNF-α and IL-1β may explain or contribute to BACE1 increases and AD-abetting effects of PKC-δ overexpression (see Figure 2).

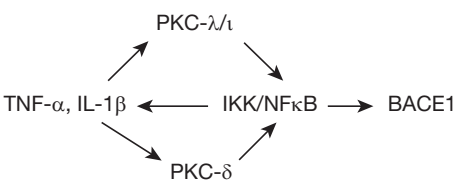


**Figure 2. Potential interplay of aPKC, PKC-λ/ι, nPKC, and PKC-δ, to operate upstream and downstream of IKKβ/NFκB and act cooperatively in a vicious cycle to increase BACE1 transcription.**

Also note that, in the previously cited PKC-δ study (27), BACE1 mRNA and protein levels were substantially increased in Tg/APP/PS1/AD mice, and inhibition of PKC-δ activity by rottlerin reduced BACE1 levels back to normal levels in Tg/APP/PS1 mice, but had little or no effect on basal BACE1 levels in WT cells. This partial reduction of elevated BACE1 levels to normal in Tg AD mice contrasts with the marked (up to 80–90%) reductions of basal/resting levels of BACE1 protein and mRNA seen in both brain and liver of mice following chemical inhibition or haploinsufficiency of PKC-λ. Thus, PKC-λ may be more potent than PKC-δ in regulating BACE1 transcription.

In this regard, further note that, in adipocytes, PKC-λ and PKC-δ were found to physically interact, and the PKC-δ inhibitor, used in the aforesaid study (27), rottlerin, was found in another study (31) to block insulin-stimulated translocation of Glut4-containing vesicles to the plasma membrane *independently of PKC-δ*, which, in fact, is *not* required for this process in adipocytes (31). Thus, rottlerin may have also inhibited PKC-λ, which is clearly required for Glut4 translocation in adipocytes (31). Or, stated differently, rottlerin is not a specific inhibitor of PKC-δ, and the ability of rottlerin to improve AD pathology in Tg/APP/PS1/AD mice may reflect inhibition of PKC-λ (or other factors that operate via NFκB), rather than PKC-δ. Alternatively, both PKC-λ and PKC-δ may work together, cooperatively or additively, to regulate BACE1 expression.

Finally, note that we have found that PKC-λ/ι activity is increased in brains of APP/PS1/AD mice, and inhibition of PKC-λ/ι with a relatively specific inhibitor, which clearly does not inhibit PKC-δ, is effective in improving memory impairments and for reducing Aβ-peptide and Aβ-plaque levels. This, of course, suggests that PKC-λ/ι is required for BACE1-dependent AD development in Tg AD mice, and AD-promoting effects of PKC-δ overexpression could conceivably involve PKC-λ/ι activation by the above-described circular pathway.

Nevertheless, as alluded to, it is possible that both PKC-δ and PKC-λ/ι are activated in the AD process, and, moreover, both may participate in the activation of NFκB, which, in turn, via TNF-α and IL-1β, may further activate both PKC-δ (32) and PKC-λ/ι (30) in the aforesaid circular "vicious" cycle. This possibility becomes even more interesting in light of the observation that increases in the activity of both PKC-δ and PKC-λ/ι/ζ, but not PKC-α, are seen in T-lymphocytes isolated from AD humans, but only when stimulated *ex vivo* with $A\beta_{1-42}$ (33,34); this suggests that neurotoxic $A\beta_{1-42}$ (Aβ42) activates both PKC-δ and PKC-λ/ι/ζ, and this brings into play an interaction between the proliferative actions of PKC-λ/ι and the proapoptotic effects of PKC-δ, a combination that, when more fully activated, may enhance and hasten AD pathology development. The latter possibility, featuring a triad of increases in PKC-λ/ι, PKC-δ, and NFκB that are put into high-gear when Aβ42 levels reach a threshold may provide an explanation for the rapid downhill phase of AD that follows a slow inductive process.

## aPKC INHIBITORS

By high throughput screening, a number of compounds have been identified, which target a site at or near the substrate and/or the ATP-binding site of aPKCs, and potently inhibit both recombinant aPKCs and aPKC activity of cultured

neurons and hepatocytes, but not myocytes. Among others yet to be studied, three such agents have been studied in detail: (i) ICAPP, which potently inhibits both recombinant PKC-λ/ι, and neuronal and hepatocyte PKC-λ/ι (IC50, 1–10 nM); (ii) ICAP, which is converted to active phosphorylated ICAPP intracellularly by adenosine kinase and is similarly potent for PKC-ι/λ in neurons and hepatocytes ($IC_{50}$, 10–100 nM); and (iii) ACPD, which comparably inhibits recombinant forms of both PKC-λ/ι and PKC-ζ, and their activities in isolated neurons and hepatocytes ($IC_{50}$, 10–30 nM) (15–17, 28, 35). Note that, whereas ACPD inhibits PKC-λ/ι and PKC-ζ with equal potency, ICAPP and ICAP preferentially inhibit PKC-λ/ι. Also note that the full-length, 70 kDa aPKC in brain, is largely if not entirely PKC-λ/ι, as, unlike peripheral non-CNS PKC-ζ, brain PKC-ζ lacks an inhibitory regulatory domain and exists largely as a shortened, 50 kDa, constitutively-active moiety, called PKMζ, which is particularly important in long-term memory (LTM) functions, as discussed later. Further note that ICAPP, ICAP, and ACPD do not inhibit recombinant forms of conventional/novel (c/n) PKCs (α,β,δ,ε,θ) (15–17, 28, 35).

During *in vivo* usage, in doses that are sufficient to effectively inhibit hepatic aPKC and largely, albeit not completely, reverse post-IR hepatic aberrations seen in mouse obesity/MetS/T2D models (15–17), the aforesaid inhibitors, ICAPP, ICAP, and ACPD, do not inhibit muscle, adipocyte, or brain aPKC, and, in all tissues, they have no inhibitory effects on Akt, or on AMPK. These inhibitors also have no effect on activities of an array of 35 other kinases independently tested by Life Technology Selectscreen Profiling, Madison, WI (15–17, 28, 34). We therefore believe that these agents have reasonable selectivity for aPKC, but as with all drugs, exclusive targeting cannot be assumed.

On the other hand, we have verified that partial heterozygous KO of total body PKC-λ in the mouse (total homozygous KO is embryonic lethal), and thus haploinsufficiency of PKC-λ, has biological effects similar if not identical to those of the aforesaid chemical aPKC inhibitors in liver (36) and in brain: for example, PKC-λ haploinsufficiency (i) in liver blocks all hepatic diabetic aberrations induced by high-fat-feeding (36) and (ii) in brain markedly diminishes BACE1 levels and insulin-stimulated production of Aβ-peptides. In addition, liver-specific KO of PKC-λ (by administration of adenovirus encoding Cre-recombinase to PKC-λ-floxed mice) or liver-specific inhibition of hepatic aPKC by adenovirus-mediated expression of kinase-inactive aPKC has biological effects in liver similar if not identical to those of the aforesaid chemical aPKC inhibitors on obesity/MetS/T2DM (18, 36, 37). We therefore are confidant that aPKC inhibition underlies all biological effects of ICAPP, ICAP, and ACPD that we have reported in both liver and brain.

However, at higher *in vivo* doses, ICAPP, ICAP, and ACPD inhibit brain, as well as liver, aPKC (13). Thus, in insulin-resistant, hyperinsulinemic states, these agents can reduce brain aPKC activity (i) at lower doses, *indirectly* via inhibition of liver aPKC and reduction of blood insulin levels, and (ii) at higher doses, by *directly* inhibiting brain aPKC. In this regard, note that, in published (38) and other pharmacokinetic/pharmacodynamic (PK/PD) studies, it was found that ICAP preferentially distributes to liver, but clearly passes the BBB to enter the brain. And, as found in other studies, ICAP is effective in liver and brain when given insufficiently increased doses by oral gavage (i.e., *per os* [PO]), as well when administered IV or SC. Moreover, when administered to mice intranasally, ICAP

inhibits brain, but not liver, aPKC, suggesting that ICAP reaches the brain through neurovascular bundles that traverse the sphenoid sinuses and the cribriform plate; this raises the possibility that nasally administered aPKC inhibitors (or other agents) may be used to selectively target brain aPKC during AD treatment.

Furthermore, in cultured human neuronal cells and/or mouse hippocampal slices, ICAPP fully inhibits insulin-stimulated increases in activities of 70 kDa PKC-ι/λ and BACE1, and increases in the production of $A\beta_{1-40/42}$ by approximately 50% at 10 nM and 90–100% at 100 nM, without inhibiting the putative memory protein, that is, constituently-active 50 kDa PKMζ (13), which was alluded to earlier and discussed more fully later. And, in cultured human neuronal cells, ICAPP, at low 10 nM concentrations, fully blocked 2–3-fold increases in BACE1 *levels* that were provoked by 24-h insulin treatment. It therefore seems clear that prolonged treatment with high-dose insulin acts directly via aPKC to induce remarkably strong increases in neuronal BACE1 levels. This bolsters our contention that hyperinsulinemia is an important risk factor for AD. It also seems very clear that aPKC inhibitors are working directly in neurons to alter BACE1 and BACE1-dependent alterations in Aβ-peptide production.

## aPKC REQUIREMENTS FOR MEMORY FUNCTION

As alluded to, a number of findings suggest that the 50 kDa, constitutively active, brain-specific form of PKC-ζ, that is, PKMζ (described earlier), functions in long-term potentiation (LTP) and LTM formation (39, 40). However, although PKMζ KO in brain does not impair LTP/LTM, this appears to be explained by a compensatory increase in PKC-λ/ι in PKMζ-null mice, as suggested by the fact that LTP/LTM is impaired by selective inhibition of PKC-ι/λ by ICAP in PKMζ-null mice, *but not in normal mice,* wherein ICAP does not inhibit PKMζ (40). And, from the lack of effect of ICAP on LTP and memory function in normal mice (40), it may be argued that PKC-λ/ι is not required for *on-going* LTP/LTM.

Indeed, in HFF mice, as reported in (13), aPKC inhibitor ACPD improved high-fat-diet-induced decreases in acute memory function (as per Novel Object Recognition [NOR]) while blocking insulin-stimulated increases in PKC-ι/λ activity, but largely sparing basal PKC-ι/λ activity. Moreover, in other studies, a 50% loss of brain PKC-λ in PKC-λ haplo-insufficient mice, and subsequent decreases in PKC-λ activity and BACE levels (80–90%) did not impair training and memory functions in Radial Arm Water Maze (RAWM) and NOR tests. It thus appears that brain aPKC activity can be partially diminished to levels that markedly reduce AD development, without impairing memory processes.

Nevertheless, it should be noted that, whereas a *marked and chronic loss* of PKC-ι/λ in a long-term, hippocampus/anterior-cortex-selective KO study showed essentially normal memory function and LTM/LTP, which was attributed to PKMζ compensation, *acute KO* of hippocampal/anterior-cortical PKC-λ (and thus lacking time for compensatory changes) suggested a need for PKC-λ, in learning/memory processes, but only in more difficult (nonstandard) learning tasks (41). Furthermore, *acute 1-month knockdown* of PKC-λ produced specifically in the hippocampus by stereotactic injection of virus encoding shRNA, suggested that hippocampal PKC-λ is needed for the initiation of LTP, short-term memory (as per contextual and trace

fear testing), and consolidation of LTM (42). Thus, these inhibitory effects on learning and memory may be exaggerated by *acute*, and thus uncompensated, and *excessive* losses of PKC-λ, since (i) as discussed, *partial* inhibition of PKC-λ/ι and simple reduction of elevated brain PKC-λ activity to normal by aPKC inhibitor treatment improve memory loss (in NOR) in hyperinsulinemic HFF mice (13) and (ii) chronic 50% loss of PKC-λ in PKC-λ-haploinsufficient mice did not impair learning/memory performance in RAWM and NOR tests.

It is, of course, interesting that brain PKC-λ/ι and PKMζ (and perhaps other PKCs) can apparently compensate for losses of each other over time in the adult mouse brain, and these compensatory changes can satisfactorily maintain relatively normal memory functions. In any case, these caveats of aPKC inhibition or deficiency need to be kept in mind.

## aPKC ACTIVATORS

Atypical PKCs are activated not only by insulin and other polypertides (IGF-1, NGF, BDNF, NMDA-receptor activators (43–47)) that activate PI3K and thereby produce PI-3.4.5-$(PO_4)_3$ ($PIP_3$) which directly activates aPKCs (48) but also by C:14 and C:16 ceramides which directly activate aPKCs (15) (higher C:20–24 ceramides may be inhibitory); PLD-derived phosphatidic acid (PA), which, like $PIP_3$, contains an acidic head group and directly activates aPKCs (49, 50); various factors, such as Aβ42 (33, 34) and certain oxidants (e.g., sorbitol (49)) and inflammatory factors (e.g., lipopolysaccharide (51)) which presumably activate PLCs and/or PLD to produce PA. Note that many of these metabolites and agonists are thought to abet AD development.

## CONTROL OF BACE1 TRANSCRIPTION BY NFκB

There is clear evidence that NFκB is a strong, if not the major, transactivator of the BACE1 gene in mouse brain (52–54). This is of considerable interest, as we have shown, that hyperinsulinemia in various O/MetS/T2D states (e.g., HFF, ob/ob, and HetMλKO mice (15–18, 36, 37) and T2D humans (28)) leads to, in liver, aPKC-dependent increases in (i) phosphorylation/activation of inhibitor of kappa-B kinase-β (IKK-β), (ii) phosphorylation and thus dissociation of the inhibitor of NFκB inhibitor (IκB) from NFκB (and, presumably, subsequent degradation of IκB), (iii) transfer of the active p65/RelA subunit of NFκB into the nucleus, (iv) p65/RelA phosphorylation and activation, as per electrophoretic mobility shift assay, and (v) NFκB-dependent transcription of genes encoding various proinflammatory cytokines, including TNF-α and IL-1β, that is, with increases in their mRNA and protein levels. These changes are clearly provoked by *an aPKC-dependent mechanism*, as shown by inhibition of aPKC by chemical inhibitors and expression of kinase-inactive-aPKC, and by various PKC-λ KO methods.

In particular, note, in normal WT mice, acute insulin treatment over 15 min increases nuclear levels of the p65/RelA subunit of NFκB, and simple feeding-induced increases in insulin provoke increases in mRNA levels of TNF-α and

IL-1β; *and* all effects of insulin and feeding are blocked or markedly diminished in littermate mice haploinsufficient for PKC-λ (35). And, perhaps most importantly, we found in both hepatocytes harvested from humans with long-term preexisting T2DM, and following acute 4-h insulin treatment of normal human hepatocytes, that there were increases in TNF-α and IL-1β mRNA levels by an aPKC-dependent mechanism (28). Note that others have also shown that aPKCs are major activators of NFκB during inflammation and lymphocyte activation (29).

We therefore strongly suspect that aPKC increases BACE1 transcription by activating NFκB in hepatocytes and neurons by the following mechanism and linear pathway:

(i) In liver, dietary caloric excesses → hepatic aPKC activity → IKKβ activity → NFκB activity → BACE1 levels → IR degradation → systemic insulin resistance → hyperinsulinemia → activation of hepatic spare IRs → post-IR hepatic aberrations → hyperinsulinemia → and so on in a vicious cycle

(ii) and, in brain, hyperinsulinemia, cellular stress/oxidant/inflammatory factors, $A\beta_{42}$, and so on → aPKC activity → IKKβ activity → NFκB activity → BACE1 levels → Aβ-peptides and p-tau, and IR degradation (that is partial and can be bypassed by hyperinsulinemia) → AD pathology.

## CONCLUSION

It seems abundantly clear that AD risk in obesity MetS/T2DM is real, and this risk at least partly results from hyperinsulinemia-induced increases in brain aPKC activity that provokes increases in BACE1 levels and production of Aβ-peptides and p-tau. Increases in brain aPKC activity and BACE1 activity/levels can also proteolytically diminish brain IR levels, but hyperinsulinemia in all mouse and monkey models of obesity/MetS/T2DM that we have examined does not diminish IR-dependent Akt activity; indeed, despite decreases in brain IR levels, brain IR and Akt activities are increased (apparently via "spare" IRs) by hyperinsulinemia and/or other factors.

On the other hand, increases in BACE1 activity and levels that occur in *nondiabetic* AD can not only increase Aβ-peptide and p-tau levels but presumably can also diminish brain IR levels, and this may diminish *insulin-stimulated* Akt activation in norm- or hypoinsulinemic subjects. On the other hand, activities of both Akt and aPKC are reportedly elevated by uncertain noninsulin factors in the brains of humans with nondiabetic AD, and this increase in aPKC activity can increase BACE1 levels and activity in these AD brains.

Accordingly, there is considerable evidence to suggest that brain aPKC activity is increased in both diabetes-related and nondiabetic forms of LO-AD. And initial findings in mice suggest the feasibility of using chemical aPKC inhibitor treatment to reduce BACE1, Aβ-peptides, and p-tau. Alternatively, treatments that target factors that activate aPKCs, or factors that operate between aPKC and BACE1, that is, at levels surrounding NFκB, may also be effective in AD treatment and should be considered. In any event, the continued elucidation of signaling pathways that are operative in AD pathogenesis is likely to open new avenues for the development of effective treatments for AD.

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7

# Modeling the Blood–Brain Barrier to Understand Drug Delivery in Alzheimer's Disease

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**Abstract:** The blood–brain barrier is a semipermeable barrier structure that lines the walls of brain microvessels. Although the blood–brain barrier plays a key role in protecting the brain from unwanted molecules, it simultaneously challenges the delivery of drugs into the brain. In addition, the blood–brain barrier has been shown to be dysfunctional in Alzheimer's disease, the most common cause of dementia for which there is no cure. Mouse models of Alzheimer's disease have played a central role in investigating disease-specific changes in the blood–brain barrier, but the translation of findings from mouse models into the human system is hindered by interspecies differences. In an effort to develop new drug delivery techniques and/or understand changes in the human blood–brain barrier in Alzheimer's disease, several human blood–brain barrier *in vitro* models have been developed. These comprise primary and immortalized human endothelial cell-based models as well as human induced pluripotent stem cell-derived brain microvascular endothelial cell models. Both two- and three-dimensional

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(2D and 3D) culture platforms have been established to better mimic the complexity of the brain. This chapter discusses the current blood–brain barrier models, their advantages and disadvantages as well as their potential to understand drug delivery in Alzheimer's disease.

**Keywords:** Alzheimer's disease; astrocyte cell culture model; blood–brain barrier; brain endothelial cell; induced pluripotent stem cell

## INTRODUCTION

The blood–brain barrier (BBB), formed by tightly-sealed brain endothelial cells (BECs), serves as a selectively permeable membrane at the blood–brain interface. It allows for the delivery of oxygen and nutrients into the brain and at the same time, maintains a highly controlled brain milieu by preventing the entry of neurotoxic blood components into the brain and transporting metabolic waste products from the brain to peripheral circulation (1). Furthermore, the brain vascular system supports the activity of neuronal networks through increased blood flow and oxygen supply, a mechanism termed as neurovascular coupling (2).

Integrity of the BEC layer is critical for the barrier function of the BBB and is achieved by the presence of tight and adherens junctions (AJ) between BECs. Tight junctions (TJ) consist of claudins-3,-5, and -12, occludin, and TJ associated *zona occludens* (ZO-1 and ZO-2) proteins (3). AJ are formed by vascular endothelial cadherin (VE-cadherin) and platelet endothelial cell adhesion molecule-1 (PECAM1) (3). Synergistically, TJ and AJ proteins ensure high transendothelial electrical resistance (TEER) of the BBB (1000–2000 ohm/cm$^2$ compared to 10 ohm/cm$^2$ in peripheral capillaries) and restrict paracellular permeability to molecules under 500 Da (4). Compared to peripheral endothelial cells, BECs also have more mitochondria and less fenestrations and pinocytic vesicles, further limiting the exchange of solutes across the BBB (4).

In addition to TJs and AJs, BECs express a unique selection of transporters, which either transport nutrients to the brain or transport molecules back to the blood. Solute carrier mediated transport (CMT) enables transendothelial exchange of organic cations and anions, carbohydrates, vitamins, fatty acids, nucleotides, amino acids, and hormones, whereas receptor-mediated transcytosis (RMT) facilitates the transport of peptides and proteins across the BBB, including insulin, transferrin, and apolipoproteins (1). ATP-binding cassette (ABC) transporters, including, P-glycoprotein (P-gp), multidrug resistance-associated proteins (MRPs), and breast cancer resistance protein (BCRP), protect the brain from the accumulation of xenobiotic compounds and drugs *via* their active efflux from BECs to the blood, and also greatly hinder drug delivery across the BBB (5).

Finally, to support BBB integrity, BECs are ensheathed by pericytes and astrocyte end-feet, and may create connections with microglia, neurons, and neural stem cells, which together form the so-called neurovascular unit (NVU) (Figure 1A) (1). Pericytes cover an approximate 1/3 of the BEC monolayer and can contract or relax cell membrane extensions to locally change cerebral blood flow in response to neuronal activity (6). Pericytes can also guide the polarisation of astrocyte end-feet, modulate TJ protein expression and permeability, participate in BBB immune responses, and regulate clearance of neurotoxic substances, such

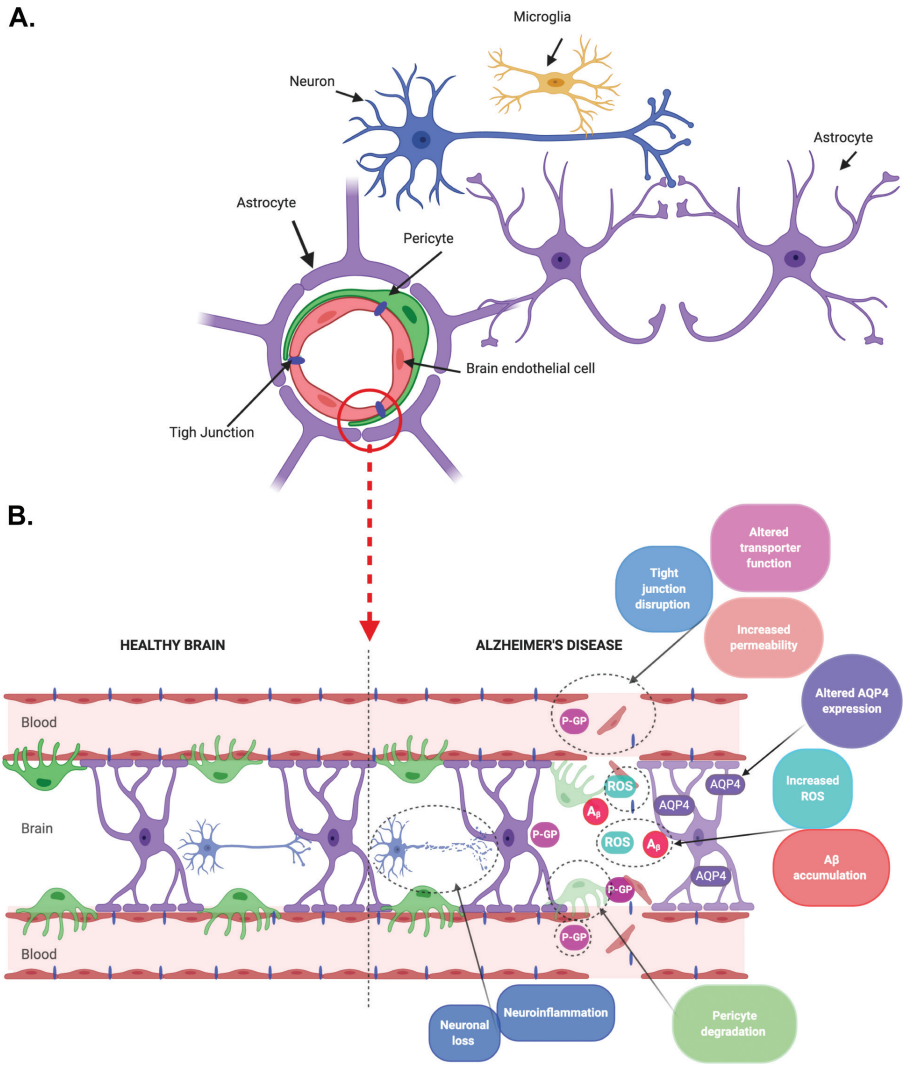


**Figure 1. Cellular structure and Alzheimer's disease (AD) specific changes in the blood–brain barrier (BBB).** (A) Schematic representation of the BBB structure. The BBB is formed by brain endothelial cells (BECs) with astrocytes and pericytes functioning as key supporting cells. The BBB and other brain cells (neurons and microglia) form the neurovascular unit. (B) AD-specific changes in the BBB include altered tight junction and transporter expression in BECs, pericyte degradation, altered aquaporin-4 (AQP4) expression in astrocytes with these changes leading to increased permeability, reactive oxygen species (ROS) and neuroinflammation, and subsequent amyloid-β (Aβ) accumulation and neuronal loss. *Created using BioRender.com.*

as amyloid-β (Aβ) (6, 7). Astrocytes mediate innate immune responses on the brain barrier, provide nutrients from the blood to neurons, and regulate BBB barrier function by secreting protective factors, which modulate BEC TJ integrity (8). Astrocytes also play a primary role in the maintenance of parenchymal water and ionic homeostasis due to high expression of aquaporin 4 water (AQP4) channels

and ion transporters, contributing to clearance of interstitial solutes, including Aβ (9, 10). Together all the components comprise a highly specialized BBB unit, which plays a major role in maintaining homeostasis, demonstrates heterogeneity in disease, and poses a major hurdle for drug delivery.

## ALZHEIMER'S DISEASE RELATED CHANGES IN THE BBB

Emerging evidence suggests that BBB dysfunction, together with Aβ plaques and hyperphosphorylated tau, is the third driving pathology underlying early Alzheimer's disease (AD) and corresponding cognitive decline (11–15). During preclinical asymptomatic stages of AD, cerebrovascular dysfunction is one of the first changes preceding any other detectable alterations characteristic of AD, including Aβ and tau (12, 16). The two-hit vascular hypothesis proposes that blood vessel impairment initiates the cascade of events by causing initial BBB dysfunction, decreased cerebral blood flow, and infiltration of neurotoxic molecules that in turn lead to neuronal loss (hit 1). BBB pathology subsequently causes impaired Aβ clearance and increased production of toxic Aβ species (hit 2), which synergistically with other vascular, genetic, and environmental risk factors, leads to progression of AD pathology (17).

Interestingly, major genetic risk factors of AD, including mutations is *APP* and *PSEN1* as well as *APOE ε4* polymorphism, have been shown to contribute to BBB leakage, brain microbleeds, BEC degeneration, pericyte injury, and abnormal Aβ clearance, linking multiple pathways of vascular- and neurodegeneration (13, 14, 18, 19). Increased BBB leakage in AD has been shown in both brain imaging studies as well as post-mortem brain tissue demonstrating an accumulation of blood-derived proteins in the brain (13, 20–23). Leakage of blood-borne factors further contributes to multiple pathological pathways in the AD brain, including BBB breakdown, pericyte dysfunction, neuronal death, neuroinflammation, and increased oxidative stress (24–27).

Defective function of nutrient and efflux transporters in the BBB has also been identified in human and animal studies of AD. Impaired glucose transport and reduced glucose transporter 1 (GLUT1) expression has been shown at the BBB of individuals with mild cognitive impairment (MCI) and in transgenic murine models of AD (28–30). The brain-to-blood efflux transporter P-gp has been implicated in AD pathology with clinical studies showing decreased P-gp activity in patients with mild AD, suggesting P-gp dysfunction and corresponding xenobiotic and Aβ build-up in the brain could contribute to AD pathogenesis (31, 32). Altered expression of efflux transporters in AD has further been demonstrated in a human induced pluripotent stem cell (hiPSC)-derived BEC model of familial AD (fAD) (33).

Cell-specific changes in the NVU have also been shown in AD. Human post-mortem and cell model studies of AD have identified reduced BEC integrity, altered BEC TJ and AJ protein expression, and reduced expression in the brain endothelium of low-density lipoprotein receptor-related protein 1 (LRP1), the main receptor facilitating removal of Aβ (11, 33–35). Pericyte loss and decreased pericyte coverage of brain capillaries has also been observed in brain samples from AD individuals—an effect being amplified in *APOE ε4* carriers (11, 13, 36).

In addition, pericytes contribute to the clearance of Aβ from the brain, and their loss has been shown to decrease the age of onset of AD and accelerate the development of Aβ and tau pathology in transgenic mice models (37). Astrocyte dysfunction is also implicated in AD, with altered APQ4 expression and localization as well as increased inflammatory and oxidative stress responses reported in human and mouse models of AD (38–40).

AD-specific changes in the BBB are summarized in Table 1 and Figure 1B. These alterations highlight severe AD-specific effects on the BBB with likely important consequences on disease progression and drug delivery.

**TABLE 1 Alzheimer's disease related changed in the BBB**

| Change | Details | Model | Ref |
|---|---|---|---|
| BBB breakdown | • Increased albumin ratio in CSF/blood<br>• Increased leakage in the hippocampus<br>• Increase in blood-borne factors in the brain | Human brain imaging (MCI and APOE4)<br>Human post-mortem tissue | (11, 13, 16, 36) |
| Vascular pathology | • Reduced cerebral blood flow<br>• Cerebral amyloid angiopathy<br>• Infarcts<br>• Haemorrhages<br>• Abnormal blood vessels<br>• Increased fibrin(ogen) deposition in cortical vessels | Human brain images (LOAD)<br>Human post-mortem tissue<br>Transgenic (*PSEN1*) AD mouse model | (11, 12, 14, 15, 18, 19, 21) |
| Brain endothelium dysfunction | • Reduced integrity<br>• Reduced LRP1 expression<br>• Altered TJ and AJ protein expression<br>• Accumulation of CypA and MMP-9 | Human post-mortem tissue<br>Human fAD iBEC model | (11, 33–35) |
| Transporter dysfunction | • Reduced P-gp activity<br>• Reduced GLUT-1 expression<br>• Reduced glucose metabolism<br>• Altered efflux transporter expression | Transgenic (*APP*) AD mouse model<br>Human brain imaging (MCI and AD)<br>Human fAD iBEC model | (28–33) |
| Pericyte dysfunction | • Loss of pericyte number and coverage in the hippocampus<br>• Increased PDGFβ in the CSF<br>• Accumulation of CypA and MMP-9<br>• Upregulated calcineurin signalling | Human brain imaging (MCI)<br>Human post-mortem tissue<br>Human APOE4 iBEC model | (11, 13, 16, 36, 64) |
| Astrocyte dysfunction | • Increased AQP4 expression<br>• Altered AQP4 distribution<br>• Altered inflammatory response<br>• Altered calcium signalling<br>• Increased oxidative stress | Human post-mortem tissue<br>Transgenic (*APP*) AD mouse model<br>Human fAD iPSC-derived astrocytes | (38–40) |

AJ, adherens junction; APOE4, apolipoprotein E allele ε4 carrier; APP, amyloid precursor protein; AQP4, aquaporin 4; BBB, blood–brain barrier; CSF, cerebrospinal fluid; CypA, cyclophilin A; fAD, familial Alzheimer's disease; GLUT1, glucose transporter 1; iBEC, induced brain endothelial cell; iPSC, induced pluripotent stem cell; LRP1, low density lipoprotein receptor-related protein 1; LOAD, late onset Alzheimer's; MCI, mild cognitive impairment; MMP9, matrix metalloproteinase-9; PDGFβ, platelet-derived growth factor β; PSEN1, presenilin 1; TJ, tight junction.

## DIFFERENCES BETWEEN ANIMAL AND HUMAN BBB AND IMPLICATIONS TO DRUG DELIVERY

Due to the challenges in studying the BBB in humans, animal models have played a central role in understanding AD-specific changes in the BBB and modeling delivery of brain targeting therapeutics (1, 41). Animal models have allowed for the in-depth investigation of BBB structure and biology, which is mostly only possible in humans using post-mortem tissue. The limitation of post-mortem tissue is that it does not allow for the analysis of BBB structure and function in a living person and at early stages of disease, critical for understanding the role of the BBB at different stages of disease progression.

Although extensive research has been conducted in animal models to identify novel therapeutics for AD, successful pre-clinical studies rarely translate into humans. In fact, over 99% of AD clinical trials with drugs which provided promising results in model animal systems, have not been successfully translated into humans (41, 42). The underlying problem is that although animal models of AD express key pathological hallmarks, including Aβ and hyper-phosphorylated tau, these models do not necessarily exhibit other biological features of AD and can be considered to not have AD (42). In addition, there is a lack of animal models for sporadic forms of AD, the most common type of AD in humans (42). Finally, interspecies differences are a central hindrance to translation of therapies from animal models to humans.

The mouse is one of the most commonly used animal models in BBB research and has been central to understanding BBB development and biology (43). Structurally mouse and human brains and BBB contain the same cell types, but distinct differences between mouse and human have been reported in properties of cells located in the neocortex, such as differential morphology and gene expression (43, 44). In addition, the human neocortex is vastly larger and more complex compared to that of the mouse, which complicates drug delivery in humans. The human neocortex also contains proportionally more astrocytes than the mouse cortex (45), which could potentially affect BBB formation and subsequent drug delivery.

Differences between the rodent and human BBB have been reported in TJ protein (TJP) and transporter expression and function. Key TJPs including claudin-5, occludin, and ZO-1 have been reported to have higher mRNA expression in mouse BECs compared to human BECs (46). In addition, comparison of protein level expression of transporters in brain microvessels identified a clearly higher expression of some transporters, including ABC (P-gp and MRP4) and solute carrier transporters (monocarboxylate transporter 1, L-type amino acid transporter, and organic anion transporter 3) in rats compared to humans (47). Real-time brain imaging also revealed differences in P-gp-dependent uptake of drugs between rat and human brains with brain concentrations of P-gp substrates found to be higher in humans than rats (48). These results indicate higher BBB permeability in humans compared to rodents and suggest that drug delivery experiments cannot be directly translated from rodents to humans. Interestingly, differences between humans and other primates have been shown to be smaller (47), with non-human primates potentially providing a more accurate model for human drug delivery than rodents.

Interspecies differences have also been found in other cell components of the NVU. Human astrocytes are larger and exhibit differences in process complexity than corresponding rat astrocytes (49). AQP4, the main water channel in the brain expressed by astrocytes, is polarized in astrocyte end-feet surrounding the BBB to a lesser extent in human astrocytes compared to mouse (50). Mislocalization of AQP4 has been linked to AD in humans (51), thus the differential expression and localization of AQP4 between mouse and human could have implications to how AD features in mouse models. Finally, primates are unique in terms of the presence of interlaminar astrocytes, which are not found in rodents (49). The disruption of processes of interlaminar astrocytes in AD has been reported (52), highlighting an important characteristic of AD humans that cannot be replicated in rodent models.

## BBB *IN VITRO* CELL MODELS

To overcome species differences in BBB modeling, *in vitro* models of the human BBB are central to enhancing our understanding of BBB biology at a cellular level in health and disease. BBB cell models have traditionally been limited to primary and immortalized BECs (53), with hiPSC-derived BBB cells emerging as a novel approach for BBB modeling (54).

### Primary and immortalized BECs

Human and mouse primary BEC isolation has been described from both fetal and adult brain tissue (Figure 2A) (55, 56). Primary BECs are reported to express BBB markers, such as TJPs and transporters (57), providing a tool for *in vitro* modeling of the BBB. BEC isolation from AD patient post-mortem tissue has also been described, revealing disease-specific differences compared to healthy BECs (56), suggesting a potential for using primary BECs for disease modeling.

The use of primary BECs is, however, associated with multiple limitations. Isolation of BECs from brain tissue is challenging as the proportion of BECs from all brain cell types is low (approximately 1–2%), easily resulting in contamination by unwanted cell types (55). In addition, the availability of tissue from patients is limited with donor-to-donor variability and ethical considerations posing challenges (57). Withdrawing BECs from their *in vivo* tissue and culturing them *in vitro* has also been reported to result in the loss of TJ markers and reduced transporter expression (58, 59). Passaging primary BECs has been reported to result in reduced TEER and an unstructured monolayer with loss of localized TJP expression (53), limiting the time that these cells can be utilized for experiments.

Immortalized human BEC lines (such as the hCMEC/D3 line) have helped overcome some of the challenges associated with primary BECs (Figure 2B) (60). Advantages of immortalized BEC lines include their ability to maintain cell properties over multiple passages, high viability, and expression of brain endothelium-specific transport systems, making them an ideal model to perform high-throughput screening of new drugs targeting specific transporters and/or receptors (61). However, immortalization has been shown to affect the cell phenotype compared to primary BECs, including highly upregulated expression of genes related to

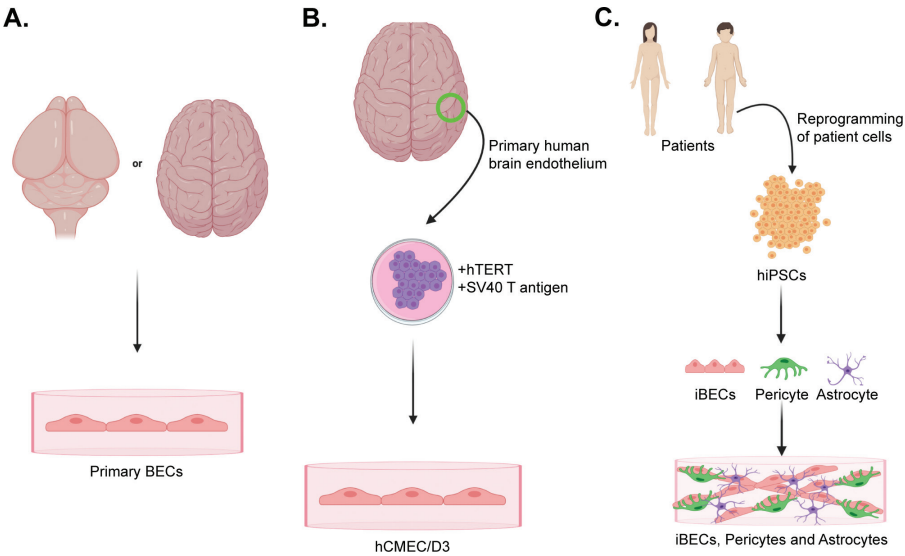


**Figure 2. Brain endothelial cell (BEC) sources for blood–brain barrier (BBB) modeling.** (A) Primary BECs are isolated directly from the brain with (B) immortalized BEC lines (e.g., hCMEC/D3) generated via the introduction of immortalization factors. (C) An alternative source of BECs are human induced pluripotent stem cells (hiPSC), which are generated by reprogramming patient-derived cells. hiPSCs can be used to generate all BBB cell types, including iBECs, pericytes and astrocytes. *Created using BioRender.com.*

nucleic acid processing and repair as well as interferon signaling (46). In addition, differential cell growth and altered gene expression of TJPs, receptors and transporters has been reported between immortalized and primary BECs (46). A major limitation of immortalized BECs is that they do not allow for the study of AD-specific changes. The overexpression of fAD mutations in *APP* and *PSEN1* in human immortalized neural progenitor cells has been described, resulting in the production of Aβ-plaques and pathological tau *in vitro* (62). A similar approach in human immortalized BECs could be possible, however, how this would alter BEC properties is unknown.

## Human-induced pluripotent stem cell derived BECs

Due to the limitations associated with primary and immortalized BEC models, hiPSC-derived models have arisen as a promising approach for *in vitro* BBB modeling (Figure 2C) (54, 63). BECs can be generated from iPSCs (iBECs) with relative ease and AD patient-derived hiPSCs allow for the study of disease-specific differences (33, 63). iBECs exhibit key characteristics of BECs, including high TEER, high expression of BBB-specific TJPs as well as expression and function of efflux transporters (33, 63). Importantly, hiPSCs from the same patient can be differentiated into other cell types of the NVU, including pericytes and astrocytes, enabling the generation of an isogenic multi-cell BBB model (Figure 2C) (64, 65). In addition, CRISP/Cas9 gene editing allows for the further examination of the

contribution of AD risk genes on BBB function (33, 64). iBECs generated from patients with familial and sporadic AD have revealed key cellular differences giving insights into AD-specific effects on the BBB and potential implications to drug delivery (33, 64).

Although hiPSC-based technologies hold enormous potential for the development of preclinical BBB models, there are a few hindrances that need to be considered. hiPSC lines often exhibit a high level of variability and their generation and maintenance is expensive (66). The genetic editing of these cells also often results in loss of patient-specific epigenetic signatures and genetic instability. Other limitations include the lack of relevant genome matched-controls and lack of maturity in iPSC-derived cells, particularly important for modeling late onset diseases (67, 68). Finally, models based on hiPSC-derived iBECs allow only for a narrow experimental window, since cells tend to de-differentiate rapidly (54). Despite these limitations, continuous advancements in hiPSC research and commercially available reprogramming kits and cell culture reagents have the ability to ensure standardized culture conditions and the production of high quality hiPSC lines for BBB research.

## *IN VITRO* CULTURE PLATFORMS FOR MODELING OF BBB STRUCTURE AND DRUG DELIVERY

*In vitro* platforms of the BBB are central to modeling the human BBB and to screening and developing BBB permeable drugs. BBB *in vitro* culture systems can broadly be divided into static 2D cultures and static or microfluidic 3D cultures.

### 2D models of the BBB

Static monolayer (i.e., 2D) culture systems are, to date, the most commonly used BBB *in vitro* model platforms. Most commonly, BECs (primary, immortalized or iPSC-derived) (69–71) are seeded inside a Transwell insert, in which cells are cultured on a permeable support as opposed to solid plastic (Figure 3A). The inside of the insert represents the luminal (blood) side, whereas the surrounding well, in which the insert is placed, represents the abluminal (brain) side (Figure 3A). The Transwell model allows for measurement of integrity as well as permeability of compounds through the BEC monolayer, from the luminal to the abluminal side (72).

BEC barrier integrity in the Transwell system can be increased via co-culture with pericytes, astrocytes, and other cells of the NVU usually resulting in increased TEER, higher TJP and transporter expression, promoting *in vivo*-like BBB phenotype (70, 73, 74). Co-cultures are achieved by culturing other BBB cells, such as pericytes or astrocytes, in the surrounding well, in which the BEC containing Transwell insert is placed, or on the underside of the BEC containing Transwell insert (Figure 3A) (63, 69). In this formation, cells are not in direct physical contact, as they are separated by a membrane, but will regulate each other via secreted factors. Direct contact Transwell models have also been described, where BECs and astrocytes, for example, are layered directly on top of each other, with this reported to result to higher TEER than indirect co-cultures (64, 70).

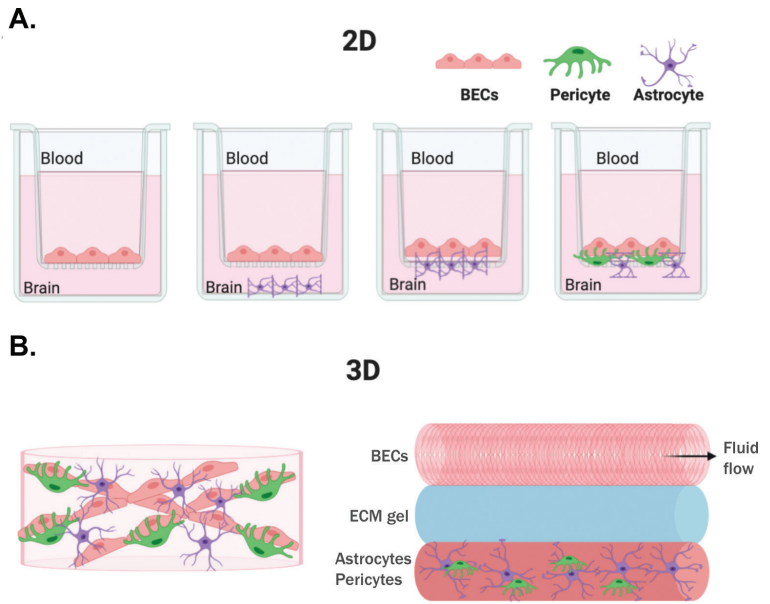


**Figure 3. Two- (2D) and three- (3D) dimensional model platforms of the blood–brain barrier (BBB).** (A) The Transwell system is the most common 2D platform for BBB modeling. In the Transwell system, brain endothelial cells (BECs) are cultured inside the Transwell insert with BBB co-cultures established by culturing other cell types (such as pericytes or astrocytes) in the surrounding well or on the underside of the Transwell insert. (B) 3D cultures can be established by embedding BBB cell types in a scaffolding matrix and allowing them to grow in a 3D conformation. In 3D and microfluidic models of the BBB, BECs are cultured in a tubular formation and the culture medium is allowed to flow through the BEC tube mimicking blood flow. Other BBB cell types (pericytes and astrocytes) are embedded in an extracellular matrix and allowed to grow adjacent to the BEC tube. *Created using BioRender.com.*

The Transwell assay has been widely used to study BBB integrity, permeability, and drug delivery (71, 72, 75). Although the Transwell assay is relatively easy to set-up and thus, widely used in BBB research, its limitations are that it lacks the complex 3D structure of the BBB *in vivo*, and BECs are cultured as a monolayer instead of a tubular structure, lacking complex cell interactions.

## 3D models of the BBB

Three-dimensional model systems of the BBB are emerging to better mimic the complexity of the BBB *in vivo*. In the brain, the BBB is a tubular structure, which is not accurately replicated using traditional 2D culture settings. Other features of the *in vivo* BBB include the complex interaction of BECs and other cells of the NVU with each other and with the extracellular matrix. In the body the BBB is also exposed to blood flow, which causes shear stress in BECs (4).

A central component of scaffold-based 3D models is using a supporting matrix (e.g., Matrigel or collagen I) that forms a gel, in which cells are able to grow in 3D conformation (Figure 3B) (76). In 3D BBB modeling, a central aim is to allow BECs to grow in a tubular formation using extracellular matrix (ECM) support (64, 77, 78).

Other cells, such as astrocytes and pericytes are then ideally cultured in direct contact with BECs, mimicking cell interactions in the body (64, 77). Vascular networks of both the healthy and AD BBB have been achieved *in vitro* by allowing BECs to self-assemble in an extracellular matrix containing 3D culture environment in co-culture with astrocytes and pericytes (64, 79). When combining microfluidic technology with a 3D growth environment, it is possible to achieve tubular *in vitro* models of the BBB that also mimic blood flow (Figure 3B). These "organ-on-a-chip" models have shown promising outcomes in understanding cellular interactions and modeling drug transport, often allowing for minimal use of cells and culture reagents (79–81). Three-dimensional and microfluidic culture conditions have been used to model the AD BBB using immortalized human BECs and neural progenitor cells, with this model revealing AD-specific BBB dysfunction (78).

The limitation of many 3D and microfluidic BBB models is that they are complex in-house made platforms, often utilising proprietary materials, which are difficult to replicate in other laboratories. Commercial 3D and microfluidic platforms are emerging, providing a means for off-the-shelf systems for BBB modeling (82, 83). Other limitations include that most of the 3D and microfluidic BBB models have been established using cell lines, such as immortalized BECs. To be able to accurately model AD- or other neurodegenerative disease-specific effects, patient-derived cells, such as primary BECs or iPSC-derived iBECs would be ideal. Likely the challenge of culturing patient-derived BECs in co-culture with other cells as well as the poor long-term survival has hindered the development of patient-derived 3D and microfluidic models of the BBB.

BBB organoids provide an additional platform of studying BBB function in a 3D format without the need for a complex device. Previously generated BBB spheroids have consisted of primary or iPSC-derived BECs and other NVU cell types which spontaneously assemble under low-attachment conditions into a multicellular 3D structure (84, 85). These spheroids have been shown to demonstrate direct cell-to-cell contacts, enhanced TJ and AJ expression, higher efflux transporters expression and reduced paracellular permeability, thus more closely mimicking the *in vivo* BBB when compared to traditional 2D cultures (84, 85). Human cortical spheroids containing BECs, pericytes, microglia, astrocytes, oligodendrocytes and neurons were shown to exhibit high expression of BBB markers as well as high viability, making them an attractive platform for drug discovery, disease modeling and long-term neuro- and cytotoxicity testing (85). The resemblance of organoid-like spheroids to the *in vivo* environment make them a promising platform for high-throughput screening of BBB penetrating drugs. Limitations of organoid research, however, include inter-sample variability, high processing time and technical difficulties in TEER measurements and permeability studies (86).

## Permeability and drug delivery assays

Integrity of *in vitro* models of BBB is usually assessed via the permeability to fluorescently-conjugated molecules, such as dextran or sodium fluorescein, which have both been used in 2D Transwell and 3D microfluidic models of the BBB (33, 72, 83). For modeling drug delivery, various methods have been studied to transiently open the BBB *in vitro* cell model. Mannitol can be used to reversibly open the BBB, based on hyperosmosis, and has been used in an *in vitro* hiPSC-derived 3D BEC model to increase paracellular permeability (87). In the clinic, mannitol

has been used to deliver antibodies to treat brain tumors (88), with its use otherwise not widely described due to possible side-effects. Human patient cell based AD BBB models may be important to allow the in-depth investigation of mannitol-dependent BBB opening (87), to develop its use for the delivery of therapeutic antibodies, such as to treat AD. Another reversible means to open the BBB is focused ultrasound (FUS) applied in-conjunction with gas-filled microbubbles (MB) (89). The safety of FUS+MB treatment in AD patients has been demonstrated, opening an avenue for potential Aβ clearance or therapeutic drug delivery (90). The effects of FUS+MB have also been investigated in a patient iPSC-derived fAD iBEC *in vitro* model, which demonstrated a differential response to FUS+MB treatment between patient and control cells, such as in FUS+MB-mediated permeability and Aβ clearance (33). These results highlight the importance of using patient-derived cell models to identify potential patient-specific differences that could affect drug delivery in the clinic.

## ENHANCING DRUG DELIVERY IN ALZHEIMER'S DISEASE THROUGH *IN VITRO* MODELS OF BBB—CHALLENGES AND FUTURE PERSPECTIVES

BBB dysfunction in AD not only underlies disease pathogenesis and progression but also serves as the main burden for successful drug delivery. Additionally, BBB impairment in AD and subsequent physiological changes lead to disruption in drug delivery by diffusion, with pathological changes in transporters contributing to minimal (or no) bioavailability of the drug in the brain (1, 91). Furthermore, infiltrating toxic blood-derived products, reactive oxygen species and increased neuroinflammation may change the tightly controlled brain milieu and lead to undesired metabolism and/or interactions of delivered drugs (Figure 1B) (1). As such, to overcome challenges associated with drug delivery in AD, an in-depth understanding of AD-related changes at the BBB and subsequent effects on drug delivery are needed, which can be addressed using accurate model systems.

The basis of an accurate AD BBB cell model is that the cells, as closely as possible, recapitulate the disease phenotype. With challenges associated with all the described BEC sources, it is important to consider the benefits and limitations of each model. AD patient-derived primary BECs provide an opportunity to examine cells obtained directly from a patient (56), but patient brain tissue is difficult to obtain with these cells often only capturing late-stage of the disease. In addition, the lifespan of primary BECs *in vitro* is short, limiting their use for large-scale experiments (53). Patient-derived iPSCs on the other hand provide a scalable approach to generate BECs *in vitro* and one patient line can be used to generate all components of the NVU, providing an isogenic patient-specific BBB model (33, 64). In addition, using CRISPR/Cas9 gene editing, the role of specific AD mutations on BBB dysfunction can be investigated (33, 64). To ensure the translatable use of iPSC-derived cells, it is important to generate standardized differentiation protocols and utilize defined xeno-free reagents to minimize variability associated with iPSC culture.

Following selection of a good BBB cell source, it is vital to consider the culture environment for accurate BBB modeling. Successful screening for drug delivery

across the BBB will be best achieved in an environment that as closely as possible mimics the brain environment in complexity, structure and chemistry. It has been shown that following a long-term culture (>3 months), AD-patient derived iPSC cerebral organoids exhibit hallmarks of AD, including Aβ plaques and accumulation of hyper-phosphorylated tau (92). Thus, to replicate physiological changes characteristic of AD, long-term cell culture models of the BBB are likely needed. Furthermore, to capture the complexity of the brain, standardized platforms that enable both 3D and microfluidic culture conditions are likely the future means to establishing an accurate BBB *in vitro* model. The challenge that still exists is developing a reproducible culture platform that is easy and cost-effective for laboratories to use, with emerging commercial platforms providing a potential solution (82, 83).

Finally, to accelerate drug discovery in AD, high-throughput testing in BBB *in vitro* models is important. Scaling down reagent and cell use (i.e., minimizing culture platform size) and scaling up the number of replicates, would help to achieve testing of a large number of drugs in a cost-effective manner. For this reason, small-scale "organ-on-a-chip" type platforms may be ideal (93). It is also vital to consider how easily drug delivery efficiency and downstream effects can be measured. Ideally, a BBB *in vitro* model that enables the simultaneous observation of drug delivery efficiency as well as effects on brain cell types and AD pathologies (Aβ and tau) would be a key step in AD drug discovery.

## CONCLUSION

BBB dysfunction is associated with AD, likely playing a central role in AD progression and drug delivery. Considering inter-species differences, accurate human BBB *in vitro* models are needed to understand AD-specific changes on the BBB and subsequent effects on drug delivery. Human BBB cell models of AD, in particular using patient-derived cells in a culture environment that accurately mimics the AD brain, are an important step to enhance drug discovery in AD.

**Conflict of Interest:** The authors declare no potential conflict of interest with respect to research, authorship, and/or publication of this chapter.



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8

# Blood–Brain Barrier Degradation and the Implication of SPARC Protein as a Potential Therapeutic Target for Alzheimer's Disease

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**Abstract:** Alzheimer's disease is a progressive neurodegenerative disorder affecting a substantial portion of the older population, with the number of afflicted individuals expected to grow with time. Although numerous contributing factors to the disorder have been identified, there is currently no cure or effective prevention method. With the situation as dire as it is, many efforts have been made to shed light on the mechanisms tying diverse contributing factors to the pathogenesis of Alzheimer's disease. One common neuropathological feature of Alzheimer's disease is the dysfunction of the blood–brain barrier, which normally maintains brain homeostasis by isolating it from the peripheral circulation and mediating the transport of various bloodborne elements in and out of the brain. An increase in the blood–brain barrier permeability has been observed in Alzheimer's disease at a level considerably above normal aging. This chapter provides an overview of the effects of aging, the neuroimmune system, inflammation, traumatic brain injury, apolipoprotein E gene ε4 allele, and secreted protein acidic and rich in cysteine (SPARC) protein on blood–brain barrier. The potential

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of SPARC as a therapeutic target for Alzheimer's disease, and the application of deep-learning-based virtual screening tools against SPARC protein are explored.

**Keywords:** Alzheimer's disease; blood–brain barrier; Hevin; secreted protein acidic and rich in cysteine; virtual screening

## INTRODUCTION

Alzheimer's disease (AD) is a progressive neurodegenerative disorder that has widespread detrimental effects on memory and cognitive abilities that worsen over time. The disease is ultimately fatal, often through complications associated with decreased cognition. It is the most common form of senile dementia, with an estimated 5.8 million Americans currently afflicted with AD, a number that is expected to increase dramatically with an aging population that is more consistently reaching the "oldest-old" phase where AD risk is at its highest (1). AD has a substantial economic impact, with projections indicating the global cost of dementia could balloon to 2 trillion US dollars by 2030 (2). Given the threat the disease poses, researchers have been tackling AD from different angles, but as of now attempts to develop treatments have been met with widespread clinical failure (3).

The blood–brain barrier (BBB) consists of endothelial cells, serving as a layer of separation between blood vessels and the brain. The endothelia that line the blood vessels of the brain serve to isolate the brain parenchyma from bloodborne molecules that lack corresponding transporters to mediate their entry, and maintain the equilibrium of the brain's environment. The barrier is also comprised of other elements interacting with endothelial cells, including astrocyte foot processes and pericytes, which together with neurons and microglia comprise the neurovascular unit. The BBB is also responsible for controlling immune surveillance within the brain, by restricting the flow of immune cells (4). Dysfunction of the BBB is implicated in AD pathogenesis. The BBB is partially responsible for the clearance of amyloid-beta (Aβ), which builds up and forms plaques in AD, and the BBB is a site of CNS inflammation, which is frequently observed in AD patients (5).

While the integrity of the BBB is tightly regulated, emerging evidence implicates the matricellular proteins, secreted protein acidic and rich in cysteine (SPARC), and Hevin, as having a role in regulating BBB permeability. This chapter discusses the effects of various AD risk factors on BBB permeability, with emphasis on SPARC, which is upregulated in AD brain tissue (6, 7). Since the SPARC protein enhances BBB permeability, promotes neuroinflammation, and prolongs pro-inflammatory M1 phase of microglia, its potential as a druggable target is also discussed.

## THE BBB IN AD

The integrity of the BBB is critical to the maintenance of brain homeostasis in health. As mentioned, BBB dysfunction is commonly seen in cases of AD, and a variety of factors may contribute to the observed disruption.

## Aging

Age is perhaps the most predominant risk factor for AD, with almost half of all individuals over the age of 85 suffering from it; conversely, less than 10% of cases under the age of 65 suffer from AD (8). BBB deterioration has been well-documented in aging (9), with nearly all its components being affected (Table 1). Degradation of the BBB is known to start early, with notable permeability increases around the hippocampal region in individuals between 23 and 47 years of age, which worsens with increasing age (9, 10). Hormones such as insulin, which are associated with aging, can affect the permeability of the BBB and contribute to leakage (11). Additionally, transporters of certain molecules such as glucose, along with various proteins and hormones, may become defective in older individuals, reducing their availability to the brain (12). Outside of the barrier, the appearance of white matter hyperintensities, which are indicative of a loss of vascular integrity, also correlates with age. Damaged vasculature may cause a corresponding decrease in BBB integrity (13). Corroborating this, the vascular density of the brain appears to experience a significant age-related decline between the ages of 57 and 90 (14). Hypertension, which is more common in older individuals, can contribute to microvascular injury, thereby increasing the incidence of BBB disruption (15, 16).

**TABLE 1** **Alterations of BBB components during physiological aging**

| BBB element | Property changes due to aging |
|---|---|
| Endothelial cells | Increased capillary wall thickness |
| | Decreased number of endothelial cells |
| | Decreased number of mitochondria |
| Tight junctions | Decreased expression of tight junction protein |
| Basal lamina | Increased thickness of basement membrane |
| | Increased concentration of collagen IV and arginase |
| | Decreased concentration of laminin |
| Astrocytes | Increased astrocyte proliferation |
| | Increased GFAP expression |
| Microglia | Changes to amoeboid morphology |
| | Production of neurotoxic proinflammatory mediators |
| Pericytes | Degeneration and loss of pericytes |
| | Vesicular and lipofuscin-like inclusions |
| | Increased size of mitochondria |
| | Foamy transformation |
| Neurons | Deterioration of synaptic plasticity |
| | Deficit in long-term potentiation |
| | Impaired neurogenesis |
| | Increased apoptosis |
| | Neuronal damage due to cytokine release |

Adapted from (9). GFAP, glial fibrillary acidic protein; BBB, blood–brain barrier.

## Neuroimmunity

The brain exists in a state that is considered "immune privileged." Due to the existence of barriers between the brain and the rest of the body's circulation, the brain is insulated against many peripheral immune events (17). The presence of immune cells derived from main circulation, such as peripheral macrophages, neutrophils, and leukocytes, in the brain is an indication of BBB breakdown (18). In addition, the brain has intrinsic immune components, and these components interact with the BBB in such a way that peripheral immune events can also invoke a response in the brain (17). There are two primary types of neuroimmune cells, microglia and astrocytes, and both interact with the BBB.

Microglia are a variant of macrophage, though they do not develop and function as peripheral macrophages do. Microglial progenitors emerge from the yolk sac, and the development of microglia occurs in phases, with each phase being regulated by different transcription factors, and exhibiting differing gene expression profiles (19). Some of their key functions include phagocytosis, synapse pruning, and mediating immune signaling through the release of cytokines and other factors (20). They play a role in AD primarily by phagocytizing abnormal Aβ amyloid and forming a barrier between the plaques and the rest of the brain through plaque envelopment, thereby limiting the expansion of the plaque (21). Microglia are known to associate tightly with the BBB. Microglia exist in a resting state until they are activated due to brain injury or another immunological stimulus. Upon activation, they release a host of cytokines and other molecules that increase the permeability of the BBB; in the case of brain injury, this allows bloodborne agents like myeloid cells to cross the BBB. They also have been found to release reactive oxygen species that impair the function of the BBB (22). Perhaps, the most significant contribution of microglia to AD pathology is their involvement in evoking inflammatory responses within the brain (23). The role of inflammation in BBB disruption is discussed further in the following section.

Astrocytes are of epithelial origin and feature a wide array of morphologies and functions within the nervous system. Aside from their roles in neural immunity, they are responsible for ion transport, removal and catabolism of neurotransmitters, and neurogenesis. Some astrocytes are noted for their vascular end-feet, which are closely associated with brain vasculature and the BBB (24). They increase the permeability of the BBB through vascular endothelial growth factor A (VEGFA) and thymidine phosphorylase (TYMP). VEGFA, along with the TYMP product 2-deoxy-d-ribose, downregulates tight-junction proteins and promotes angiogenesis and BBB permeability (25). The release of these two factors is induced by interleukin-1 beta (IL-1β), an inflammatory cytokine (25). Both astrocytes and microglia produce and react to inflammatory responses that can impact BBB health primarily through inflammatory cytokines (26).

## Neuroinflammation

Neuroinflammation has been found to be relevant to AD pathology in a variety of ways. Inflammation in the brain has widespread effects on vasculature, cell signaling, neural function, and other immune responses. The effect of cytokines and other inflammatory mediators released during an inflammatory event involve some of the key components of the neuroimmune system and have been found to regulate the

clearance of Aβ (27). Many of these mediators have also been shown to influence BBB permeability. A summary of some of the mediators and their effects on the BBB are given in Table 2 (28). Neuroinflammation is a common effect of aging and notably includes an increase in the production of inflammatory cytokines by microglial cells (29). These mediators, in general, are not directly responsible for modulating the permeability of the BBB. Instead, they influence the expression or activation of other factors that, in turn, disrupt the BBB function (30).

## Traumatic brain injury

Traumatic brain injury (TBI) has been shown to be a significant risk factor for AD. Individuals who had experienced a mild traumatic brain injury (mTBI) are more vulnerable to early-onset cognitive impairment (31) than those that have not experienced such an event. The effects of a TBI are often immediate, with force-induced injury resulting in what is considered secondary brain damage, which includes an increase in BBB permeability. Following TBI and mTBI, bloodborne substances accumulate in various regions of the brain, due to a breach in the BBB. While, in most cases, the effects of mTBI appear to be relatively short-lived, in rats with preexisting hypertension, mTBI can induce persistent disruption of the BBB (32). These rats experienced an increase in fibrin accumulation and neuronal expression of inflammatory cytokines (32). Generally, a focal breach following mTBI has been observed in rats to persist for

**TABLE 2 Inflammatory mediators and their effect upon the BBB**

| Inflammatory mediator | Observed effects on BBB |
|---|---|
| TNF-α | Increase in BBB permeability in *in vivo* and *in vitro* models |
| | Increased efflux of albumin from brain to blood |
| | Decreased ZO-1 expression |
| | Increased MMP-9 protein expression |
| IL-1β | Increase in BBB permeability in *in vivo* and *in vitro* models |
| | Decreased TEER of primary cultures of brain endothelial cells and human brain endothelial cells |
| | Increased production of PGE and COX |
| | Decreased ZO-1 expression |
| IL-6 | Decreased TEER in cerebrovascular endothelial cells from rats at higher doses but not at lower doses |
| | Decreased BBB permeability in ischemic brain in rodents |
| IL-17A | Increase in BBB permeability in *in vivo* and *in vitro* models |
| CRP | Increase in BBB permeability in *in vivo* and *in vitro* models |
| | Increase in ROS production in brain endothelial cells |

Adapted from (28). BBB, blood brain barrier; COX, cyclooxygenase; CRP, c-reactive protein; IL, interleukin; MMP-9, matrix metalloproteinase-9; PGE, prostaglandin E; ROS, reactive oxygen species; TEER, transepithelial/transendothelial electrical resistance; TNF-α, tumor necrosis factor alpha; ZO-1, zonula occludens-1

approximately 24–48 h (33, 34). In rare occurrences, singular instances of BBB disruption via mTBI in humans, typically measured by the cerebrospinal fluid/serum albumin quotient, have been found to persist for months or even years (35). A meta-analysis of studies conducted from 1995 to 2012 found that TBI and mTBI events are substantial risk factors for AD (36). The BBB disruption at the onset of TBI is relatively short-lived; however, the subsequent events lead to structural degeneration in the brain causing long-lasting cognitive impairments (37). Disruption of the BBB has been observed to be a marker of mild cognitive impairment independently of the neurofibrillary tangles (NFTs), tau protein, and Aβ amyloid plaques, indicating that substantial breakdown of the BBB itself contributes to cognitive decline in addition to exacerbating other neurodegenerative processes in AD (38).

### Apolipoprotein E gene ε4 allele (APOE ε4)

The APOE ε4 allele has been identified as the most significant genetic risk factor for AD (39). It is also associated with other dementia subtypes, such as Parkinson's (40) and frontotemporal dementias (41, 42). Individuals homozygous for APOE4 (ε4/ε4) experience a 10-fold higher risk of dementia, and individuals heterozygous (ε3/ε4) for the variant experience a 1.7-fold higher risk of dementia (43). APOE4 is attributed to reduced clearance of Aβ amyloid, which contributes to the formation of the Aβ amyloid plaques that are a hallmark of AD (44). Possession of at least one APOE4 allele increases the leakage of the BBB (45, 46). The role of APOE in maintaining the integrity of the BBB is confirmed by experiments involving APOE deficient ($APOE^{-/-}$) mice, which exhibit signs of increased BBB permeability, such as the leakage of exogenous tracers, starting at 2 weeks old (18). The allele may also have relevance to TBI. When assessing the BBB repair ability of APOE3 and APOE4 mice, the APOE3 mice experienced a significant reduction in permeability between the 3-day and 10-day measurements, indicating substantial BBB repair. APOE4 mice, however, did not experience a significant reduction in permeability in the same time period; APOE4 was also expressed at lower levels than APOE3 at both 3 and 10 days (47). The role of APOE in BBB integrity is further reinforced by postmortem studies of both AD and normal humans with and without the APOE4 allele. AD-afflicted APOE4 carriers experienced a 3.1-fold increase in fibrin perivascular deposits in the brain relative to APOE3 carriers, indicating an increase in BBB permeability. The same study also found that APOE3 carriers still had a 6.9-fold increase in Aβ amyloid deposits relative to normal controls, indicating that BBB disruption is indeed a significant component of AD. In addition, pericytes, which are constituents of the BBB, have substantially reduced coverage in both AD and normal individuals (48).

## SECRETED PROTEIN ACIDIC AND RICH IN CYSTEINE

SPARC belongs to a family of matricellular proteins that modulate cell interaction with the extracellular environment. There are currently six known members of the SPARC family. These members, along with some key features of them, are shown

in Table 3. While their structures and functions are not identical, each member of the family possesses shared motifs and is secreted into the extracellular space where they influence the structure of the extracellular matrix and modulate various signaling pathways (58) such as the TGF-β pathway (30, 58). Two particular members of this family, SPARC and Hevin/SPARCL1, are notable in that they have collagen binding domains in addition to the calcium binding domains exhibited by all of the other members of the protein family (30). The SPARC-collagen binding interaction is depicted in Figure 1. Nullification of SPARC expression decreases the expression of the proinflammatory cytokines IL-6, IP-10, and FAS/CD95 in rats (59). Hevin is a member of the SPARC protein family, which is most commonly expressed in the brain along with SPARC (30). Studies have found that SPARC has an antiadhesive effect on brain endothelial cells and decreases cerebral endothelial transepithelial/transendothelial electrical resistance (TEER), indicating decreased BBB integrity (60).

## SPARC expression in AD

Within the brain, SPARC and Hevin are attributed to a variety of functions, such as the regulation of synaptogenesis and tissue remodeling following an injury. The proteins are primarily expressed in immune cells. While both SPARC and Hevin are produced by microglia (7) and astrocytes (61), Hevin is produced only by some neurons (62). Postmortem examination of the brains of AD and control individuals found that there is a notable upregulation of SPARC and downregulation of Hevin in the AD brains. As indicated in Figure 2, SPARC is expressed by microglia found in close proximity to pathological Aβ amyloid plaques (7). Interestingly, while it seems that SPARC has a destructive effect on the BBB, which would exacerbate the AD condition, it appears to support the Aβ amyloid clearance process, which should have the opposite effect (7, 60). Hevin's role in BBB health is unclear, though it may be responsible for the initiation of the repair process by microglia (7).

**TABLE 3 Members of the SPARC protein family**

| SPARC family member | Significant brain expression | Binds to | Impact on cell adhesion |
|---|---|---|---|
| SPARC | Yes (7, 30) | Collagen + calcium (30) | Antiadhesion (49, 50) |
| Hevin/SPARC-like 1 (SPARCL1) | Yes (7, 30) | Collagen + calcium (30) | Antiadhesion (51) |
| Smoc-1 | Yes (52) | Calcium (30) | Unidentified |
| Smoc-2 | No (53) | Calcium (30) | No effect on non-epithelial cells (53) |
| Testicans/spocks | Yes (54, 55) | Calcium (30) | Antiadhesion (testican-1) (56) |
| Follastatin-like 1 (FSTL1) | Yes (57) | Calcium (30) | Unidentified |

SARC, secreted protein acidic and rich in cysteine.

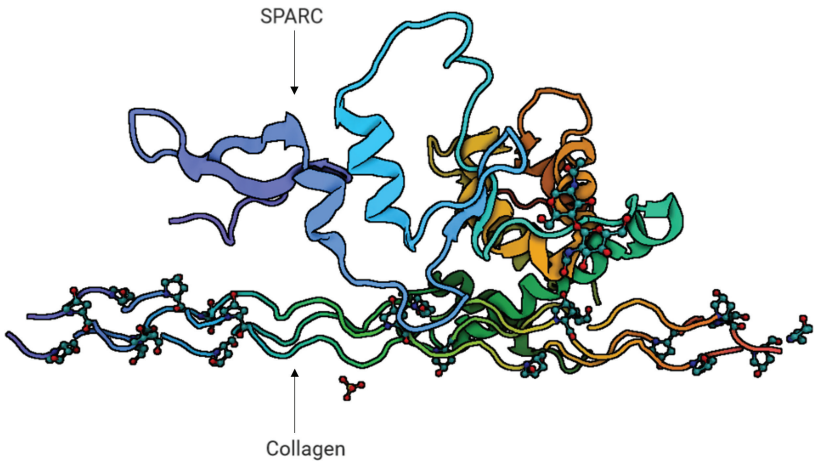


**Figure 1. Secreted protein acidic and rich in cysteine (SPARC)-collagen binding.** The bottom part of the structure diagram represents a collagen alpha-1(III) chain; the top part of the structure diagram represents SPARC. The SPARC-collagen binding site represents a potential target for agents that modify SPARC activity. *Created with BioRender.com*.

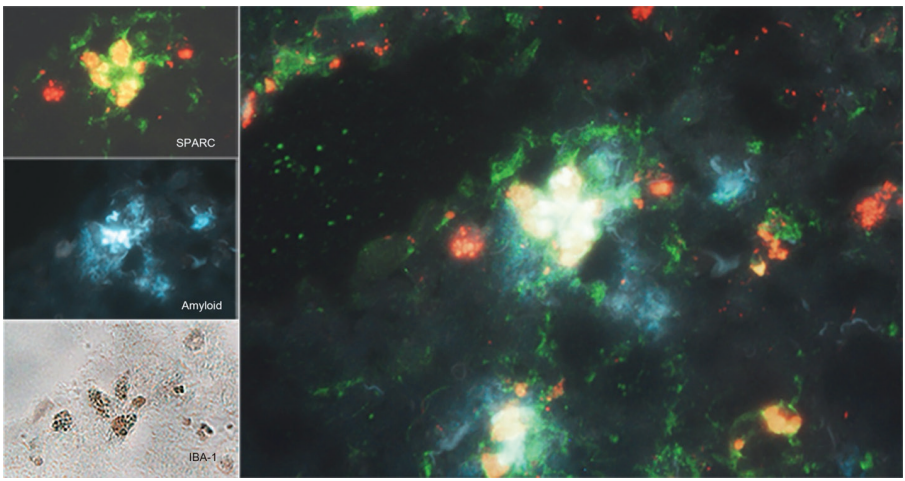


**Figure 2. Secreted protein acidic and rich in cysteine (SPARC) is expressed by microglia found in close proximity to pathological Alzheimer's Aβ amyloid aggregates.** Analysis of cortical tissue from Alzheimer's disease patients reveals the presence of SPARC (in green) in and around glial cells within Aβ amyloid (ThioS in blue) plaques. These SPARC-associated glial cells were identified as microglia (IBA-1 in HRP brown). Adapted from (7) under CC BY-NC 4.0 (https://creativecommons.org/licenses/by-nc/4.0/) license. Reproduced with permission.

## SPARC and the BBB

Expression of SPARC is also associated with inflammatory responses. When testing the effects of various cytokines on SPARC expression and BBB permeability in hCMEC/D3 cell culture, it was found that TNF-α caused an upregulation in SPARC only in the absence of IFN-γ that negated the effects of TNF-α (63).

In the brain, SPARC is typically localized to astrocytic end-feet and cerebral endothelium. SPARC was experimentally determined to increase transendothelial permeability and affect the differentiation of endothelial cells through protein tyrosine kinase signaling (63). A particular area of interest is the SPARC-collagen binding domain. Increased levels of collagen IV, as well as general thickening, in the basement membrane of brain microvessels are noted in cases of AD (64); increases in SPARC show a corresponding increase in the levels of collagen (65, 66), and SPARC acts as a chaperone for collagen IV (67). Furthermore, abnormalities in the vasculature that surround the Aβ amyloid plaques are associated with aberrant levels of collagen IV (68). The interactions between SPARC and collagen have been linked to inflammation and pathological fibrosis (69), as well as induction of a pro-inflammatory response in brain monocytes (64). The differential effects of SPARC may mean it can serve as an effective broad-spectrum therapeutic target.

### Rationale for SPARC protein as a potential Alzheimer's therapeutic target

Although a better understanding of the disease and its mechanisms have provided avenues for druggable targets, attempts to develop effective ways to treat or reverse AD progression have been met with failure thus far. Perhaps targeting AD from multiple treatment angles may be the key. As vascular dysfunction is a substantial component of AD, SPARC and other members of the protein family may be druggable targets for AD (70). Information on SPARC modifiers on the central nervous system is limited; however, such modifiers have been studied to a degree in the context of cancers (71, 72). Currently, the translational aspects of these drugs for AD are largely speculative. The SPARC-collagen binding site represents a reasonable start to the search, given the detailed research surrounding the structure and mechanism of the SPARC-collagen binding domain and knowledge of collagen binding with other molecules (73). A general diagram of how SPARC and Hevin, in particular, interact with the BBB is shown in Figure 3.

## FUTURE DIRECTIONS

Research on the SPARC protein and molecules that can modify its activity is limited. Given that the process of drug discovery is capital-intensive and time-consuming, it may be prudent to establish what molecules modify the activity of SPARC and its relative such as Hevin. High-throughput screening has been applied to other molecules, in which batteries of mini-scale experiments assaying the activity of the target when introduced to a library of molecules are conducted (74). However, conducting this procedure on a compound library that can contain hundreds of thousands of molecules, with a generally low hit rate of modifying compounds, is costly; samples of every compound must first be synthesized before it can be tested. The use of screening tools that conduct the filtering of molecules *in silico* has become increasingly popular, as large numbers of molecules can be processed quickly and cheaply, so long as one has access to sufficient computational power (75). Molecules must be first tested both *in vitro* and *in vivo* and, ultimately, in humans before a drug

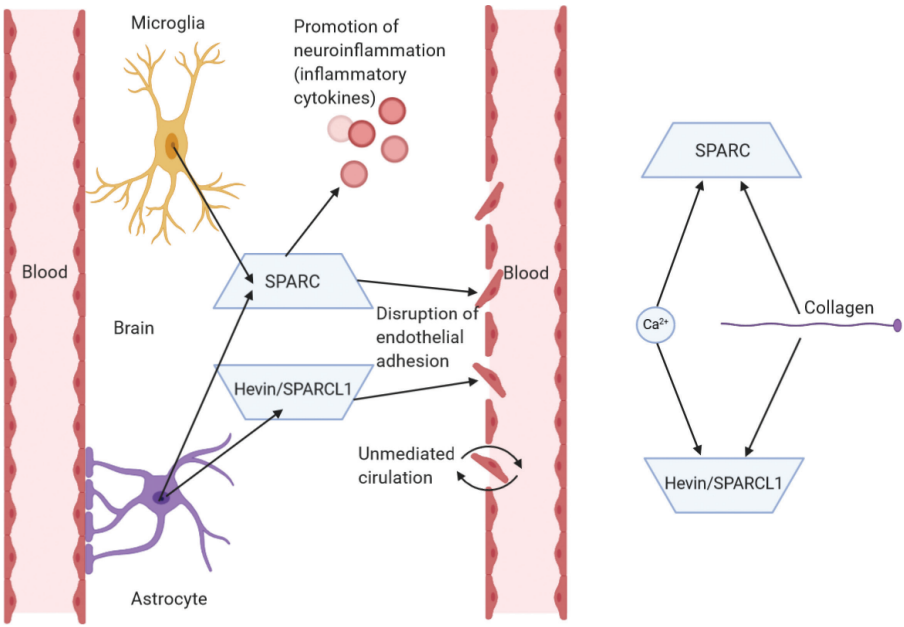


**Figure 3. Secreted protein acidic and rich in cysteine (SPARC) and Hevin interactions with the blood–brain barrier (BBB).** SPARC is produced primarily by microglia and astrocytes, while Hevin is primarily produced by astrocytes. SPARC and Hevin exert antiadhesive effects, and SPARC promotes the release of inflammatory cytokines in certain conditions. Both SPARC and Hevin are distinct from the other members of the SPARC protein family because they bind to collagen as well as calcium; both binding sites represent potential targets for Alzheimer's disease-modifying agents. *Created with BioRender.com*.

can be considered successful. The fast *in silico* screening compound hits can significantly reduce the time and costs of drug development (76).

We thus propose that such methods be used to identify inhibitors for SPARC-collagen binding. A variety of machine learning (ML) algorithms have been applied to the problem of drug discovery and molecular screening with considerable success; decision trees, support vector machines, and other classifiers have been applied to either structural or ligand-based virtual screening (VS) (77–79). Ligand-based approaches take the similarities of different molecules to other compounds that are known to be active against a target protein. Information on the molecules is generally taken from compound databases, which are filtered based on certain properties, such as those that influence pharmacokinetics and toxicity, in order to make the problem of screening a large number of molecules more computationally feasible (76). As the name implies, structure-based VS involves structural information, either obtained from techniques such as X-ray crystallography or, more commonly, data obtained from computational models. The molecular structures of the protein target and those of the structural databases are examined, in order to determine which will interact in the desired manner (76). Structural methods also encompass the development of novel molecules,

as generative models, which are notable for their ability to use information gleaned from training data for the purpose of classification or prediction and create novel data for a novel sample of the given type.

Deep learning (DL), which involves ML algorithms that feature multiple neural network (NN) layers, has become prevalent in a variety of fields. NNs form the primary basic structure of DL models. Notable examples of NN-based drug discovery platforms include "AtomNet," developed by Wallach et al., a structure-based virtual screener based on a convolutional neural network (CNN) algorithm. Though typically applied to image processing and linguistic applications, a CNN model, which features layers of feature-reduction (convolution) and pooling operations, was trained on a set of molecular structures and tested against a set of benchmark decoy-structures. Performance of the CNN was found to be better than other ML methods (80). Thus, it may be the case that more research into some unorthodox NN strategies may provide a helpful performance boost for VS tools; such a tool could be useful in VS against SPARC protein target for AD drug discovery, as the limited data on SPARC target make *in silico* methods a practical predecessor to future *in vitro* and *in vivo* work and beyond.

## CONCLUSION

Current evidence shows that the BBB plays a crucial role in a variety of neurological disorders, and its disruption is evident in AD. There is a great deal of interplay between the various known hallmarks of AD, such as the buildup of amyloid plaques, NFTs, and BBB degradation. Many risk factors tie into multiple facets of the disorder; APOE4, the most significant genetic risk factor for AD discovered to date, diminishes Aβ clearance and inhibits BBB repair. TBI/mTBI and neuroinflammation contribute to AD pathogenesis and BBB damage. Gradual erosion of the BBB is a common part of the aging process, increasing an individual's vulnerabilities to further breakdown and neurodegenerative diseases. Given the importance of the health and stability of the BBB, and the wide array of factors that can be detrimental to it, such as SPARC, more research into its mechanics, maintenance, and recovery pathways may be vital to understanding AD and how to treat it. DL-based VS tools may be employed to identify inhibitors of SPARC-collagen binding for AD drug discovery.

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# The Therapeutic Potential of Epigenetic Modifications in Alzheimer's Disease

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**Abstract:** Alzheimer's disease is characterized by the formation and deposit of abnormal peptides such as amyloid plaques and neurofibrillary tangles in the brain. Therapeutic strategies aimed at preventing the formation of such deposits have not been successful. Currently, there are no effective treatments for the disease. Since numerous epigenetic changes have been detected in Alzheimer's disease, treatments aimed at reversing these changes by intervening in DNA methylation, histone acetylation, and microRNA expression may constitute promising lines of research in the future. This chapter provides an overview of the epigenetic changes and the potential epigenetic therapies in Alzheimer's disease.

**Keywords:** Alzheimer's disease; DNA methyltransferase; epigenetic changes; histone acetylation; noncoding RNA

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## INTRODUCTION

Alzheimer's disease (AD) is the most common cause of dementia. It usually occurs in people over 60 years of age and presents with progressive loss of memory and cognitive capacity, language disorders, inability to translate ideas into actions (ideomotor apraxia), impaired planning and judgment, apathy, depression, and, in later stages, psychosis with paranoid delusions. AD is characterized by the presence of abnormal peptide deposits in the brain. The most characteristic lesions are neuritic extracellular plaques of the amyloid β (Aβ) peptide, which consists of 33–40 amino acids derived from the proteolysis of the transmembrane protein amyloid precursor protein or APP. These neuritic plaques contain a large number of distorted neuronal expansions, known as dystrophic neurites. Activated microglial cells are observed at their center.

Some evidence suggests that amyloid deposits may be neurotoxic and may cause neuronal dysfunction and even neuronal death. In the normal brain, APP is fragmented into functional segments by the α-, β-, and γ-secretase enzymes. Occasionally, there is an increase in β- and γ-secretase relative to α-secretase, leading to the accumulation of peptides with 40 and 42 amino acids, known as amyloid β40 ($A\beta_{40}$) and amyloid $\beta_{42}$ ($A\beta_{42}$). The $A\beta_{42}$ peptide appears to have greater neurotoxic properties. Aβ oligomers, small aggregates of 2-12 peptides, appear to be especially toxic (1). Diffuse plaques, another kind of plaque, lack a dense center of amyloid and dystrophic neurites. Unlike neuritic plaques, they are not associated with either neuronal destruction or cognitive dysfunction (2).

Neurofibrillary tangles are twisted aggregates of abnormal intraneuronal fibers that have a helical structure, typically paired helical filaments, made up of hyperphosphorylated tau protein. The tau protein is involved in stabilizing microtubules, maintaining the integrity of the cytoskeleton and axoplasmic transport. Neurofibrillary tangles are found in the areas of association of the neocortex, hippocampus, limbic system, substantia nigra, raphe nuclei, locus coeruleus, and the nucleus basalis of Meynert (3). In AD, there is also a significant synaptic loss in certain areas of the neocortex and in the hippocampus, as well as the disappearance of dendritic spines.

AD occurs frequently in humans over 65 years of age. In those aged over 85, the prevalence of AD ranges between 20 and 40% in developed countries (3). In 2010, the prevalence of AD in China among people aged between 85 and 89 was 18.54% (4). In 2006, the number of patients with AD was 26.6 million worldwide. In the United States, the prevalence of AD in people over 70 years of age is 9.51%, and the incidence is 14.26 per 1000 person-years (4). However, AD is not an inevitable consequence of old age. A relatively high number of elderly people show neither cognitive decline nor lesions typical of AD with age. The causes of AD are still not well understood. In a small percentage of cases, AD can be attributed to mutations in genes located on chromosomes 1, 14, and 21. These cases are usually of early onset and are transmitted in an autosomal dominant manner. Most AD cases appear to be caused by the interaction of multiple genetic and environmental factors that are not yet well understood (1).

Areas of association, phylogenetically more recent areas of the human brain, have simpler organization and greater immaturity in the adult than phylogenetically older primary areas. Thus, in the neurons belonging to the areas of

association, myelination occurs very slowly and many neurons belonging to these areas remain incompletely myelinated—that is, immature even in adulthood. Poorly myelinated neurons are chronically subjected to high-energy turnover, which makes them more vulnerable to the influence of oxidative stress. There are, therefore, extensive cortical areas in the human brain that remain structurally immature throughout life (5).

Various studies show that there has been an increase in the expression of genes related to aerobic metabolism and, more importantly, to synaptic plasticity and activity in the human cerebral cortex relative to nonhuman primate brains (6, 7). Learning and memory take place through the formation of new synapses and remodeling of preexisting synapses, suggesting that the increase in the expression of genes related to these functions has occurred in humans, as well as the selection of genes that encode proteins capable of increasing neuroplasticity. The apolipoproteins E (ApoEs) are proteins of 299 amino acids synthesized in the astrocytes of the central nervous system. They influence the transport and reuptake of cholesterol and the stabilization of the neuronal cytoskeleton, contributing to the preservation of synaptic integrity (3).

Humans present a polymorphism for ApoE with three alleles: ε2, ε3, and ε4. Possession of allele ε4 of the ApoE is the most important risk factor for the development of AD, after advanced age (3, 6). The most common allele is ε3, whose frequency is 60% or higher in all the populations studied. Possession of the ε4 allele is associated with lower neuroplasticity and lower synaptic repair capacity, and seems to promote the relatively early appearance of brain deposits of neurotoxins, such as Aβ and neurofibrillary tangles, whose excess is associated to AD.

There has been an increase in the expression of genes associated with neuronal plasticity in the human cerebral cortex, resulting in an increased capacity for learning and memory, neurotransmission, axonal transport, aerobic metabolism, and neuroprotection, all of which are adaptations that promote high neuronal activity over a long life (6, 7). The human brain has a high need for glucose, especially during its development. A child's brain consumes more than 40% of the body's basal energy requirements. Most of the glucose is oxidized to produce ATP. This process is upregulated in anaerobic conditions. Aerobic glycolysis is increased during childhood and is synonymous with high rates of synaptic formation and the growth and remodeling of synapses. Aerobic glycolysis is associated with the persistence of genetic expression associated with childhood, especially genes active in youth, and especially those related to the growth and formation of new synapses (transcriptional neoteny) (8). In the adult human brain, aerobic glycolysis is especially elevated in cortical areas related to cognitive functions that have undergone significant modifications during the evolution of the human species, such as the dorsolateral prefrontal cortex and the brain's default mode network (BDMN), related to the coordination of activity between different cortical areas and to planning and autobiographical memory capacities, which allow "mental travel in time," remembering and planning.

The brain regions where most of the Aβ deposits are located almost exactly match the regions that make up the BDMN, which suggests that the high synaptic turnover that occurs in these areas predisposes the formation of abnormal peptide deposits characteristic of AD (8). Multiple studies show that AD appears to be associated with oxidative stress (9). Increased aerobic metabolism in neurons that retain juvenile characteristics in adulthood could subject these neurons to high

oxidative stress. It appears that oxidative stress could induce epigenetic changes, reducing the expression of certain genes, including those related to synaptic plasticity.

## EPIGENETIC CHANGES AND AD

Epigenetic changes modulate the expression of certain genes without altering the DNA sequence. Epigenetic factors include DNA methylation, histone modification, and the regulation and modification of chromatin by noncoding RNA (ncRNA) (10). DNA methylation modifies cytosine residues by adding methyl groups in regions rich in cytosine-guanine. DNA methyltransferases, such as DNA methyltransferase 1, DNA methyltransferase 2, DNA methyltransferase 3, and DNA methyltransferase 3,6, are involved in the process.

Some cytosines, for example those located in the promotor region of the APP gene, have been found to exhibit methylation with age, which can lead to the formation of Aβ deposits. Methylation of the gene coding for the microtubule-associated protein tau (MAPT) can lead to the suppression of MAPT, which can end up affecting the level of the tau protein. Further, methylation in the promotor region of the brain-derived neurotrophic factor (BDNF) gene seems to play a significant role in the appearance of mild cognitive impairment (11).

Methylation of certain loci of specific genes, such as sortilin-related receptor 1 (*SORL1*), ATP binding cassette subfamily A member 7 (*ABCA7*), HLA class II histocompatibility antigen DRB5 beta chain (*HLADRB5*), solute carrier family 24 member 4 (*SLC24A4*), and box-dependent-interacting protein 1 (*BIN1*), has also been associated with AD (11). The protein encoded by *SORL1* controls the production of Aβ, so the methylation of the DNA that codifies this protein could lead to increased levels of Aβ.

Reelin is an extracellular matrix glycoprotein that, together with ApoE, shares the LRP and VLDLR/ApoER2 membrane receptors. During embryonic development, this protein regulates neuronal migration and, in the adult brain, intervenes in synaptic plasticity, interacting with ApoE. Binding of this protein to the membrane receptors activates a series of proteins that constitute the signaling pathway of reelin, inducing changes in the neuronal cytoskeleton. In transgenic mice that have lesions similar to those of AD, reelin counteracts early-phase synaptic dysfunction induced by the Aβ peptide (12).

*In vitro* studies have shown that oxidative stress alters the activation of proteins that are part of the reelin signaling pathway, resulting in the hyperphosphorylation of tau, which precedes the formation of neurofibrillary tangles in AD (13). Depletion of brain reelin has been detected in patients with AD prior to the formation of Aβ deposits (14). Thus, there seems to be a relationship between dysfunction of the reelin signaling pathway and AD.

Some reelin genotypes have been found to be associated with mild cognitive impairment and AD. The reelin single nucleotide polymorphism 2299356 (RELN-rs2299356) guanine–guanine genotype is associated with cognitive decline, while the adenine–adenine genotype triples the risk of developing AD. The reelin single nucleotide polymorphism 528528 (RELN-rs528528) cytosine–cytosine genotype, on the other hand, reduces the probability of mild cognitive

impairment by two thirds. These variations are located in the promoter region of the gene, which seems to play a regulatory role in its expression (15).

Reelin is involved in neuroplasticity, a process that has increased during the evolution of the human brain. Oxidative stress and probably other factors seem to induce epigenetic changes capable of reducing the expression of genes involved in synaptic plasticity. In some cases, such as that of the carriers of certain reelin genotypes, dysfunction of the proteins involved in the reelin signaling pathway caused by a reduction in reelin related to epigenetic changes could increase the probability of having the abnormal peptide deposits that characterize AD (15). It cannot be ruled out that certain alleles are more vulnerable than others to oxidative stress, toxins, inflammation, and other factors possibly related to AD.

Histones are proteins that serve as structural support for the DNA of the cell nucleus. Nuclear DNA associates with histones to form nucleosomes. The distribution and compaction of nucleosomes determines the structure of the chromatin and the accessibility of DNA to factors involved in the transcriptional machinery. Histones are also susceptible to epigenetic changes that can cause an increase or decrease in genetic expression. Nucleosomes are mainly regulated by posttranslational modifications that occur in the N-terminal region of histones.

Both methylation and acetylation can occur in histones through the antagonistic action of histone acetyltransferases (HATs), histone deacetylases (HDACs), histone methyltransferases, and histone demethylases. The acetylation of histones results in increased genetic activity by reducing the compactness of the nucleosomes and thus facilitating access of the transcriptional machinery to DNA. During senescence, mammalian cell cultures develop highly condensed regions of chromatin that may be associated with transcriptional decline (16).

Various HDAC inhibitors, like valproic acid and sodium butyrate, seem to improve memory in animal models and some neurodegenerative diseases like Parkinson's and even AD (16). Among the epigenetic changes described are the alteration of expression of the ncRNA. ncRNA is involved in genetic silencing as well as other functions, including the regulation of the activity of retrotransposons, genes that are capable of moving from one location in the genome to another. Short fragments of ncRNA, such as microRNA (miRNA), are involved in transcriptional gene regulation. ncRNA is primarily expressed in the brain, where it is involved in neuronal development, control of regions of the genome, which are involved in neuronal migration, homeostasis, and plasticity (17).

Epigenetics has improved our understanding of the evolution of the human brain, synaptic plasticity and neuronal diversity. Several studies have identified DNA methylation, changes in histones and chromatin, and changes in ncRNA expression in various neurological diseases, including AD. A large proportion of the genes that compose our genome are expressed in the central nervous system, where a substantial amount of miRNA is also synthesized. Several factors that have been associated with AD, such as diabetes mellitus, high blood pressure, obesity, diet, excessive sedentary lifestyle, smoking, and even a low educational level, are capable of inducing epigenetic changes (18).

There is currently no effective treatment for AD. However, cholinesterase inhibitors, such as donepezil, rivastigmine, and galantamine, together with *N*-methyl-D-aspartate (NMDA) receptor antagonists, such as memantine, produce moderate and transient symptomatic benefits in the early stages of the disease. Various treatments targeting the supposed causes of the disease are being developed, all still in the

experimental phase. One such treatment, active immunotherapy with Aβ fragments, which has been effective in transgenic mice (19), has not only been clinically ineffective in human patients but has also caused encephalitis in some cases (20). Passive immunotherapy with antibodies to Aβ has shown some benefits in transgenic mice and is being tested in humans. These clinical trials have shown that the clearance of Aβ in humans does not appear to produce significant cognitive improvements, which has led to some researchers questioning the role that Aβ plays in the cognitive decline associated with AD (20).

Attempts are also being made to develop drugs that prevent hyperphosphorylation or aggregation of the tau protein, although less effort has been made to this end than in inhibiting the formation of Aβ deposits. Most researchers support the hypothesis that amyloid plaques and the neurofibrillary tangles are neurotoxic. The amyloid cascade hypothesis has led to the development of treatments that promote Aβ clearance or prevent the formation of plaques. Such treatment has thus far been ineffective. A relatively high number of elderly people develop Aβ deposits without presenting with cognitive decline, which calls into question the amyloid cascade hypothesis.

Recent studies show that the Aβ peptide has antimicrobial properties, and that the absence of this peptide leads to an increased vulnerability to infection. Although the immune system has limited access to the central nervous system, it could fight invading pathogens with antimicrobial peptides like Aβ. The abnormal accumulation of Aβ observed in AD could be caused by persistent subacute infection or by noninfectious factors, such as trauma, ischemia, toxins, and anesthetics (21). Some researchers defend the hypothesis that Aβ acts as an antioxidant in response to the oxidative stress that takes place in regions of the brain subjected to high synaptic turnover, like that which occurs in the phenomenon of neuronal neoteny, where certain neurons retain a high synaptic plasticity in adulthood.

The generation of the Aβ peptide may have an adaptive function in its initial phases, and the same could be assumed about the hyperphosphorylation of tau. This would explain why drugs that reduce Aβ production have not been effective so far. Attempting to reverse the epigenetic changes that occur in AD could perhaps be of therapeutic value in the future.

## EPIGENETIC THERAPIES IN AD

As previously discussed, the treatments currently approved for AD are acetylcholinesterase inhibitors (donepezil, rivastigmine, and galantamine) and the glutamate NMDA receptor antagonist memantine, drugs indicated for the specific treatment of memory disorders. Acetylcholinesterase inhibitors increase the levels of the neurotransmitter acetylcholine, which is decreased in brains with AD, and NMDA receptor antagonists prevent aberrant stimulation (22). These drugs achieve a discrete and transient improvement in cognitive and functional capacities, but do not delay the progression of the disease. Nevertheless, observational studies suggest that the combination of these treatments prolongs the time until patients need to be admitted to a residence (23). As a result, there is a great deal of interest in researching new treatments for the disease.

The main line of research in AD is that of anti-amyloid therapies (24). Despite the serious complications associated with active immunotherapy and the repeated failures of passive immunotherapy, novel anti-amyloid antibodies such as aducanumab and BAN2401 have brought fresh hope in this line of research, since they have been shown to be capable of reducing amyloid load in preliminary clinical trials (25). Other treatments within the amyloid cascade hypothesis have been developed, which promote Aβ clearance or prevent plaque formation. However, not only have none of these treatments within this line of research been shown to be effective, but some of them have led to clinical worsening (26, 27).

Another line of research is that of anti-tau therapies, drugs that prevent the hyperphosphorylation or aggregation of the tau protein, or antibodies that reduce the levels of the protein in the cerebrospinal fluid. Lastly, other avenues of research that are currently unsuccessful or under investigation are anti-APOE4 drugs, antioxidants, anti-inflammatory drugs, cardiovascular drugs, mitochondrial protectors, hormone therapy, and antiviral drugs (28–30).

Due to the difficulty in finding effective drugs for AD, it is crucial that other possible therapeutic avenues are explored, such as that of epigenetic drugs. This line of research is based on the fact that epigenetic changes take place during neurodevelopment and aging, and that epigenetic alterations are common in various neurodevelopmental and neurodegenerative diseases.

In the case of AD, more than 20 epigenetic mechanisms have been identified, most of which involve direct DNA modifications (as in the case of methylation), modifications in chromatin structure (as in the case of histone modifications), or modification of mRNA-related processes, including ncRNA and miRNA.

With regard to changes in methylation in AD, a recent study has established reference maps of the genome-wide distribution of the three possible states of DNA methylation (5mC, 5hmC, and 5fC/caC) in this disease (31). The results of this study, based mainly on cortical neurons obtained from induced pluripotent stem cells, suggest that the changes detected in DNA may precede the appearance of the disease, rather than appear later as a consequence of its progression. These results could mean these markers could be very useful in reaching early molecular diagnosis and therapy.

In addition, it has been detected in AD and frontotemporal dementia that the levels of an important transcriptional repressor—repressor element 1-silencing transcription factor (REST)—do not increase adequately with age (32). Consequently, transcriptional changes occur, and decreases in the expression of neuroprotector genes are found, including forkhead box protein class O (FOXO), which contributes to resistance to oxidative stress. In contrast, increased expression levels of genes that promote AD pathology, such as presenilin 2, are found. Taken together, these changes would increase neuronal fragility in these diseases. Furthermore, in animal models, such as the K-p25 AD mouse model, an increase in the expression of genes associated with the immune response has been detected, along with decreases in the expression of genes involved in synaptic functions and learning (33).

Several changes in the histone acetylation process, which is heavily involved in the consolidation of memory, have been detected in AD. For example, the levels of histone H4 with acetylation at the 16th lysine residue protein (H4K16ac), a histone marker located in enhancers and promoters generally associated with active gene expression, are duplicated in the cerebral cortex in healthy aging but

are barely detectable in the cerebral cortex of people with AD (34). Levels of histone deacetylase 2 (HDAC2), which increase in cultured cells after neurotoxic insults, are also found to be increased in the hippocampus and prefrontal cortex of AD mouse models and in the hippocampus of people with AD (35). Increases in deacetylase lead to worsening of synaptic function. It should be noted that blocking HDAC2 increases synaptic density and alleviates the loss of memory, but does not improve neuronal survival. This means that deficits in AD are caused not only by neuronal loss but also by epigenetic blocking of the functions of neuronal survivors (36). In addition to the reduction in expression of genes important for neuronal function, an increase in aberrant expression of genes that are normally silenced or expressed at low levels has also been observed in AD (37, 38).

Furthermore, the expression of miRNA in the brain is altered in AD. For example, reduced levels of miRNA-29a/b-1 and miRNA-132, and increased levels of miRNA-34c have been detected. The decrease in miRNA-29a/b-1, which is a beta-site amyloid precursor protein cleaving enzyme 1 (BACE1) inhibitor, correlates with an increase in the production of Aβ (39). A decrease in miRNA-132, which targets the tau protein, HAT-associated protein 300 (EP300), sirtuin deacetylase 1, and FOXO1a, would jeopardize neuronal growth, the integration of newborn neurons, synaptic structure, and plasticity (40). Dysregulation of miRNA expression has also been detected in biofluids, suggesting that these molecules could be used as both biomarkers and therapeutic targets (39, 40).

Finally, an acceleration of epigenetic age has also been observed in AD, especially in the prefontal cortex. Epigenetic age is estimated from DNA methylation levels in 353 CpG sites, and its acceleration with respect to chronological age is associated with, in addition to AD, higher mortality, cognitive impairment, and other neurodegenerative diseases (41, 42). Epigenetic age could also explain differences in the onset age of AD in members of the same family that share the same gene mutation (43). That is, those who have an accelerated epigenetic age would develop AD symptoms at an earlier age.

The goal of epigenetic therapies is to reverse at least some of the epigenetic changes caused by AD. Such therapies have several advantages. First, specific drugs can be designed because the epigenetic changes are induced by enzymes that act at the DNA or histone level. Second, they act on reversible mechanisms since the epigenetic changes at the DNA and histone level are both regulated by enzymes. Finally, these therapies enable us to unite physiology and pathology, because epigenetics influence gene expression throughout life, and thus epigenetic drugs would be effective in both neurodevelopmental and neurodegenerative diseases. In addition, epigenetic therapies can target any component of the epigenetic machinery.

In the last decade, several epigenetic drugs have been designed for the treatment of neurological diseases. The most promising are DNA-demethylating agents and HDAC inhibitors (HDACis). In fact, there are already drugs of these two therapeutic groups that have been approved by the US Food and Drug Administration for the treatment of hematological cancer. In the former group, there is 5-azactydine and the 5-aza-2'-deoxycytidine (or decitabine), and in the latter group, suberoylanilide hydroxamic acid (SAHA or vorinostat), romidepsin, belinostat, panobinostat, and chidamide have been approved.

HDAC aims to regulate imbalances in protein acetylation levels and transcription. Their use in neurodegenerative diseases is based on their neuroprotective,

neurotrophic, and anti-inflammatory properties. In the case of AD, HDACs play an important role in memory consolidation and could be useful as therapeutic targets. For example, HDAC2 and HDAC3 have been shown to play a repressive role in memory formation, while HDAC5 has a memory-enhancing effect (44). As a result, HDAC2-inhibiting drugs, such as CI-994, and HDAC5-enhancing drugs could be useful in AD. The therapeutic potential of HDACis has been demonstrated in studies with animals. In APP/PS1 mice, acute treatment with HADCi trichostatin A (TSA), sodium valproic acid, SAHA, sodium butyrate (NaB), butyrate, vorinostat, 4-phenylbutiric acid, MS-275, and crebinostat improved the cognitive performance of these animals (45–49).

Sulforaphane could also be useful in AD. Sulforaphane is an HDACi that decreased HDAC2 levels in the triple-transgenic mouse model of AD (3 × Tg-AD). This was accompanied by an increase in the acetylation of histones H3 and H4 in the BDNF promoter and resulting in an increase in its expression (50).

There are HDACis that affect multiple genes involved in AD, which could be advantageous given the multifactorial etiology of AD. The disadvantage of these compounds is that their wide spectrum theoretically broadens the possibilities of adverse effects with their use. This group includes M344 {4-(dimethylamino)-*n*-[7-(hydroxyamino)-7-oxoheptyl]benzamide}, CM-414 {3-[[4-ethoxy-3-(1-methyl-7-oxo-3-propyl-6*H*-pyrazolo[4,3-d]pyrimidin-5-yl)phenyl]methyl]-*N*-hydroxycyclobutane-1-carboxamide}, and RGFP-966 {(E)-*N*-(2-amino-4-fluorophenyl)-3-[1-[(E)-3-phenylprop-2-enyl]pyrazol-4-yl]prop-2-enamide}. Their chronic use in animal models showed cognitive benefits (51–57).

In addition to the HDACis described, there are other HDACis that have been specifically designed. In this way, HDACi W2 was obtained, which features a longer half-life and better penetration of the blood–brain barrier than the HDACis currently available. HDACi W2 has been shown to be capable of significantly reducing Aβ levels in hAPP 3×Tg AD mice by reducing the expression of genes involved in Aβ production and increasing the expression of Aβ degradation enzymes. This HDACi is also capable of decreasing phosphorylation of the tau protein, promoting the formation and growth of dendritic spines, and improving learning and memory in these mice, which makes it a potential candidate for the treatment of AD.

Due to the observed benefits of using HDACis in animal models of AD, there are several ongoing clinical studies with these compounds. One of the compounds under study is valproate, which is a class I HDAC inhibitor. It has already been approved for treating epilepsy, migraine, and bipolar disorder, and since its activity as a HDACi was discovered, it is also being studied to evaluate its effectiveness in neurodegenerative disease. In preclinical studies, it was shown to be effective in reversing cognitive impairment in a mouse model of AD (58, 59).

Another drug under study is vitamin B3 or nicotamide, which is also a class III NAD-dependent sirtuin HDAC inhibitor. This compound can delay aging in mouse oocytes and delay cognitive impairment in a mouse model of AD (60). A phase I clinical trial in patients with AD demonstrated its safety. It is currently in a phase II clinical trial. Another HDACi, vorinostat, is being evaluated in a phase I clinical trial in patients with AD. Finally, RDN-929, which is a CoREST-selective HDAC inhibitor that could reactivate neuronal gene expression, strengthen synaptic function, and promote new synapses, has been studied in two phase I clinical trials as a possible treatment for AD.

Another mechanism for enhancing acetylation is the use of drugs that enhance HATs. The increased expression of an enzyme of this type, Tip60, in drosophila overexpressing human APP was able to restore the benefits of environmental enrichment (61).

With regard to miRNA, utility of the drug gemfibrozil has been studied. Gemfibrozil is capable of modifying miR-107 levels, the reduction of which may accelerate the progression of AD by regulating the expression of BACE1. A phase I clinical trial showed the drug was safe and reduced miR-107 in plasma and in CSF to undetectable levels.

Other possible epigenetic therapeutic targets not yet explored in AD are drugs directed against HATs, ten-eleven-translocation methylcitosine-dioxygenases enzymes (which catalyze the conversion of 5-methylcytosine to 5-hydroxymethylcytosine), DNA demethylation, chromatine remodelers, and other histone modifications.

Finally, it should be noted that there are also nonspecific epigenetic therapies that can be useful in AD. The first is blood plasma therapy from young subjects. In a recent study, in which aged mice were treated with blood plasma from young mice, it was observed that the treatment halved the epigenetic ages of blood, heart, and liver tissue, and also rejuvenated the hypothalamus. The treatment also improved the functioning of these organs as well as cognitive functions (62). The second nonspecific epigenetic therapy is cognitive stimulation, which has been shown to be capable of causing epigenetic changes (63). The third nonspecific epigenetic therapy is physical exercise. In animals, physical exercise is capable of reversing age-related reduction of adult neurogenesis and cognitive function in the aged hippocampus (64). In addition, a recent study showed that the administration of circulating blood factors in the plasma of aged mice subjected to exercise was capable of transferring the beneficial effects to sedentary-aged mice. These investigations led to the discovery that glycosylphosphatidylinositol (GPI)-specific phospholipase D1 (Gpld1), a GPI-degrading enzyme derived from liver, was probably responsible for these effects.

Although epigenetic therapies have a promising future role in AD, there are still problems that must first be addressed. First, we need to bear in mind that perhaps not all epigenetic changes can be reversed with these types of therapy. Second, epigenetic changes are extremely complex, and the therapies could have any number of side effects that are difficult to control. Third, different regions of the same gene can have antagonistic epigenetic changes, so the effect of an epigenetic therapy could be unpredictable. With respect to pharmacological properties, current therapies lack specificity and are not selective for specific brain regions, cell types, or genes. This limitation could be addressed with the use of siRNA or the use of chromatin-modifying enzymes, transcription activation-like effectors or clustered regularly interspaced short palindromic repeats/Cas System, and the use of artificial transcriptional factors of the silencing or promoter type.

The development of new study techniques will also be essential to better understand how epigenetic therapies work and to be able to design future drugs. First, laboratory techniques such as cell-type specific analysis of transcription and DNA methylation in the brain, single cell analysis of DNA–protein interactions, and chromatin 3D structure might be applied in future studies to uncover neuronal cell-type specific chromatin structure and interneuronal variations. Furthermore,

new models for studying the effect of these therapies, such as neuronal cultures or other brain cell types derived from human stem cells or induced pluripotent stem cells, could be better than the animal models used so far. The application of innovative imaging techniques, such as positron emission tomography using radiochemical [$^{11}$C] martinostat, which binds specifically to certain HDAC isoforms, could help to discover the gene expression patterns regulated by chromatin-modifying enzymes in the live brain. Finally, establishing epigenetic biomarkers for diagnosis, prognosis, and therapy would be important to test the efficacy of epigenetic drugs and to classify patients according to the particular therapy they would most benefit from.

## CONCLUSION

At present, there are no treatments for AD. Given that several epigenetic changes occur in this disease, treatments aimed at reversing these changes may constitute promising lines of research in the future.

**Conflict of Interest:** The authors declare no potential conflicts of interest with respect to research, authorship, and/or publication of this chapter.

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# 10

# Chimeric Conjugates for Alzheimer's Disease

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**Abstract:** Alzheimer's disease is a complex, progressive, neurodegenerative disorder with a multifactorial etiology. More than one mechanism appears to be involved in its pathogenesis. Current treatment targeting only a single mechanism provides only symptomatic relief and is unable to stop the progression of the disease. There is a substantial unmet medical need to develop more efficacious drugs that can address all the causative factors that lead to the development and progression of Alzheimer's disease. One of the strategies which has emerged is the development of chimeric conjugate compounds, in which multiple bioactive components are combined to form novel molecular entities, that can simultaneously regulate multiple mechanisms effectively. This chapter presents an overview of the various factors contributing to the pathophysiology of Alzheimer's disease. Chimeric strategies that are being developed to supplement the single-mechanism targeting acetylcholinesterase drugs, which are currently available for the treatment of Alzheimer's disease, are also exemplified.

**Keywords:** Alzheimer's disease; chimeric compounds; cholinesterase inhibitors; hepatotoxic; neuroinflammation

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## INTRODUCTION

Dementia can be defined as a clinical syndrome characterized by cognitive impairment that often leads to dependence on others for carrying out basic functions of daily life (1, 2). Alzheimer's disease (AD) is one of the most prevalent neurodegenerative disorder seen in older individuals, accounting for over 80% of all dementia cases worldwide (1, 2). AD causes structural damage to the brain, which results in substantial functional loss (3). It is a neurological disorder, often characterized by short-term memory impairment, which progresses into cognitive and physical disabilities (4). The etiology of AD is multifactorial with genetic, environmental, behavioral, and developmental components playing a role (5). The greatest risk factor is advancing age, while others include positive family history, head trauma, female gender, history of depression, diabetes mellitus, hyperlipidemia, and vascular factors (5).

In developed countries, the incidence of AD is increasing rapidly along with the aging of populations. The prevalence of AD among 60-year-old individuals is about 1%. This frequency doubles approximately every 5 years, becoming 2% at the age of 65 years, 8% at 75 years, and 16 and 32% at 85 years (4). This disorder may be classified as early and late-onset AD. Early onset AD is typically seen between 30 and 60 years of age and accounts for less than 6% of all cases. Late onset AD accounts for approximately 90% of cases and has an age at onset of more than 60 years (5). The estimated number of patients is 7–8 million in Europe, 4–5 million in the USA, and 24 million worldwide (6). This number is expected to reach around 100 million (one out of every 85 people) by 2050 (7).

AD is a multifaceted disease related with multiple risk factors that have an impact on the emotional and financial status of the patients and their families (3, 8). As per the World Alzheimer Survey 2015, the total health care expenses of the disease including medical services, social support, and informal care were 818 billion dollars with a rise of 35.4% compared with the same survey conducted in 2010. The expense for the treatment of AD for the year 2018 was $1 trillion, which is expected to rise about 2 trillion by the year 2030 (9).

## DISEASE AND PATHOLOGY

AD is a complex, self-escalating neurodegenerative disease, which is marked by the presence of beta-amyloid (Aβ)-rich senile plaques and neurofibrillary tangles (NFTs) in the brain (10, 11). The disease is characterized by impairments of memory and cognition, depression, and psychiatric and behavioral changes (12). The diagnosis of AD is confirmed by brain histopathological examination and relies on many clinical factors (4). Cognitive and functional declines are spread over 5–8 years as the disease progresses clinically from mild to moderate to severe AD (4). The mild stage is marked by short-term memory loss and generally lasts for about 2–3 years, which is often followed by symptoms of anxiety and depression (4). Neuropsychiatric manifestations, such as visual hallucinations, false beliefs, and reversal of sleep patterns, are prominent during the moderate stage (4). Motor signs, such as motor rigidity, mark the severe stage of AD (4). Cognitive and functional declines are seen in all three stages of the disease (4). The gross pathology

of AD includes generalized cortical atrophy, usually most prominent in the medial temporal lobe and hippocampus (13). AD pathology includes positive and negative signs (14, 15). Positive signs manifest in the form of cerebral Aβ plaques (the major peptide component being Aβ42) and NFTs of paired helical filaments made of hyperphosphorylated microtubule-associated protein tau (MAPτ) (4). These signs are quantified and shape the foundation for diagnostic criteria. Negative signs include neuronal and synaptic losses (14). The chronology of neuronal loss occurs on two levels. There is a local impact after the accumulation of Aβ and aggregation of tau, and there is selective impact on the regions affected by tau pathology (14). Synapse loss is another factor that contributes toward atrophy of the brain cortex. Synaptic loss has been demonstrated in AD patients through immunohistochemical studies, where immunoreactivity to antibodies of pre- or postsynaptic proteins (generally the presynaptic protein synaptophysin) is quantified using electron microscopy studies (16). Negative signs are difficult to evaluate and are not included in the diagnostic criteria, even though they have great physiopathological relevance (14). In recent years, several hypotheses have been proposed in an attempt to explain the pathogenesis of AD. These include the amyloid hypothesis, tau hypothesis, cholinergic hypothesis, oxidative stress hypothesis, and metal ion hypothesis, as depicted in Figure 1.

## Amyloid hypothesis

Histopathologically, the two hallmarks of the disease process in AD are extracellular amyloid plaques and intraneuronal tau NFTs, which characterize the dominant amyloid cascade hypothesis (9, 17). Aβ is formed after the proteolytic cleavage of a larger protein, known as the amyloid precursor protein (APP) (3).

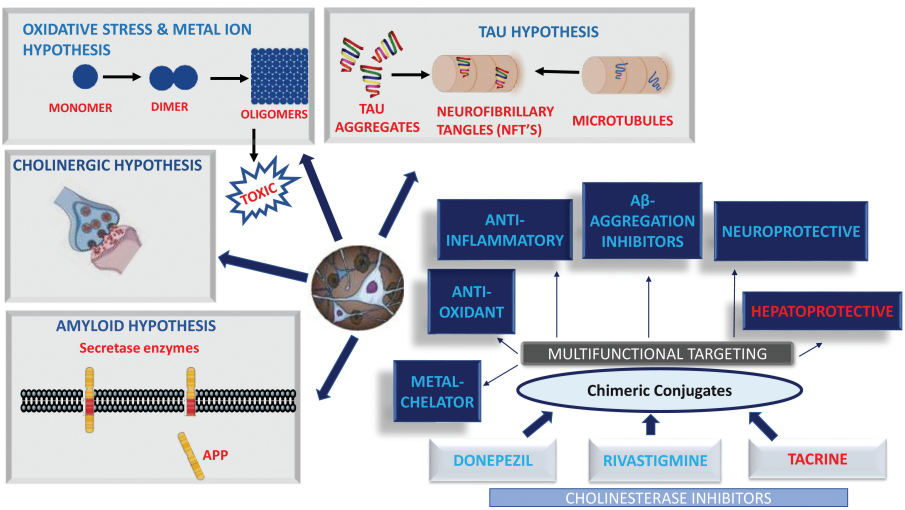



**Figure 1. Pathophysiological mechanisms and multifunctional targeting as an approach for Alzheimer's disease (AD) treatment.** This figure depicts the various hypotheses involved in AD pathology and the role of chimeric conjugates in multifunctional targeting as a new therapeutic strategy for AD.

APP is a glycoprotein comprising of about 770 amino acids and is found primarily in the CNS neurons (9). Activation of APP and subsequent cleavage leads to the formation of oligomers, fibrils, plaques, and β-sheets, ultimately resulting in Aβ aggregation, disruption of cellular communication, and generation of neuroinflammation (3, 18). There are several forms of Aβ, including lower and higher order oligomers and fibrils such as $A\beta_{40}$ and $A\beta_{42}$ (3). $A\beta_{42}$ is the first form that accumulates in the brain and forms amyloid plaques and deposits (3, 19). The two enzymes responsible for APP cleavage are alpha-secretase (α-secretase) and beta-secretase (β-secretase) (3, 20). The α-secretase enzyme combines with the C-83 subunit (non-amyloidogenic pathway) and produces APP-α precursors, which is thought to have neuroprotective effects (3, 21). On the other hand, the β-secretase enzyme combines with the C-99 β-subunit and produces APP-β (amyloidogenic pathway) (3).

Genetic factors also influence Aβ production and deposition. Genetic mutation and polymorphism of presenilin (PSEN1 and PSEN2) elevate the production of Aβ (3, 22).

Amyloid plaques are extracellular deposits of Aβ, abundant in the cortex of AD patients (23). There are two forms of plaques: neuritic and diffuse. Neuritic plaques mainly consist of Aβ and form part of tau-containing dystrophic neurites (13). Neuritic plaques are useful in the pathological diagnosis of AD because their appearance indicates the extent of cognitive impairment (23). Diffuse plaques consist mainly of Aβ-protein (13). Diffuse plaques are generally non-neuritic and are not linked with synaptic loss (23). These forms of plaques are also normally found in the brains of elderly patients with normal cognition, and their presence is not indicative of AD (23). In extracellular regions, Aβ accumulates and forms deposits in the parenchyma and vascular walls, which is denoted as cerebral amyloid angiopathy (CAA) (15). These deposits lead to a threefold increase in the concentration of soluble Aβ and comprise the visible part of Aβ aggregates (15). These soluble, oligomeric Aβ assemblies are highly toxic as well as difficult to identify and analyze (14, 15). These deposits, which are comprised of Aβ42, can be further classified as diffuse, focal, or stellate (15).

Diffuse deposits are large, about 50 μm in size or larger, and are generally seen in patients where there is no cognitive impairment, leading to the conclusion that these lesions are not directly toxic (15). Such deposits are typically seen in the striatum and the molecular layer of the cerebellum (14, 16). Focal deposits are characterized by dense, spheroid aggregation of Aβ. Few microglial cells are located in the area around the focal deposit that contains the amyloid plaque core. Astrocytes are located far away from the core and are involved in the processes of differentiation of neuronal stem cells without affecting neuronal or oligodendrocyte differentiation (16, 24). Stellate deposits are generally linked to astrocytes and are seldom observed and rarely examined (15). Aβ may build up in the walls of blood vessels, primarily in the arteries and capillaries, but seldom in the veins leading to CAA. CAA is characterized mainly by the presence of Aβ-40, which is frequently seen in the parenchymal deposits and is more soluble than Aβ-42 (14). Some degree of CAA, usually mild, is present in approximately 80% of AD patients (23).

## Tau hypothesis

Tau is an MAP found in the axon, where it attaches and stabilizes the microtubules, thereby physiologically promoting axonal transport (23). In AD, tau is

translocated to the somato-dendritic component, where it hyperphosphorylates, misfolds, and forms aggregates, leading to the formation of NFTs and neuropil threads (23). The effects of tau in neurodegeneration are less well established (25). However, aggregation of phosphorylated tau in dendritic spines appears to disrupt synaptic plasticity (14). In the cellular body of the neurons, tau aggregates form NFTs and neuropil threads in the dendrites and axons, which surround the core of the senile plaque (14). NFTs are hyperphosphorylated and misfolded tau intraneuronal aggregates, which become extraneuronal or "ghost tangles" with the death of the neurons carrying the tangles. NFT progression occurs in a stereotypical spatiotemporal way, which is linked with the decline in cognitive function (23). These are axonal and dendritic segments consisting of aggregated and hyperphosphorylated tau and are typically related with the NFTs in the brain (23).

The tau protein has a significant role to play in microtubule stabilization, which is important for maintaining cell integrity (3). The major structural domains of the tau proteins include the N-terminal projection domain, a microtubule-binding domain at the C-terminus, and a short sequence encompassing the tail domain (3). Tau proteins are hyperphosphorylated and form insoluble intracellular NFTs in AD, which lose the tenacity to bind the microtubules of brain cells. These hyperphosphorylated forms bind to each other, tying themselves in knots called NFTs that disrupt neuronal plasticity and cause neurodegeneration. Tau–tau interactions and its hyperphosphorylation in AD trigger a cascade of events in the microglial cells and astrocytes, activating the NF-kB pathway and overproduction of proinflammatory mediators such as TNF-α and interleukins (ILs), resulting in inflammatory reactions in brain (3). The elimination of tau is far more complicated compared to others like Aβ (3). It has been shown via PET imaging that deposition of tau has a greater correlation with the decline of cognitive functions than deposition of Aβ (26).

## Cholinergic hypothesis

The cholinergic hypothesis was the first established theory proposed to explain the pathogenesis and development of AD (9). In addition to the histopathological markers, the brain of AD patients is usually characterized by atrophy, synaptic loss, and decline in central neurotransmission (9) along with degeneration of neurons of the basal forebrain (9, 27). Cholinergic neurons are affected in the initial phase of the disease with greater than 90% of cholinergic neurons being lost in the advanced stages (9). Per this theory, the development of all symptoms related to impaired cognition in AD is due to the disruption of cholinergic neurons in the basal forebrain, along with loss of central cholinergic transmission (9).

## Oxidative stress hypothesis

Free radicals play an important role in the progression of neurodegeneration (3). Neuronal cells are more susceptible to free radical damage because of greater oxygen content and lack of antioxidant enzymes when compared with other organs (3). There is clear evidence that oxidative stress induced by Aβ is critical to the pathogenesis and progression of AD, leading to exacerbation of inflammatory processes, which is a characteristic of many multifactorial diseases including AD (9). The mitochondrial membranes of the AD postmortem brain have

demonstrated that Aβ and APP cause disruption of electron transport chain, thereby promoting irreversible neurodegeneration and cellular damage (3, 28).

### Metal ion hypothesis

Metal dyshomeostasis is involved in the progression of AD (29). Development of Aβ plaques and NFT is aggravated by the aberrant accumulation of metals in the brains of AD patients (30). High concentrations of Cu and Fe in the brain trigger the production of reactive oxygen species, which further exacerbates oxidative stress, thereby leading to worsening of AD (31, 32). Thus, a useful therapeutic strategy to mitigate AD would be to decrease the abnormal load of metal ions in the brain by chelating them (33).

## CURRENT LINE OF TREATMENT

AD is a complex disease, and hence difficult to treat with a single medication or therapy (34). The current line of treatment functions by modulating the levels of specific brain neurotransmitters such as acetylcholine and glutamate (34). These are helpful in retaining thoughts, cognitive functions, and social skills and can mitigate behavioral issues to a certain extent (34). However, these approaches do not address the root cause of the disease. The U.S. Food and Drug Administration (US-FDA) has approved several drugs to provide symptomatic relief in AD (34). Existing drugs employed for the symptomatic treatment of AD can be divided into two major classes: acetylcholinesterase (AChE) inhibitors such as donepezil, rivastigmine, galantamine, and tacrine and *N*-methyl-D-aspartate (NMDA) antagonists (glutamate inhibitor) such as memantine.

### Acetylcholinesterase inhibitors

Cholinesterase inhibitors (ChEIs) are generally used for long-term symptomatic treatment for AD (35). ChEIs are the only class of drugs approved by FDA for the symptomatic treatment of AD that can alter cholinergic neurotransmission and these include donepezil, rivastigmine, galantamine, and tacrine (4). To date, ChEIs are the only drugs that have shown significant improvements in cognition of AD patients by improving the cholinergic transmission in neuronal synapses. ChEIs slow down the degradation of the choline neurotransmitters at the synaptic clefts by inhibiting the cholinesterase enzymes, AChE, and butyrylcholinesterase (BuChE), which are responsible for the choline neurotransmitter degradation (9). These enzymes are abundant in neuritic plaques and can be inhibited by ChEIs; this may alter the build-up of Aβ, which is a critical part of AD pathophysiology (4). ChEIs increase cholinergic functions in AD at the postsynaptic cholinergic neuron (35). This class of drugs decreases AChE-induced destruction of ACh in the synaptic cleft, elevates the intrasynaptic residence time of acetylcholine, and promotes interaction between acetylcholine and the postsynaptic cholinergic receptor (35). Thus, to inhibit them, ChEIs increase the availability of these neurotransmitters in the synaptic cleft, thereby reducing the symptoms of AD (9).

AChE is also partially involved in the production of amyloid plaques and neurofibrillary tangles (34). AChE acts as an influencer to help in aggregating clusters of Aβ peptides, resulting in the formation of complexes with mature fibrils (34). The newly formed complexes are more cytotoxic in comparison to Aβ fibrils alone. ChEIs increase the levels of ACh in the brain of AD patients (34). Evidence derived from clinical trials, imaging, and basic science studies indicates that ChEIs are useful for symptomatic treatment but have limited disease-modifying effects (35).

Donepezil is a piperidine-derivative AChE inhibitor drug, which increases the levels of acetylcholine in the CNS (9, 36). It has shown moderate benefit in the treatment of AD patients due to its modest and transient outcomes (36, 37). It is effective in managing the symptoms of AD-associated dementia. However, it does not alter the progression of AD (38). Donepezil is metabolized via the cytochrome P-450 system and has the tendency of being involved in drug–drug interactions, especially when used in combination (9).

Rivastigmine, a physostigmine-derived drug, is the only carbamate containing AChE inhibitor approved for the treatment of mild to moderate AD (9, 39). It improves cognition and shows neuroprotective effects, but does not alter the course of disease and only leads to a modest improvement in cognitive functions. This drug shows good activity and tolerance in AD patients and is not involved in the cytochrome P-450 system metabolism, thereby decreasing the chances of drug–drug interactions (9).

Galantamine is a tertiary alkaloid extracted from various species of *Amaryllidaceae* (9). It is a selective, competitive, and reversible inhibitor of AChE with nicotinic-modulating properties, has low hepatotoxicity (9), and reduces APP metabolism in animal models of AD (35). However, its involvement in cytochrome P-450 metabolism makes it prone to interaction with other drugs (9).

Tacrine, a dual AChE and BuChE inhibitor, was the first of its kind to get FDA approval for the treatment of AD. It was withdrawn from the market shortly after FDA approval due to serious hepatotoxicity (40). Tacrine is a noncompetitive, reversible inhibitor of AChE, which has a short half-life (9).

## N-methyl D-aspartate receptor antagonism (NMDA antagonists)

Overstimulation of the NMDA receptor by the neurotransmitter glutamate is implicated in neurodegenerative disorders (4). Glutamate is the principal excitatory neurotransmitter in the brain (4). The overstimulation of glutamate has been known to contribute to neuronal damage, which is termed as excitotoxicity (4). Such excitotoxicity eventually contributes to neuronal calcium overload and has been implicated in neurodegenerative diseases (4). Glutamate activates several postsynaptic receptors, including the NMDA receptor, which have a direct impact on the memory processes, dementia, and in the pathogenesis of AD (4).

The FDA-approved NMDA antagonist memantine decreases glutaminergic excitotoxicity by influencing neuronal activity in the hippocampus and can be used in the treatment of moderate to severe AD (35). Memantine is approved as an alternative to ChEIs in treatment of moderate to severe AD (25). Memantine at high concentrations can suppress synaptic plasticity, which is believed to have an effect on learning and memory. However, at lower concentrations, memantine can

promote synaptic plasticity, thereby enhancing memory in animal models of AD (4). Memantine also has the potential to enhance long-term potentiation and decrease tau hyperphosphorylation (17). The neurobiological basis for the therapeutic action of memantine in AD is not clearly known. Memantine is a noncompetitive NMDA receptor antagonist with moderate affinity and fast on/off kinetics (4). These attributes are vital for memantine action as it is able to balance the effects of excessive glutamate levels while preserving physiologic activation of NMDA receptors necessary for learning and memory (4). Memantine inhibits the effects of excessive glutamate production, which contributes to cell death and cognitive impairment (4).

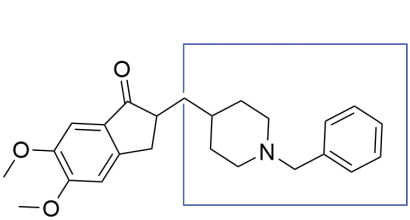
## RATIONALE FOR CHIMERIC CONJUGATES

The current FDA-approved drugs addressing a single mechanism have turned out to be palliative rather than curative. By addressing just the cholinergic hypothesis, they only provide temporary relief for the patients by improving their cognitive functionality throughout the time period of usage (41, 42). There is a critical need to add antioxidant, metal chelation, neuroprotective, $A\beta_{1-42}$ amyloid anti-aggregation, and anti-inflammatory activities into the compounds. Such molecules that can address all the hypotheses of AD will likely yield significant disease-modifying outcomes.

The multifaceted, complex nature of AD has limited the treatment options in the battle against the disease. One of the ways of tackling this problem is to expand the scope of single-mechanism targeting drugs to form multi-targeting chimeric entities (43). The multi-target directed ligand (MTDL) design strategy is a method where a molecule is designed by simultaneously integrating multiple functionalities into it that can target different mechanisms crucial to the disease pathology (44). These chimeric conjugates are created by molecular hybridization of different biologically relevant pharmacophores (45). Each pharmacophore of the new chimeric molecule retains the ability to interact with its own target while the chimeric molecule simultaneously modulates multiple molecular targets, thereby producing a range of diverse pharmacological responses (45–47). Such engineered chimeric compounds simultaneously target many of the implicated pathways of the disease, thereby yielding a disease-modifying effect (48, 49). In addition, these compounds potentially have a lower risk of triggering drug–drug interactions and facilitate pharmacodynamic and pharmacokinetic roles of drug administration (46, 47). AChE inhibitors like donepezil, rivastigmine, and tacrine have been used as starting points and combined with several bioactive molecules to generate chimeric series, which have demonstrated expanded efficacy and safety profiles. The hybrid series were designed so as to retain the key structural features essential for retaining AChE inhibition of the parent molecules.

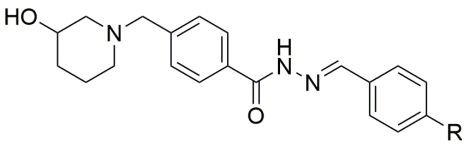
### Donepezil-related derivatives as multifunctional compounds for AD

The structure of donepezil shows the presence of benzyl piperidine and substituted indanone fragments linked via a methylene bridge (Figure 2, left panel). The binding pose of donepezil within the AChE pocket as seen in the X-ray




Benzyl piperidine

Donepezil
[pharmacophoric unit (boxed)]




Donepezil-aryl acyl hydrazone hybrids

| Compound | R |
|---|---|
| 1a | nitro |
| 1b | N-piperidino |
| 1c | F |
| 1d | Br |
| 1e | Cl |
| 1f | methoxy |
| 1g | morpholino |

**Figure 2.** ***Left panel*—donepezil with its pharmacophoric unit boxed in blue; *right panel*—donepezil-aryl acyl hydrazone hybrids.** Various donepezil-aryl acyl hydrazone chimeras have been constructed by conjugating the pharmacophoric unit of donepezil with substituted phenyl acyl hydrazones.

diffraction-elucidated crystal structure (PDB id 4EY7) shows the interaction of the benzyl piperidine fragment with the catalytic site of AChE (50). This fragment has been nominated as a crucial pharmacophoric subunit of donepezil. The indanone moiety binds to the peripheral anionic site of the AChE pocket and forms aromatic stacking interactions (50). The benzyl piperidine fragment of donepezil has been hybridized with various bioactive groups in order to introduce other activities into the molecule so as to make them more effective for the holistic treatment of AD. Aryl acyl hydrazones possess anti-inflammatory activity, which has been shown to slow down the progression of AD. Donepezil-aryl acyl hydrazone chimeras were constructed (51) by attaching the aryl-acyl hydrazone sidechain to the benzyl end of *N*-benzyl piperidine of donepezil (Figure 2, right panel).

The donepezil-derived hybrid molecules were screened for AChE inhibition according to the spectrophotometric method developed by Ellman (52). Replacement of the dimethoxy indanone fragment of donepezil with various ring-substituted aryl acyl hydrazones gave molecules that exhibited comparable AChE inhibition. In-depth structure–activity correlation studies showed that compounds that had the 3-hydroxy piperidine moiety were more active than the molecules where the 3-hydroxy group was acetylated. Substitutions on the aromatic ring on the other side of the molecule with groups like nitro (1a) and piperidine (1b) resulted in molecules that were more potent relative to the unsubstituted compound (51). Halogen substitution gave a threefold increase in activity as compared to the unsubstituted molecule with the fluoro analogue (1c) being more potent than bromo (1d) and chloro (1e) derivatives (51). The placement of the benzyl piperidine fragment in the chimeric series with the piperidine ring placed at the terminal end was opposite to that in donepezil. It is possible that by reversing the placement of benzyl piperidine fragment, the hybrid molecules were able

to find an alternate binding orientation in the AChE binding pocket, which resulted in their enhanced activity profiles.

Suppression of the neuroinflammation process is an effective therapeutic approach against AD. The donepezil-aryl acyl hydrazone chimeric molecules were tested for their *in vivo* anti-inflammatory activities using classical animal models such as mechanical allodynia test, formalin-induced hyperalgesia, and carrageenan-induced paw oedema assays (51). Halogens (1c, 1e) and methoxy-substituted hybrid molecules (1f) as well as compounds substituted with rings such as piperidine (1b) and morpholine (1g) (Figure 2) significantly reduced mechanical hyperalgesia index, decreased licking time in the formalin test pointing to an analgesic effect, and reduced oedema volume, thereby confirming an anti-inflammatory effect (51).

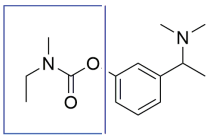
The halogen-substituted compounds (1c, 1e) were found to inhibit the release of TNF-α and IL-1β induced by lipopolysaccharides (LPS) in THP-1 cells, which are representative of human microglial cells (51). THP-1 cells were treated with 10 μM of the compounds and LPS (1 μg/ml) for 24 h. At the end of the treatment, it was concluded that both halogen-substituted compounds reduce evoked neuroinflammation (51).

## Rivastigmine-related derivatives as multifunctional compounds for AD

Rivastigmine, a carbamate-containing AChE inhibitor, has been found to provide only mild to moderate benefit in patients with AD (39). The carbamate moiety is the pharmacophore subunit of rivastigmine (Figure 3, left panel), which binds to the catalytic site of AChE and is responsible for its ChEI activity (53). Rivastigmine chimeras with amino chalcones with promising cholinesterase inhibition activity are shown in Figure 3 (right panel). Xiao et al. worked on developing rivastigmine-4 amino chalcone hybrids and conducted in-depth structure–activity correlation studies for the series (54).

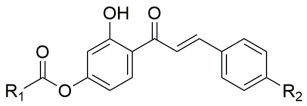
Several of the rivastigmine-derived compounds showed potent AChE inhibition. Structure–activity correlation studies revealed that compounds with cyclic amine groups (2b, 2c, 2d) at one or both extremities of the molecule show better inhibitory activity as compared with those with noncyclic amine groups (2a). Pyrrolidine ring-substituted compound 2b was twice as potent as rivastigmine, while morpholine ring-substituted compound 2e (Figure 3) was the weakest inhibitor of AChE (54). The authors surmised that the electron-withdrawing inductive effect of the oxygen atom on the morpholine ring may decrease the electron density of the ring nitrogen, thereby influencing its protonation at physiological pH, which could diminish the cation–π interaction between the nitrogen and residues of the catalytic active site of AChE (54).

The antioxidant activity for rivastigmine-amino chalcone hybrids was evaluated by the oxygen radical absorbance capacity assay involving fluorescein. Compounds containing a pyrrolidine ring (2b), *N*-methylethaneamine (2f), and benzyl piperazine ring (2g) as substituents were found to exhibit the most potent antioxidant activities. 4-Dimethylamine chalcone–rivastigmine hybrid molecule (2a) showed moderate antioxidant activity, while replacement with other amino alkyl groups resulted in loss of antioxidant activity. The authors concluded that dimethylamine substitution at para position is favorable for antioxidant potency (54).



Carbamate

Rivastigmine
[pharmacophoric unit (boxed)]



Rivastigmine-aminochalcone hybrids

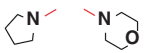
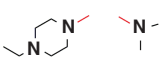
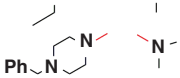
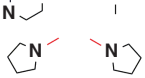
| Compound | $R_1$ | $R_2$ | Compound | $R_1$ | $R_2$ |
|---|---|---|---|---|---|
| 2a |  | | 2e |  | |
| 2b |  | | 2f |  | |
| 2c |  | | 2g |  | |
| 2d |  | | | | |

**Figure 3.** ***Left panel*—rivastigmine with its pharmacophoric unit boxed in blue; *right panel*—rivastigmine-aryl acyl hydrazone hybrids.** Various rivastigmine-aminochalcone chimeras have been constructed by conjugating the pharmacophoric unit of rivastigmine with substituted amino chalcones.

The 2-hydroxy and ketone groups of rivastigmine-amino chalcone chimeras undergo intramolecular hydrogen bonding as they exhibit metal-chelating properties. Xiao et al. found that compound 2b (Figure 3) exhibited selective metal chelation for copper and aluminium but not iron and zinc metal ions (54).

The effects of the rivastigmine-amino chalcone hybrid molecules on Aβ aggregation were evaluated by performing thioflavin T (ThT) fluorescence assay using curcumin as a reference standard. Structural studies concluded that noncyclic amines 2a and 2f (Figure 3) exhibited the most potent inhibition effects on self-induced Aβ aggregation, while the *N*-benzyl piperazine containing analogue (2g, Figure 3) showed the best $Cu^{2+}$-induced Aβ aggregation inhibition. Besides this, bulky substituents on the hydroxy group at 2-position on the chalcone fragment lowered the anti-aggregation effects (54).

## Tacrine analogues with decreased liability of hepatotoxicity

Tacrine, an acridine analogue, was the first centrally acting ChEI approved for the treatment of AD. It is a reversible, noncompetitive inhibitor of AChE and BuCHE. It also possesses the ability to reduce Aβ-induced neurotoxicity. Despite it benefits, tacrine is poorly tolerated and often causes reversible abnormalities in liver enzymes. Nevertheless, its inherent efficacy and small molecular weight have attracted a lot of research directed toward the development of MTDLs. The whole molecule of tacrine (Figure 4, left panel) has been used as a starting point and fused with hepatoprotective scaffolds, leading to the development of safe, efficacious tacrine hybrids (55). Tacrine derivatives coupled to fragments that help counter its hepatotoxicity are shown in Figure 4 (right panel).

Zha et al. developed tacrine-benzofuran chimeric molecules in an attempt to combine the AChE inhibitory properties of tacrine and the *in vitro* inhibitory

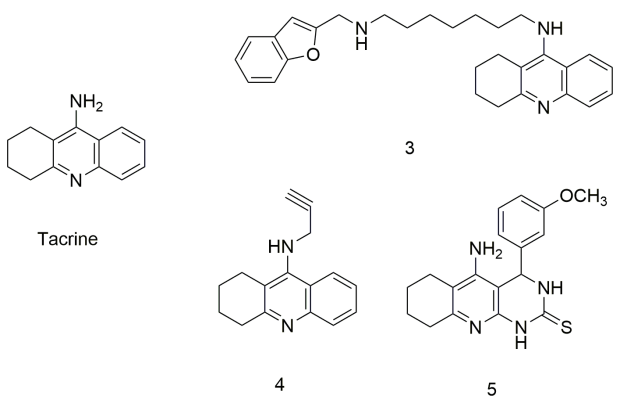



**Figure 4. Tacrine hybrids with reduced hepatotoxicity.** Several tacrine chimeras have been constructed by conjugating tacrine with various hepatoprotective moieties.

effects on Aβ fibril formation and aggregation reported for the benzofuran nucleus (56). The most active designed molecule, compound 3 (Figure 4), showed nanomolar inhibitory potency against AChE, as well as an ability to partially inhibit AChE-induced Aβ fibril formation and amyloid self-aggregation (56). This compound demonstrated mixed inhibitory behavior in an enzyme kinetics study pointing to dual binding interaction sites (56). This was confirmed by the authors by determining the three-dimensional form of the AChE-bound structure of this compound through X-ray diffraction studies (56). The tacrine fragment was seen to occupy its place at the catalytic site, engaging in stacking interactions with Trp 84 and Phe 330 and hydrogen bonding interactions with His 440 of the catalytic triad of AChE, while the methyl benzofuran was found to make contact with the peripheral anionic site where it is accommodated within a pocket of hydrophobic residues (56). In addition, compound 3 had a better safety profile and showed significantly lower hepatotoxicity than tacrine when tested with the alanine aminotransferase and aspartate aminotransferase activity assays (56).

Mao et al. synthesized a number of tacrine-propargylamine derivatives, inspired by propargylamine-containing compounds, which exhibit neuroprotective effects (40). These compounds were tested for AChE inhibition and neurotoxicity (40). In addition, they were evaluated for hepatotoxicity in human hepatic stellate cells using the colorimetric MTT assay (40). It was reported that compound 4 (Figure 4) exhibited superior AChE inhibition and lower neurotoxicity than tacrine. In addition, it almost eliminated the hepatotoxicity of tacrine (40). Kinetic studies were also carried out on this compound, which pointed to a mixed type of enzyme inhibitory behavior. It is possible that extending the tacrine amine with the lipophilic propargyl group endowed the molecule with additional binding opportunities within the AChE enzyme, resulting in a twofold improvement in AChE inhibition. Such tacrine chimeras, which have excellent AChE inhibition and neuroprotective effects without the hepatotoxicity of tacrine, can be used as potential lead compounds for the treatment of AD (40).

Chioua et al. designed a series of tacripyrimidines by coupling the ChEI tacrine moiety to derivatives of 3,4-dihydro dihydropyrimidin-2(1H)-thiones, which are known calcium channel blockers (CCBs) (57). CCBs enhance cerebrovascular

perfusion and attenuate amyloid-β-induced neuronal decline and neurotoxicity, improve cell survival in the presence of Aβ *in vitro*, and show neuroprotective effects (57). Derivatives bearing halogens (Br, Cl) at meta and para position of the aromatic ring of the dihydropyrimidine-thiones demonstrated the highest inhibitory potencies toward AChE, while the presence of a 4-dimethylamino group or 3-nitro group was found to be the best CCBs, with potencies higher than that of the reference CCB drug nimodipine (57). Tacripyrimidine compound 5 (Figure 4) had the most balanced overall biological profile. It had low micromolar AChE inhibitory potency as well as calcium channel blocking activity and had no significant hepatotoxicity toward HepG2 cells up to 300 mM and excellent predicted oral absorption and BBB permeability (57).

## CONCLUSION

The currently available FDA-approved drugs for the treatment of AD are limited by the fact that they target only a single mechanism in the development of this multifactorial disease with extremely complex pathophysiology. Several molecules with antioxidant, anti-inflammatory, and neuroprotective properties are known. Conjugation of these molecules with the currently available, FDA-approved ChEIs to form molecular chimeras has been shown to expand their anti-AD spectrum, thereby creating entities that have the potential for development as disease-modifying therapies for AD.

**Conflict of Interest:** The authors declare no potential conflict of interest with respect to research, authorship, and publication of this chapter.

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# 11

# Multifunctional Ligand Approach: Search for Effective Therapy Against Alzheimer's Disease

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**Abstract:** Alzheimer's disease is a progressive, incurable, and complex neurodegenerative disease. Currently, an effective treatment that can slow down or stop the damage and death of neurons, which is a characteristic of Alzheimer's disease, is lacking. Taking into account the complex nature of the disease, a multitarget design approach has been developed for the production of new potential anti-AD agents. The goal of this approach is to create a single molecule that can interact selectively with several desired molecular targets relevant to the disease. This strategy was successfully developed two decades ago and has been improved in recent years. This chapter describes the progress made in the discovery and design of selected multitargeted drugs based on molecular targets, which can be used for treating Alzheimer's disease. The most promising among these drugs are the molecules having properties that are valuable not only in the symptomatic therapy but also in the causal treatment of the disease. The main hypotheses of Alzheimer's disease, such as β-amyloid (Aβ), tau, and cholinergic, suggest that compounds capable of inhibiting the aggregation of neurotoxic Aβ-amyloid peptide and tau protein, and improving the cholinergic neurotransmission, may possess such properties.

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Examples of such multifunctional molecules, which have been recently reported in the literature, are presented in this chapter.

**Keywords:** Alzheimer's disease; disease-modifying strategies; multimodal compounds; polypharmacology; symptomatic strategies

## INTRODUCTION

Alzheimer's disease (AD) is a progressive, incurable, and complex neurodegenerative disorder. The two main neuropathological hallmarks of AD are extracellular amyloid plaques composed of β-amyloid (Aβ), and intracellular neurofibrillary tangles (NFTs) containing the tau protein, leading to nerve cell death. In addition, AD is characterized by a low level of the neurotransmitter acetylcholine and loss of cholinergic neurons, excitotoxicity, impairment of other neurotransmitter systems, extensive oxidative stress, chronic neuroinflammation, mitochondrial dysfunction, calcium and metal dyshomeostasis, and other factors (1). This complex and unclear pathogenesis of AD has led to the creation of several theories. The oldest one is the cholinergic hypothesis, followed by others such as the Aβ hypothesis, the tau hypothesis, the oxidative stress theory, the metal imbalance theory, the mitochondrial cascade, and the inflammation hypothesis were developed. The available AD therapy is based only on medications that are capable of treating the cognitive symptoms: three cholinesterase inhibitors (donepezil, rivastigmine, and galantamine) and one *N*-methyl-D-aspartic acid (NMDA) receptor antagonist (memantine). Currently, an effective treatment that may slow down or stop the damage and death of neurons in AD is lacking.

Complex diseases such as AD require a more intricate treatment (2, 3), and hence, agents acting on only one particular target or modulating only one process are not sufficient. This problem can be overcome by two possible approaches. The first, polypharmacy, involves combination therapies with the use of two or more drugs (4). An alternative way is to use multifunctional compounds that exhibit many effects by interacting with more than one biological target (5, 6). Such molecules, which are referred to as multifunctional or multidirectional compounds, multitargeted ligands (MTLs), or multitarget-directed ligands (MTDLs), provide additive or synergistic effects, called polypharmacology (7).

The issues related to the use of combination therapies for AD have been recently described by Cummings et al. (8). Pharmacodynamic combination therapies are based on two or more symptomatic agents that can improve the behavioral and cognitive symptoms of AD and/or disease-modifying therapies (DMTs) that affect the causes of the disease. In 2014, Namzaric, a combination of two symptomatic medications—the cholinesterase inhibitor, donepezil and the NMDA receptor antagonist, memantine—was approved for the treatment of AD patients with moderate-to-severe dementia (9). Currently, various clinical trials are ongoing on combination therapies involving a standard-of-care medication like a cholinesterase inhibitor or memantine combined with another agent. Selected add-on clinical trials of combination treatment for AD, using a small molecule with a standard-of-care medication, are presented in Table 1.

**TABLE 1** **Selected add-on clinical trials of combination treatment for AD using a small molecule with a standard-of-care medication**

| Agent | Biological target | Phase | Baseline Therapy | Clinical trials |
|---|---|---|---|---|
| Masitinib | Selective tyrosine kinase inhibitor | III | Rivastigmine and/or memantine | NCT01872598 |
| Telmisartan | Angiotensin II receptor antagonist | II | AChE inhibitor and/or memantine | NCT02085265 |
| Saracatinib | Src/Abl kinase family inhibitor | II | AChE inhibitor and/or memantine | NCT02167256 |
| UE2343 | β-Hydroxysteroid dehydrogenase inhibitor | II | AChE inhibitor and/or memantine | NCT02727699 |
| ANAVEX2-73 | Sigma-1 chaperone agonist | II | AChE inhibitor and/or memantine | NCT02244541 |
| Neflamapimod | P38 MAPK alfa inhibitor | II | AChE inhibitor or memantine | NCT03402659 |
| CT1812 | Antagonist of the sigma-2 receptor | II | AChE inhibitor or memantine | NCT02907567 |
| Idalopirdine | 5-$HT_6$ receptor antagonist | III | Donepezil or donepezil and memantine | NCT01955161 |
| Citalopram | Selective serotonin reuptake inhibitor | III | AChE inhibitor and/or memantine | NCT00898807 |
| Rasagiline | MAO-B inhibitor | II | AChE inhibitor or memantine | NCT02359552 |
| Piromelatine | Melatonin and serotonin receptor agonist | II | AChE inhibitor or memantine | NCT02615002 |

AD, Alzheimer's disease; AChE, acetylcholinesterase; MAO, monoamine oxidase; MAPK, mitogen-activated protein kinase.

## MULTITARGET LIGANDS APPROACH

Combination therapy has some disadvantages resulting from the possible accumulation of drug side effects, pharmacokinetic complexity, drug–drug interactions, and decreased compliance. Moreover, older patients might feel that using one tablet is more convenient than using two or more. Multifunctional compounds are free of the above-mentioned disadvantages. Since these agents can be considered as a form of multicomponent therapy, the creation of new molecules is based on similar principles. Multifunctional agents can be designed by combing two or more structures of active agents or their pharmacophore fragments that interact with the desired targets. Possible strategies for designing multifunctional agents include combining molecules that can interact with two symptomatic targets or symptomatic targets and disease-modifying targets or with two disease-modifying targets. The targets of DMT are neurotoxic aggregates of Aβ and tau

protein, as well as various processes associated with neuroprotection or neuroinflammation. The targets of symptomatic therapy are the cholinergic system, including acetylcholinesterase (AChE) and butyrylcholinesterase (BuChE) enzymes, NMDA neurotransmission, G protein-coupled receptors (GPCRs), and monoamine oxidase (MAO) enzymes (Table 2).

A multitarget design approach was successfully developed two decades ago and has been improved in recent years. There are basically three ways to combine pharmacophores to form an MTL structure, which can interact with appropriate biological targets (10). The simplest way is to connect two pharmacophores through a linker. Another way is to combine two pharmacophore fragments without a linker, forming condensed molecules. The third and best way is to create merged pharmacophores that result in small molecules with low molecular weight and thus with good drug-like properties. Over the years, various potential multifunctional anti-AD agents have been designed, synthesized, and developed (11–13). Based on the structures of currently used anti-AD drugs or their

**TABLE 2 Therapeutic strategies of AD applied for the creation of multifunctional agents**

| Therapeutic strategies for AD treatment | Target for AD treatment | Agents |
|---|---|---|
| Disease modifying therapies | Aβ aggregates | β-Secretase inhibitor<br>Aβ aggregation inhibitor<br>Metal chelator |
| | Tau aggregates | GSK-3β inhibitor<br>Tau aggregation inhibitor |
| | Neuroinflammation<br>Neuroprotection<br>Various | COX-2 inhibitor, LOX inhibitor,<br>Antineuroinflammatory agent,<br>S1R modulator,<br>Antioxidant agent,<br>PDE4 inhibitor |
| Symptomatic therapies | Cholinergic neurotransmission | AChE inhibitor<br>BuChE inhibitor |
| | NMDA neurotransmission | NMDA receptor antagonist |
| | GPCRs<br>Serotonin receptor | $5\text{-HT}_4$ receptor agonist<br>$5\text{-HT}_6$ receptor antagonist |
| | GPCRs<br>Histamine receptor | $H_3$ receptor antagonist/inverse agonist |
| | GPCRs<br>Cannabinoid receptor | $CB_1$ receptor antagonist<br>$CB_2$ receptor agonist<br>FAAH enzyme |
| | Oxidative damage | MAO-A inhibitor<br>MAO-B inhibitor |

AD, Alzheimer's disease; AChE, acetylcholinesterase; BuChE, butyrylcholinesterase; COX, cyclooxygenase; FAAH, fatty acid amide hydrolase; GPCR, G protein-coupled receptors; GSK, glycogen synthase kinase; LOX, lypoxygenase; MAO, monoamine oxidase; NMDA, *N*-methyl-D-aspartic acid; PDE4, phosphodiesterase 4.

pharmacophoric fragments, multimodal compounds are produced. Thus, ligands containing fragments of tacrine (as a strong inhibitor of cholinesterases), donepezil, or rivastigmine are the most numerous. Of these, only ladostigil has entered phase II/III clinical trials in patients with mild cognitive impairment. Its structure represents a merged pharmacophore, which is formed by combining a propargylamine moiety derived from an MAO-A/B inhibitor rasagiline with a pharmacophoric carbamyl moiety derived from rivastigmine. Ladostigil is a dual AChE/BuChE and a brain-selective MAO-A/B inhibitor possessing *in vivo* neuroprotective properties (14) and, as such, is an example of a multifunctional agent created by combining two pharmacophores that act on the symptomatic targets of AD.

Among the various multifunctional agents, the largest group that focuses on the targets of symptomatic therapy is cholinesterase inhibitors combined with other targets (15). Dual binding site cholinesterase inhibitors capable of interacting with the catalytic active site and the peripheral anionic binding site of AChE can inhibit Aβ aggregation. Based on this action, many such ligands were obtained, which showed potency toward both symptomatic targets (AChE/BuChE) and disease-modifying targets (Aβ aggregation) (16–20). Herein, the recent advances made in the design of multifunctional agents for AD treatment based on molecular targets and selected examples of novel multimodal ligands are presented.

## MULTIFUNCTIONAL AGENTS FOCUSING ON DISEASE-MODIFYING TARGETS: Aβ AND OTHERS

The Aβ hypothesis assumes that the primary cause of neuron loss is the formation of senile plaques due to abnormal processing of amyloid precursor protein (APP) (21). The key role in this process is played by the β-secretase enzyme (BACE-1, Aβ precursor protein-cleaving enzyme 1), which together with γ-secretase cleaves APP, producing Aβ peptides consisting of 38–42 amino acids. Because of their fibrillogenic and hydrophobic nature, Aβ peptides accumulate easily. These peptide aggregates induce oxidative stress, neuroinflammation, hyperphosphorylation, and the aggregation of tau protein, ultimately resulting in loss of neurons and dementia. Based on this theory, some biological disease-modifying targets have been identified and are used in the search for new anti-AD agents. Inhibition of BACE-1 and amyloid aggregation is considered as the most important objective of these agents. Currently, 38 (29%) Aβ-targeting agents are undergoing clinical trials, of which four BACE-1 inhibitors are gaining continued interest as biological targets in the field of drug discovery and development (22). BACE-1 inhibition has also been applied for the generation of MTDLs having other beneficial properties.

### BACE-1 inhibitors with other properties

The structure of donepezil is widely used as a pharmacophoric moiety for developing multifunctional agents (19). Examples of compounds formed using the donepezil structure are *N*-benzylpiperidine analogs acting as BACE-1 and AChE inhibitors with antioxidant and antiaggregating properties, which were described

by Sharma et al. (23). As a core group, *N*-benzylpiperidine moiety present in donepezil and capable of inhibiting BACE-1 was selected. Virtual screening led to the emergence of the hit compound SEW06622. Based on its structure, a series of new anti-AD agents was designed. The compounds differed in the substitution of benzylamine and the presence of a double bond close to the nitrogen atom. The design of these compounds is presented in Figure 1. Among them, compound **1**

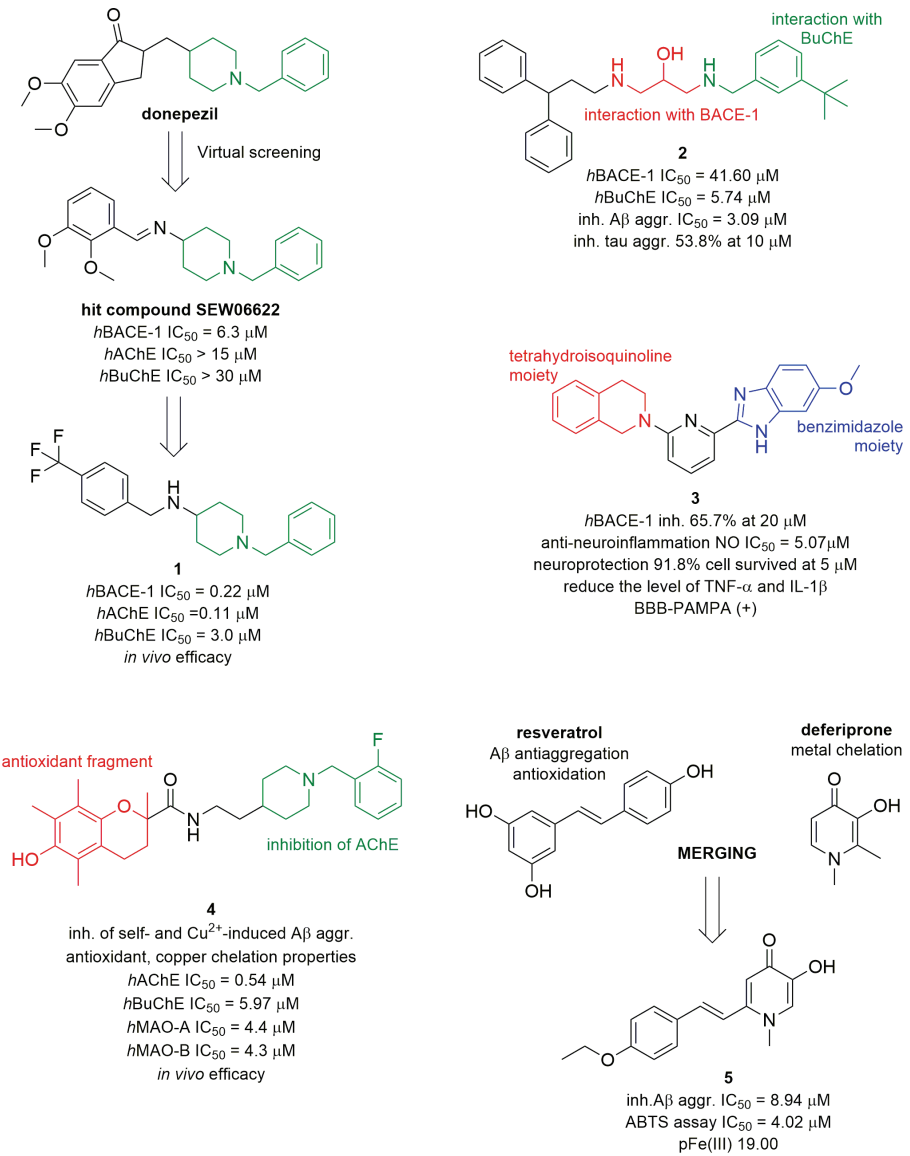



**Figure 1. Multifunctional agents focusing on disease-modifying targets: BACE-1, Aβ, and others.**

was found to be the most promising. Besides inhibiting both BACE-1 and AChE enzymes with $IC_{50}$ values in the submicromolar range, it showed blood–brain barrier (BBB) permeability in the parallel artificial membrane permeability assay (PAMPA) and inhibited self-induced and AChE-induced Aβ aggregation, while proving to be safe in SH-SY5Y neuroblastoma cell lines at a concentration of up to 80 μM. Most importantly, it alleviated cognitive impairment in rat models of scopolamine-induced amnesia and by continuous intracerebroventricular infusion of a pathogenic dose of Aβ peptides. In addition, compound **1** displayed good pharmacokinetic parameters after oral administration. Therefore, it can be considered as a potential drug candidate for AD treatment (23).

The approach to obtain disease-modifying and symptomatic multifunctional ligands inspired the design and synthesis of a group of 1-benzylamino-2-hydroxyalkyl derivatives (24). The new multifunctional agents contained substituted benzylamine, responsible for inhibiting BuChE, connected to aromatic fragments: benzyl, phenyl, and benzhydryl through a 2-hydroxyethyleneamine linker. This linker was suggested to confer the agents with BACE-1 inhibitory activity. Among these ligands, the diphenylpropylamine derivative **2** (Figure 1) was the most interesting, which was capable of inhibiting BACE-1 and BuChE, as well as Aβ and tau aggregation. Due to its broad biological profile, it is regarded as a potential multifunctional compound (24).

## Multifunctional agents combining Aβ antiaggregation effects with other activities

A series of tetrahydroisoquinoline–benzimidazole derivatives was developed, which represent multifunctional anti-AD agents focusing only on the disease-modifying effects (25). To design this group, two well-known pharmacophores, benzimidazole, present in BACE-1 and inflammation inhibitors, and a tetrahydroisoquinoline moiety possessing anti-inflammatory, antioxidation, and neuroprotection properties, were chosen. Benzene or pyridine ring was used as a linker. Among the obtained derivatives, compound **3** showed the most balanced profile (Figure 1). It inhibited nitric oxide (NO) production in lipopolysaccharide (LPS)-induced BV2 microglia cells with an $IC_{50}$ value of 5.07 μM and BACE-1 activity by 65.7% at a concentration of 20 μM. Additionally, it showed a strong neuroprotective effect against glutamate-induced cell death at 5 μM (91.8% cell viability) and reduced the level of proinflammatory cytokines such as TNF-α and IL-1β. Furthermore, the ability to penetrate the BBB demonstrated that compound **3** is a promising multiple anti-AD ligand.

Suppressing the formation of senile plaques is feasible not only by the inhibition of enzymes involved in the pathological Aβ cascade but also by direct inhibition of Aβ aggregation. Kong's group (26) designed compounds that displayed this property as well as other anti-AD activities, based on two structures: donepezil and Trolox, an antioxidative analog of vitamin E. The Trolox fragment was fused with a differently substituted *N*-benzylpiperidine moiety derived from donepezil by an amide bond. The designed compounds were supposed to bind to both active sites of AChE and inhibit AChE, Aβ aggregation, and MAOs, while also acting as antioxidants. The biological evaluation of the effect of these compounds on all proposed targets revealed that compound **4**, which had a fluor

atom in position 2 of benzene ring, was a promising MTDL (Figure 1). This compound inhibited both cholinesterases ($IC_{50}$ *h*AChE = 0.54 μM, $IC_{50}$ *h*BuChE = 5.97 μM). Moreover, it displayed nonselective inhibitory activity against MAO-A and MAO-B at a micromolar range. Its antioxidant property compared to Trolox was confirmed in three different experiments. Furthermore, compound **4** exhibited a metal-chelating effect, especially with copper ions, which are associated with AD pathogenesis. The results of self-induced and metal-induced Aβ aggregation assays also indicated that compound **4** can inhibit both types of Aβ aggregation. The promising outcomes of the *in vitro* assays were translated successfully to *in vivo* experiments, in which multifunctional ligand **4** was found to significantly improve cognitive decline in scopolamine-, D-galactose-, and $AlCl_3$-induced memory deficit models. All these results suggested that this compound is an excellent anti-AD candidate.

Another antioxidant and antiaggregating molecule that can serve as a multifunctional compound is the well-known natural derivative resveratrol. For designing new hybrid structures, deferiprone, metal-chelating drug was used (27). These two structures were merged by replacing one of the aromatic rings of resveratrol by deferiprone moiety (Figure 1). Among the newly synthesized hybrids, compound **5** possessing an ethoxy group at position 4 showed triple anti-AD functions—inhibition of Aβ aggregation, antioxidation, and metal chelation. Such properties make it a potential disease-modifying drug candidate.

## MULTIFUNCTIONAL AGENTS FOCUSING ON DISEASE-MODIFYING TARGETS: TAU PROTEIN AND OTHERS

Besides the presence of senile plaques in the brain of patients suffering from AD, the occurrence of NFTs consisting of hyperphosphorylated tau protein aggregates is discerned as the second hallmark of the disease. Based on this observation, the tau hypothesis was formulated to explain the development of AD (28). Tau is a microtubule-associated protein, which, in the physiological condition, stabilizes the microtubules and takes part in axonal transport. During the pathological process, kinases, especially glycogen synthase kinase (GSK-3β) and GSK-3α, excessively phosphorylate this protein, resulting in a loss of function. Hyperphosphorylated tau proteins aggregate and easily create intracellular lesions—primarily paired helical filaments and further NFTs. As indicated by the tau theory, the presence of these aggregates contributes to all the processes related to the AD pathomechanism leading to dementia, including Aβ aggregation. Therefore, in drug discovery and development, the tau-centered approaches, which involve the interaction with kinases, mainly the GSK-3β enzyme, and the direct inhibition of tau aggregation process, are especially important (29).

### Tau and Aβ inhibitors

One of the valuable strategies applied in the search of new anti-AD agents is the dual inhibition of Aβ and tau aggregation for reducing simultaneously the level of both lesions. An example of these agents is 1,2,3,4-tetrahydro-1-acridone

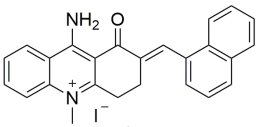
analogs, which were designed by combining the structure of tacrine with quinolone moiety, and the antiaggregating compound cinnamaldehyde containing α, β-unsaturated carbonyl fragment (30). Among the derivatives that differed in the length of linker, degree of saturation, substitution of cinnamaldehyde moiety, and methylation of the nitrogen atom of tacrine, five compounds inhibited $A\beta_{1-42}$ and tau aggregation by 84.7–99.5% and 71.2–101.8%, respectively, at the screening concentration of 20 μM. All these compounds had a quaternary amine considered crucial for the observed inhibitory activities. Compound **6** with a naphthalene residue (Figure 2), which was identified as a noncovalent inhibitor of both neurotoxic proteins and predicted to penetrate the BBB as well as prevent tau aggregation in living cells, was selected as the most promising dual agent.

Due to its rich biological properties, the curcumin scaffold is highly preferred in the design of multiple antiaggregation agents. It is a natural polyphenol, reported to have antioxidant, antiaggregating, and anti-inflammatory activities, and is used as a yellow spice. Because of its poor pharmacokinetic parameters, especially bioavailability, the scaffold is not applied in the treatment of AD. However, its structure was included in a series of potential multifunctional ligands (31). Compound **7** (PE859) is a perfect example of such ligands (32) (Figure 2), which was found to display higher dual antiaggregating properties compared to the parent compound. Moreover, it penetrated the BBB, improved memory *in vivo*, reduced the amount of both aggregated lesions in mice brains, and showed a promising pharmacokinetic profile.

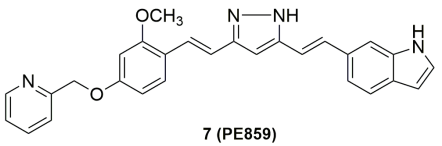
Dual Aβ and tau antiaggregating properties were also combined with anticholinesterase activity in order to achieve disease-modifying and symptomatic effects. However, it should be noted that such hybrid molecules were designed as dual binding site cholinesterase inhibitors with potential Aβ antiaggregating properties. The hybrids described by Muñoz-Torreo et al. (33) were formed by the fusion of 6-chlorotacrine, a strong AChE inhibitor, and the previously described tetrahydrobenzonaphthyridine derivatives capable of interacting with both active sites of AChE and displaying weak Aβ aggregation inhibitory activity. This combination allowed obtaining a group of compounds that can target tau, Aβ, and cholinesterases. The most potent derivative **8** (Figure 2) inhibited *h*AChE with an $IC_{50}$ value of 2.06 nM and *h*BuChE with 0.286 μM, as well as $A\beta_{42}$ and tau aggregation by 77.5% and 68.7%, respectively, in recombinant *Escherichia coli* cells at a concentration of 10 μM. However, a major disadvantage of this compound is its weak drug-likeness. Similarly, a series of shogaol–huprine hybrids displaying antioxidant properties, in addition to anticholinesterase and antiaggregating activities, was developed, which was characterized by poor drug-likeness (34).

## GSK-3β inhibitors with other activities

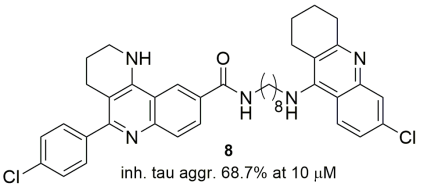
The inhibition of tau protein phosphorylation by interaction with GSK-3β is an important tau-centered approach. A group of researchers from the University of Bologna (35) developed a series of 2,4-thiazolidinedione derivatives, which were capable of inhibiting not only GSK-3β but also directly the tau aggregation process. Compound **9** possessing a substituted indole moiety (Figure 2) was the most potent GSK-3β inhibitor ($IC_{50}$ = 0.89 μM). It inhibited the AcPHF6 aggregation peptide (306VQIVYK311) up to 80% in a model system at 10 μM concentration.



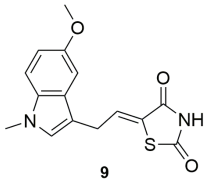
**6**
inh. tau aggr. 101.8% at 20 μM
inh. Aβ aggr. 86.7% at 20 μM



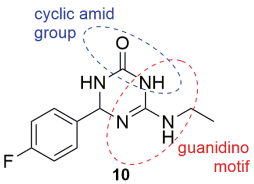
**7 (PE859)**
inh. tau aggr. $IC_{50}$ = 0.66 μM
inh. Aβ aggr. $IC_{50}$ = 1.2 μM
*in vivo* efficacy



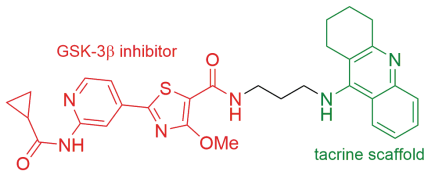
**8**
inh. tau aggr. 68.7% at 10 μM
inh. Aβ aggr. 77.5% at 10 μM
*h*AChE $IC_{50}$ = 2.06 nM
*h*BuChE $IC_{50}$ = 286 nM



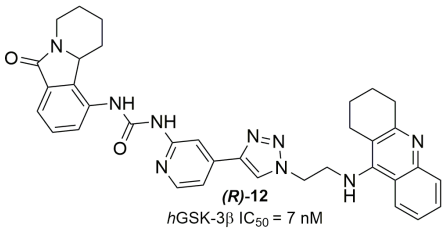
**9**
*h*GSK-3β $IC_{50}$ = 0.89 μM
inh. AcPHF6 aggr. 80% at 10 μM



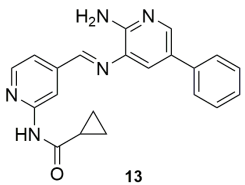

**10**
*h*GSK-3β $IC_{50}$ = 7.11 μM
*h*BACE-1 $IC_{50}$ = 16.05 μM
neuroprotective effect



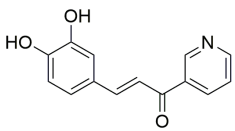

**11**
*h*GSK-3β $IC_{50}$ = 66.41 nM
*h*AChE $IC_{50}$ = 6.45 nM
*in vivo* efficacy



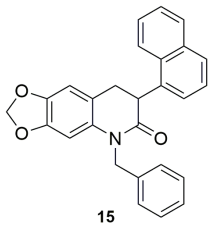
***(R)*-12**
*h*GSK-3β $IC_{50}$ = 7 nM
*h*AChE $IC_{50}$ = 9.5 nM
*h*BuChE $IC_{50}$ = 395 nM



**13**
*h*GSK-3β $IC_{50}$ = 49 nM
metal chelating, antioxidant,
Aβ aggregating properties



**14**
*h*GSK-3β $IC_{50}$ = 24.36 μM
inh. Aβ aggr. $IC_{50}$ = 9.0 μM
metal chelating, antioxidant properties



**15**
*h*GSK-3β $IC_{50}$ = 6.4 μM
*h*AK $IC_{50}$ = 13.6 μM
antioxidant properties

**Figure 2. Structures of small molecules targeting tau protein and other targets.**

The BBB permeability of the derivatives revealed by PAMPA, their safety, and ability to inhibit truncated and full-length tau aggregation allow regarding them as tau-centric multitarget agents.

A new disease-modifying strategy that suppressed both amyloid and tau cascades by the inhibition of two enzymes, GSK-3β and BACE-1, was proposed by Bolognesi's group (36, 37). A series of triazinones was designed based on two fragments: a guanidine motif interacting with BACE-1 and a cyclic amide group binding to GSK-3β. Among the obtained derivatives, compound **10** (Figure 2) was recognized as a novel well-balanced multiple anti-AD agent ($IC_{50}$ GSK-3β = 7.11 μM, $IC_{50}$ BACE-1 = 16.05 μM) because it showed neuroprotective and neurogenic effects and a good ADME (Adsorption, Distribution, Metabolism, Excretion) profile. Thus, it seems to be a promising lead structure for further development.

The inhibitory activity against GSK-3β, together with AChE inhibition, was proposed as a new strategy by Chinese researchers (38). Figure 2 presents the most interesting example (compound **11**) for simple combination of tacrine scaffold with the structure of a selective GSK-3β inhibitor using an alkyl linker. Among the ligands developed by the above team, compound **11** exhibited a well-balanced biological profile with the $IC_{50}$ values in the nanomolar range ($IC_{50}$ *h*AChE = 6.5 nM, $IC_{50}$ *h*GSK-3β = 66 nM). The efficacy of this dual strategy was verified in the animal model where compound **11** ameliorated the cognitive decline. Moreover, the examined compound did not demonstrate any hepatotoxicity for tacrine.

Oukoloff et al. (39) also used the same AChE inhibitor fragment for creating hybrids, which showed a similar mechanism of action. They connected tacrine pharmacophore by a linker containing 1,2,3-triazole with the structure of the GSK-3α/β inhibitor valmerin. The *in vitro* results revealed that compound **12** in the form of an *R* enantiomer (Figure 2) was the most potent, which exhibited inhibitory potential with an $IC_{50}$ value of 9.5 and 7 nM for AChE and GSK-3α/β, respectively. Additional advantages of the developed novel tacrine–valmerin multifunctional ligands were low cytotoxicity and good BBB permeability.

The metal hypothesis of AD indicates that excess levels and dysregulation of biometal ions, such as $Cu^{2+}$, $Fe^{2+}$, $Zn^{2+}$, and $Ca^{2+}$, in the brain cause Aβ aggregation, generation of reactive oxygen species (ROS), and oxidative stress, leading to cell death (40). Metal chelators are potential anti-AD agents and are also used in the development of multifunctional agents (41). A novel approach based on combing GSK-3β inhibitors with agents exhibiting metal-chelating and Aβ-antiaggregating properties was recently presented. The newly formed compounds included 2,3-diaminopyridine derivatives (42) and hydroxy-substituted *trans*-cinnamoyl derivatives (43). The most promising representative compound **13** from the first group (Figure 2) inhibited the GSK-3β enzyme with an $IC_{50}$ value of 49 nM and acted as a selective $Cu^{2+}$ and $Al^{3+}$ chelator, showing antioxidant and Aβ-antiaggregating properties. On the other hand, compound **14** from the second series (Figure 2) exhibited lower inhibitory potency against GSK-3β ($IC_{50}$ = 24.36 μM), but displayed a broad profile of anti-AD activities.

In turn, two series of compounds, benzoxazinone and indole derivatives, were designed as dual kinase inhibitors (44). Besides inhibiting GSK-3β, they targeted human adenosine kinase (*h*AK), which also induces the development of AD by

regulating the level of adenosine and cytoprotective effects. Based on three well-known *h*AK/*h*GSK-3β inhibitors, the structures of novel agents were designed and developed. Among them, **15** (Figure 2) displayed dual kinase inhibitory activity ($IC_{50}$ *h*AK = 13.6 μM, $IC_{50}$ *h*GSK-3β = 6.4 μM) with antioxidant and neuroprotective properties, and thus identified as an appropriate lead structure for the development of multifunctional ligands that can exhibit an innovative mechanism of action.

## MULTIFUNCTIONAL AGENTS FOCUSING ON SYMPTOMATIC THERAPIES WITH ADDITIONAL BENEFICIAL PROPERTIES

In the course of Alzheimer's disease, it is important to both treat the causes and symptoms of the disease. Dementia changes in patients are also associated with other disease symptoms, such as depression, anxiety, psychosis, and personality changes. According to the oldest theory of AD, dementia changes and memory disturbances are associated with damage to cholinergic neurotransmission. Other symptoms result from disturbances in other neurotransmitter systems, including GPCRs, such as serotonin, $H_3$ histamine receptors, and the cannabinoid system or with impaired functioning of enzymatic systems such as MAOs. Hence, the design of multifunctional molecules aimed at the treatment of disease symptoms is based on combining the inhibitory activity of cholinesterase with the influence on the other molecular targets mentioned earlier.

### Multifunctional ligands influencing serotonin and cholinergic neurotransmission

Among all GPCRs, serotoninergic receptors are the most attractive as they are targeted for designing MTDLs as potential treatment of AD. Their particularly desired actions are the activation of 5-$HT_4$ receptors (5-$HT_4$Rs) and inhibition of 5-$HT_6$ receptors (5-$HT_6$Rs) (45). The first action leads to the modulation of APP metabolism and redirects the protein to a nonamyloid pathway. In turn, 5-$HT_6$ blockage improves cognitive performance by increasing the release of glutamate, acetylcholine, and catecholamine in the cortical and limbic areas.

The first class of multifunctional ligands formed by combining 5-$HT_4$Rs with cholinesterases was presented by Dallemagne's research team (46). They described a compound named donecopride (compound **16**, Figure 3) as a new preclinical multifunctional drug candidate showing both *in vitro* AChE inhibitory activity and 5-$HT_4$R agonistic potency. Moreover, donecopride also displayed *in vivo* procognitive and antiamnestic activities in mice. The same research group (47) described novel MTLs with an interesting biological profile, which were capable of restoring cholinergic transmission through the activation of 5-$HT_4$R, blockage of 5-$HT_6$R, and inhibition of AChE. This class of compounds was developed by merging the previously developed dual compound with the benzyl analog of donecopride displaying *in vitro* 5-$HT_4$R agonist and 5-$HT_6$R antagonist properties. The most interesting compound was **17** (Figure 3), a fumaric acid salt exhibiting well-balanced activities toward all the three mentioned targets. Compound **17** was found to act

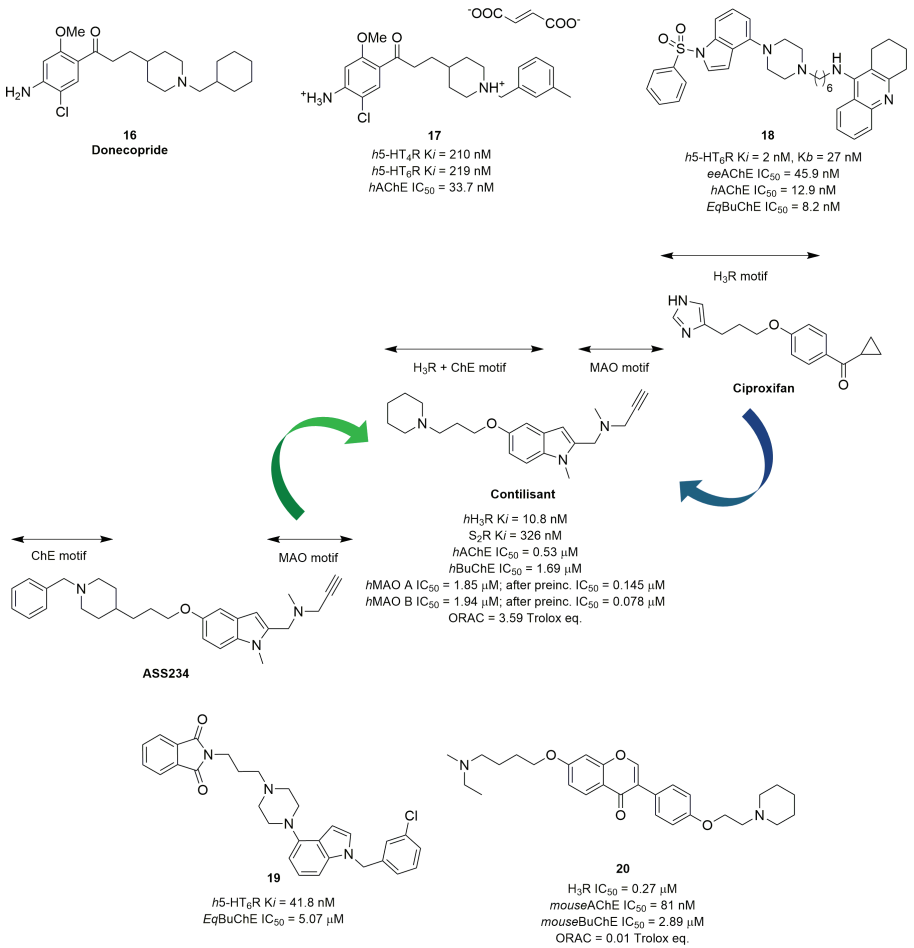



**Figure 3. Multifunctional ligands influencing serotonin or histaminergic neurotransmission and cholinesterases.**

as a partial agonist of *h*5-HT$_4$R (K*i* = 210 nM), an inverse agonist of *h*5-HT$_6$R (K*i* = 219 nM), and an inhibitor of *h*AChE ($IC_{50}$ = 33.7 nM). Additionally, the compound was tested *in vivo* in a model of scopolamine-induced working memory deficit, where it displayed antiamnestic effects at a dose of 0.3 mg/kg.

Several multifunctional ligands based on the combination of pharmacophores that are dedicated to 5-HT$_6$R blockage and cholinesterase inhibition were developed by Więckowska et al. (48, 49). These novel hybrid ligands were obtained by combining the pharmacophores directed against 5-HT$_6$R (1-(phenylsulfonyl)-4-(piperazin-1-yl)-1*H*-indole) and cholinesterases (tacrine or *N*-benzylpiperidine analogs). Among them, compound **18** (Figure 3) was the most interesting as it displayed potent and balanced antagonist activity toward 5-HT$_6$R (K*i* = 27 nM) and inhibitory effect against both cholinesterases ($IC_{50}$ AChE = 12 nM, $IC_{50}$

BuChE = 29 nM). The compound also showed good *in vitro* BBB permeability (proved by PAMPA). Additionally, an *in vivo* study with the use of a scopolamine-induced hyperlocomotion rat model confirmed the central cholinomimetic activity of the compound (48). The further development of this series of hybrids resulted in multifunctional ligands with Aβ antiaggregation properties (49).

Indole-based multifunctional ligands were designed, synthesized, and evaluated for the treatment of AD as 5-$HT_6$R blockers with inhibitory abilities toward *eq*BuChE and antioxidant properties (50). Based on the biological screening of the 5-$HT_6$R antagonists against BuChE, two compounds were selected. In order to improve their BuChE inhibitory activity, structural modifications were introduced in the next stage in the two series of compounds. Of them, ligand **19** (Figure 3) displayed beneficial, dual activity targeting 5-$HT_6$R (*Ki* = 41.8 nM) and *eq*BuChE ($IC_{50}$ = 5.07 μM), in addition to favorable antioxidant properties that were comparable with the reference ascorbic acid.

## Multifunctional ligands combining $H_3$R antagonism and cholinesterase inhibition

Histamine $H_3$ receptor ($H_3$R) belongs to the GPCRs family. In the central nervous system (CNS), $H_3$R acts as a presynaptic autoreceptor and is involved in inhibiting the release of histamine and the modulation of other neurotransmitters such as acetylcholine. Numerous *in vivo* studies have proven that both antagonist and inverse agonists of $H_3$R improve cognitive deficits, memory, and spatial orientation (51). Based on their results, $H_3$R is frequently chosen as a biological target for anti-AD multifunctional ligands (52–54).

An international multiteam group discovered new multifunctional ligands with a broad and well-balanced spectrum of activities against $H_3$R, AChE, BuChE, and MAO-A/B (55, 56). These new indole derivatives were designed by combining the neuroprotectant ASS234 with cholinesterase- and MAO-inhibiting motifs and ciproxifan containing $H_3$R-blocking and MAO-inhibiting pharmacophore fragments (Figure 3). Among the newly formed hybrids, contilisant was identified as the most promising multifunctional agent. It inhibited *h*MAO-A ($IC_{50}$ = 1.85 μM, after 30 min of preincubation $IC_{50}$ = 0.145 μM), *h*MAO-B ($IC_{50}$ = 1.94 μM, after 30 min of preincubation $IC_{50}$ = 0.078 μM), *h*AChE ($IC_{50}$ = 0.530 μM), and *h*BuChE ($IC_{50}$ = 1.690 μM), as well as blocked $H_3$R (*Ki* = 10.8 nM). Moreover, contilisant demonstrated *in vitro* antioxidant neuroprotective effects and the ability to penetrate the BBB in PAMPA. Due to its original *in vitro* biological profile, contilisant was selected for *in vivo* studies and tested using the novel object recognition test in mice with LPS-induced cognitive deficit. Based on the excellent *in vitro* data supported by positive *in vivo* activity, contilisant was studied further with the aim of exploring new pharmacological properties that may be potentially beneficial for AD therapy (57). The studies revealed another valuable activity of the compound: selective agonistic effect on Sigma 1 receptor (S1R) (*Ki* = 65.2 nM). S1R is associated with learning and memory processes, and hence, its agonists are used as antiamnestic agents in a variety of pharmacological models, probably due to the improvement of glutamatergic and cholinergic neurotransmissions. Additional *in vivo* studies including Y-maze and radical arm-maze tasks in mice with cognitive impairment induced by $A\beta_{1-42}$ oligomers showed that contilisant exhibited higher activity in comparison with the

commonly used anti-AD drug donepezil. Due to its multifunctional profile as well as *in vitro* activities, which were reflected in *in vivo* tests, contilisant thus seems to be an interesting multifunctional ligand for further development as a disease-modifying anti-AD agent (57).

A number of anti-AD multifunctional ligands have been developed based on naturally occurring substances. Wang et al. (58) synthesized a series of multifunctional ligands based on isoflavone, which was recently proven to exhibit inhibitory properties toward cholinesterases. *In vitro* studies revealed that some of these ligands showed a multifunctional profile including blockage of $H_3R$, neuroprotective effect, and antineuroinflammatory properties. Among them, compound **20** (Figure 3) is noteworthy since it displayed moderate $H_3R$ antagonist property ($Ki$ = 270 nM) and potently inhibited AChE ($IC_{50}$ = 80 nM). In addition, the compound **20** demonstrated neuroprotective and antineuroinflammatory properties. The *in vivo* study also confirmed that it did not induce acute toxicity even at high doses (1000 mg/kg), but penetrated through the BBB into the CNS and caused significant improvements in mice with scopolamine-induced cognitive deficit, which were revealed by passive avoidance test (58).

Ismaili et al. (59) described new small molecules combining activities against three biological targets including $h$H$_3$R. The most promising lead showed high affinity toward $h$H$_3$R ($Ki$ = 0.565 μM), $Ca^{2+}$ channel blockade activity ($IC_{50}$ = 21 μM), and moderate selective $h$BuChE inhibition ($IC_{50}$ = 7.83 μM), besides strong antioxidant properties and ability to restore cognitive impairment induced by LPS.

## Multifunctional ligands targeting endocannabinoid system and cholinesterases

Since the discovery that the activation of cannabinoid receptors ($CB_1R$, $CB_2R$) causes a reduction in the production of neurotoxic factors (ROS, proinflammatory mediators) leading to decreased neuroinflammatory processes, the receptors were considered as another biological target in the search for anti-AD agents as well as multipotent ligands (60, 61). Consequently, Decker's group (62) developed a series of benzimidazole-based dual-acting ligands that can activate $CB_2R$ and inhibit BuChE. Among them, the authors highlighted compound **21** as the most promising hybrid (Figure 4) as it activated $h$CB$_2$R with an $IC_{50}$ value of 0.763 μM and $h$BuChE with an $IC_{50}$ value of 1.6 μM. It is worth noting that this compound **21** was selective over $h$CB$_1$R and AChE. Compound **21** was further evaluated *in vivo* which revealed that it improved cognitive functions in mice showing neuroinflammation and cognition deficits after $A\beta_{25–35}$ administration at doses ranging from 1 to 3 mg/kg.

In turn, Montanari et al. (63) synthesized multifunctional ligands that indirectly enhanced endocannabinoid signaling by inhibiting fatty acid amide hydrolase (FAAH), an enzyme responsible for the degradation of crucial endocannabinoid signaling molecules: *N*-arachidonoylethanoloamine and 2-arachidonoyl glycerol. In addition to FAAH, the compounds inhibited cholinesterases. Some of the compounds exhibited very high potencies toward single targets; however, the most interesting MTL was compound **22** (Figure 4). It potently inhibited FAAH ($IC_{50}$ = 157.2 nM, after preincubation $IC_{50}$ = 27.9 nM), $h$AChE ($IC_{50}$ = 922 nM), and $h$BuChE ($IC_{50}$ = 42.7 nM).

### Enzyme inhibitors—MAO-A/B and cholinoesterases

MAOs (MAO-A and MAO-B) are the enzymes responsible for the degradation and inactivation of monoamine neurotransmitters. The inhibition of MAOs leads to neuroprotective effects, not only due to an increase in monoaminergic neurotransmission but also due to limitations in the production of neurotoxic substances, which are by-products of a reaction catalyzed by these enzymes. Due to its multiactive nature, the chromone scaffold is commonly applied as a structural motive in the development of multimodal ligands. Based on its structure, Reis et al. (64) reported a series of dual MAO and cholinesterase inhibitors. Among the tested compounds, the most promising was found to be compound **23** (Figure 4), which selectively inhibited *h*AChE ($IC_{50}$ = 210 nM) over BuChE and nonselectively inhibited both the isoforms of MAO ($IC_{50}$ MAO-A = 0.94 μM, $IC_{50}$ MAO-B = 3.81 μM). Moreover, the additional pharmacokinetic and toxicological studies of compound **23** showed its lack of cytotoxicity at a concentration below 25 μM, and PAMPA predicted its penetration through the BBB. Thus, the established well-balanced activities, low toxicity, and predicted permeability of the tested compound make them interesting multifunctional ligands for AD therapy.

Another group of dual inhibitors of MAOs and cholinesterases was developed by connecting *N*-benzylpiperidine fragment derived from donepezil with di-*tert*-butylated hydroxytoluene, which is a fragment responsible for antioxidant, anticancer, and anti-inflammatory properties in numerous compounds (65). These inhibitors were analyzed by studies divided into two parts: the first aimed to find an appropriate linker to connect the two pharmacophores and the second aimed to decorate the aromatic ring of the benzylpiperidine scaffold. Among the tested compounds, **24** was recognized as a double AChE ($IC_{50}$ *ee*AChE = 75 nM, $IC_{50}$ *h*AChE = 750 nM) and MAO-B ($IC_{50}$ = 7.5 μM) inhibitor with noticeable antioxidant properties and ability to reduce both self-induced and *h*AChE-induced Aβ aggregation (65).

## MULTIFUNCTIONAL AGENTS FOCUSING ON VARIOUS DISEASE-MODIFYING AND SYMPTOMATIC THERAPIES

The complexity of AD etiopathogenesis forced a search for new biological targets in the CNS and even beyond. Among the numerous molecular targets discovered, some have been used to create multifunctional ligands.

In pathological conditions, S1R modulates regulatory proteins, restores calcium homeostasis, and controls the production of ROS, thereby contributing to the overall neuroprotection effect (66). Based on their previous study, Rui et al. (67) developed multifunctional agents capable of modulating S1R and inhibiting AChE. The chemical structure of the new multifunctional ligands was developed through an interesting combination of RRC-33 (S1R agonist), curcumin (having antioxidant properties), and donepezil. The newly developed compounds exhibited a high affinity to S1R, but very weak AChE inhibitory properties. Among them, compound **25** (Figure 4) showed binding affinity to S1R with a Ki value of 15 nM; however, it inhibited AChE only by 64.80% at a concentration of 50 μM. Its neuroprotective effect associated with S1R modulation was confirmed by the neurite outgrowth observed in the dorsal root ganglia in the *in vitro* model (67).

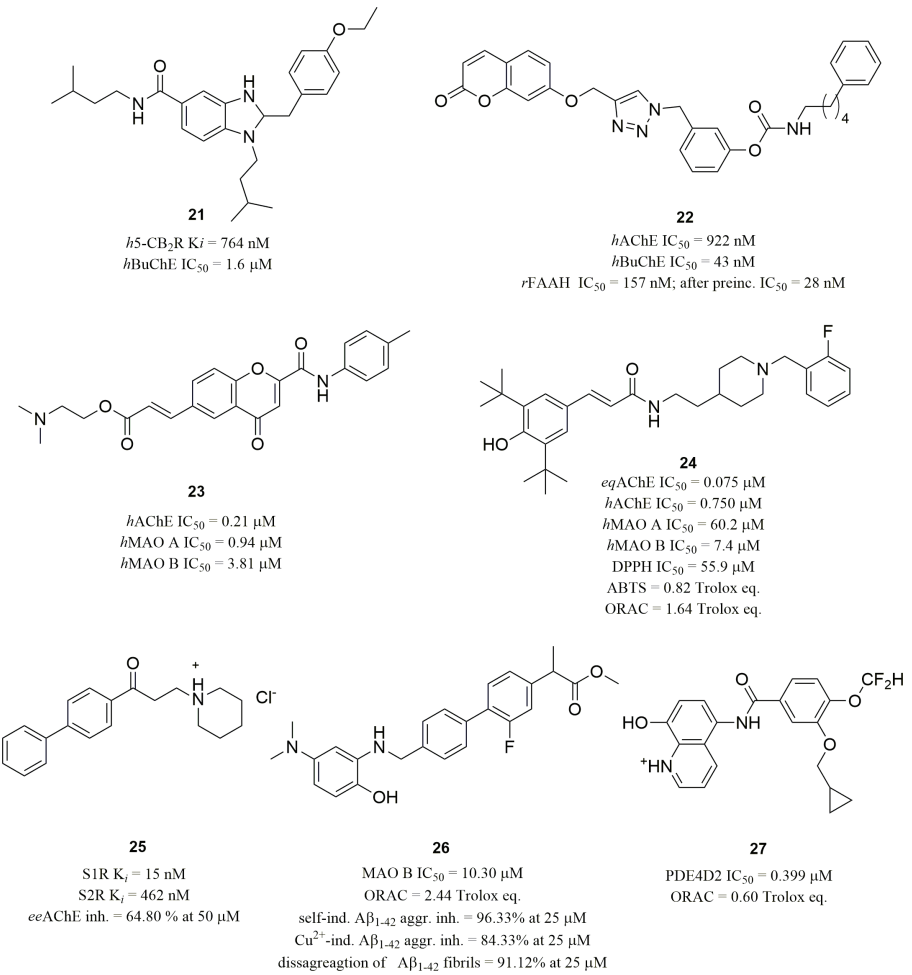



**Figure 4. Multifunctional agents focusing on various targets.**

Interesting biological activities were also presented by flurbiprofen–clioquinol hybrids (68). Flurbiprofen is a known potent nonsteroidal anti-inflammatory drug, which also inhibits platelet aggregation and Aβ aggregation and reduces tau phosphorylation. On the other hand, clioquinol is a metal chelator with proven antioxidant properties. Among the obtained hybrids, compound **26** (Figure 4) was able to inhibit both self-induced and $Cu^{2+}$-induced Aβ aggregation and MAO-B. In addition, it presented biometal-chelating abilities as well as antioxidant and antineuroinflammatory activities and appropriate BBB permeability.

In turn, Hu et al. (69) developed a novel group of multifunctional compounds by combining clioquinol with rolipram or roflumilast. These compounds inhibited phosphodiesterase 4D (PDE4D), an enzyme participating in the process of memory consolidation and long-term potentiation. The most interesting of them

was compound **27** (Figure 4), which inhibited not only the PDE4D with an $IC_{50}$ value of 0.399 μM but also the metal-induced aggregation of Aβ, chelated metal ions, and exhibited antioxidant properties (ORAC [oxygen radical absorbance capacity] = 0.60 Trolox eq.). In addition to *in vitro* activity, compound **27** showed *in vivo* activity by demonstrating procognitive effects in the Morris water-maze test.

Another example of multifunctional compounds is hybrids formed by the combination of pharmacophore cyclooxygenase-2 and 15-lipoxygenase enzymes with the structure of tacrine (70). These compounds are endowed with antineuroinflammatory and anticholinesterase activities.

## CONCLUSION

The lack of an effective treatment or DMT capable of influencing the causes of AD has led to a constant need to search for new solutions and drugs. Undoubtedly, an important issue here is the identification of new disease biomarkers, which can indicate early enough the pathological changes in the brain that may lead to irreversible processes and symptoms over time. The search for new anti-AD agents is challenging due to the complexity of the disease and the corresponding lack of appropriate animal models useful in preclinical studies. In addition, there is a need for properly planned clinical trials to demonstrate the effectiveness of the drugs. These problems concern small molecules targeting single biological target as well as combination treatment and multifunctional compounds. The clinical trials of combination therapy for AD focus on combining a cholinesterase inhibitor or memantine with other medications having various therapeutic indications. In this context, multifunctional ligands seem to be a much better strategy as they combine effects aimed at both causal and symptomatic treatment of the disease.

The examples presented in this chapter show that the search for new anti-AD drugs is based on the combination of pharmacophores acting not only on cholinesterases and Aβ aggregation but also on tau protein aggregation (GSK-3β inhibitors), neuroinflammation, and antioxidation. The ligands that regulate the influence on GPCRs or MAOs are particularly interesting, taking into account that the symptoms of depression or other mental disorders often coexist in AD. This may indicate that the proper design of a multifunctional molecule can facilitate the discovery of an effective therapy for this devastating disease.

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# 12

# Natural Products for Neurocognitive Disorders

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**Abstract:** Neurocognitive disorders are devastating. In 2016, 43.8 million people were estimated to have Alzheimer's disease worldwide. By 2050, this figure is expected to rise by 56%. Despite the extreme importance of the disease, the weapons available to us to combat it are very scarce. Natural substances may be a worthwhile option for the treatment and management of neurocognitive disorders. Some of these natural products have been shown to be capable of positively impacting memory, behavior, and functions of patients with Alzheimer's disease. These substances act on the disease mainly through antioxidant properties, the ability to eliminate oxygen radicals, the capacity to influence cell survival and programmed cell death, and the potential to condition amyloidogenesis. This chapter provides an overview of our current knowledge on the potential of natural products to be effective neuroprotective agents for Alzheimer's disease. Current evidence on Ginkgo biloba, bacopia, resveratol, curcumin, quercetin, kaempferol, capsaicin, and berberine, along with their adverse effects and drug interactions are discussed.

**Keywords:** bacopa; ginkgo biloba; natural products for Alzheimer's disease; neurocognitive disorders; resveratrol

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## INTRODUCTION

In 2013, the Diagnostic and Statistical Manual of Mental Disorders (DSM–5), introduced the term "Neurocognitive Disorders". It groups neurocognitive disorders into "major" and "mild" categories. A major neurocognitive disorder (MaND) is a disorder characterised by a decline in intellectual function due to disease of the brain, caused by a variety of acquired conditions such as cerebrovascular disease, Alzheimer's disease, infections, adverse drug reactions, and trauma. Alzheimer's disease is the most common form of MaND. The key distinction between a MaND and a mild neurocognitive disorder (MND) is that individuals with MaND experience substantial degradation in function resulting in loss of independence due to profound cognitive impairment, whereas subjects with MND experience only modest cognitive decline and can therefore function relatively independently. Reduced mental capacity may involve problems with complex attention, executive functioning, learning and memory, expressive and receptive language, perceptual-motor abilities, changes in behavior, and trouble performing everyday tasks (1).

A growing number of herbal remedies, dietary supplements, and "medical foods" are advertised as having beneficial neuroprotective effects. Compounds such as *Ginkgo biloba,* resveratrol, curcumin, capsaicin, berberine, kaeperol, quercetin as well as others are promoted as memory enhancers or as treatments to delay or prevent MaND (2). Many of these substances are found naturally in the diet, in vegetables and fruits, as well as in some spices. Some epidemiological investigations revealed that high consumption of certain foods was inversely associated with the incidence of Alzheimer's disease. These foods contain antioxidants, especially polyphenols. Polyphenols are excellent antioxidants both as reactive oxygen species (ROS) scavengers and transition metal chelators. While the neuroprotective effects of these foods in neurocognitive disorders are largely attributed to their antioxidant potential, some antioxidants derived from these foods go beyond modulating ROS (Figure 1). Several natural products are used alone or in combination with other treatment modalities to improve memory and cognition in both AD and MND (3). This chapter discusses the current scientific data on the potential of Ginkgo biloba, bacopa, resveratol, curcimun, quercetin, kaempferol, capsaicin, and berberine as effective neuroprotective agents for Alzheimer's disease along with their toxicities and drug interactions.

## GINKGO BILOBA

*Ginkgo biloba*, also known as the Maidenhair tree, is an ancient plant whose origins date back to 250 million years ago to the Permian period. The extracts of its leaves have been used since antiquity in traditional Chinese medicine to treat various pathologies. Nowadays, extracts of this plant are used in Europe, especially in Germany and France, for the treatment of memory and concentration problems, depressive anxiety disorder, dizziness, headache, and many other issues. As highlighted by a systematic review and meta analysis, the extract of *Ginkgo biloba*, EGb761, which contains about 22% of glycosides

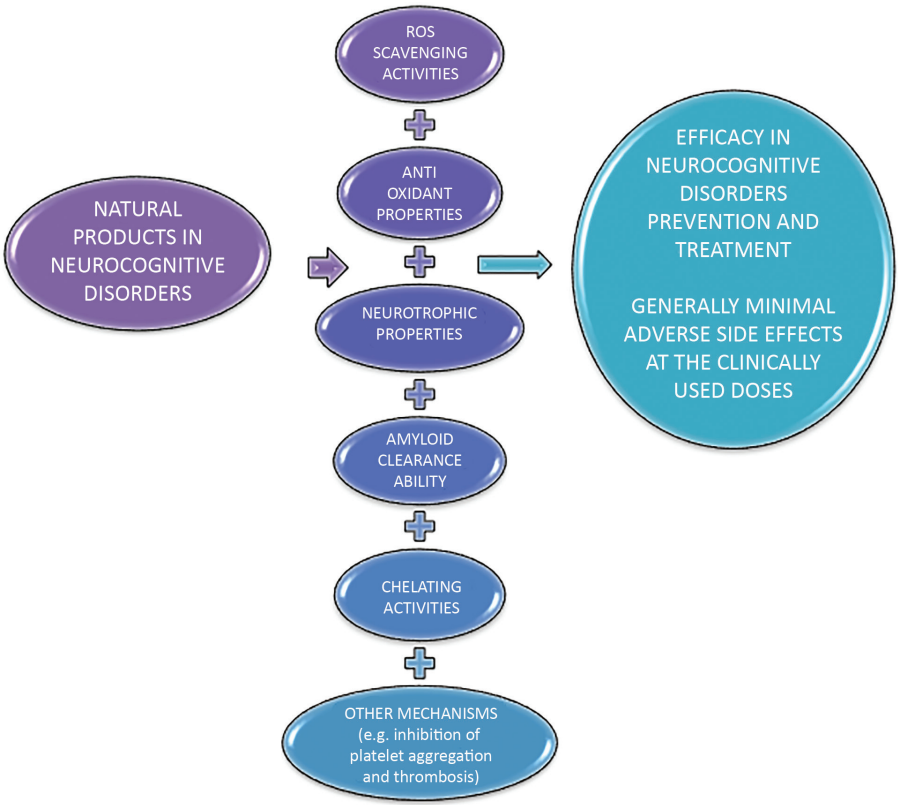


**Figure 1. Natural products for neurocognitive disorders.** The neuroprotective effects of natural products are largely attributed to their free radical scavenging abilities. In addition, they can chelate metals, inhibit amyolid beta formation, and enhance its clearance. In general, the natural products exert minimal or lower-grade adverse events.

and flavonoids and about 5–7% of terpene lactans (ginkgollids and biloballids), is capable of stabilizing or slowing down cognitive decline, functional decline, and behavior disorders at a dose of 240 mg/day. Furthermore, it is able to achieve global changes in cognitive impairment and neuro cognitive disorders in 22–26 weeks. These changes are significant for patients with neuropsychiatric symptoms (4, 5).

## Neuroprotective Effects of Ginkgo Biloba

The *in vitro* effects of *Ginkgo biloba* extracts are manifold and are widely documented. They contain significant amounts of polyphenols. The effects of the extracts are largely attributed to their free radical scavenging abilities and metal chelation properties, especially chelation of copper and iron. *In vitro* studies conducted on PC12 cell lines have shown that *Ginkgo biloba* extracts

prevent the production of ROS and reduce the cytotoxicity of $A\beta_{(1-42)}$ by inhibiting apoptosis and cellular glucose absorption (6). *Ginkgo biloba* extracts were also found to prevent the formation of diffusible neurotoxic ligands derived from $A\beta_{(1-42)}$.

The *in vivo* activities of *Ginkgo biloba* have been studied in various animal models. In the nematode *Caenorhabditis elegans*, EGb761 alleviated the pathological behavior connected to the presence of Aβ, inhibited beta oligomerization and Aβ deposits, and attenuated basal and induced levels of ROS in models of neurodegenerative pathology. In a trial conducted on TgCRND8 AD mice (mice that overexpress the amyloid precursor protein (APP) particularly at a neuronal level), treatment with EGb 761 for 5 months significantly improved cognitive function, as measured by Barnes Maze test. The results clearly showed inhibition of neuroinflammation, reduction in cognitive deficits, reduction in synaptic damage, improvement of autophagy, and inhibition of $A\beta_{1-42}$-induced microglial inflammatory activity (7). The doses given were designed to maintain plasma concentrations comparable to those reached by humans who take 240 mg of product per day (recommended dose).

The effects of the treatment with EGb761 extract of *Ginkgo biloba* have been the subject of a meta-analysis that collected data from three clinical studies. The meta-analysis showed that a dose of 240 mg per day of *Ginkgo biloba* extract is able to delay the deterioration of basal activities of daily life. An interesting fact that emerged from this analysis was that treatment with EGb761 had the same efficacy as acetylcholinesterase inhibitors, at a lower cost (8). The weak point of the cited meta-analysis was that it took into account only daily life activities and treatment costs, not the behavioral and psychological symptoms of dementia. The behavioral and neuropsychiatric effects were explored in a randomized controlled double-blind multicenter trial, which was designed to investigate the safety and efficacy of a daily dose of 240 mg of EGb761 in patients with mild to moderate neurocognitive disorders, using the neuropsychiatric inventory scale. Treatment with EGb761 improved cognitive function and the neuropsychological symptoms of dementia. Significantly, the quality of life of patients and their caregivers improved compared to placebo-treated patients (9). A randomized double-blind phase III trial conducted on 3069 community volunteers of at least 75 years of age with normal cognitive functions or mild cognitive impairment has shown that a dose of 120 mg twice daily of *Ginkgo biloba* is unable to prevent or delay the overall incidence rate of MaND or cases of Alzheimer's disease (10). On the contrary, a systematic review and meta-analysis investigating the clinical efficacy of EGb761 in minor and major cognitive decline, established that 240 mg per day was able to stabilize or slow down the decline in cognitive functions, functional skills, and behavior, resulting in overall positive effects, especially in patients with behavioral and psychological symptoms of dementia (11).

The Paquid study, a population-based cohort with a follow-up period of 22 years, examined the effect of *Ginkgo biloba* in the elderly. There were 3777 community participants, at least 65 years of age at the time of recruitment. The patients were visited at home by psychologists at baseline, and then every 2 years. They were grouped into three categories: consumers of *Ginkgo biloba* extracts, consumers of other medicines, and controls that did not receive any treatment. Consumers of *Ginkgo biloba* extracts showed lower mortality rates

and longer dementia-free lifespans than subjects taking other drugs for the same indications (12).

*Ginkgo biloba* extracts are mainly indicated for the treatment of "age-associated" cognitive decline and for improving the quality of life in patients with mild neurocognitive disorders (MND). Long-term use of these extracts is most effective in patients over 50 years of age. According to European Pharmacopoeia (Ph. Eur.), the extract should contain 22.0–27.0% of flavonoids, 2.8–3.4% of ginkgolides A, B, and C, and 2.6–3.2% of bilobalide (active constituents), and a maximum of 5 ppm ginkgoid acids (potential allergens) (13). Treatment with *Ginkgo biloba* extracts is safe, but close attention should be paid to patients being treated with anticoagulants, as the pharmacokinetics of some may be altered by certain plant extracts (14).

### Adverse Effects of Ginkgo Biloba

*Ginkgo biloba* is currently considered to have one of the highest rates of adverse side effects and interaction (Table 1) with conventional drugs (15). Cases of intoxication have been reported in Japan and China, where Ginkgo seeds and nuts have been a common food since ancient times. Poisoning causes tonic–clonic epileptic seizures, vomiting, and loss of consciousness. These effects are mainly related to a neurotoxic compound known as ginkgotoxin. Rare cases of death related to Ginkgo poisoning have been described, especially in the years following the first and second World Wars, probably due to food shortages. Now-a-days, cases of *Ginkgo* poisoning are much rarer, perhaps due to an increase in knowledge of the toxicological profile of these substances. Another adverse effect attributable to *Ginkgo biloba* extracts is spontaneous bleeding, especially in patients taking Ginkgo and warfarin together. Rarer adverse effects include a proarrhythmic effect, nocturnal palpitations, cerebrovascular events (ischemic stroke and transient ischemic attacks), and allergic reactions such as contact dermatitis. Drug interactions of *Ginkgo biloba* with conventional medicinal substances have also been reported. It interacts with drugs widely used in clinical practice such as omeprazole, midazolam, and tolbutamide.

## BACOPA

Brahmi or *Bacopa monnieri* is a plant used in Ayurvedic medicine with neuroprotective and nootropic properties. The neuroprotective effect of this plant seems to derive from numerous bioactive components such as bacoside A, bacoside B, bacosaponins, and betulinic acid. The mechanisms by which these compounds exert a neuroprotective effect and offer improvement of cognitive and learning abilities include reduction of ROS, anti-inflammation, and inhibition of beta amyloid aggregation. Bacopa seems to play a relevant role in the treatment of not only of Alzheimer's disease but also of other neurological pathologies (16).

## TABLE 1 Interactions and side effects of natural products

| Natural product | Indications | Drug interactions and side effects |
|---|---|---|
| *Ginkgo biloba* | • Age associated cognitive decline<br>• Mild neurocognitive disorder<br>• Multi infarct dementia<br>• Traumatic brain injury<br>• Normal aging<br>• Stroke<br>• Tinnitus<br>• Intermittent claudication<br>• Senile macular degeneration | • Pay attention in association with anticoagulants<br>• Intoxication: tonic clonic epileptic seizures, vomiting, loss of consciousness<br>• Spontaneous bleeding (when administered with warfarin)<br>• Rare: proarrhytmic effect, allergic reactions, acute generalized exhantematous pustolosis |
| *Bacopa monnieri* | • Improve cognition in the elderly and in patients with neuro cognitive disorders<br>• Beneficial effects in Parkinson disease, depression, neoplastic pathologies<br>• Beneficial properties on gastrointestinal tract | No significant harmful side effects is currently known |
| Resveratrol | • Improves adult cognitive abilities<br>• Reduces cognitive decline in the healthy elderly<br>• Slows down the decline in pathological states such as Alzheimer's and Parkinson's disease | • Leukopenia<br>• Decreases circulating levels of TNF and IL 6<br>• Mild to moderate diarrhea, nausea, hypersensitivity and annoying itching in the anal area. |
| Curcumin | • Neurodegenerative diseases, multiple sclerosis, prion diseases, stroke<br>• Autism, Down syndrome, Amyotrophic Lateral Sclerosis, depression, anxiety and aging<br>• Beneficial properties in diabetes mellitus, arthritis, liver, kidney and cardiovascular pathologies | • High doses can produce toxic and even carcinogenic effects<br>• Mild nausea and diarrhea<br>• Subclinical iron deficiency |
| Quercetin | • Prevention and treatment of Alzheimer's disease and other types of dementia | • Adverse effects are rare and usually very mild<br>• In animal models it shows toxic effects on kidney |
| Kaempferol | • Protective action against cognitive decline<br>• Beneficial effects in experimental models of Alzheimer's disease | No significant harmful side effects is currently known |

Table continued on following page

**TABLE 1 Interactions and side effects of natural products (Continued)**

| Natural product | Indications | Drug interactions and side effects |
|---|---|---|
| Capsaicin | • Beneficial effects both on cognitive functions of middle aged adults and advanced age subjects<br>• Beneficial effects on biomarkers of Alzheimer's disease<br>• Anti cancer properties<br>• Anti obesity properties<br>• Osteoarthritic pain<br>• Cannabinoid hyperemesis syndrome<br>• Useful as a dermal patch in peripheral neurotrophic pain | • At very high doses, well above those clinically useful, capsaicin can lead to death due to respiratory paralysis<br>• It is hypothesized that capsaicin may have carcinogenic effects linked to overdose<br>• Capsaicin in the form of pepper spray is likely to induce a form of acute polyneuropathy that resembles Guillain Barrè syndrome |
| Berberine | • Neurodegenerative pathologies and, in particular, Alzheimer's disease<br>• Ischemic stroke<br>• Vascular dementia | • When administered for prolonged periods of time, causes a deterioration of dopaminergic neurons<br>• Several drug interactions (tetrandine, levodopa, doxorubicin, beta lactam antibiotics and hydroxycamptotecin, Panax ginseng, cisplatin, fluconazole, cyclosporin A, warfarin and thiopental) |

## Neuroprotective Effects of *Bacopa*

*Bacopa's* mechanisms of action (Table 2) have been studied on the microglial cell line N9. Infusions, teas, and extracts of bacopa and bacoside A are able to significantly inhibit the release of mediators such as TNF-α and interleukin 6 by microglial cells *in vitro*. In cell-free systems, teas, infusions and alkaloid extracts of Bacopa can inhibit caspases 1 and 3, and matrix metalloproteinases 3. Thus the fundamental mechanism of action of bacopa appears to be inhibition of the release of pro-inflammatory cytokines by microglia cells and inhibition of enzymes that perform pro-inflammatory actions in the central nervous system. The net effect of all these pharmacological actions of bacopa evidently consists of keeping inflammation under control in the brain (17). In animal models, *Bacopa monnieri* extracts improve cognitive functions mainly through protective action on hippocampal neurons and partly through promoting neuroregeneration in the dentate gyrus (18, 19). Furthermore, studies on APP/PS1 mice have shown that *Bacopia monnieri* extracts can reduce plaque load and enhance plaque clearance, possibly via phagocytosis (20). *Bacopa monnieri* prevents senescence and has an anti-apoptotic effect in brain astrocytes and, therefore, is thought to combat brain pollution and age-related neurological disorders (21).

## TABLE 2 Mechanism of action

| Natural products | Mechanisms of action |
|---|---|
| *Ginkgo biloba* | • Antioxidant power<br>• Copper chelating ability<br>• Radicals scavenging properties<br>• Prevents formation of diffusible neurotoxic ligands derived from $A\beta_{(1–42)}$<br>• Prevents apoptosis |
| *Bacopa monnieri* | • Inhibits the release by microglial cells *in vitro* of mediators such as TNF α and IL 6<br>• Inhibits caspases 1 and 3 and metalloproteinases 3<br>• Overall, keeps inflammation under control into the brain<br>• Expresses amyloid clearance capacity<br>• Prevents senescence and has anti apoptotic effect in brain astrocytes<br>• Reduces the share of Reactive Oxygen Species |
| Resveratrol | • Improves metabolic functions<br>• Acts as a neuroinflammation modulator<br>• Shows protective effects against oxidative stress<br>• Seems to inhibit the mechanism of apoptosis in brain cells<br>• Anti aggregating activity against $A\beta_{(1–42)}$ |
| Curcumin | • Powerful antioxidant, anti inflammatory, anti cancer, anti microbial actions<br>• Neurotrophic properties<br>• Stimulates amyloid clearance in Alzheimer's disease |
| Quercetin | • Anti inflammatory, anti oxidant, anti cancer properties<br>• Beneficial properties for dyslipidemia, hypercolesterolemia, cardiovascular diseases, diabetes<br>• ROS scavenging ability<br>• Neuroprotective properties |
| Kaempferol | • Antiamyloidogenic activity<br>• Destabilizing activity against amyloid fibrils<br>• Chelates metal ions<br>• Keeps oxidative stress under control<br>• Inhibits platelet aggregation and thrombosis |
| Capsaicin | • Mitigates amyloid induced synaptic loss<br>• Performs neuroprotective functions against stress induced cognitive impairment<br>• Able to reduce the hyperphosphorylation degree of tau protein |
| Berberine | • Anti inflammatory activities<br>• Anti oxidant properties<br>• Inhibits acetyl cholinesterase<br>• Inhibiting properties against amyloid formation |

Taken together, the neuroprotective actions and positive effects of bacoside A on memory, mental, and intellectual functions can be largely attributed to its ability to reduce beta amyloid aggregation and toxicity (22), lower inflammation in the brain, scavenge ROS, and increase cerebral blood flow (23, 24). Promising indications for use in humans include improving cognition in the elderly and in patients with neurodegenerative disorders (25). Bacopa is considered by some authors as a kind of

"bulletproof vest" against Alzheimer's disease (26). It seems that *Bacopa monnieri* extracts are able to reduce the formation of transthyretin fibrils (TTR) by attenuating their disassembly from the tetrameric form to the monomeric form. This suggests bacopa has a role in preventing transthyretin amyloidosis, a very debilitating and often fatal disease in humans (27). The anti-inflammatory properties of bacopa have been beneficial in the treatment of many neurological diseases of the central nervous system. For example, they have shown promising results in some experimental models of Parkinson's disease (28). In addition, bacopa has proven beneficial in the treatment of depression, anhedonia, various inflammatory diseases, and neoplastic pathologies. In the rat, bacopa has exhibited beneficial properties on the gastrointestinal tract as an antidiarrheal, as a protector of the gastric mucosa, and as an anti-ulcer drug (29, 30). Currently, no significant harmful side effects of *Bacopa monnieri* are known.

## RESVERATROL

Resveratrol is a phenol predominantly found in grapes and wine as well as in berries, peanuts, and soybeans (31). Resveratrol is effective in reducing the risk of dementia in mouse models; this perhaps is due to the fact that improving metabolic function improves brain health during senile age (32). Resveratrol acts as a brain neuroinflammation modulator (33), shows protective effects against oxidative stress (34), inhibits the mechanism of apoptosis in brain cells (35), and exerts anti-aggregating activity against $A\beta_{(1-42)}$ (36). Resveratrol is a promising molecule for improving cognitive abilities in adults, reducing cognitive decline in healthy elderly, and slowing decline in pathological states such as Alzheimer's and Parkinson's disease (37, 38). The toxic effects of resveratrol seem to be closely associated with a hormetic effect; low doses are generally associated with antioxidant effects while higher doses can have a pro-oxidant effect. Resveratrol can cause leukopenia, decrease circulating levels of TNF and interleukin 6, and increase plasma levels of alanine aminotransferase. In addition, it can cause mild to moderate diarrhea, nausea, hypersensitivity, and irritation of the anal area. Through the dose-dependent ROS increase, resveratrol can also cause proteolysis and DNA damage (39).

## CURCUMIN

Curcumin is an extract of turmeric widely used in Asia, especially in the culinary sector. This substance seems to have multiple beneficial effects, particularly with respect to various neurological pathologies and cancer. Curcumin exerts its effects through powerful antioxidant, anti-inflammatory, anti-cancer and antimicrobial actions. In addition, the molecule has neurotrophic properties and appears to stimulate amyloid clearance in Alzheimer's disease (40). Curcumin shows beneficial effects in neurodegenerative diseases such as Alzheimer's disease, Parkinson's disease, Huntington's chorea (41), multiple sclerosis, prion diseases, and stroke. In addition, the molecule can attenuate age-related severity of autism and Down syndrome, and prevent the progression of amyotrophic lateral sclerosis, depression, anxiety, and pathological aging by reducing the expression of IL-1α, IL-6, and TNF-α, and by the activation of mitochondrial protection and anti-apoptotic

mechanisms (42). Currently, there is still insufficient clinical data to support the beneficial effects of curcumin in patients with Alzheimer's disease. In addition, curcumin administered orally is poorly bioavailable (43). The pharmacological effects, whether therapeutic or toxic, are dose-dependent. At high doses, the molecule can produce toxic and even carcinogenic effects (44). Sometimes curcumin, due to metabolic activation, can exert pro-oxidant effects (45). Other side effects include mild nausea and diarrhea (46). Curcumin also chelates iron and suppresses hepcidin, leading to a subclinical iron deficiency (47).

## QUERCETIN

Quercetin is a flavonoid present in numerous plants, as well as in certain fruits and vegetables. It is also present in red wine and green tea. Quercetin has antioxidant, anti-inflammatory, anti-cancer, and ROS scavenging properties, and has been shown to be beneficial for the prostate, dyslipidemia hypercholesterolemia, cardiovascular diseases, diabetes, viral infections, kidney transplantation, asthma, and lung disease. Furthermore, it seems to have neuroprotective effects in schizophrenia, Alzheimer's disease, and other forms of dementia (48, 49). The adverse effects associated with the use of quercetin are rare and typically mild; however, animal studies have shown that quercetin has toxic effects on the kidney, promote tumor development especially in estrogen-dependent cancers, and can interact with various drugs to alter their bioavailability (50).

## KAEMPFEROL

Kaempferol is a flavonoid present in many plants and in vegetables such as cabbage, spinach, beans, broccoli, and tea (51). Kaempferol has antiamyloidogenic activity. It can destabilize amyloid fibrils, chelate metals, and keep oxidative stress under control (52). Kaempferol has also been shown to inhibit platelet aggregation and thrombosis, an effect that could prove particularly interesting in vascular forms of cognitive decline (53). Like the other main dietary flavonoids (myricetin and quercetin), Kaempferol protects against cognitive decline (54). All flavonoids (myricetin, morin, rutin, quercetin, fisetin, kaempferol, apigenin, and glycitein) have shown beneficial effects in experimental models of Alzheimer's disease (55). There is currently insufficient data in the literature regarding the adverse effects of Kaempferol in humans.

## CAPSAICIN

Capsaicin is a well-known alkaloid present in chilli pepper and is responsible for its spiciness. In experimental mouse models, capsaicin has been shown to mitigate amyloid-induced synaptic loss (56). Furthermore, capsaicin has proven to be capable of performing neuroprotective functions against stress-induced cognitive impairment (57). Finally, it has been shown in experimental animals that capsaicin is able to reduce the degree of hyperphosphorylation of the tau protein, a protein

involved in the pathogenesis of Alzheimer's disease (58). A capsaicin-rich diet has been shown to have beneficial effects both on the cognitive functions of middle aged adults and advanced age subjects, and on the blood biomarkers of Alzheimer's disease ($A\beta_{40}$, $A\beta_{42}$, and their ratio) (59). In addition to its neuroprotective properties, capsaicin has anti-cancer (60), antiobesity properties (61) and can counteract metabolic syndrome (62). It is useful as a topical applicant to ease osteoarthritic pain (63), particularly in that of the knee (64). It can be used in the treatment of cannabinoid hyperemesis syndrome (65), and as a dermal patch for peripheral neuropathic pain (66). At very high doses (well above those clinically useful), capsaicin can lead to death due to respiratory paralysis (67, 68). Capsaicin in the form of pepper spray can induce a form of acute polyneuropathy that resembles Guillain-Barrè syndrome (69).

## BERBERINE

Berberine is an alkaloid extracted from various plants of the Berberis species and is widely used in Asia. Berberine exhibits anti-inflammatory, antioxidative, and anti-amyloid activities (70). It can also inhibit acetylcholinesterase (70). Berberine is useful in neurodegenerative pathologies such as Alzheimer's disease (71), stroke (72), and vascular dementia (73, 74).

Berberine, when administered for prolonged periods of time, causes a deterioration of dopaminergic neurons due to the cytotoxic effect exerted by 6 hydroxydopamine (75–77).

Berberine stimulates uterine contractions and should therefore be used with caution in pregnancy. It also appears to exacerbate jaundice and kernicterus (Kernicterus or Bilirubin encephalopathy or Nuclear jaundice) in infants with glucose-6-phosphate dehydrogenase deficiency. Berberine is likely to be able to cross the placenta and harm the developing fetus. It can be transferred to breast milk and should therefore also be used with caution when breastfeeding. However, berberine does not have known genotoxic, cytotoxic, mutagenic actions, or other adverse effects at clinically relevant doses.

Various drug interactions (Table 1) of berberine have been reported: (i), tetrandineh worsens the hypoglycaemic properties of berberine; (ii), the activity of levodopa is antagonized by berberine; (iii), with doxorubicin, Panax ginseng, cisplatin, and fluconazole berberine exerts synergistic effect; and (iv), berberine increases the circulating levels of cyclosporin A, warfarin, and thiopental (78).

## CONCLUSION

Neurocognitive disorders are multifactorial pathological conditions that require a multidisciplinary therapeutic approach. The pharmacological weapons at our disposal are extremely limited and any aid is a welcome measure for the patients, their families, and caregivers. Given the available evidence, it is reasonable to assume that natural products will gain an increasingly important role in the future alongside traditional and experimental pharmacological therapies for the management of patients with neurocognitive disorders, including Alzheimer's disease.

**Conflict of Interest:** The authors declare no potential conflicts of interest with respect to research, authorship, and/or publication of this chapter.

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