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Small-molecule inhibitors of BACE1

AS ALZHEIMER DISEASE TREATMENT

STEFAN BUSSCHER

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Stefan Busscher
Student number: S4569059

Rijksuniversiteit Groningen, Biomedical sciences premaster

Supervisors: Prof. Dr. Uli Eisel
Prof. Dr. Martina Schmidt

Rijksuniversiteit Groningen

Preface

Here in front of you lies my report 'Small-molecule inhibitors of BACE1 as Alzheimer disease treatment. This report was written for the completion of my Premaster for the master Biomedical sciences. Due to the coronavirus, the entire report was written from the comfort of my home.

I would like to thank Prof. Dr. Uli Eisel for supervising me over the course of time that I needed to write this report. Thank you for helping me write this report as well as possible me through our online meetings during this pandemic. Also thank you Prof. Dr. Martina Schmidt for being the second examiner for my report.

I hope you will enjoy reading this report.

Stefan Busscher
Groningen, the Netherlands
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Summary

Alzheimer disease (AD) is the most common aging-related neurodegenerative disease worldwide that gets worse over time. Characteristic for AD are the extracellular senile plaques and intracellular neurofibrillary tangles that consist of aggregated hyperphosphorylated tau proteins that can be found in the brains and damage the brain. These plaques mostly consist of Amyloid-β, which is produced by the cleavage of the amyloid precursor protein by an enzyme called β-site amyloid precursor protein cleaving enzyme 1, or BACE1. Since BACE1 is thought to play a crucial role in AD pathogenesis, BACE1 inhibitors are studied as a potential treatment against this disease. The small-molecule inhibitors LY2811376 and Verubecestat have been studied in animal models and in clinical trials. LY2886721 successfully inhibited BACE1 in animal models and in healthy volunteers where reduced amyloid-β levels were measured in their cerebrospinal fluid. LY2886721 went onto stage 2 trials where it was tested on patients with AD. This trial was however terminated since some patients that were administered with LY2886721 showed abnormal liver biochemistries. The first study of Verubecestat showed that the drug reduced amyloid-β levels in the CSF of healthy volunteers. In a larger study, Verubecestat reduced Aβ and sAPPβ levels of the plasma, CSF and brain in monkeys and rats, while also reducing Aβ and sAPPβ levels of humans in a dosage dependent manner. In a 104-week long, phase 3 trial where Verubecestat was tested on patients with AD, cognitive functioning was not improved after treatment. Because of this, Verubecestat trials was also terminated. Up until now, no BACE1 inhibitors have improved cognition in AD patients or improved AD pathology. Because of this, future research should not only focus on the amyloid cascade hypothesis and BACE1 inhibitors, but also other types of treatments such as monoclonal antibodies that target amyloid-β or hyperphosphorylated tau.

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Introduction

Alzheimer disease (AD) is an aging-related neurodegenerative disease that gradually gets worse over the course of time (Raskin et al., 2015). AD was first discovered in 1906 by Dr. Alois Alzheimer, who noticed changes in the brain tissue of a woman who had died of an unknown illness. Clumps of protein and tangled fibres were found in her brain. These abnormalities are now known as amyloid plaques and neurofibrillary tangles and these are characteristic for Alzheimer's (Tiwari et al., 2019). AD is also the most common cause of dementia, accounting for >80% of all dementia diagnoses (Raskin et al., 2015). Dementia is the term used for the progressive cognitive decline in memory, language and behaviour, interfering with a person's ability to function independently. AD symptoms are different depending on the stage of the disease. Early on, the most prominent symptom would be the loss of short-term memory because the hippocampus is usually the first part of the brain that is damaged by AD. AD progressively gets worse, eventually also damaging other parts of the brain, affecting the patients problem-solving and normal functioning and finally the motoric tasks (Duong et al., 2017; Robinson et al., 2015).

Alzheimer disease is the most common neurodegenerative disease worldwide and also the 6th most 47common cause of death in the United States (Apostolova, 2016). Globally, approximately 47 million people suffer from dementia, and 60-70% of these people have Alzheimer's. It is estimated that the prevalence of AD will double every 10 years (Mayeux & Stern, 2012; Tiwari et al., 2019). Since dementia and AD mainly affect older people, this increase in people suffering from Alzheimer's is largely due to the population of the elderly (65+) that is increasing and growing at a rapid rate. People are getting older and older because of the improved health care and people are overall living a healthier life than was the case a hundred years ago (Qiu et al., 2009). In 2019, an estimate of 47 million people suffered from dementia, and this number is thought to be tripled by 2050, meaning that come that time, more than 130 million will suffer from dementia (Tiwari et al., 2019). Even though it is mainly the elderly that are affected by dementia and AD, about 5% of the patients are younger than 65 years old. This form of AD is called early-onset, or familial Alzheimer's disease (Mendez, 2017). Familial AD is driven by a patient's genetics, meaning that it is inherited from one of the persons parents. The other, more common type of AD is sporadic Alzheimer's, in which the disease is not necessarily inherited, but other environmental or genetic risk factors may play a role (Bekris et al., 2010). Old age is of course the biggest risk factor for developing AD, but there is some evidence that leads to believe that a number of vascular risk factors such as obesity and smoking may also lead to an increased chance in developing dementia and Alzheimer's. Studies have also shown that high blood pressure, high cholesterol, and excessive alcohol consumption also may increase the risk of developing AD (Helzner et al., 2009; Mayeux & Stern, 2012).

Mutations in 3 different genes are mainly associated with the development of early-onset Alzheimer disease. These 3 genes being the *APP*, *PSEN1,* and *PSEN2* genes (Bird, 2008). The *APP* gene encodes for the Amyloid Precursor Protein (APP). The Amyloid Precursor Protein is a membrane protein that is expressed in many tissues, where it functions as a cell-surface receptor. APP is involved in several processes such as synaptogenesis and synaptic plasticity (Gralle & Ferreira, 2007). APP is also the precursor protein of Amyloid-β (Aβ), which are the peptides that are the main component of the plaques that damage the brains of AD patients (Tcw & Goate, 2017). Studies have shown that mutations in this *APP* gene may cause a change in the generated Amyloid-β to a more amyloidogenic isoform or increase the tendency to form a plaque of aggregated Amyloid-β (Li et al., 2019; Weggen & Beher, 2012). Since the *APP* gene is located on chromosome 21, people with Down Syndrome are more

susceptible to AD. People with Down Syndrome, also called Trisomy 21, have 3 copies of chromosome 21. This means that people with Down Syndrome have an overexpression of *APP,* leading to more APP expression and overall more production of Aβ, which forms plaques in the brain (Castro et al., 2017; Strydom et al., 2018). Presenilin 1 and presenilin 2 are encoded by the *PSEN1* and *PSEN2* genes. Presenilin is one of the four components of the γ-secretase complex that cleaves its substrate APP, generating Amyloid-β. The 179 *PSEN1* and 14 *PSEN2* mutations that are associated with familial AD are thought to impair the cleaving of the γ-secretase protease, favouring the increased production of Aβ42. Aβ42 seems to be more toxic, less soluble and more prone to aggregate into senile plaques when compared to the otherwise produced Aβ40 (O'Brien & Wong, 2011; Shen & Kelleher, 2007).

Another genetic risk factor for developing AD is the presence of the *APOE E4* allele of the *APOE* gene. The *APOE* gene encodes for apolipoprotein E (O'Brien & Wong, 2011). This is an important protein in the lipid metabolism and tissue repair, binding cholesterol and other lipids and transporting them through the body. APOE also bind to Amyloid-β, helping with clearing soluble and build up Aβ (Van Cauwenberghe et al., 2016). There are three allelic variant of the *APOE* gene known that encode for three different isoforms known as ApoE2, ApoE3, and ApoE4 (Huang & Mahley, 2014). Presence of the *APOE E4* allele increases the risk of developing familial AD (Kim et al., 2009). The risk of developing AD is three times higher for people carrying a copy of this allele and this risk is increased to 15-fold for homozygous carriers of this *APOE E4* allele (O'Brien & Wong, 2011). This is because the *APOE E4* allele seems to be not as efficient at clearing Aβ (Van Cauwenberghe et al., 2016). Globally, about 13.7% of the population carry this allele of the *APOE* gene, which means a lot of people are at risk of developing AD (Liu et al., 2013).

Characteristic for AD are the extracellular senile plaques and intracellular neurofibrillary tangles that consist of aggregated hyperphosphorylated tau proteins that can be found in the brains (Tiwari et al., 2019). These plaques mostly consist of Amyloid-β, that is produced by cleavage of APP (Swerdlow, 2007). One theory describes that AD probably results from the overproduction and inability to clear this Amyloid-β peptide from the brain, and the following events such as tau hyperphosphorylation also contribute to the typical AD features, like brain atrophy and synaptic loss. This is called the amyloid cascade hypothesis (Apostolova, 2016; Swerdlow, 2007). Under normal, non-pathogenic circumstances, APP is cleaved in a non-amyloidogenic manner where no Amyloid-β is produced. In this non-amyloidogenic pathway, the transmembrane APP protein is first cleaved within the Amyloid-β domain of APP by an enzyme family called the α-secretases (Coronel et al., 2019). ADAM10 and ADAM17 are two secretases that belong to this family and are believed to be involved in the cleaving of APP in neurons (Tiwari et al., 2019). Cleavage of APP by this α-secretase produces a N-terminal fragment called sAPPα, which is released to the extracellular medium, and a C-terminal transmembrane fragment called C83. The generated sAPPα can start Notch signalling and has neuroprotective functions. C83, which is still stuck on the cell's membrane, is subsequently cleaved by another secretase called γ-secretase. This generates a small extracellular peptide called p3 and a larger intracellular fragment called AICD that is released into the cytoplasm. AICD may act as a transcription factor, regulating the expression of several target genes, but it seems that it is mostly degraded under non-pathological conditions (Coronel et al., 2019; Haass et al., 2012; Tiwari et al., 2019). The AD pathogenesis starts with an altered cleavage of APP in what is called the amyloidogenic pathway. Here, APP is cleaved by a β-secretase instead of an α-secretase. β-secretase cleaves APP outside of its Amyloid-β domain, generating an extracellular N-terminal fragment called sAPPβ and a membrane bound C-terminal fragment called C99. C99 is subsequently cleaved by γ-secretase, releasing Amyloid-β to the extracellular space and AICD into the cytoplasm (Chow et al., 2010; Coronel et al., 2019).

Amyloid-β, especially Aβ42, is toxic and sometimes not broken down. It can clump together between neurons, forming the characteristic plaques (Swerdlow, 2007). These plaques disrupt cell-cell signalling of the neurons, thereby impairing brain function. These Amyloid-β plaques may also activate microglia, inducing an immune response that can further damage the surrounding neurons (Guillot-Sestier & Town, 2013). AICD is released into the cell, where it will migrate into the nucleus and function as a transcription factor, regulating expression of genes like glycogen synthase kinase 3β (GSK-3β). GSK-3β is a serine/threonine kinase that is one of the enzymes responsible for the hyperphosphorylation of tau (Coronel et al., 2019; Hooper et al., 2008). These hyperphosphorylated tau protein form the neurofibrillary tangles that are also characteristic for AD. In contrast to the plaques, these tangles are formed intracellularly. Normally tau associates with tubulin to form microtubules. These microtubules are very important, as they provide stability to the cell and also form bridges by which information and nutrients can be transported (Iqbal et al., 2010; Mazanetz & Fischer, 2007). However, when tau gets hyperphosphorylated by a kinase such as GSK-3β, it becomes oligomerized. Tau is disassociated from tubulin, making the microtubules unstable. The hyperphosphorylated tau aggregate, forming neurofibrillary tangles. These tangles are insoluble and in the cytoplasm, along with the unstable microtubules, lead to a loss of communication between neurons and possibly apoptosis (Mazanetz & Fischer, 2007; Tiwari et al., 2019).

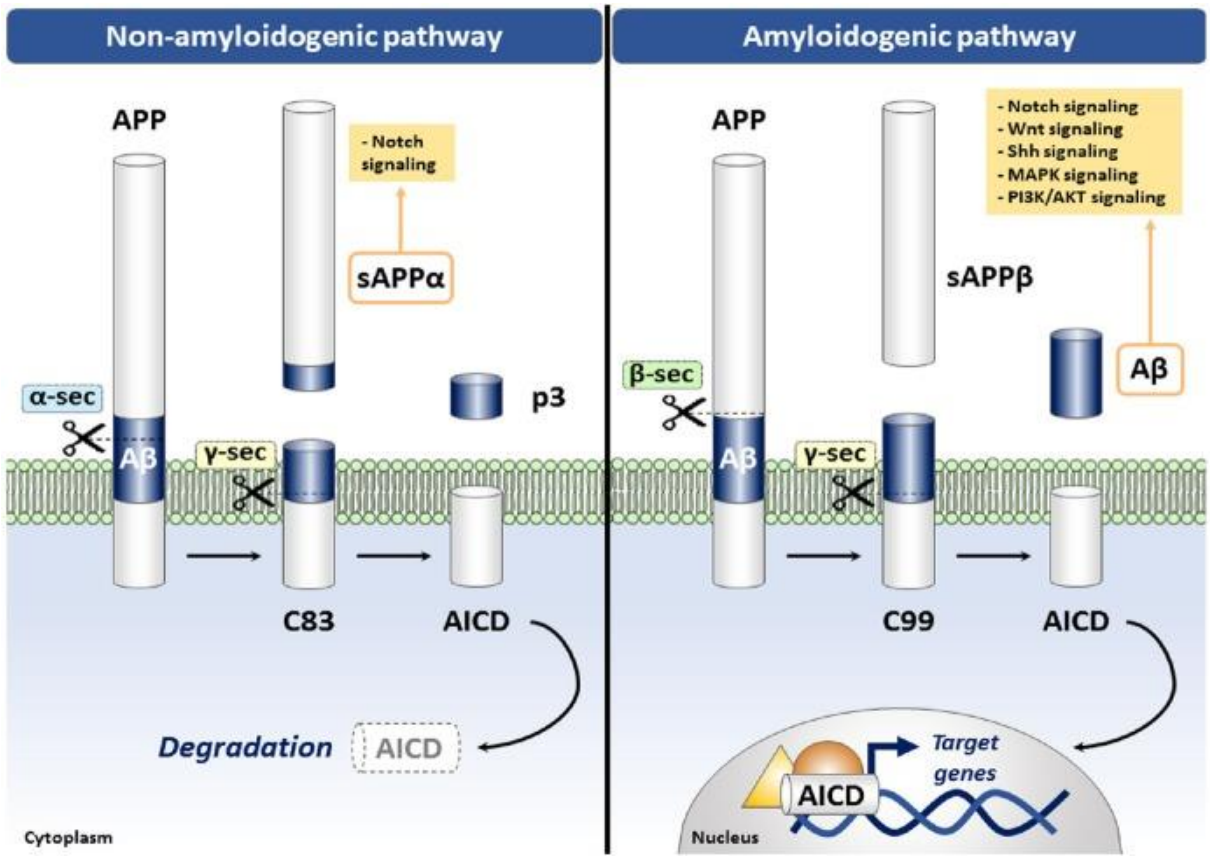

Figure 1: Schematic overview of the non-amyloidogenic cleavage of Amyloid Precursor Protein (APP) and the amyloidogenic cleavage of APP that leads to Amyloid-β production. In the non-amyloidogenic pathway, APP is cleaved by α-secretase, generating a sAPPα and C83 fragment. Subsequently, the C83 fragment is cleaved by γ-secretase, generating p3 and AICD, which is degraded in the cytoplasm. In the amyloidogenic pathway, APP is cleaved by β-secretase instead, generating sAPPβ and C99. C99 is subsequently cleaved by γ-secretase, producing extracellular Aβ and intracellular AICD. Aβ forms the extracellular plaques characteristic for Alzheimer disease. AICD acts as a transcription factor. From Physiological effects of amyloid precursor protein and its derivatives on neural stem cell biology and signalling pathways involved, by R. Coronel, 2019 (https://www.ncbi.nlm.nih.gov/pmc/articles/PMC6585543/)

Treatment of Alzheimer's is difficult since the exact mechanism of developing this disease is not yet fully understood. The diagnosis of AD also comes in too late, since the patient won't be showing any symptoms until it is too late. Most of the therapies that are currently available for AD patients focus on ameliorating symptoms and reducing damage and progression of the disease. Since none of these currently available therapies reverse the course of AD, prevention of developing it is, if possible, the better solution. A healthy lifestyle and diet can come a long way in AD prevention (Mendiola-Precoma et al., 2016). For pharmacological treatments against AD there are a few available options. The most used for AD dementia are cholinesterase inhibitors such as donepezil, rivastigmine, and galantamine (Weller & Budson, 2018). Acetylcholine is a neurotransmitter that is known to be very important in mediating learning and memory in the brain. In patients with AD a deficiency of acetylcholine is often found, and it is thought that this deficiency may also be responsible for the reduced cognitive ability that is typical for Alzheimer's (Mendiola-Precoma et al., 2016; Schachter & Davis, 1999). The neurotransmitter acetylcholine is released into the synaptic cleft in response to an action potential and is subsequently interacts with receptors present on the post-synaptic neuron. Acetylcholine in the synaptic cleft that is not interacted with is hydrolysed by the enzyme cholinesterase into choline and acetate (Dvir et al., 2010). Inhibiting this breakdown of acetylcholine via cholinesterase inhibitors may increase the duration and concentration of acetylcholine in the synaptic cleft, making up for the deficiency found in AD (Schachter & Davis, 1999).

A lot of different types of therapeutics are still tested, some promising drugs make it to clinical trials. A promising type of drug against AD are the monoclonal antibodies. Since the toxic plaques seem to be critical for the AD pathology, research has been conducted to create monoclonal antibodies that target the amyloid-β in these plaques. These monoclonal antibodies would provide some passive immunity for people with AD and target amyloid-β that has aggregated in plaques for removal by microglia (Mendiola-Precoma et al., 2016; Weller & Budson, 2018). These antibodies that target amyloid-β seemed very promising, but only a few of the created anti-amyloids showed meaningful results in trials. The most promising monoclonal, Aducanumab, has been submitted for approval in late 2020. Aducanumab did however not show a significant improvement in AD patients in the phase 3 trial, but since a small subgroup of patients did show improvement, the drug was still submitted for approval (Schneider, 2020). Monoclonal antibodies that target phosphorylated tau in tangles have also been created, with some of them going into phase 2 trials (Weller & Budson, 2018).

Another possible treatment for AD that is currently investigated are the β-secretase inhibitors. Since β-secretase, or β-site amyloid precursor protein cleaving enzyme 1 (BACE1), is the enzyme that initiates the production of amyloid-β by cleaving APP, so inhibiting the function of this enzyme may inhibit the formation of the toxic plaques (Ghosh & Osswald, 2014). By lowering the amyloid-β concentration and plaque formation, the disease could, in theory, be prevented from doing too much damage in the brain (Yan & Vassar, 2014). Development of a few of these β-secretase inhibitors is begin pursued. It has been proven difficult to have these drugs be able to enter the brain through the blood-brain barrier (BBB), but a few β-secretase inhibitors have been able to reach the brain (Vassar, 2014). Some BACE1 inhibitors, such as LY2811376 and Verubecestat have been studied extensively and undergone clinical trials (Weller & Budson, 2018).

Targeting BACE1 and inhibiting this protein for AD therapeutics might also come with some risk. Research has shown that knockout of BACE1 in mice puts an end to Aβ generation, but these mice have complex phenotypes. BACE1 knockout mice have shown to be less exploratory and smaller. They have also shown neuronal problems such as hypomyelination, impaired synaptic plasticity, retinal

pathology and schizophrenia-like phenotypes (Barão et al., 2016). These phenotypes in BACE1 knockout mice are likely caused by other substrates of BACE1 that are not processed by BACE1 in these knockout models and therefore do not function properly. This means BACE1 is involved in other processes besides the cleavage of APP, and inhibition of BACE1 could possible result in other deficiencies and side-effects. One of the substrates of BACE1 besides APP is Neuregulin-1 (NRG1) (Cheret et al., 2013). NRG1 is a trophic factor that interacts with its receptor ErbB3, which is a tyrosine kinase receptor that are present on Schwann cells of the peripheral nervous system (PNS). The *Nrg1* gene encodes for over 15 isoforms, most of which are membrane-bound proteins. The type III isoform of NRG1 is a transmembrane protein that requires cleaving by BACE1 to become active. After cleavage by BACE1, NRG1 activates ErbB3 on Schwann cells. This activation results in the myelination of the peripheral nerves (Cheret et al., 2013; Willem et al., 2006). This means that inhibiting BACE1 functions as AD treatment might have a side effect of hypomyelination in the PNS. L1 and CHL1, which are neural cell adhesion protein, are also physiological substrates of BACE1. L1 and CHL1 are both important molecules in axonal guidance and they also maintain and remodel neural circuits in the central nervous system. Knockout L1 and CHL1 mice showed a similar phenotypes to BACE1 knockout mice, with impaired cognitive function and schizophrenic behaviour (Zhou et al., 2012).

Many substrates of BACE1 have been identified and their physiological functions could be impaired by BACE1 inhibitors. This loss of function of these substrates could result in side effects after treatment for Alzheimer's disease by BACE1 inhibitors. This is why it is also important to understand the physiological functions of BACE and it could also be the biggest drawback to small-molecule BACE1 inhibitors as a treatment for Alzheimer's disease (Barão et al., 2016).

Research findings

According to the amyloid cascade hypothesis, cleavage of APP by β-secretase instead of α-secretase leads to production of amyloid-β and AD pathologies such as amyloid plaques and eventually neurofibrillary tangles (Swerdlow, 2007). To justify the time and effort that would go in to creating a specific inhibitor for BACE1, the involvement of this enzyme in the formation of amyloid plaques needed to be proven. To confirm the importance BACE1 in amyloid-β formation and to see if BACE1 inhibition could actually prevent the formation of amyloid-β plaques in the brain, BACE1 knockout mouse models were analysed. Lisa McConlogue et al. deleted the BACE1 gene in PDAPP mice to determine if deletion of BACE1 would prevent the formation of plaques (McConlogue et al., 2007). This PDAPP mouse line have a high expression of human APP and aggressive extracellular amyloid-β plaque aggregation and is therefore considered a good model for Alzheimer disease (Games et al., 1995). Sections of PDAPP/BACE1 knockout mice were stained with amyloid-β specific antibodies (3D6) to observe the amyloid plaques. Figure 2A shows the results from the histochemistry. In the PDAPP/BACE1(+/+) sections amyloid-β plaques are clearly visible, which is typical for this line of mice. In the brain sections of the BACE1 knockout mice (PDAPP/BACE1(-/-)) however, the staining did not show any build-up of amyloid-β (McConlogue et al., 2007). This complete lack of presence of plaques would suggest a critical role of BACE1 in the formation of amyloid-β plaques. Yi Luo et al. also showed a lack of plaque formation in APP transgenic BACE1 knockout mice (Luo et al., 2003). In their experiments they also performed an enzyme-linked immunosorbent assay (ELISA) to detect amyloid-β in these mice. Their results shown in figure 2B showed a large difference in amyloid-β concentration between the BACE1 knockout mice and mice with functional BACE1. The BAC1 knockout mice even showed a complete absence of Aβ-42 and a very little amount of Aβ-40. In contrary, the mice with functional BACE1 gene showed a higher concentration of both Aβ-42 and Aβ-40 (Luo et al., 2003). These combined results of the staining and the ELISA suggest that amyloid-β formation and aggregation into plaques requires BACE1, and therefore a BACE1 knockout or inhibitor may prevent the formation of amyloid plaques in the brain.

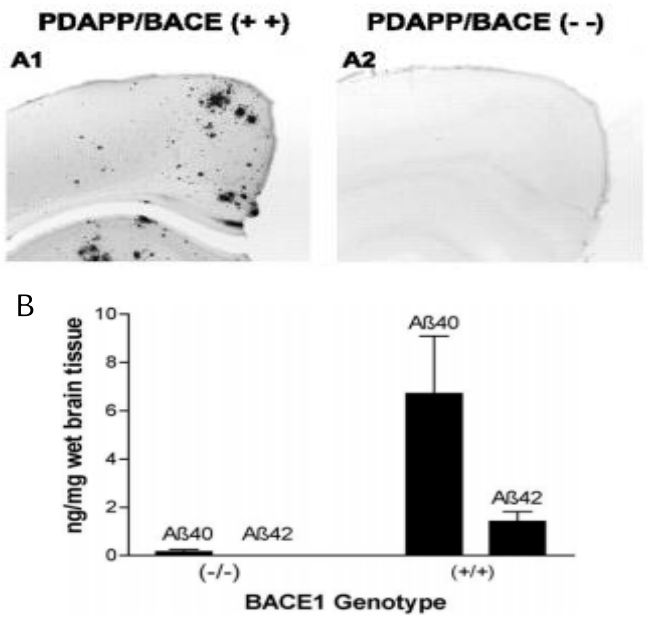

Figure 2: Histochemistry staining's (A) and ELISA (B) against Aβ in PDAPP/BACE (+/+) and PDAPP/BACE (-/-) mice. BACE1 knockout mice show no amyloid plaques in the staining and Aβ concentration are greatly decreased and Aβ-42 is not detected. Mice with functional BACE1 showed plaque formation and high Aβ concentrations. Figure A obtained from (Luo et al., 2003), Figure B was obtained from (McConlogue et al., 2007)

Since BACE1 has been confirmed to be crucial in the generation of beta-amyloid plaques and inhibition of the β-secretase could provide protection from developing AD, a lot of different BACE1 inhibitors have been created and tested. The first generation of created BACE1 were large molecules that were designed to contain the APP sequence where BACE1 cleaves. These large peptidomimetic molecules showed promising results in *in-vitro* studies, but were unable to pass the blood-brain barrier due to their size. This, along with other problems such as long serum half-life made these BACE1 inhibitors not suitable as drugs against AD. Instead, focus has been on small molecules BACE1 inhibitors that can penetrate the blood-brain barrier and gain access to the active site of BACE1 to inhibit its function (Vassar, 2014; Yan & Vassar, 2014). Two of such inhibitors, LY2886721 and MK-8931 are described here.

LY2886721

One of the first small-molecule BACE1 inhibitors, called LY2811376, was created by the company Eli Lilly. This small molecule showed very promising results in animal models, reducing amyloid-β and sAPPβ levels in mice. In healthy volunteers, LY2811376 also reduced Aβ-40 concentrations in the plasma, which would slowly return to their normal values after treatment. LY2811376 was discontinued however, because toxicology studies in rats showed off-target associated pathology in the retina and brain of the treated rats with the inhibitor (May et al., 2011). Even though LY2811376 turned out to have toxic effects in rats, it was successful in reducing Aβ concentrations and therefore the drug LY2886721 was created. LY2886721 is a promising small-molecule active site inhibitor of BACE1. This inhibitor has a core of a bicyclic amino thiazine with fluoropyridine and fluorophenyl groups that are connected by an amide group. LY2886721 binds to the active site of BACE1, where its bicyclic core engages with the catalytic dyad of BACE1 and the fluorophenyl group of the inhibitor also binds in the S1 pocket of BACE1 (May et al., 2015). Patrick C. May et al. tested the anti-amyloid effects of LY2886721 on PDAPP mice that were treated with 3, 10, or 30 mg/kg doses of the inhibitor drug. Treatment with all doses of the inhibitor significantly reduced Aβ levels in the hippocampus and cortex of the mice when compared to the control. Treatment with LY2886721 also significantly reduced the C99 and sAPPβ levels in the cortex and hippocampus of the mice. C99 and sAPPβ are both products of APP cleavage by BACE1, so their reduction means LY2886721 successfully inhibits BACE1 in these mice. Plasma Aβ in healthy humans was also measured after receiving 5, 15, 35 and 70 mg doses of LY2886721. The healthy subjects got a daily dose of LY2886721 for 14 days, where-after samples were taken for up to 216 hours. Figure 3 shows that the plasma Aβ40 and Aβ42 are reduced by LY2886721 in a dose-dependent manner when compared to the placebo. Administration with 70 mg LY2886721 even leads to an 80% reduction in plasma Aβ40 and Aβ42. Over time, the plasma Aβ returned to baseline. About 72 after administration, the Aβ reduction stopped being significant for the 5 mg dose of LY2886721. For the highest, 70 mg dose the effect would stop at around 120 hours after treatment (May et al., 2015).

LY2886721 advanced into phase 1 and later phase 2 trials because of these positive results. Two separate phase 1 study designs have been done (NCT01227252, NCT01534273). These trials consisted of 47 and 30 participants respectively and took 14 days. The participants were treated with either a multiple ascending dose, from 5 mg to 15 mg to 35 mg LY2886721, or a single dose of 70 mg of the drug followed by the ascending doses. During these trials, LY2886721 was found to be non-toxic and well tolerated, and a half-life of roughly 12 hours was reported. The phase 1 clinical trials showed a decrease in Aβ40 levels in the plasma and Cerebrospinal fluid (CSF). This decrease got larger when treated with the 70 mg dose of LY2886721. Here, a 74% reduction in Aβ40 had been found. Aβ42 and sAPPβ were also decreased in the CSF of the participants after administration of LY2886721. During

these trials, sAPPα was found to be increased after treatment with LY2886721. This makes sense since this is a product of APP cleavage by α-secretase and these enzymes normally have to compete with β-secretase. But since LY2886721 inhibits BACE1, the α-secretase has no competition and more sAPPα is produced (Vassar, 2014; Yan & Vassar, 2014).

Because of the promising results of the phase 1 trials, a phase 2 trial was started (NCT01561430). In this phase 2 trial, 130 participants with mild AD where treated with daily doses of 35 or 70 mg LY2886721 over the course of 6 months. This phase 2 trials was however stopped prematurely since some participants developed abnormal liver biochemistries after begin administered LY2886721 (Vassar, 2014).

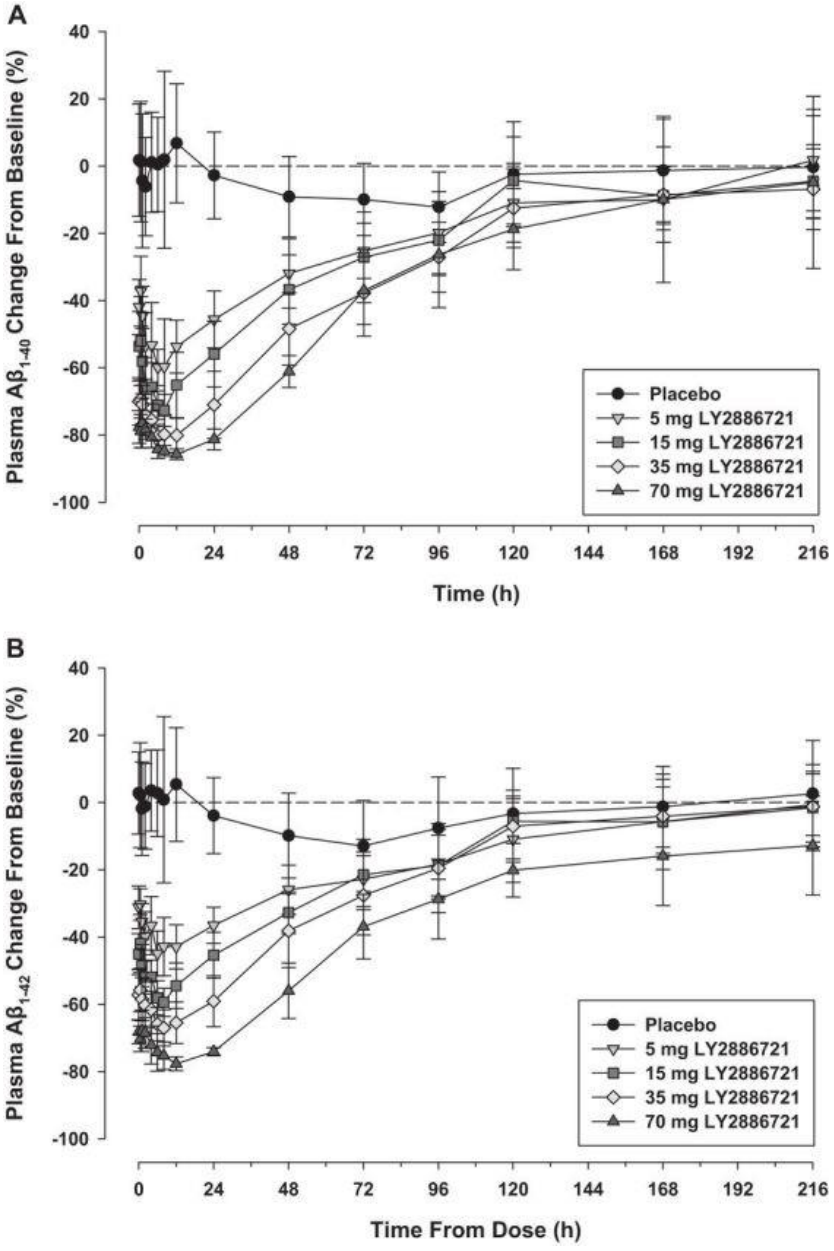

Figure 3: A) Plasma Aβ40 and B) Aβ42 levels in healthy participants after administration of a placebo or 5, 15, 35, or 70 mg of LY2886721 for 14 days. Plasma was collected for up to 216 hours after last administration of the drug. Values shown are percentage change from baseline. Both Aβ40 and Aβ42 levels were decreased in a dose-dependent manner compared to the placebo and Aβ levels returned to baseline after about 216 hours. Reduction of Aβ40 and Aβ42 were around 86% and 80% for the 70 mg dose. Figure obtained from (May et al., 2015)

Verubecestat

Verubecestat, also called MK-8931, was a small molecule inhibitor of BACE1 that was developed by the company Merck. Verubecestat consists of an iminothiadiazinane core and a fluoroaryl ring connected to a fluoropyrimidine ring by an E-amide group (Saravanan et al., 2019). In 2012, Mark Foreman et al. tested Verubecestat in 88 healthy volunteers that were between 18 and 45 years old. In this randomized, double-blind, placebo-controlled trials, the participants were treated with a single dose of Verubecestat of 20, 100 and 550 mg or a rising multiple dose of 10 to 250 mg Verubecestat every day for 14 days. Treatment with Verubecestat was well-tolerated in the participants, with side effects being mild. Amyloid-β levels in the CSF were reduced in a dose-dependent manner. After treatment with the highest, 550 mg dose of Verubecestat, Aβ CSF levels were decreased by 92% 36 hours after treatment. Both single and multiple dose treatment of this small-molecule inhibitor were well-tolerated and showed a reduction in Aβ40, Aβ42 and sAPPβ in the participants' CSF (Forman et al., 2012).

Another study by Matthew E. Kennedy et al. tested the effects of Verubecestat in animal models and in patients with AD. Rats and monkeys were treated with 10 mg/kg and 30 mg/kg doses of Verubecestat for 6 and 9 months respectively to test for toxicity. Rats and monkeys treated with Verubecestat showed no signs of treatment related toxicology. Rats treated with Verubecestat showed a dosage-dependent reduction of Aβ40 in the plasma, CSG and cortex and monkeys also showed this reduction in the CSF and cortex. Oral administration of 20, 100 or 550 mg Verubecestat in healthy, non-elderly participants also resulted in a decrease of Aβ40, Aβ42 and sAPPβ. Figure 4A shows the decrease in Aβ after treatment. Aβ is decreased in a dose-dependent way, with the 550 mg dose showing only 8% Aβ42 when compared to baseline. Treatment with 20 mg Verubecestat only showed a slight decrease in Aβ levels, and Aβ levels returned to baseline after 28 hours. Aβ40 and sAPPβ behaved similarly. Patients with mild to moderate AD were also orally administered with 12, 40 or 60 mg of Verubecestat for 7 days, where-after Aβ levels were measured in the CSF and blood. Figure 4 shows the Aβ42 levels in the CSF of AD patients after treatment with Verubecestat. The figure shows that Aβ42 levels are decreased in a dosage-dependent manner. Patients treated with 12 mg of Verubecestat had roughly 40% Aβ42 in the CSF when compared to baseline. Patients treated with the highest, 60 mg dose of Verubecestat even showed a greater reduction, at around 18% Aβ42 compared to baseline. CSF levels of Aβ40 and sAPPβ were reduced in a very similar manner (Kennedy et al., 2016).

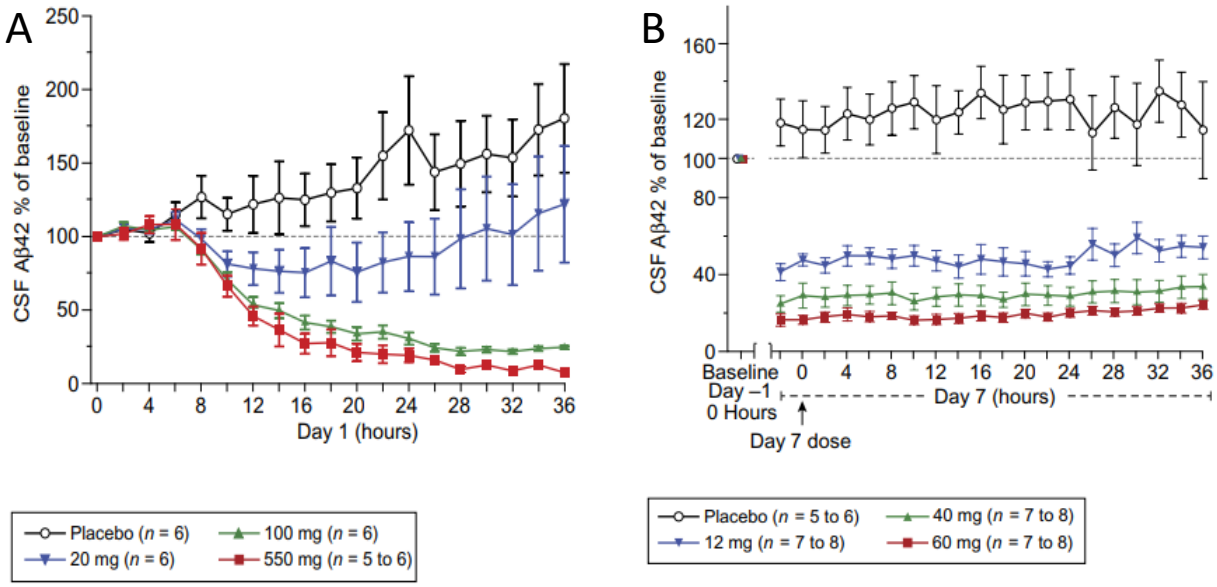

Figure 4: Aβ42 level in A) healthy non-elderly adults and B) AD patients CSF. Healthy participants were treated with 20, 100 or 550 mg Verubecestat and AD patients were treated with 12, 40 or 60 mg for 7 days. Aβ42 levels are expressed as percentages compared to baseline. Treatment with Verubecestat reduced CSF Aβ42 levels in healthy and AD patients. Healthy participants treated with 20 mg Verubecestat returned to baseline levels after 28 hours. From (Kennedy et al., 2016)

Back in 2013, Michael F. Egan et al. conducted a large, randomized, double-blind trial. In this trial that was supposed to take 104 weeks, patients with memory impairment and elevated levels of Amyloid-β in the brain were administered with daily oral doses of 12 and 40 mg Verubecestat (Egan et al., 2019). The change from baseline in Clinical Dementia Rating Scale-Sum of Boxes (CDR-SB) were scored from the participants. The CDR is a scale widely used for dementia scaling. On this scale, scores can range from 0 to 18, with the higher scores indicating worsening of cognition in the patient (O'Bryant, 2008). Results of this trial suggested that treatment with 12 or 40 mg of Verubecestat did not improve the scores on the CDR-SB and thus did not improve cognition or daily function. There were even some measurements that suggested that patients that were treated with Verubecestat scored worse on the tests than patients that received the placebo. Because of these results, the company Merck terminated the trials since Verubecestat did not improve the cognitive decline in patients with memory impairment (Egan et al., 2019).

Discussion

Alzheimer disease is the most common aging-related neurodegenerative disease worldwide and it is characterized by clumps of protein and tangled fibres in the brain known as amyloid plaques and neurofibrillary tangles (Apostolova, 2016; Tiwari et al., 2019). The most researched and accepted theory for AD pathogenesis is the Amyloid cascade hypothesis, where the production and aggregation of amyloid-β plays a central role. According to this hypothesis, cleavage of the amyloid precursor protein (APP) by the β-secretase BACE1 instead of the normal α-secretases results in the production of the peptide amyloid-β. Amyloid-β subsequently builds up in the brain forming the characteristic senile plaques in AD that can cause synaptic loss (Coronel et al., 2019). Since BACE1 plays a crucial role in the formation of amyloid-β, it has been theorized that BACE1 targeting therapeutics could be used as a potential treatment against Alzheimer disease (Ghosh & Osswald, 2014). The aim of this thesis was to explore small-molecule inhibitors of BACE1, such as LY2886721 and Verubecestat, and their current standings as potential drugs against Alzheimer disease after several clinical trials.

To justify the needs and time that would go into the creation and testing of small-molecule BACE1 inhibitors, BACE1 first had to be identified as a required protein for amyloid-β formation. This was made possible by studying the levels of Aβ in BACE1 knockout mice. Using a BACE1 knockout model of Tg2576 APP mice, Luo et al. discovered that the knockout of BACE1 resulted in no formation of amyloid-β plaques on their histochemistry sections and their ELISA showed that Aβ-40 levels were reduced by a very large margin while Aβ-42 levels were not even measured in the knockout mice. They also theorized that in these BACE1 knockout mice, a different enzyme such as its close homolog BACE2 could possibly take over the functions of BACE1 in aged mice. To disprove this theory, the BACE1 knockout mice were given time to age to roughly 14 months to see if BACE2 or a different enzyme would substitute the functions of BACE1 and generate amyloid plaques. Their results showed that a BACE1 knockout was sufficient in stopping the amyloid-β plaque formation in both the aged mice and the mice that were not given the time to age. These results indicate a crucial role of BACE1 in Aβ formation and that other enzymes such as BACE2 are not capable of replacing BACE1 functions (Luo et al., 2003).

Lisa McConlogue et al. wanted to confirm that BACE1 knockout would result in an absence of amyloid-β build-up. Instead of using the same Tg2576 APP transgenic mice that were used in previous experiments, they used BACE1 knockout PDAPP mice instead since these mice would give rise to more aggressive plaques. Their results also showed that there was a complete lack of amyloid-β build up in their histochemistry sections in the BACE1 knockout mice. Since the histochemistry sections of mice with fully functional BACE1 did show amyloid plaques, BACE1 is likely a critical player in the formation of amyloid-β plaques. They also described that the BACE1 knockout mice displayed abnormal behaviour and electrophysiological alterations, which led to believe that other substrates of BACE1 were being affected by its absence. To investigate this, heterozygous BACE1 knockout mice were generated. These heterozygous knockout mice also caused a significant decrease in Aβ levels and plaque formation. The mice also showed no abnormal behaviour, suggesting that other BACE1 substrates are affected by a complete knockout of the gene (McConlogue et al., 2007).

There are several other physiological substrates of BACE1 besides APP. BACE1 inhibitors could therefore not only prevent amyloid-β formation but also inhibit the functions of other BACE1 substrates, which could cause toxic effects. Neuregulin-1 (NRG1), for example, is a physiological substrate of BACE1 that is involved in the myelination of neurons. Inhibition of BACE1 would result in NRG1 not getting activated, leading to hypomyelination (Barão et al., 2016; Moussa-Pacha et al., 2020).

Since myelination is complete in adulthood and patients that would require BACE1 inhibitor drugs are mostly the elderly, this particular example of NRG1 does not really affect a patient, but many other physiological processes BACE1 is involved in continue throughout life (Barão et al., 2016). It has also been reported that APP cleavage by BACE1 occurs in the early endosome, whereas other interaction with nonamyloidogenic substrates is not endocytosis dependent. This means that selective inhibitors can be designed that only target endosomal BACE1, which would prevent other processes from being affected (Moussa-Pacha et al., 2020).

Many small-molecule BACE1 inhibitors have been tested and have advanced to clinical trials. After the LY2811376 inhibitor was cancelled after various side-effects were observed, LY2886721 was created and researched. LY2886721, which acts on the active site of BACE1, was shown to be a very potent BACE1 inhibitor, reducing levels of Aβ, C99 and sAPPβ in PDAPP mice. Oral administration of LY2886721 in beagle dogs also led to a significant drop of Aβ in the cerebrospinal fluid. Since the central nervous system is protected by the blood-brain barrier, it was important for the BACE1 inhibitor to be able to penetrate this barrier and reach the brain. LY2886721 was shown to have the ability to penetrate the BBB, because the concentrations of LY2886721 in the CSF matched that of the concentrations in the plasma. After LY2886721 had shown to be a potent inhibitor of BACE1 and was able to cross the BBB without any significant side-effects in animal models, the drug advanced to clinical trials. In healthy volunteers, LY2886721 reduced plasma concentrations of Aβ40 and Aβ42 in a dose-dependent manner. The concentrations of the drug in the CSF also matched that of the concentration in the plasma, proving again that LY2886721 could cross the blood-brain barrier and perform its function in the brain. Furthermore, other products of APP cleavage by BACE1, such as sAPPβ and C99, were also reduced in the CSF while products of APP cleavage by α-secretases were increased. This suggests that LY2886721 successfully inhibits BACE1 in healthy subjects and that nonamyloidogenic cleavage of APP by α-secretase is enhanced upon BACE1 inhibition (May et al., 2011, 2015).

After these positive results in the first trials of LY2886721, the BACE1 inhibitor went onto stage 2 clinical trials where its safety and tolerability was tested in patients with mild AD. The company responsible for this trials did however terminate their study, because some of the patients that were treated with LY2886721 showed abnormal liver biochemistries (May et al., 2015). Since BACE1 knockout mice did not show any toxic effects on the liver, its likely that the abnormal liver biochemistries that was detected in these patients is not related to the pathway BACE1 operates in. Small molecule drugs are known to sometimes cause irregular liver phenotypes during clinical trials. Even though LY2886721 trials were terminated, BACE1 inhibitors could still be a viable option for AD treatment (Vassar, 2014).

Another small-molecule inhibitor that acts on the active site of BACE1 was Verubecestat. In one of the first studies of Verubecestat in healthy humans, the drug reduced amyloid-β level in the CSF for up to 94%, without causing any side-effects. This first small study of Verubecestat showed promising results, so larger scale experiments were started not shortly after (Forman et al., 2012).

Verubecestat was also shown to reduce Aβ40, Aβ42 and sAPPβ levels of the plasma, CSF and cortex in animal models with monkeys and rats. Reduction of these products of APP cleavage by BACE1 suggests that BACE1 function is not substituted by a different protease. Aβ levels were reduced the most in the plasma when compared to the CSF or brain cortex. This can be explained by the fact that Verubecestat is a substrate of P-glycoproteins (Kennedy et al., 2016). These P-glycoproteins are proteins that are present in the endothelial cells of the blood-brain barrier, where they transport drugs or different toxins out of the brain and into the plasma (Van Assema et al., 2012). In rabbits and rodents,

hypopigmentation was observed after treatment with Verubecestat. Since BACE2 has a known role in the pigmentation process of rodents, it can be said that treatment with Verubecestat not only affects BACE1, but also BACE2 (Kennedy et al., 2016; Rochin et al., 2013).

In healthy individuals, treatment with Verubecestat reduced levels of Aβ and sAPPβ in a dosage dependent manner in both the plasma and CSF. Since Verubecestat lowered Aβ levels in the CSF in a similar way as it did plasma Aβ levels, it seems that the inhibitor is able to penetrate the BBB and can overcome getting transported out of the CNS by P-glycoproteins. Significant reductions in Aβ lasted for up to 24 hours, making Verubecestat suitable for daily treatments. Similar reductions in Aβ and sAPPβ were observed in AD patients that were treated with Verubecestat, meaning that the disease does not cause Verubecestat to lose its ability to inhibit BACE1. In both the healthy participants and the AD patients, Verubecestat was well-received and did not cause any harmful side-effects. There was no hypomyelination, retinal deficiencies or hypopigmentation found in any of the human participants, even though BACE1 knockout in animal models sometimes showed these different phenotypes (Kennedy et al., 2016).

Since Verubecestat was well-received in healthy volunteers and AD patients, it advanced to phase 3 clinical trials. In this 104-week long trials, patients with AD were treated with 12 or 40 mg Verubecestat. Cognition and daily functioning were measured using the CDR-SB score. Unfortunately, Verubecestat did not improve cognitive functioning of the AD patients compared to the placebo. In some cases, Verubecestat even caused a worse decline than patients that were treated with the placebo. These results concluded Verubecestat as a drug against AD, and Verubecestat research was terminated (Kennedy et al., 2016).

For now, none of the small-molecule BACE1 inhibitors have shown to be able to reverse AD progression and cognitive decline, even though animal models showed promising results and Aβ levels were reduced. These BACE1 inhibitors were based on the amyloid cascade hypothesis where the cleavage of APP by BACE1 plays a crucial role. Since inhibiting BACE1 shows no beneficial effects in AD patients, BACE1 inhibitors as a drug against AD might need to be reconsidered and future research on AD treatment should not only focus on BACE1 inhibitors, but other types of treatment as well. Many new treatments against AD are researched, such as GSK-3β inhibitors and RIPK1 inhibitors. Possibly the most promising type of treatment are the immunotherapies. Many monoclonal antibodies against amyloid-β and tau are already researched and tested in clinical trials. Since AD is a very complicated disease with a complex pathology, future treatments against Alzheimer disease might consist of multi-drug treatments (Coimbra et al., 2018; Moussa-Pacha et al., 2020).

Conclusion

The effect of the small-molecule BACE1 inhibitors LY2886721 and Verubecestat on Alzheimer disease was tested. The results of several trials show that both inhibitors successfully inhibit BACE1 and thereby reduce levels of amyloid-β in both animal models and in humans. LY2886721 showed to not be a viable drug option since patients treated with this drug received abnormal liver biochemistries. Verubecestat showed very promising results, but in phase 3 trials it did not improve cognitive functioning in AD patients meaning it was not successful in treating Alzheimer disease. No BACE1 inhibitors have yet improved AD pathology, so other treatment options such as immunotherapy should also be investigated.

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