Universiteit Utrecht

Crossbeta Biosciences

Alzheimer's Disease

From Target to Drug

Mapping the Developmental Stages

Master thesis

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December 2009

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1. Introduction

Alzheimer's Disease (AD) is a neurodegenerative disease, with dementia as the main clinical manifestation. AD is characterised by a progressive loss of neurological and cognitive function. There are three known forms of AD; early-onset Alzheimer's (EOAD), late-onset Alzheimer's (LOAD) and familial Alzheimer's disease (FAD). EOAD is a rare form of AD in which diagnosis of the disease occurs before age 65 and is commonly linked with more genetic defects than other types of AD. LOAD is the most common form of AD, accounts for about 90% of diagnosed patients and usually occurs after age 65. LOAD may and may not be hereditary. FAD is the hereditary form of AD in which at least three early onset genes are causative of the disorder. Increasing age is the greatest risk factor for AD, as demonstrated by its prevalence: less than 10% of persons over the age of 65 years are affected, while after the age of 85 years, almost 50% suffers from AD (Ferri et al 2005) (Mount and Downton 2006). Within 5-10 years, the patient goes through different stages of AD: mild, moderate, severe, profound and finally terminal giving rise to a social burden in countries with a growing number of elderly individuals. In 2006, 26,6 million people were affected worldwide (Brookmeyer et al 2007), also leading to an estimated economic burden of $ 100 billion annually for direct and indirect patient care in the US alone (NIA, NIH 2001-2002) (Alzheimer's Drug Discovery Foundation). The rate of occurrence of the disease increases exponentially with age and considering the average age of the demographic population will shift towards a more increasing age in time, interventions are thus strongly recommended.

The major microscopic abnormalities in AD are neurofibrillary tangles and senile (neuritic) plaques, together with a degeneration of the neurons and synapses. Neurofibrillary tangles are bundles of paired helical filaments, composed of hyperphosphorylated tau protein. Neuritic plaques are dystrophic neurites, surrounding a plaque core mainly consisting of the amyloid β protein. Because neuritic plaques and neurofibrillary tangles are specific for AD, their presence indicates an AD- positive diagnosis. However, their presence can only be definitely confirmed post-mortem after dissection of an affected brain using the NIA-Reagan criteria for the post-mortem diagnosis of AD (Hyman and Trojanowski 1997). Definitive diagnosis is therefore only possible post-mortem. To exclude alternative causes of dementia, neuroimaging techniques such as computed tomography (CT), magnetic resonance imaging (MRI), single photon emission computed tomography (SPECT) or positron emission tomography (PET) play an important part in the early diagnosis of Alzheimer's disease. Because ante-mortem diagnosis enables clinical intervention, cognitive tests are being developed to aid an earlier diagnosis. Until now, cerebrospinal fluid analysis of amyloid β or tau proteins and cognitive tests are being used to calculate the risk for AD and assess the intellectual functioning. These are based on the criteria of the Diagnostic and Statistical Manual of Mental Disorders, fourth edition (DSM-IV-TR)1 and the National Institute of

Neurological Disorders and Stroke–Alzheimer Disease and Related Disorders (NINCDS–ADRDA) working group; e.g. the score on the Alzheimer's disease assessment scale, cognitive subscale (ADAS-Cog), the score on the mini-mental state exam (MMSE), and the score on the Dementia Severity Rating Scale (DSRS) (Blennow and Zetterberg 2006)(Mount and Downton 2006). Recently, Dubois et al proposed a new AD diagnostic framework, in which new research criteria should allow an earlier and more specific AD diagnosis than the NINCDS–ADRDA system (Dubois et al 2007). Although this article was positively received, the article was also commented upon by several papers (Foster 2007) (Foster et al 2008) (Gauthier et al 2008). Foster et al 2008 stated that Dubois et al 2007 should have taken several more associated criteria into account when revising the NINCDS–ADRDA system. Foster et al 2008 proposed that new research criteria should also involve other biomarkers such as presence of the APOE ε4 allele and blood biomarkers with or without genetic and cerebrospinal fluid markers. Much evidence also pointed towards involvement of olfactory dysfunction which should also be incorporated into a revised framework for the reliable diagnosis of AD. Therefore further validation is needed.

In the search for a disease modifying drug (DMD) against AD, many players are active in the same field. In this master thesis, an attempt is made to survey the consensus in this field, enlighting all aspects starting from compound library to final drug. The thesis can thus be formulated as follows:

"Can the developmental stages commonly used by pharmaceutical companies or institutions be mapped in the development of a small compound drug against Alzheimer's Disease?"

2. Biology of Alzheimer's Disease

Mechanisms that underlie the pathophysiological abnormalities seen in AD have been intensively studied. Two proteins are merely involved in the pathogenesis of AD: Amyloid β and Tau protein.

2.1 Tau Protein

Tau is a normal axonal protein that binds to microtubules through its microtubule-binding domains, thereby promoting microtubule assembly and stability. Tau phosphorylation is regulated by the balance between multiple kinases and phosphates. Tau hyperphosphorylation in Alzheimer's disease results in the formation of neurofibrillary tangles (NFT) inside nerve cell bodies (fig. 1), causing disassembly of microtubules and thus impaired axonal transport.

2.2 Amyloid β

Amyloid β (Aβ) is derived from Amyloid Precursor Protein (APP) by sequential proteolytic cleavages by cleaving enzymes such as β-secretase and γ-secretase. Cleavage by these enzymes renders peptide fragments ending at amino acid 42 ($A\beta_{42}$) and/or ending at amino acid 40 ($A\beta_{40}$), which is normally more produced by cells than $A\beta_{42}$. Although the two species are found colocalized together in the neuritic (senile) plaque (fig 1), much of the fibrillar Aβ found in neuritic plaques is $A\beta_{42}$, the slightly longer, more hydrophobic form that is more prone to aggregation (Selkoe 2001b). Upon cleavage from the precursor protein APP it renders Aβ peptide fragments, the major component of amyloid plaques in the brain of individuals with AD (figure 1) (Haass and Selkoe 2007). Under specific conditions, soluble forms of Aβ can be converted into highly ordered fibrillar, neurotoxic, β-sheet containing aggregates (Blanchard et al 2004) (Di Carlo 2009). Although neuronal degeneration occurs near the amyloid plaques, some studies have suggested that intermediates such as protofibrils or soluble oligomers are also involved in AD pathogenesis and even appear to be the more dangerous species in the onset of AD pathology (Di Carlo 2009). These structures attack particular neurons in the CNS, causing them to become dysfunctional and die in large numbers, leading to neurotoxicity (Yankner et al 1990). As Aβ oligomers accumulate in the brain, neurotransmission can be impaired— ultimately leading to the clinical manifestations seen in AD; cognitive decline and dementia (Tanzi et al 2008).

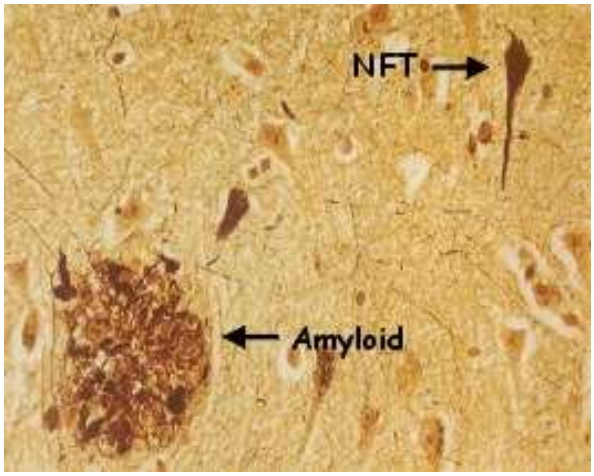

Fig 1. Amyloid plaque and Neurofibrillary tangle (NFT). Microscopic analysis of an amyloid plaque and a neurofibrillary tangle (NFT) as seen in post-mortem diagnosis of AD. Silver impregnation is used as visualisation technique. A senile plaque consists of a large, compacted deposit of extracellular amyloid surrounded by a halo of dilated, structurally abnormal, or dystrophic, neurites. NFT start by forming accumulations inside neurons of paired helical filaments that are semi-crystalline aggregates of hyper-phosphorylated protein tau. Image courtesy of Experimental Genetics Group, Katholieke Universiteit Leuven, Belgium.

Aβ is produced constitutively during normal cell metabolism. Recent studies have revealed its physiological role to enhance learning and memory (Morley et al 2009) and the relation between Aβ levels and learning and memory effects has shown to follow an inverted U dose response curve. Too low amounts of Aβ causes learning impairment, low but significant levels enhances memory, and excess Aβ leads again to memory deficits. It is suggested that in AD it is an excess of Aβ that is responsible for the learning and memory impairments, as shown previously in an animal model of AD (Morley et al 2009) (Christensen et al 2008) (Farr et al 2003). This also explains the failure of the effectiveness of an Aβ vaccine; the vaccine

lowers Aβ levels below endogenous levels which also induces memory and learning impairments (Castellani et al 2009).

The levels of endogenous Aβ peptides vary, as does the concentration of Aβ in the synaptic cleft of brain synapses. This presynaptic concentration is determined by the balance of Aβ production, release and degradation. Recent findings suggest that endogenous Aβ peptides are involved in activity-dependent regulation of synaptic vesicle release, responsible for the compensatory synapse loss in AD. Several enzymes are identified that can regulate Aβ concentrations in the synaptic cleft, for example several members of the M13 zinc-dependent metallopeptidase family such as neprilysin, and that are involved in the degradation of endogenous Aβ in the rodent brain. This is of importance considering that synapse loss is the strongest structural correlate of the cognitive decline seen in patients with AD (Abramov et al 2009).

2.3 Amyloid Hypothesis

A central hypothesis for the cause of Alzheimer's disease is the amyloid hypothesis, which states that an imbalance between the production and clearance of Aβ from the brain through the blood brain barrier (BBB) is the initiating event, ultimately leading to accumulation of Aβ in the brain. Regarding this imbalance between production and clearance, several studies suggest that Aβ accumulation at individuals with AD is probably not due by increased production, but rather by deficient clearance from the brain (Zlokovic et al 2000) (Selkoe 2001) (Tanzi et al 2004) (Holzman and Zlokovic 2007). Gradual accumulation of aggregated Aβ initiates a complex, multistep cascade that includes gliosis, inflammatory changes, neuritic/synaptic change, tangles and transmitter loss. There have been several observations in literature that support Aβ as the initiating factor in AD (Blessed et al 1968) (Hardy and Selkoe 2002).

2.4 Genetics of Alzheimer's Disease

Several studies have been published that have performed research about the genetic factors involved in AD. To date genetic studies have revealed four genes that may be linked to FAD. These four genes include: amyloid precursor protein (APP), apolipoprotein E (ApoE), presenilin 1 (PS1) and presenilin 2 (PS2) (Parihar and Hemnari 2004). Missense mutations of the APP gene on chromosome 21 are described to be implicated in the pathophysiology of FAD. These mutations are considered to be located within or very next to the Aβ region of β-APP. It consists of a double mutation: Lys to Asn at residue 595 plus Met to Leu at residue 596, although the Met to Leu at position 596 is more responsible for the found 6-8 fold increase in Aβ expression in comparison to wild type cells (Citron et al 1992). The ApoE gene on chromosome 19 is considered to be a missense mutation. ApoE exists in three

allelic variants; E2, E3 and E4, of which the E4 allele is associated with a fourfold higher prevalence of AD, an earlier onset of AD and more Aβ deposition than the other alleles (Marques and Crutcher 2003). However, the majority of FAD cases are caused by mutations within the PS genes. The presenilin 1 and presenilin 2 genes are both described to be critical for Aβ production out of APP (Wolfe et al 1999), seeming to function as essential cofactors for γ-secretase, or are itself γ-secretases. More than 40 mutations have been described in the gene for PS1 that can subsequently result in FAD (Citron 2000). Besides contributing to a better understanding of the aetiology of AD, insights in the genetics of this disorder can provide researchers with a better view towards new research possibilities in the search for a drug against AD.

3. Materials and Methods

A search for Alzheimer's Disease related literature was conducted using internet and studybooks. Academic studybooks were available from my own collection, the university library but also in the subsection "books" of the PubMed website. A search for scientific literature was primarily conducted on PubMed using combinations of the following keywords: alzheimer, alzheimer's disease, AD, amyloid, amyloid β, tau, tau protein, drugs, acetylcholinesterase inhibitors, N-methyl-D-aspartate receptor antagonists, RAGE, LRP, APOE, flavonoids, biomarkers, HTS, compound library, in silico, in vitro, in vivo, clinical trial, patent application, IND (Investigational New Drug Application), NDA (New Drug Application), tacrine, rivastigmine, galantamine, donezepil, memantine. A search on the internet for current pipelines in AD drug discovery and treatment was conducted by a search on the internet by browsing through the different pharmaceutical companies' websites but also using the specific search engine alzforum.org, in which the discovery and developmental phases were possible as search criteria. Results from these searches were then incorporated into a new search within specific patent databases for the current status en claims: wipo.int, nl.espacenet.com and hspto.gov, but also clinicaltrials.gov and clinicalstudyresults.org. An information search within patent database nl.espacenet.com was conducted using keywords Alzheimer, amyloid as well as the combination Alzheimer and amyloid. The patents achieved were selected according to relevance, from which the matching ECLA numbers were incorporated into a new search.

4. Therapeutic Strategies

Until now, no cure is available. Treatment is only based on diminishing the neurological symptoms, no disease-modifying drug is available yet. Annually, much is spent on drugs against AD, while there are no beneficial effects on the neurological condition of the affected patient. Therefore, AD is an interesting focus for pharmaceutical companies. Until now, two

classes of drugs have been approved by the Food and Drug Administration (FDA) for treatment against AD: acetylcholinesterase (AChE) inhibitors and N-methyl-D-aspartate (NMDA) receptor antagonists. AChE inhibitors and NMDA antagonists will continue to form the backbone of symptomatic relief of dementia in Alzheimer disease for the near future. However, none of the currently approved drugs stops the underlying degeneration of brain cells or reverses the progression of Alzheimer disease. Therefore, research against AD stays in development. There are many targets for drugs against AD, the most promising drug would be a drug that can act as a disease modifier and delay or prevent progression to later stages. A drug that can act early in this disease, would be highly valuable (Mount and Downton 2006).

4.1 Acetylcholinesterase Inhibitors

Levels of the neurotransmitter acetylcholine are very low in patients with AD. AChE inhibitors reduce the rate at which acetylcholine is broken down, increasing the concentration of ACh in the brain and synapses, compensating for the loss of ACh caused by the death of cholinergic neurons. This most widely used strategy to compensate the low levels of ACh in the brain results in improvements of cognition, mood and behaviour (Mount and Downton 2006). ACh drugs currently approved by de Food and Drug Administration (FDA) and European Medicines Agency (EMEA) are tacrine, rivastigmine, galantamine and donezepil (Melnikova 2007).

Tacrine (brand name Cognex) is manufactured by Warner-Lambert Co. of Morris Plains; rivastigmine (brand name Exelon) is manufactured by Novartis Pharmaceuticals; galantamine (brand name Razadyne) is manufacturedby Ortho-McNeil Neurologics; donepezil (brand name Aricept) is manufactured by Eisai Inc. and co-promoted with Pfizer Inc (www.FDA.gov).

4.2 N-methyl-D-aspartate Receptor Antagonists

Excessive amounts of the excitatory neurotransmitter glutamate in the brain can lead to cell death through a process called excitotoxicity. Excitotoxicity occurs in part because of overactivation of N-methyl-d-aspartate (NMDA)-sensitive glutamate receptors, thought to be involved in preventing the death of certain cells in the brains of patients with AD (Lipton 2006). NMDA receptor antagonists regulate levels of glutamate in the brain by binding to this receptor (Mount and Downton). A NMDA drug currently approved by de FDA and EMEA is only memantine. Memantine (brand name Namenda) acts on the glutamatergic system by blocking NMDA receptors and inhibiting their overstimulation by glutamate, and is manufactured by Forest Pharmaceuticals, Inc. (www.FDA.gov) (Lipton 2006).

4.3 Amyloid β Modificators

These drugs are aimed at amyloid β. Targeting Aβ is considered the most promising therapeutic strategy for the future. Nonetheless, targets for treatment aimed at Aβ can be very divers; preventing Aβ production, blocking Aβ aggregation into plaques or lowering its soluble levels in the brain are examples of strategies to slow the progression of AD (Raffii and Aisen 2009). A strategy to prevent Aβ production would be to develop small compounds that cross the blood-brain barrier and inhibit Aβ production by decreasing the activity of β- and γ- secretase, the enzymes involved in proteolytic cleavage of Aβ. This is difficult, because the enzymes are also involved in Notch processing. Therefore, inhibitors must be used that specifically interfere only with the Aβ production and not with other substrates (Schroeter et al 1998) (Wolfe 2008). A strategy in blocking Aβ aggregation would be to use small molecules to bind Aβ monomers and prevent their assembly into potentially cytotoxic oligomers. However, this anti-oligomerization strategy is still under observation, because prevention of Aβ assembly would theoretically lead to accumulation of intermediate compounds, enhancing the disease course (Selkoe 2001b). A strategy to lower the soluble Aβ levels in the brain offers an array of therapeutic strategies: increasing the clearance of Aβ from the brain by either receptor mediated export using LRP and RAGE- multiligand cell surface receptors that mediate clearance by directly transporting cerebral Aβ across the BBB into the plasma, or by peptidolytic and proteolytic degradation of cerebral Aβ using IDE and NEP- two major Aβ degrading peptidases (Tanzi et al 2004) (Deane et al 2009) (Zlokovic 2008b). Lowering the soluble levels of Aβ levels in the brain could also be performed by immunotherapy acting at Aβ in which molecules that bind Aβ peptide in the blood could draw the peptide from the brain through the blood-brain barrier, possibly by a receptor-mediated process. These molecules are thought to trap Aβ peptide in the blood and so lower the soluble Aβ level in the brain, reducing the change of brain Aβ accumulation (Raffii and Aisen 2009).

Although therapies based on these strategies are mainly in development, other strategies are also being developed, of which preliminary results are also promising. E.g.: neuroprotection studies: mechanisms that protect neurons from degeneration using antioxidants and anti-inflammatory treatments (Raffii and Aisen 2009) or targeting serotonin receptors is investigated as a use for symptom-treatment in AD because a decline in serotonin function is associated with cognitive decline in AD (Schechter et al 2005).

4.4 Tau Modificators

In addition to senile plaques evolved from Aβ accumulation, neurofibrillary tangles derived from hyperphosphorylation and aberrant aggregation of the tau protein are characteristic hallmarks of brain pathology in AD. Few therapeutic programs have aimed at reducing tau

phosphorylation and/ or aggregation (Raffii and Aisen 2009), but a truly effective disease-modifying therapy will have to reduce both amyloid and tau-related pathology.
Because tau phosphorylation is regulated by the balance between multiple kinases and phosphates (Iqbal et al 2005), a strategy based on a single kinase/ phosphatase will not be sufficient. Therapeutic strategies are being developed using activation of degradation pathways or anti-tau immunotherapy. Even alternative strategies are being investigated such as stabilizing microtubule networks, which cannot bind hyperphosphorylated tau, or modulating the splicing machinery to decrease levels of four-repeat tau, which is more prone to aggregation (Tanzi 2008).

4.5 Multiligand Receptors

As mentioned in the paragraph for Aβ modificators, RAGE and LRP can possibly be used to lower the soluble Aβ levels by enhancing the clearance from the brain.

4.5.1 RAGE

The receptor for advanced glycation end products (RAGE) is normally expressed at low levels in the brain (Zlokovic 2008). In AD, RAGE expression is increased (Yan et al 1996, Giri et al 2000, Deane et al 2003, LaRue et al 2004, Donahue et al 2006). RAGE binds different isoforms of Aβ, making it an important therapeutic target. The interaction between RAGE and different forms of Aβ mediates re-entry of circulating Aβ into the brain across the BBB, followed by Aβ binding to neurons. RAGE–Aβ interaction on neurons can kill neurons directly by producing oxidative damage. Aβ- RAGE interaction also induces activation of endothelium with expression of proinflammatory cytokines and adhesion molecules and secretion of endothelin-1 resulting in reductions of the cerebrospinal fluid (Zlokovic 2008). Inhibition of a RAGE–Aβ interaction in the affected blood vessels blocks Aβ influx across the BBB and the associated oxidant stress and neuroinflammation (Deane et al 2003), making it an interesting target (Tanzi et al 2004).

4.5.2 LRP

Aβ peptides not only exist in the form of monomers and oligomers, but also in the form of complexes with Aβ-binding molecules, such as the low density lipoprotein receptor related protein 1 (LRP-1) ligand. LRP, a member of the LDL receptor family, is a major clearance receptor for Aβ at the BBB (Shibata et al 2000). LRP is involved in AD pathogenesis by altering the catabolism of LRP ligands and/or influencing Aβ metabolism and accumulation (Bu et al 2006). Such LRP ligands can be: activated α2-macroglobulin (α2M), apolipoprotein E2 (apoE2), apolipoprotein E3 (apoE3) and apolipoprotein E4 (apoE4). These ligands are described to act as carriers, facilitating Aβ uptake by neurons in vitro (Qiu et al. 1999) (Gylys

et al. 2003). For example ApoE2 and ApoE3 are described to bind well to Aβ and therefore have a high clearance. However, ApoE4 binds less and therefore has a less clearance (Ito et al 2007). Aβ binding to LRP is the first step in Aβ clearance from brain mediated by transvascular Aβ transport across the BBB (Deane et al 2004, Shibata et al 2000, Cirrito et al 2005, Bell et al 2007). In AD, reduced expression of LRP was found, together with the presence of $A\beta_{40}$ and $A\beta_{42}$ in cerebral vessels (Deane et al 2004) (Donahue et al 2006) (Shibata et al 2000). Soluble LRP binds 70-90% of the Aβ in human plasma (Sagare et al 2007). In AD, these soluble LRP levels are decreased, leading to higher levels of brain Aβ.

LRP also mediates Aβ systemic clearance from the liver (Tamaki et al 2006). Thus, LRP fragments may have therapeutic potential as novel Aβ clearance agents or in soluble LRP replacement therapy for AD (Zlokovic 2008).

4.6 Other Interesting Therapeutic Targets

The therapeutic strategies revised here are the main focus of drug development, although several other strategies also have interesting objectives.

Reactive oxygen species (ROS) caused by oxidative stress is described to be associated with neurodegenerative diseases such as AD. Several authors suggest that ROS are responsible for the dysfunction or death of neuronal cells that contributes to AD disease pathogenesis (Barnham et al 2004) (Lipton et al 2007). In that view, antioxidants such as vitamin E are often recommended in combination with AChE inhibitors, although it is controversial for use in patients with cardiovascular diseases (Mount and Downton 2006).

Maintaining normal calcium homeostasis is necessary for proper cell functioning within an array of different processes (Carafoli 1988). The β-sheet-containing fibrils and protofibrils formed by Aβ42 in patients with AD exert their cytotoxic effect by promoting a deleterious influx of external Ca^{2+} ions and thereby disturbing cytosolic Ca^{2+} ion homeostasis, which can impede with Ca^{2+} function as an ubiquitous cytosolic messenger. This immediate Ca^{2+} influx renders the activation of Ca-permeant α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors in certain neurons leading to cell dysfunction and cell death. This influx can be completely blocked by its specific antagonist 2,3-dihydroxy-6-nitro-7-sulfamoyl-benzo [f]quinoxaline-2,3-dione (NBQX), offering another perspective towards future interesting therapeutic strategies (Blanchard et al 2004).

The histochemical dye Congo Red was primarily used as an amyloid fibril-binding dye. Lorenzo and Yankner found that the neurotoxicity of Aβ is mediated by the amyloid fibril, and that Congo red binding to these fibrils renders them nontoxic. These results suggest that Congo red is a general inhibitor of amyloid fibril toxicity, which offers a potential new therapeutic approach to AD (Lorenzo and Yankner 1994) (Frid et al 2006).

Flavonoids are compounds occurring naturally in food, which have been described to contain anti-inflammatory, anti-allergic, anti-mutagenic, anti-viral, anti-neoplastic, anti-thrombotic, and vasodilatory properties (Hendriks et al 2004). Flavonoids have been proposed to exert their biological activity as free radical scavengers, although much more evidence begins to emerge that they act on signalling mechanisms (Williams et al 2004). Several researchers have made observations that different polyphenols inhibit Aβ aggregation (Conte et al 2003) (Lashuel et al 2002) (Ono et al 2003) (Ono et al 2004) (Yang et al 2005). Proposed working mechanism of flavonoids are: their capacity to act as antioxidants, the direct scavenging of free radicals, a reduction in ischemia-reperfusion injury by interfering with inducible nitric-oxide synthase activity, the xanthine oxidase pathway after ischemia-reperfusion damage, the immobilization and firm adhesion of leukocytes to the endothelial wall leading to the formation of oxygen-derived free radicals, or through interaction with various enzyme systems (Nijveldt et al 2009). Considering these many mechanisms of action, flavonoids are also an interesting therapeutic target in the search for a drug against AD. Flavonoids can be subdivides into glycosides, flavonoids connected to one or more sugar molecules and flavanols, flavonoids that are not connected to sugar molecules. Plants and most foods that we consume consists of glycosides. After consumption most flavonoids or flavonoid metabolites reach the intestines intact where they will be metabolized by enzymes for absorption. A person's flavonoid absorption in the intestines may vary and depends on a person's microflora present in the colon. In general, the bioavailability of flavonoids is relatively low due to limited absorption and rapid elimination. Since flavonoids are rapidly and extensively metabolized, the low biological availability of flavonoids (metabolites) is not always useful for therapeutic interventions (Linus Pauling Institute).

Evidence of a role for the apolipoprotein- E (ApoE) epsilon 4 allele in relation to AD is widespread found in literature. ApoE is described to play critical roles in regulating brain Aβ peptide levels, as well as the deposition and clearance (Holtzman 2001) (Zlokovic et al 2005). Thus, processes that regulate ApoE expression and functional state could affect its ability to influence brain Aβ homeostasis. Several researchers report of a significant association between APO-E and facilitated proteolytic clearance of soluble Aβ from the brain (Jiang et al 2008), offering another interesting target against AD (Patterson et al 2008).

Thus, several targets are possible in the pursuit for a disease modifying drug (DMD) against AD. Crossbeta Biosciences is participating in this query by performing it's own quest.

5. Drug Development by Crossbeta Biosciences/ Outline of Thesis

At the time of writing this master thesis, on the road to drug discovery against Alzheimer's disease there is a traffic jam. As was stated earlier, AD is already a major health care topic and it will only become more prominent as the world population ages. There still is a need for

a disease-modifying drug against AD. For this development, there has to be an analysis performed of the current track that pharmaceutical companies and institutions follow in selecting such a drug, which leads to the outline of this master thesis:

“Can the developmental stages commonly used by pharmaceutical companies or institutions be mapped in the development of a small compound drug against Alzheimer’s Disease?”

In this master thesis, it is tried to survey the consensus in this field, enlighting all aspects from compound library to drug.
Crossbeta Biosciences is an active player in this field in trying to develop small compound drugs against AD. Crossbeta Biosciences (CBB) aims at protein misfoldings, especially crossbeta structures, hence the name Crossbeta Biosciences. The aim of CBB at misfolded proteins offers a wide range of diseases for drug discovery. All proteins eventually lose their unique shape and become misfolded, which can then results in the formation of disease-associated fibrils, enriched in crossbeta structures which are called amyloid (Herczenik and Gebbink 2008). These misfolded proteins can contribute to the progression of a disease, for example AD. Studies on amyloid fibrils in AD indicated the presence of this β structure in the fibrils, oriented so that the β strands are perpendicular to the fibre axis, the so called ‘cross-*β*’ structure (Blake and Serpell 1996) (Pauling and Corey 1951). CBB especially aims at these crossbeta structures, in order to develop selective therapeutics. CBB has defined several druggable targets in the development of a new drug: e.g. human amyloid-beta peptide 1-42 (inhibition of cytotoxic effects of amyloid, prevention/reversion of amyloid formation), alpha-synuclein, тau protein and inhibition of inflammatory activity (Crossbeta, small compounds and drug discovery program).

5.1 Report of Progression Beyond CBB

Not only CBB is in the search for a drug against AD, also universities and pharmaceutical companies are eager to develop a drug against AD. About 250 compounds are in the discovery/ developmental phase within pharmaceutical companies.
This search begins with target formulation. Concerning AD, many targets are possible, e.g.: amyloid β production inhibitors, amyloid aggregation inhibitors, fibril formation inhibitors, Aβ ligands, or other therapies such as combination therapy or delaying progression of AD.
Modern therapeutic interventions are mostly amyloid-associated, probably because the amyloid hypothesis is strengthened by literature (Kola and Landis 2004) (Roberson and Mucke 2006). A grasp of AD-related target findings *as claimed in patents* are given in the overview of table 1. In table 2, a selection is shown of AD related target findings as published *in literature*, now used for the search towards the development of a disease modifying drug

against Alzheimer's Disease. In table 3, a selection is shown of AD related target findings as now tested *in clinical studies*, used for the search towards the development of a disease modifying drug against Alzheimer's Disease. All tables are listed in the appendix.

5.2 Compound Libraries

Irrespective on which strategy the search for a drug against AD is based, a clear target must be stated. From collecting the data for tables 1, 2 and 3, it is clear that there are several ways to go into the search and development for a disease modifying drug against AD.

Computer-aided drug design (CADD) and structure-based drug design (SBDD) have become essential tools on the road for drug discovery. Usually, design of a High-throughput screening (HTS) assay of a small compound library follows (Ma et al 2002) (Han et al 2008) (Haugabook et al 2001). The design of an HTS is dependent on the mentioned strategy, e.g. focussed on metal chelation, amyloid deposition, genetic factors, serotonin receptors, etc.

Compound libraries are the hart of screening. Efforts to synthesize, collect, and characterize compounds for a ready-to-screen library are an essential and costly part of drug discovery, because they have to fit selection criteria previously stated, matching the strategy. There are several sources for compounds:

– Natural products (NPs) from microbes, plants, or animals. NPs are usually tested as crude extracts first, followed by isolation and identification of active compounds.

– (Random) collections of discretely synthesized compounds.

– Focused libraries around certain pharmacophores.

– Random libraries exploring "chemical space."

– Combinatorial libraries (Tutorial drug discovery- by Jens Eckstein)

CBB has focused on a TimTec natural compound library, with 290 compounds previously selected by crossbeta binder ELISAs. As a control, known fluorescent crossbeta probes were used, e.g. Congo red, ThT, ThS, K114 and Chrysamine G. Once a library of potential cadidates has been established, a HTS of the small compound library follows.

Several companies offer commercial libraries with a specific set of compound collections between a range of e.g. 0-100k or 100-300k or 300-500k structure range.

The HTS is aimed to rapidly assess the activity of a large number of compounds or extracts on a given target. The term HTS is used when assays are run in a parallel fashion using multi-well assay plates (96-, 384-, 1536-well). The design of an HTS includes e.g. incubation times, temperature, buffers, pH, concentrations, excipients, etc. While the actual assay is in most cases run in a few days, the design may take up to several weeks. But this fine-tuning of the HTS assay before the implementation is necessary to achieve sufficient speed, efficacy and save costs. After successful tuning of an assay, screening follows. Output of an HTS screening will point to areas of success and failure in terms of identifying hits and leads

respectively (Chen et al 2007). After such a primary screens, subsequent confirmation screens and counter screens will identify leads out of the pool of hits. This winnowing process is commonly referred to as "hits-to-leads." This process is followed by lead optimization which is the complex, non-linear process of refining the chemical structure of a confirmed hit to improve its drug characteristics with the goal of producing a preclinical drug candidate. This stage frequently represents the bottleneck of a drug discovery program. Animal pharmacokinetics (PK), pharmacodynamics (PD), and absorption, distribution, metabolism, and excretion (ADME) assess the general pharmacology and mechanisms of action of drugs. This is a good example why flavonoids are not useful and thus not on the market for disease treatment purposes; considering their previously discussed rapid ADME dynamics. PK/PD/ADME studies are an integral part of lead optimization. The lead optimization process continues for as long as it takes to achieve a defined drug profile that warrants validation of the new drug in vitro using a cell-based bioassay.

5.3 In Vitro Based Selection Assays

After having potential candidates from the HTS screen, drug candidates must be tested in an in vitro assay setting. Some in vitro assays that will be discussed here are given in table 4.

A protein involved in AD related pathology is the tau protein, so an option for an in vitro based selection assay is a tau aggregation assay. Prior to the assay, an HTS screen had to be performed which screens for both inhibition of tau aggregation and for induced disassembly of tau aggregates. The in vitro assay should then be performed in the presence of thioflavin S and a tau construct. This tau aggregation assay is well described by Bulic et al (Bulic et al 2009). As discussed previously, there are now four FDA-approved AChE inhibitory drugs against AD available against one NMDA drug. Therefore, ACh offers an opportunity in which there is a higher probability of finding a disease modifying drug against AD. An in vitro assay which is aimed at AChE inhibitors is well described by Ellman et al (Ellman et al 1961). Following the protocol as described by the authors, the AChE inhibition can be determined for each potential drug compound as achieved from the previous HTS round. Also, to determine the amyloid-beta$_{(1–42)}$ aggregation inhibition, a thioflavin T (ThT) assay can be performed in comparison with Congo-red, tacrine and donepezil as reference compounds (Levine 3rd 1993). Another possibility for an in vitro assay is to screen potential compounds that interfere with amyloid precursor APP processing. Such an assay is

described by Neurodetective International. In this assay, NT2N cells are used because these cells differentiate into neurons and process APP in a similar way as human cells do. A supplemental assay thereby that is used for detection of neurotrophic as well as neuroprotective effects is an Aβ-induced neurotoxicity assay using mesencephalic neuronal cells and MAP2 (neuron-specific) quantification (Neurodetective International 2009). Another in vitro assay is described by Girigoswami et al. The researchers developed a 'surface-based' lawn in vitro system which can be applied for the high-throughput analysis of amyloid toxicity and drug screening. This offers benefits, because this assay can be used in an high throughput setting, which considerably saves time and effort because it creates outcomes in a shorter timeframe (Girigoswami et al 2008). Another good in vitro assay to identify compounds in establishing a drug for patients with AD, is an assay claimed by Memory Pharmaceuticals Corporation (US) in Patent Application # WO/2003/091694. In this patent application, an assay is described in which an electrical stimulation protocol is used to generate an electrophysiological output that lasts for several hours. This method aims to identify compounds, which can be used for enhancing long term potentiation (LTP) in animal neuronal tissue. By identifying such compounds, it is tried to enhance cognitive performance and memory and ultimately for developing pharmacological treatments for learning or memory impairments in e.g. aged humans with pathological memory impairments such as AD. A specific protocol for the assay can be consulted in the patent application. A last in vitro tissue culture-based assay is claimed by California Biotechnology Inc (US). It comprises of the claim for an amyloid deposition assay, specific for Alzheimer's disease which is also suitable for routine drug screening analysis. Immunological diagnostic reagents for Alzheimer's disease are also provided in this invention. The specific protocol of the assay can be consulted in the patent application.

Aimed at	**assay**	**Source**
Tau aggregation	Tau Aggregation Inhibition assay	Bulic et al 2009
enhancing cognitive performance and memory as well as pharmacological treatments for AD	Method for assay of cognition and memory based on low frequency stimulation	Patent Application #WO/2003/091694.
Amyloid deposition	in vitro tissue culture-based assay for amyloid deposition specific for Alzheimer's disease	Patent Application # WO/1991/004339
AChE inhibitors	AChE inhibition assay in vitro	Ellman et al 1961.
Inhibition of Aβ1–42 peptide aggregation	Thioflavin T-based fluorometric assay	Levine 3rd1993
APP processing	Aβ-induced neurotoxicity (NT2N cells)	Neurodetective International 2009
Neurotrophic + neuroprotective effects	Aβ-induced neurotoxicity (mesencephalic cells)	Neurodetective International 2009
'surface-based' lawn in vitro system	Amyloid toxicity	Girigoswami et al 2008

Table 4. Some in vitro based selection assays. Shown are the aim of the assay, the subject of the assay itself and the source where to find specific protocols of the assays.

The cell based selection assays mentioned here are just a handful of possibilities to demonstrate the available options. In order to determine the best suitable assay to be used by an institution or pharmaceutical company, it depends on several factors. First, it depends on the line of approach used. Is the aim for example at the tau protein, at the amyloid protein, aimed at neurotransmitters or aimed at other receptors. Second, it depends on the subsequent outcome from HTS screens to determine what the new objective and the next step will be. In literature, a wide array of possibilities can be found for a suited follow-up assays. Dependent on the outcome of an HTS screen, the name of remaining possible compounds can be incorporated in the search term into a literature search to find the most suitable assay.

These selected in vitro assays mentioned are a logical follow up after the HTS techniques and can lead to potential AD modifying drug compounds that can enter a subsequent round of testing in a cell based selection assay.

5.4 Cell Based selection assays

First, before we can test compounds from the in vitro assay into in vivo assays, we have to test for cytotoxic effects in cell-based selection assays.

Cytotoxic effects of the potential AD drugs can be screened in a human cell line, for example as described in the assay by Pollack et al (Pollack et al 1995). In the article, the authors suggest a method to study the cytotoxic effects as well as neuroprotective effects of a certain compound on a human cell line. A detailed protocol can be found in the referred literature. GE Healthcare (GB) claims in patent application # WO/2009/090215 to offer methods of multiplex cell-based assays for compound screening, useful in

Aimed at	assay	source
Cytotoxic effects	Neuroprotective effects on a human cell line	Pollack et al 1995
Information about cellular toxicity of potential drug candidates	Multiplex cell signalling assay	patent application # WO/2009/090215
screening and testing of AD-modulating compounds and therapeutic agents	cell culture assay system in which cells, expressing human recombinant tau with Aβ causes the formation of Alzheimer-like paired helical filaments upon cell-cell contact	patent application # WO/2004/017072
identifying compounds that enhance memory in normal and memory impaired individuals.	Neurite outgrowth as an assay for memory enhancing compounds	patent application # WO/2009/086532
identifying agents that modulate phosphoinositide levels and thereby treat a variety of diseases	differentiated stem cell-based assay systems that may be used to identify agents that modulate phosphoinositide levels and thereby treat a variety of diseases.	patent application # WO/2006/118630

Table 5. Some cell- based selection assays. Shown are the aim of the assay, the subject of the assay itself and the source where to find specific protocols of the assays.

obtaining high content information relating to cellular toxicity of potential drug candidates.

As discussed earlier in the section of the in vitro based selection assays, the formation of neurofibrillary tangles is associated with neurodegenerative diseases and thus forms an interesting drug screening subject. The University of Zürich claims in patent application # WO/2004/017072 a cell culture assay system useful for the screening and testing of modulating agents of neurodegenerative diseases associated with the formation of neurofibrillary tangles, in particular AD. The claim is based on their finding that cell-cell contact of the cells growing in culture that express human recombinant tau with b-amyloid causes the formation of Alzheimer-like paired helical filaments in these cells. Another interesting cell-based selection assay is mentioned in the patent application # WO/2009/086532 from Helicon Therapeutics. The claim relates to neurite outgrowth as a cell based screening assays that is useful to identify compounds that enhance memory in normal and memory impaired individuals. A final interesting cell-based selection assay is the differentiated stem cell-based assay system, claimed by the trustees of Colombia University in the city of New York in the patent application with # patent application nr WO/2006/118630. The applicants hereby claim an assay system to identify agents that modulate phosphoinositide levels and thereby identifying genes associated with AD. It is based on the discovery that edelfosine, an agent that increases PIP2 levels by inhibiting an enzyme that catalyzes PIP2 breakdown, decreases levels of neurotoxic $A\beta_{42}$ peptide, particularly in cells expressing a mutant presenilin gene associated with Familial Alzheimer' s Disease.

These found selection of cell-based selection assays merely have a common objective, that is to identify cytotoxic effects as well as identifying therapeutic possibilities of a tested compound.

Concluding from this selection with common objectives it is clear that the specific assay in this cell-based selection stadium will not be decisive for the further outcome in the search for a disease modifying drug against AD. Thus these cell-based selection assays *are* a necessary step in the further refinement of a potential drug compound against AD, although the specific assay may be variable. Found compounds that could withstand all testfases until now are thus suited for final testing in in vivo assays.

5.5 In Vivo Assays

When in vitro assays and cell based selection assays are successfully completed, in vivo assays follow. In vivo assays are comprised of murine assays, other animal assays and human clinical studies.

Tanzi (Tanzi 1995) offers a promising animal model of AD. The article describes a transgenic mouse; the Athena mouse strain, termed PDAPP (platelet-derived growth factor promoter

expressing amyloid precursor protein). This transgenic mouse has pathological features also seen in AD: amyloid plaques, dystrophic neurites, activated glia and loss of synapses in the hippocampus and frontal cortex, two regions of the brain that are particularly affected in Alzheimer's disease. Price et al (Price et al 1998) reviews some transgenic murine models which show several neuropathological features of AD, like Aβ deposits and neurofibrillary tangles. The Tg mice that Price et al review are the amyloid precursor protein transgenic mouse line, the APP V717F and/or APPSWE transgenic mouse line, and the amyloid precursor protein and presenilin 1 gene-targeted mouse line. The generation of these murine models are extensively reviewed by the authors, therefore it is not necessary to review them here. Morgan et al (Morgan et al 2000) also used a transgenic mouse model for AD in the year 2000. The authors bred Tg 2576 APP transgenic mice16 with PS1 line 5.1 transgenic mice17, resulting in nontransgenic, APP, APP+PS1 and PS1 transgenic mice as described previously by Holcomb et al (Holcomb et al 1998). Furthermore, Yan et al (Yan et al 2003) used the APP transgenic Tg2576 mice at 11 months old as an animal model for AD. In the patent application # WO/2003/017918, the University of Zürich claims to have invented a useful in vivo assay system for the identification and testing of modulating agents for use against AD. Finally, the most recent literature is the review article from Kokjohn et al (Kokjohn et al 2009). The authors review Tg mice as a model for FAD that are based on the transfection of mutant APP, PS and tau genes, alone or in combination. However, these Tg mice only reproduce some of the pathological changes seen in FAD. A major advantage in using these murine in vivo assays is that these Tg mice models have clearly showed that although Aβ plays a central pathologic role in AD, these Tg mice affirm that AD entails more than alterations in APP/Aβ overproduction, clearance, and deposition. Therefore it still stays difficult to extrapolate such data to the not equivalent human.

These data stress the urgence of human clinical trials, of which there are several. Treatment trials test experimental treatments, new combinations of drugs, or new approaches to surgery or radiation therapy. Prevention trials look for better ways to prevent disease in people who have never had the disease or to prevent a disease from returning. These approaches may include medicines, vaccines, vitamins, minerals, or lifestyle changes. Diagnostic trials are conducted to find better tests or procedures for diagnosing a particular disease or condition. Screening trials test the best way to detect certain diseases or health conditions. Quality of Life trials (or Supportive Care trials) explore ways to improve comfort and the quality of life for individuals with a chronic illness.

Clinical trials are conducted in phases. The trials at each phase have a different purpose and help scientists answer different questions: In Phase I trials, researchers test an experimental drug or treatment in a small group of people (20-80) for the first time to evaluate its safety, determine a safe dosage range, and identify side effects. In Phase II trials, the experimental

study drug or treatment is given to a larger group of people (100-300) to see if it is effective and to further evaluate its safety. In Phase III trials, the experimental study drug or treatment is given to large groups of people (1,000-3,000) to confirm its effectiveness, monitor side effects, compare it to commonly used treatments, and collect information that will allow the experimental drug or treatment to be used safely. In Phase IV trials, post marketing studies delineate additional information including the drug's risks, benefits, and optimal use. At his time, about 250 clinical trials are running concerning a treatment against AD. Forty-four studies are in phase I, 69 studies are in phase II, 37 studies are in phase III and 32 studies are in phase IV (ClinicalTrial.gov).

5.6 End-points

In clinical studies, health outcomes caused by the tested disease are called endpoints. Endpoints of clinical trials must be accurately represented, which can be achieved through many and different validations. Usage of well defined endpoints is the most important decision to be made in clinical studies, because size and type of population to be targeted, the clinical relevance of the drug therapy, the rationale for its use in clinical practice, and cost-effectiveness all depend on the primary determined endpoints.

Vellas et al have defined the most important considerations to be made when deciding on endpoints in clinical trials (Vellas et al 2008). A clear and consensual definition of endpoints is crucial for the success of further clinical trials in the field and will allow comparison of data across studies. It is often the MMSE score that is used as a primary endpoint, although it is not suited for multiple testing as for comparison (Mount and Downton 2006). Another end-point used is ADCS-ADL. For clinical trials, end points used are Calculation of primary endpoint using ADAS-Cog , ADCS-ADL and CDRSB scores (Mount and Downton 2006)

5.7 Biomarkers

Although AD has a late onset age, the development of neurological decline occurs many years prior to visible clinical manifestations. Therefore, biomarkers that can help predict the risk for AD will be very helpful for early interventions from a therapeutic perspective. In the absence of completely specific biomarkers, the clinical diagnosis of AD can still be only probabilistic (Dubois et al 2007). Biomarkers that enable earlier diagnosis are now investigated to visualize amyloid pathology in living Alzheimer patients. Researchers described a newly discovered compound called Pittsburgh Compound-B (PIB) (Dubois et al 2007) (Blennow and Zetterberg 2006) used for in vivo imaging of pathology-specific proteins (Klunk et al 2004) (Shoghi- Jadid et al 2002). Distinctive markers of the disease are now recognised including structural brain changes visible on MRI with early and extensive involvement of the medial temporal lobe (MTL), molecular neuroimaging changes seen with

PET with hypometabolism or hypoperfusion in temporoparietal areas, and changes in cerebrospinal fluid biomarkers. Also, PET with fluorodeoxyglucose (FDG) is sensitive and specific in detecting AD in its early stages. (Dubois et al 2007). Cerebrospinal fluid biomarkers used amyloid β1–42 (Aβ42), total tau (t-tau), and phospho-tau (p-tau) (Hulstaert et al 1999), CSF examination is also recommended as an exclusion procedure for non-AD dementia, due to inflammatory disease, vasculitis, or demyelination (Dubois et al 2007). In AD, the concentration of Aβ42 in cerebrospinal fluid is low and that of t-tau is high compared with those in healthy controls. Concentrations of different phosphorylated tau epitopes may also be high. Measurement of the concentration of p-tau, notably p-tau 231, increases the specificity for AD. Combinations of abnormal markers (low Aβ42, high t-tau, high p-tau 181) reached a hazard ratio of 17 to 20 for predicting AD in a follow-up of 4–6 years. Recently, Song et al has published an overview of Biomarkers in different tissues (Song et al 2009)

6. Conclusion/ Advice for Future Research

Giving this overview, it is clear that Alzheimer's Disease is an established topic in the discovery and development of a disease-modifying drug. At this time, many different companies and institutions are on this same road towards therapeutic intervention.

With this Master Thesis, it is tried to map the developmental stages currently used by other institutions/ companies in the search for a drug against AD.

As is clear from the larger amount of scientific literature about the role of the amyloid peptide in the pathogenesis of AD, more researchers are focussed on targeting amyloid which offers a multitude of possibilities. However, AD offers more possibilities and opportunities for scientists and drug designers. Considering the complexity of the disease, this automatically generates an overwhelming amount of possibilities for drug discovery. If one looks clearly and critically at all knowledge an data gathered, a conclusion that can be drawn is that the most promising drug against Alzheimer's disease should and could be a multidrug therapy. It cannot take long before such a multidirectional medicine is discovered. If all developmental stages of AD drug discovery are properly conducted as denoted in this Master Thesis, it cannot take long before the development of hopefully THE disease modifying drug against Alzheimer's disease so we can live in a future without cognitive impairment among our population.

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* International patent application published under the patent cooperation theaty (PCT). Publication date 18 january 2007, publication number WO 2007/008072 A2.

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* indicates key literature provided by CBB

8. Appendices

btype	Compound/Method	Mode of action	Developed by	Status	Source
Amyloid β production inhibitors	(2R)-2-[[(4-chlorophenyl)sulfonyl][[2- fluoro-4-(l,2,4-oxadiazol-3-yl)phenyl]methyl]amino]-5,5,5-trifluoropentanamide	inhibition of Aβ production	Bristol-Myers Squibb	R&D	WO/2009/058552
	P25/CDK5 complex inhibitor	Inhibition of BACEl phosphorylation and reduction of secretion of β-amyloid and the hyperphosphorylation of Tau.	Inje University - Korea	unknown	WO/2006/075808
	a mono-amine neurotransmitter re-uptake inhibitor	inhibition of Aβ (Aβ40 and Aβ42)-generation	Neurosearch A/S (DK).	Pre-clinical, patent prolongation	WO/2005/117874
	use of pyridinyl-pyridinylamino-benzamide derivatives	used for treatment of amyloid related disorders and/or prevention of e.g. AD	Novartis (CH).	unknown	WO/2005/039586
	- indolyl- pyrroledione derivatives	Used for treatment of neurological and vascular disorders related to Aβ generation and/or aggregation	Novartis (CH).	Pre-clinical, patent prolongation	WO/2005/039549
	- a method of treating Aβ precursor disorder	a method of treating Aβ precursor disorder	Andrx Corporation (US).	withdrawn	WO/2002/062824
Amyloid aggregation inhibitors:	cyclohepta(B)pyridine compounds, as well as preparation method, usage and pharmaceutical composition	Amyloid aggregation inhibitor	Shanghai Institute of Materia Medica, Chinese academy of sciences	unknown	WO/2008/125008
	amino- heterocyclic compounds	Amyloid aggregation inhibitor	Pfizer Inc (US)	Examination requested	WO/2008/139293
	an Aβ protein aggregation inhibitor	Amyloid aggregation inhibitor	National University Corporation Nagoya University Japan	withdrawn	WO/2006/083019
	Prevention of Aβ deposition in the brain	a combination of GH secretagogues and PDE4 inhibitors	Merck Sharp en Dohme Limited (GB)	published	WO/2004/087157
Fibril formation inhibitors:	amorphine inhibitors of Aβfibril formation	and their use in amyloidosis based disease	Cytokine PharmaSciences, INC	withdrawn	WO/2003/053356
Aβ ligands:	A protein kinase C inhibitor that binds ß-amyloid and its peptide derivatives	A protein kinase C inhibitor that binds ß-amyloid and its peptide derivatives	the National Research Council Of Canada (CA).	granted	WO/2006/133566
	Polypeptide transportation across BBB	nerve growth factor (NGF) delivery into the brain	I.A. Ferguson	published	WO 2003/091387.

Combination therapy:	comprising of a cyclohexanehexol, especially a scyllo-inositol compound, and a secretase inhibitor, especially a beta-secretase inhibitor	employing different mechanisms to achieve maximum therapeutic efficacy	Waratah Pharmaceuticals Inc (CA).	published	WO/2008/061373
	an acetylcholinesterase inhibitorial therapy with an acetylcholinesterase inhibitor and (3AR)-1,3A,8-trimethyl-1,2,3,3A,8,8A-hexahydropyrrolo[2,3-B]indol-5-yl phenylcarbamate	treating, reducing or delaying cognitive impairments associated with Aβ +/- cholinomimetic replacement therapy or as a prophylactic treatment	Axonyx Inc (US).	published	WO/2005/079789
Delaying progression of AD:	a HMG CoA reductase inhibitor	for delaying progression of Alzheimer's Disease (phenserine.) Axonyx Inc (US).	Axonyx Inc (US).	withdrawn	WO/2005/123068

Table 1. Shown are a selection of AD related target findings claimed in patents, now used for the search towards the development of a disease modifying drug against Alzheimer's Disease. Shown are the subtype of the therapeutic strategy in which the compound or method can be subdivided, the compound or method, the mode of action of the compound or method, the responsible developer, the status (still in Research and Development, withdrawn, granted, published, examination requested, pre-clinical, clinical phase I, II, III, IV, marketed, stopped, unknown), and the source.

Subtype	Compound/Method	Mode of action	Developed by	Status	Source
Fibril formation inhibitors:	Congo Red	Fibril formation inhibitors	Lorenzo and Yankner 1994	published	Reference Lorenzo and Yankner 1994
	tetracycline	Fibril formation inhibitors	Lorenzo and Yankner 1994	published	Reference Lorenzo and Yankner 1994
	thioflavin T	Fibril formation inhibitors	Lorenzo and Yankner 1994	published	Reference Lorenzo and Yankner 1994
	ANFLVH	completely inhibit the amyloidogenic folding of synthetic human IAPP	Potter et al 2009	published	Reference Potter et al 2009
	Phenolsulfonphthalein	Fibril formation inhibitors	Levy et al 2008	published	Reference Levy et al 2008
	furansulfonic acid derivatives, small polyphenol molecules, benzofuran derivatives	Fibril formation inhibitors	Dolphin et al 2008	published	Reference Dolphin et al 2008
	NDGA	Decrease in fluorescence of ThT associated with new Aβ fibril extensions	Ono et al 2003	published	Reference Ono et al 2003
Amyloid aggregation inhibitors:	curcumin and NDGA	Amyloid aggregation inhibitor	Dolphin et al 2008	published	Reference Dolphin et al 2008
Genetic	Apo-ε gene	Apo-ε gene offers protection against age-associated oxidative damage	Choi et al 2004	published	Reference Choi et al 2004
immunization	Immunization with ab's	Aβ accumulation inhibition	Schenk et al 1999	published	Reference Schenk et al 1999
Metal chelation	Chelation (i.e., sequestering and inactivation) of zinc and copper	Slows or even reverses growth of amyloid plaques	Bush 2002 Atwood et al 2004 Cherny et al 2001	published	References Bush 2002 Atwood et al 2004 Cherny et al 2001
Amyloid deposition	acyl-CoA:cholesterol acyltransferase (ACAT)	Modulates peripheral cholesterol	Seabrook et al 2007	published	Reference Seabrook et al 2007
	Nuclear receptor LXRβ	Stimulation of transcription genes involved in efflux from the brain	Seabrook et al 2007	Published	Reference Seabrook et al 2007
	LRP-APO-E	inhibition of the low-density lipoprotein receptor–related protein (LRP)–APOE interaction	Seabrook et al 2007	Published	Reference Seabrook et al 2007
	cytochrome P450-(CYP)-46 enzyme	cytochrome P450-(CYP)-46 eliminates brain cholesterol by	Seabrook et al 2007	published	Reference Seabrook et al 2007

		oxidation to 24-hydroxycholesterol which then diffuses out of the brain			

Table 2. Shown are a selection of AD related target findings as published in literature, now used for the search towards the development of a disease modifying drug against Alzheimer's Disease. Shown are the subtype of the therapeutic strategy in which the compound or method can be subdivided, the compound or method, the mode of action of the compound or method, the responsible author, the status of the published article and the source of reference.

Compound or method	Generic name	Mode of action	Developed by	status	source
Seroquel®	Quetiapine Fumarate	blocking of the dopamine type 2 (D2) and serotonin type 2 (5-HT2) receptors.	Astellas Pharma Inc	Clinical Phase II	ClinicalStudyResults.org ID# FJ-949C-AH02
Aricept	Donezepil Hydrochloride	AChE inhibitor	Eisai Medical Research	Clinical Phase II	ClinicalStudyResults.org ID# E2020-J081-231
Aricept	Donezepil Hydrochloride	AChE inhibitor	Eisai Medical Research + Pfizer Inc.	Clinical Phase IV	ClinicalStudyResults.org ID# E2020-A001-410
Aricept	Donezepil Hydrochloride	AChE inhibitor	Eisai Medical Research + Pfizer Inc.	Clinical Phase II	ClinicalStudyResults.org ID# E2020-J081-232
Celebrex	celecoxib	Blocking the enzyme for prostaglandins (cyclooxygenase 2)synthesis, resulting in lower conc. of prostaglandins	Pfizer Inc	Clinical Phase II	Not registered to ClinicalTrials.gov
Aricept	donepezil	AChE inhibitor	Pfizer Inc.	Clinical Phase III	ClinicalStudyResults.org ID# NCT00630851
Lipitor	atorvastatin	By lowering the level of cholesterol, lower the level of Aβ	Pfizer Inc.	Clinical Phase III	ClinicalStudyResults.org ID# NCT00151502
Strattera	atomoxetine hydrochloride	selective norepinephrine reuptake inhibitor	Eli Lilly and Company	Clinical Phase II	ClinicalStudyResults.org ID# 7951
avandia	rosiglitazone	Class of thiazolidinediones (TZDs) drugs, PPARγ ligand	GlaxoSmithKline	Clinical Phase II	ClinicalStudyResults.org ID# 100193
avandia	rosiglitazone	Class of thiazolidinediones (TZDs) drugs, PPARγ ligand	GlaxoSmithKline	Clinical Phase III	ClinicalStudyResults.org ID# 105640
Flurizan	MPC-7869, R-flurbiprofen	an R-isomer offlurbiprofen, a nonsteroidal anti-inflammatory drug (NSAID)	Myriad genetics	clinical phase III	Study results still awaited
Memryte	Leuprolide acetate	affects levels of luteinizing hormone, which modulate cognition and amyloid-β deposition.	Voyager Pharmaceuticals	Clinical phase III	completed, no study outcome published yet.

neramexane		NMDA receptor antagonist		clinical phase III	Failed due to not meeting its primary objectives of (SIB) and (ADCS-ADLsev).
TTP488	PF-04494700	RAGE inhibitor	Transtech Pharma/ Pfizer Inc	Clinical phase II	Not registered to ClinicalTrials.gov

Table 3. Shown are a selection of AD related target findings as now tested in clinical studies, used for the search towards the development of a disease modifying drug against Alzheimer's Disease. Shown are the subtype of the therapeutic strategy in which the compound or method can be subdivided, the compound or method, the mode of action of the compound or method, the responsible developing company/ institution, the status of the trial and the source.