TOPICS IN MEDICINAL CHEMISTRY

02

Volume Editors Lit-Fui Lau · Michael A. Brodney

Alzheimer's Disease

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Topics in Medicinal Chemistry

Alzheimer's Disease

Volume Editors: Lit-Fui Lau · Michael A. Brodney

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Volume Editors

Dr. Lit-Fui Lau

CNS Discovery
Pfizer Global Research & Development
Groton, CT 06340
USA
lit-fui.lau@pfizer.com

Dr. Michael A. Brodney

CNS Discovery
Pfizer Global Research & Development
Groton, CT 06340
USA
michael.a.brodney@pfizer.com

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Preface to the Series

Medicinal chemistry is both science and art. The science of medicinal chemistry offers mankind one of its best hopes for improving the quality of life. The art of medicinal chemistry continues to challenge its practitioners with the need for both intuition and experience to discover new drugs. Hence sharing the experience of drug discovery is uniquely beneficial to the field of medicinal chemistry.

The series *Topics in Medicinal Chemistry* is designed to help both novice and experienced medicinal chemists share insights from the drug discovery process. For the novice, the introductory chapter to each volume provides background and valuable perspective on a field of medicinal chemistry not available elsewhere. Succeeding chapters then provide examples of successful drug discovery efforts that describe the most up-to-date work from this field.

The editors have chosen topics from both important therapeutic areas and from work that advances the discipline of medicinal chemistry. For example, cancer, metabolic syndrome and Alzheimer's disease are fields in which academia and industry are heavily invested to discover new drugs because of their considerable unmet medical need. The editors have therefore prioritized covering new developments in medicinal chemistry in these fields. In addition, important advances in the discipline, such as fragment-based drug design and other aspects of new lead-seeking approaches, are also planned for early volumes in this series. Each volume thus offers a unique opportunity to capture the most up-to-date perspective in an area of medicinal chemistry.

Dr. Peter R. Bernstein
Prof. Dr. Armin Buschauer
Dr. John Lowe
Dr. Hans Ulrich Stilz

Preface to Volume 2

It was one hundred and one years ago that Alois Alzheimer presented at a scientific meeting a case of progressive dementia in a 51-year-old patient Auguste D. Postmortem analysis revealed two pathologies, namely, senile plaques and neurofibrillary tangles. These findings were published the following year in 1907. In 1910 Emil Kraepelin, Alzheimer's mentor, named this disease after its discoverer. The two initial pathological findings remain the postmortem diagnostic features of Alzheimer's disease (AD) today. At the time, however, Kraepelin made the distinction between AD and senile dementia (> 65 years old) despite their similarities in pathologies and clinical symptoms [1, 2]. In 1976 Robert Katzman argued in an editorial in the April issue of *Archives of Neurology* that this distinction be removed. AD has thus morphed from a rare orphan disease to one with a much bigger socioeconomic threat. This nosological shift has brought AD into the lime light and exponentially—and thankfully—hastened the pace of research. Enormous strides have been made in understanding the root causes and risk factors of the disease. Analogous to the discovery of new cancer treatments over the past 20 years (see Volume 1), advances in understanding the underlying molecular biology are providing novel drug targets for future research. These efforts have resulted in greater than 500 ongoing clinical trials focused on novel mechanisms and intervention points in the disease. These trials will hopefully lead to the first approval of a disease-modifying agent for AD and pave the way for an arsenal of new medications.

October, 2007, Groton Connecticut, USA

Lit-Fui Lau and Michael A. Brodney

1. Ballenger JF (2006) J Alzheimers Dis 9:5
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Therapeutic Approaches for the Treatment of Alzheimer's Disease: An Overview

Lit-Fui Lau (✉) · Michael A. Brodney (✉)

CNS Discovery, Pfizer Global Research and Development, Groton, CT 06340, USA
Lit-fui.lau@pfizer.com, Michael.a.brodney@pfizer.com

Abstract Alzheimer's disease (AD) is a neurodegenerative disease that robs the minds of our elderly population. Approximately one in every eight adults over the age of 65 and nearly half of those over 85 are afflicted with this disease. Aging and other risk factors (e.g. cardiovascular diseases, obesity and diabetes) in developed societies will impose an ever increasing socioeconomic threat in the future. Current medicines for AD patients are mainly symptomatic treatments and a huge unmet medical need exists to slow, stop or reverse the progression of this disease. A great deal of research has been dedicated to understanding the pathogenesis of AD from which come many ideas for intervening in its progression. They can be grossly categorized into those targeting the amyloid pathology, tau pathology, microgliosis (neuroinflammation) and functional deficits. Some of these ideas have been fast-tracked to clinical trials due to the availability of medicines with proven clinical efficacies for other diseases while others represent novel chemical entities. Our continued commitment in searching for efficacious treatments together with a healthier lifestyle will be important in fighting against the growing threat of this deteriorating disease.

Keywords Aβ · Alzheimer's disease · Amyloid · Microgliosis · Neurodegeneration · tau

Abbreviations

AD	Alzheimer's disease
apoE	apolipoprotein E
APP	amyloid precursor protein
Aβ	β-amyloid
BACE	β-site APP-cleaving enzyme
CCL2	chemokine (C–C) motif ligand-2
CCR2	chemokine (C–C) motif receptor-2
Cdk5	cyclin-dependent kinase 5
CK-1	casein kinase-1
ECE	endothelin converting enzyme
ERK	extracellular signal-regulated kinase
FDA	Food and Drug Administration
FTDP-17	frontotemporal dementia and parkinsonism linked to chromosome 17
GAB2	GRB associated binding protein 2
HTS	high throughput screening
IDE	insulin degrading enzyme
LRP	lipoprotein receptor-related protein
LTP	long term potentiation
MARK	Microtubule-affinity regulating kinase
MCI	mild cognitive impairment
NFT	neurofibrillary tangle
NMDA	*N*-methyl-D-aspartate
NSAIDs	non-steroidal anti-inflammatory drugs
PPAR-γ	Peroxisome proliferator-activated receptor-γ
PS1	presenilin-1
PS2	presenilin-2
RAGE	receptor for advanced glycation end products

1 Introduction

Since the first report describing Alzheimer's disease (AD) in 1906 by Alois Alzheimer, AD has become the most common form of dementia, accounting for 50–70% of all cases. According to a 2007 report from the Alzheimer's Association (http://www.alz.org/national/documents/Report_2007FactsAndFigures.pdf) there are currently 5.1 million people in the U.S. and over 30 million worldwide afflicted with the disease. Every one in eight people over the age of 65 and every nearly half of those over 85 have AD. With its aging population, the number of AD patients in the U.S. will climb to 16 million by 2050. Combination of direct and indirect cost in the treatment and care of AD patients is already a staggering $138 billion dollars each year. It is hoped that a better understanding of the symptoms, pathologies and etiology of AD can slow, stop, reverse or even prevent the disease. Even the slightest intervention could have an enormous impact. It has been estimated that by simply postponing the onset of AD five years can reduce the number of AD patients by 50 percent by 2050 [1].

2
AD Symptoms and Neutodegeneration

AD is a chronic progressive neurodegenerative disease. The progression of symptoms varies from patient to patient but can be roughly divided into three stages: mild, moderate and severe (Mayo Clinic, http://www.mayoclinic.com/health/alzheimers-stages/AZ00041). The progression of symptoms can be ascribed to the sequential and progressive loss of neuronal functions and synaptic connections, and neuronal cell death in different regions of the brain. In the mild stage AD is first manifested with loss of memory as neurons in the region for memory formation, the hippocampus, are first affected. Patients may forget words and names with increasing frequency and get lost even in familiar places. Some believe that these incipient cases of AD are equivalent to a clinical condition known as mild cognitive impairment (MCI). Not all MCI patients will convert to AD; a 36 month study shows that the conversion rate from amnestic MCI to AD is about 16% per year [2]. In the moderate stage cortical regions responsible for reasoning become affected and AD patients may begin to lose their logical thinking and experience confusion. They may need help putting on proper clothing appropriate for the season. They may have difficulty recognizing and identifying family members. Changes in personality may also occur, e.g., making accusations of theft and fidelity, cursing, and inappropriate kicking and screaming. In the severe stage additional brain regions are damaged resulting in loss of control of many normal physiological functions and responses to the external environment. AD patients are unable to take care of their daily living and lose their ability to speak coherently. They may need help with feeding, toilet use and walking. Once diagnosed, the median survival time is 4 to 6 years [3] although some individuals can live up to 20 years. The cause of death comes from deterioration of the brain's control of vital physiological functions resulting in deadly complications including pneumonia, urinary tract infections or a physical fall.

3
Pathological Features of AD

3.1
Amyloid Pathologies

Neurodegeneration is an important but not a unique characteristic feature of AD. What distinguishes AD from other neurodegenerative diseases is the presence of its telltale pathologies in the brains of these patients: amyloid plaques and neurofibrillary tangles (NFTs). Amyloid plaques consist of mainly extracellular β-amyloid (Aβ) peptide while neurofibrillary tangles are

mainly composed of intracellular hyperphosphorylated tau in the form of paired helical filaments. A great deal of research has been done to understand the relevance of these pathologies to AD. According to the amyloid cascade hypothesis [4, 5], Aβ is the main culprit triggering a whole cascade of events, including formation of NFTs, eventually leading to neurodegeneration and loss of brain function in AD patients. The key argument for this hypothesis is that familial mutations causing AD with a 100% penetrance are found on genes that all encode proteins involved in regulating Aβ levels. These include mutations on the amyloid precursor protein (APP, substrate for Aβ production), and presenilin 1 and 2 (components of the γ-secretase complex that cleaves APP to Aβ). AD pathologies are invariably detected in Downs syndrome which is caused by having an additional copy of chromosome 21 where the APP gene is located. The major genetic risk factor for AD, apoE4 [6], has also been found to affect amyloid plaque deposition in mice [7–9]. One of the main criticisms on the amyloid cascade hypothesis is the inability of high levels of Aβ in animal models to recapitulate the other key AD pathologies (e.g. NFTs and neuronal cell loss). This shortfall has been addressed by a number of studies showing that Aβ is able to augment formation of NFTs [10–12] and that removal of Aβ subsequently reduces an early tau pathological marker [13]. What is still missing is evidence that Aβ can induce, not simply augment, tau pathologies. Despite the lack of neuronal cell loss in APP transgenic mice, Aβ peptides have been shown to impair hippocampal long term potentiation (LTP) – a cellular process believed to be the basis of learning and memory – both in vitro and in vivo [14]. The amyloid cascade hypothesis has been the foundation of many drug discovery efforts towards a treatment for AD.

3.2 Tau Pathologies

Although believed to be downstream of Aβ, tau pathologies may play an important role in the deterioration of neuronal health in AD. NFTs have been classified into six stages (I–VI) by Braak and Braak [15]. The different stages describe the progression of tau pathologies from the transentorhinal region (I–II) to the limbic region (III–IV) and finally to the cerebral cortex (V–VI). During stages I–II the affected subject remains clinically silent. Stages III–IV pathologies are found in incipient AD when there is loss of cognitive functions and subtle personality changes. In the final stages of V–VI patients have fully developed AD. The sequential evolution of tau pathologies and its correlation with neurodegeneration and clinical manifestations suggests that tau pathologies, although not the ultimate trigger of AD, may play a prominent role in the demise of neurons. In fact, genetic mutations on tau have been shown to be sufficient to cause neurodegeneration in frontotemporal dementia and parkinsonism linked to chromosome 17 (FTDP-17) [16]. A recent

study showed that genetic polymorphism of GRB associated binding protein 2 (GAB2) was associated with a higher risk of developing AD [17]. In the same study interference of GAB2 expression was shown to increase the amount of hyperphosphorylated tau – a key component of the NFTs. Finally, cognitive deficits in APP transgenic animals [18] and toxic effects of Aβ [19] can be dramatically reduced by eliminating tau expression. In PS1 $\times$ APP $\times$ tau triple transgenic mice, reduction of Aβ alone by Aβ immunization is insufficient to rescue cognitive deficits but reduction of both amyloid and tau pathologies are necessary [13]. These studies suggest that tau plays an important role in the manifestation of Aβ-induced deficits.

There are at least two schools of thoughts on how tau pathologies may cause neurodegeneration: loss of essential functions and gain of toxic functions [20]. As mentioned earlier, NFTs consist mainly of hyperphosphorylated tau. Tau is a microtubule stabilizing protein. When tau is hyperphosphorylated, it dissociates from microtubules causing their disassembly (loss of essential functions). The disintegration of microtubules then leads to disruption of axonal transport, atrophy of distal neurites and eventually neuronal cell death. In addition to loss of microtubule stabilizing activity, hyperphosphorylated tau tends to aggregate and sequester additional normal tau from binding microtubules. The aggregated tau continues to form paired helical filaments and straight filaments approximately 10 and 15 nm in diameter, respectively. The former constitutes about 95% and the latter about 5% of the tau filaments found in AD brains. The gain of toxic functions school proposes that these abnormally aggregated tau species (not limited to NFTs) may be toxic to neurons. In fact, a number of transgenic animals overexpressing FTDP-17 tau have been able to recapitulate formation of tau filaments and NFTs. These animals, unlike APP transgenic mice, display clear signs of neuronal cell loss. A recent study in conditional transgenic mice overexpressing a mutant form of tau suggests that NFTs are not sufficient for causing neurodegeneration and cognitive deficits [21]. Nevertheless, tau phosphorylation appears to be responsible for the toxic functions of tau as elimination of all the proline-directed phosphorylation sites in tau drastically reduces toxicities [22]. The exact mechanisms by which tau overexpression induces degeneration and dysfunctions of neurons remains to be elucidated. Reduction of tau pathologies and/or tau toxicities is certainly an important area in the fight against AD.

3.3 Microgliosis

Surrounding the amyloid plaques in AD brains are clusters of reactive microglia, a phenomenon known as microgliosis. Microglia are immune cells of the brain derived from bone marrow. They are equivalent to macrophages in blood whose function is to cleanse by phagocytosis. Activation of microglia, however, could be a double-edged sword. On one hand, activated

microglia can release a variety of toxic substances detrimental to neurons, e.g., proinflammatory cytokines, reactive oxygen species, proteases and complements [23–27]. On the other hand, activated microglia may be one of the defense mechanisms to clean up amyloid plaque deposits. *In vitro* it has been shown that microglial cells can scavenge deposited amyloid [28–30]. Recent studies have provided strong evidence that microglia can remove amyloid plaques *in vivo* as well. In fact, it is the bone marrow-derived microglia – not resident microglia – that are critical in eliminating brain amyloid deposits [31]. Attraction of blood microglia cells to the brain depends on the microglial surface chemokine (C–C) motif receptor, CCR2, in response to its ligand, chemokine (C–C) motif ligand 2 (CCL2). Mice deficient in CCR2 crossed with APP transgenic mice display a dramatic reduction in the number of microglial cells in the brain, a concomitant elevation of amyloid plaques and increased mortality [32]. Interestingly, overexpression of the ligand CCL2 in APP transgenic mice appears to lead to inactivation or desensitization of microglia and exacerbates plaque deposition [33]. Finally, recent success in using Aβ immunotherapy to reduce amyloid plaque deposition in mice has been shown to depend at least in part on the activation of microglial cells [34].

4 Etiology and Environmental Risk Factors for AD

The etiology of AD remains largely elusive. A small percentage (5–10%) of all AD cases can be ascribed to genetic mutations and has an early onset age (< 65). The large majority (90–95%), however, is considered sporadic and has a late onset. Thus, aging is the biggest risk factor for AD. A myriad of environmental factors that increase with age may play an important role in AD. It is unclear if these factors can be converged to one common underlying mechanism or if they are independent events leading to a heterogeneous disease. Preclinical experiments exposing animals to some of the risk factors have shown an increase in AD pathologies. Understanding the causal relationship between these associations may not only provide clues to treat AD but also prevent it.

Environmental risk factors linked to AD include, but are not limited to, cardiovascular risk factors, metabolic and energy disorders and traumatic brain injury. Problems with the cardiovascular system can lead to reduced cerebral blood flow – an important feature of the AD brain. It is possible that a compromised delivery of nutrients and oxygen to the brain may in turn cause impairment of neuronal functions. Mid-life hypercholesterolemia has been shown to increase the risk of AD by about three fold [35, 36]. The cholesterol transport protein, apoE4, is the single most reliable genetic risk factor for late onset AD [6]. Usage of cholesterol lowering agents has been associated with a reduced incidence [37, 38] and progression [39] but the causal relationship between statin usage and AD risk has been debated [40–42]. How

mid-life hypercholesterolemia is linked to the development of AD a decade or more later is not completely understood. Preclinical studies have shown that cholesterol is an important component of lipid rafts where APP processing and production of Aβ peptides occur. Animals fed on high cholesterol diets develop more amyloid plaques [43] whereas statin treatment can reduce brain Aβ levels [44]. Hypertension is another cardiovascular factor known to elevate the risk of AD [45]. Hyperhomocysteinaemia, associated with an increased risk of cardiovascular diseases, is also linked to an increase in AD risk [46–50].

Type 2 diabetes has been associated with cognitive impairment and AD [51–55]. Since diabetes usually precedes AD, it is believed that conditions in diabetics are conducive to the development of AD but the mechanism(s) by which this occurs is/are poorly understood. First, diabetics are more likely to suffer from cardiovascular diseases that may enhance the occurrence of AD as discussed above. Second, type 2 diabetes is characterized by insulin resistance. The reduced ability of neurons to respond to insulin and metabolize glucose could lead to neuronal functional impairment [56]. Indeed, disruption of insulin signal transduction in rat brains by intracerebroventricular injection of streptozotocin has been shown to recapitulate many pathological features reminiscent of AD [57–59]. Third, insulin insensitivity can cause hyperinsulinemia as the body is trying to compensate. Insulin is brain permeable and degraded by the insulin degrading enzyme (IDE). It happens that IDE is one of the enzymes responsible for the clearance of Aβ peptides [60]. A high insulin level may reduce clearance of Aβ peptides by competing for IDE. Administration of insulin in peripheral circulation has been shown to increase Aβ peptides in the cerebrospinal fluid [61]. Accumulation of brain Aβ will, according to the amyloid cascade hypothesis discussed above, lead to other AD pathologies and eventually manifestation of AD symptoms. Since obesity may increase the likelihood of developing diabetes and cardiovascular diseases, it is not surprising to find that obesity is another risk factor for AD [62]. According to the Centers for Disease Control and Prevention, in 2006 only four states in the U.S. have a prevalence rate of obesity of less than 20%. Twenty two states have a prevalence rate of equal to or greater than 25%; in two of these states almost one in three adults has obesity. The increasing trend of obesity in the U.S. will pose an extra burden to the already gargantuan task of controlling AD in light of an aging population.

In addition to aberrant internal metabolism, external insult such as traumatic brain injury can increase the risk of AD [63–65]. The mechanisms by which head trauma may augment the risk of AD is unknown. Repetitive head trauma experienced by professional boxers may lead to "punch drunk" syndrome or dementia pugilistica later in life [66]. This syndrome is characterized by progressive dementia and parkinsonism and the presence of senile plaques and neurofibrillary tangles [67, 68]. Aβ deposition has been detected in the brains of victims of even a single head injury [69]. In preclinical models head trauma can exacerbate the formation of plaques or tangles, induce neu-

ronal cell death, and impair cognitive functions [70–73]. However, under certain experimental conditions, it has been shown that traumatic brain injury may actually cause regression of amyloid plaques [74].

In addition to the risk factors, epidemiological studies have revealed protective factors associated with AD. An engagement in physical, mental and social activities has been shown to reduce the risk of developing AD. The mechanisms for the associations are not well understood. Physical exercise or caloric restriction may indirectly curb the risk for AD by reducing one's risk for cardiovascular diseases, obesity and diabetes. It has been postulated that mental activities can build cognitive "reserve" so that some cognitive decline will not adversely affect normal functions. Interestingly, recent preclinical studies have suggested that these protective factors may have a more direct role in AD pathologies. For instance, exercise in APP transgenic mice can enhance cognition and ameliorate amyloid plaque deposition [75]. Caloric restriction [76] or learning in the Morris water maze [77] not only can improve cognitive functions but also dampen development of amyloid and/or tau pathologies. Enrichment of housing has been shown to reduce amyloid pathologies [77] and improve cognition [78]. Interestingly, the improvement in cognition in the latter study is associated with increased amyloid plaque deposition.

5
Therapeutic Strategies and Approaches for the Treatment of AD

5.1
General Strategies

Our improved understanding of AD has generated a number of potential therapeutic approaches for this disease. Some of the approaches are symptomatic while others are believed to be disease modifying, i.e., being able to slow, halt or reverse the progression of AD. In general, the strategy to treat the disease aims either at ameliorating the pathologies or compensating for the functional deficits to restore normal functions. Current therapeutic approaches can be classified into those targeting amyloid pathology, tau pathology, microgliosis (or neuroinflammation), metabolic aberrations and neurodegeneration in AD. Distinction among these approaches is often not clear cut, however, as different pathologies may be interwoven with each other through complicated and murky signal transduction pathways. This can be exemplified by an Aβ vaccine that not only reduces amyloid but also tau pathologies in the PS1 $\times$ APP $\times$ tau triple transgenic mice [13].

There are at least two approaches from which therapeutic agents are discovered that fuel the AD clinical trial pipeline. They are the "bottom up" and "top down" approaches. The "bottom up" approach is the traditional approach adopted by pharmaceutical companies. A drug target is first identi-

fied followed by high throughput screening (HTS) of their chemical libraries. The HTS hits are optimized to give potent and selected leads, which have to demonstrate both efficacy and safety in appropriate animal models (see Chapter 5). This approach enables the pharmaceutical companies to pursue novel targets with no existing chemical matter but can be an arduous and time consuming process. The "top down" approach takes advantage of epidemiological findings, anecdotal evidence or preclinical studies that suggest that an FDA approved compound on the market can be beneficial for AD. Since these medicines developed for other indications have proven safety profiles in humans, they can often be fast tracked to clinical trials in AD patients (see Chapters 2 and 3). The advantage of this approach is its speed – it can potentially cut precious years typically required for research and development in the "bottom up" approach. Epidemiological and anecdotal association ,however, is not necessarily causative. Clinical trials may fail due to the lack of a causal relationship. The available medicines that could be used for AD as an alternative indication may also be limited. Their properties, designed for their original indication, may not be appropriate for AD (e.g. brain permeability). Nevertheless, there is increasing interest in tapping these opportunities. The candidates for the "top down" approach may not be limited to marketed drugs but may include those in ongoing clinical trials and those terminated for lack of efficacy for other indications.

5.2 Specific Approaches

Below we will highlight some of the therapeutic approaches in research and development. Due to the vast amount of literature and information available, this is by no means intended to be a comprehensive review but it will highlight key areas under investigation, especially those that have entered clinical trials.

5.2.1 Targeting Symptoms

It is known that symptoms in AD patients fluctuate. The rapid fluctuation cannot be accounted for by changes in structural damage. It suggests that surviving neurons in an AD brain, under the right circumstances, still retain the ability to carry out the once lost brain functions. In fact, preserving and maintaining functional balance of the surviving neuronal systems has been shown to provide benefits for AD patients. However, the full potential of symptomatic relief remains to be elucidated. To date the FDA has approved five drugs for the symptomatic relief of AD: four cholinesterase inhibitors and an *N*-methlyl-D-aspartate (NMDA) receptor antagonist. The four approved cholinesterase inhibitors are tacrine (Cognex), donepezil (Aricept), ri-

Table 1 Selected compounds in Phase III trials for the treatment of AD Sources: http://www.alzforum.org/ & http://www.clinicaltrials.gov/

Drug(s)	Mechanism of action	Sponsor
Neramexane	NMDA receptor antagonist	Forest Laboratories
Xaliproden[a]	Nerve growth factor agonist/ 5HT1A receptor agonist	Sanofi-Aventis
AAB-001	Humanized monoclonal anti-Aβ antibody	Wyeth/Elan
R-flurbriprofen (Flurizan)	γ-modulator; Nonsteroidal anti-inflammatory durg (NSAID)	Myriad Genetics
Tramiprosate[a] (Alzhemed)	Inhibit Aβ oligomerization by binding and reducing soluble Aβ	Neurochem
Simvastatin (Zocor)	HMG-CoA reductase inhibitor	Merck/NIA
Atorvastatin (Lipitor)	HMG-CoA reductase inhibitor	Pfizer
Rosiglitazone (Avandia)	PPAR-γ agonist	GlaxoSmithKline
AIT-082 (NeoTrofin)	Neurotrophic agent	NeoTherapeutics
Cerebrolysin	Neuroprotection, neurotrophic agent	Ebewe

[a] Recent results show that both xaliproden and tramiprosate have failed to demonstrate significant efficacy in AD patients

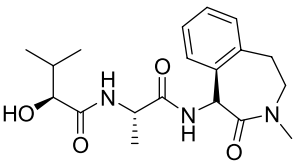
Table 2 Selected compounds in Phase II trials for the treatment of AD Sources: http://www.alzforum.org/ & http://www.clinicaltrials.gov/

Drug(s)	Mechanism of action	Sponsor
Huperzine A	Acetylcholinesterase inhibitor	Alzheimer's Disease Cooperative Study Group, Neuro-Hitech
Sabcomeline	Muscarinic M1 receptor agonist	GlaxoSmithKline
CX516	AMPAkines	Cortex
Lecozotan	5HT1A receptor antagonist	Wyeth
Paliroden	5HT4 receptor agonist	Sanofi-Aventis
MEM 1003	Neuronal L-type calcium channel modulator	Memory
SGS-742	GABA receptor agonist	Saegis/Novartis
Phenserine	Acetylcholinesterase inhibitor/ beta amyloid precursor protein inhibitor	TorreyPines
Bapineuzumab	Antibody to Aβ	Elan/Wyeth
AN-1792	Antibody to Aβ	Elan/Wyeth
LY-450139	γ-secretase inhibitor	Eli Lilly
PBT-2	Inhibits Aβ oligomer formation, disaggregates plaques	Prana Biotechnology

vastigmine (Exelon) and galantamine (Razadyne) (see Chapter 1, this BOOK). The NMDA receptor antagonist is memantine (Namenda). After their initial approval, some of these agents are undergoing Phase IV clinical trials to determine their efficacy in other stages of the disease. Since cholinesterase inhibitors and NMDA receptor antagonists are targeting different mechanisms, they can be used simultaneously for symptomatic relief. New cholinesterase inhibitors (e.g. herperzine) and NMDA receptor antagonists (e.g. Neramexane) are being tested in the clinic (Tables 1 and 2). Other therapeutic agents represent novel ways of regulating the cholinergic and glutamatergic systems (e.g. nicotinic agonists and amapkines). Finally, manipulations of the serotonergic system are being tested to determine if cognitive decline and personality changes can be corrected.

5.2.2 Targeting Amyloid Pathologies

According to the predominant amyloid cascade hypothesis, Aβ peptides are the ultimate perpetrator of AD. Understandably, most of the drug discovery efforts are targeted towards reducing their levels in the brain. This is achieved by either decreasing production or increasing clearance. Aβ peptides are produced by concerted proteolytic cleavage of their substrate, amyloid precursor protein (APP), by BACE1 (β-secretase) and γ-secretase. These two proteases have thus become the targets of intensive drug discovery efforts. To date multiple companies have advanced γ-secretase inhibitors (Fig. 1)



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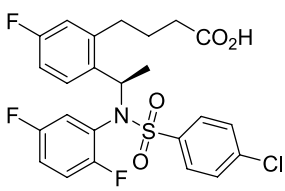

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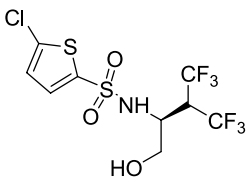

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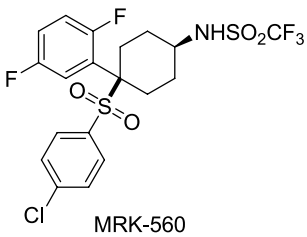

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Fig. 1 Chemical structures of γ-secretase inhibitors

into clinical trials with numerous others in preclinical development [79–81]. A major concern over using γ-secretase inhibitors is inhibition of signal transduction mediated by many other γ-secretase substrates. For instance, inhibition of Notch signaling by γ-secretase inhibitors may affect differentiation of tissues regulated by Notch. Indeed, γ-secretase inhibitors have been shown to induce morphological changes in the gastrointestinal tract and alter differentiation of intestinal globet cells and lymphocytes [82–84]. A subset of γ-secretase inhibitors, known as the γ-modulators, can selectively inhibit Aβ42 production without significantly diminishing Aβ40 or processing of Notch [85]. It is hoped that these compounds may circumvent the potential liabilities of conventional γ-secretase inhibitors. The most advanced compound of this type is R-flurbiprofen (Flurizan) (Fig. 2) which is currently in Phase III clinical trials (http://www.myriad.com/alzheimers/flurizan.php), although there are conflicting data on its effect on modulating γ-secretase activity [86–88]. The second generation of γ-modulators is moving forward in preclinical and early clinical development (Fig. 2) [89]. Development of small molecular weight BACE1 inhibitors appear to be challenging in light of the large catalytic binding pocket [90] but its potential safety concern is lessened by the apparent lack of adverse phenotype in BACE1 knockout mice [91]. Recent advances in the identification of non-peptidic inhibitors of BACE [92] have focused on overcoming the poor brain penetration and efflux liability associated with classical peptidic inhibitors (Fig. 3). These discover-

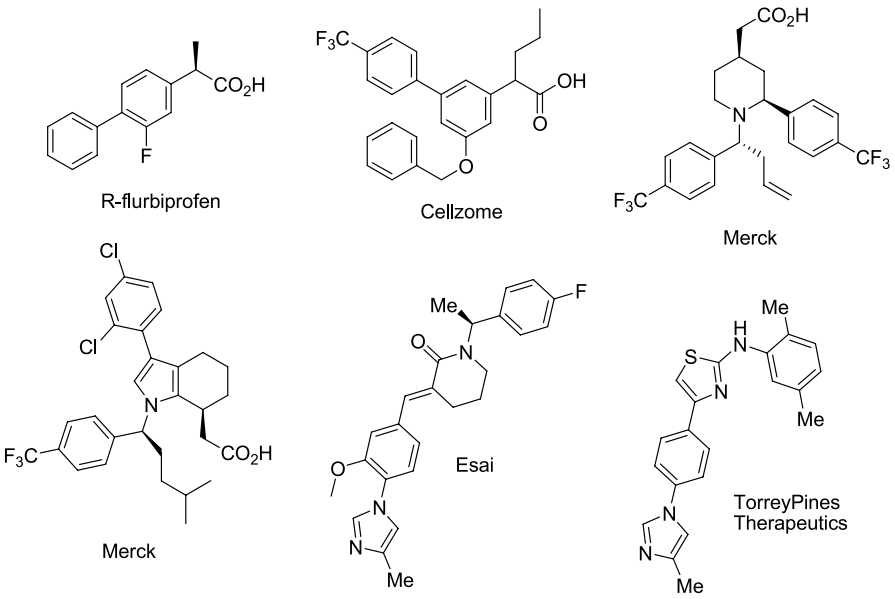

Fig. 2 Chemical structures of γ-modulators

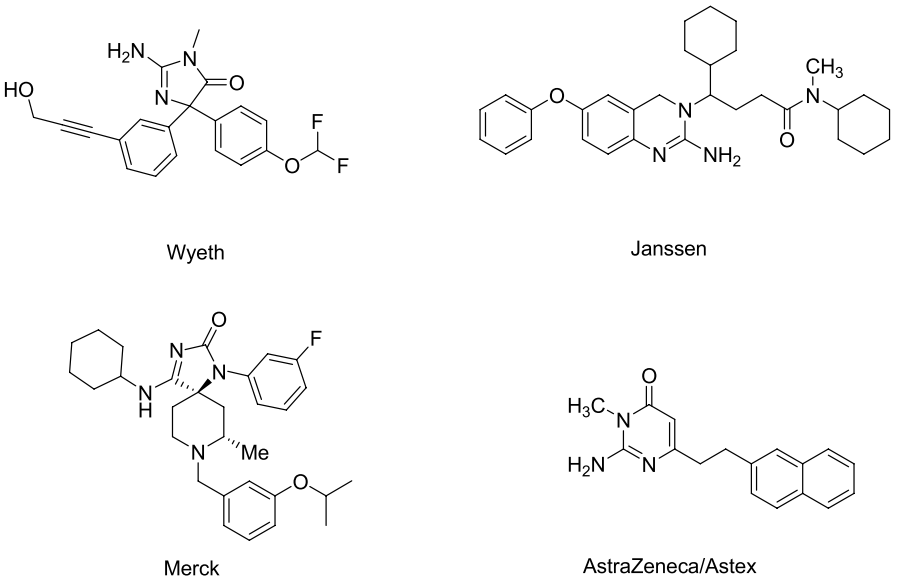

Fig. 3 Chemical structures of BACE inhibitors

ies should help further test the amyloid hypothesis in human clinical trials. In addition to secretase inhibitors and modulators, Aβ production can be indirectly reduced by augmenting the non-amyloidogenic cleavage mediated by α-secretase. Treatments that elevate the α-secretase enzyme level or activity will indirectly dampen Aβ production. Some of these treatments, e.g., statins [93] and muscarinic M1 agonists [94] are being tested in Phase III and II clinical trials, respectively, for the treatment of AD (Tables 1 and 2). Other hypotheses for the inhibitory effects of statins on Aβ levels is through reduction of cholesterol-enriched lipid raft where the amyloidogenic processing of APP occurs [95] and by regulation of trafficking of APP and its processing machinery [96].

In addition to turning off production, brain Aβ levels can be diminished by increasing clearance. Clearance of brain Aβ is regulated by protease degradation, receptor-mediated efflux from brain to blood and phagocytosis by microglia. Aβ proteases include neprilysin [97], insulin degrading enzyme (IDE) [60, 98], endothelin converting enzymes (ECE) [99], angiotensin-converting enzyme [100] and plasmin [101]. Efflux of Aβ from brain to blood has been suggested to be mediated by the lipoprotein receptor-related protein (LRP) [102–104] and P-glycoprotein [105, 106] while transport in the opposite direction has been shown to be mediated by RAGE [107, 108] and glycoprotein 330/megalin [109]. Potential therapies aimed to increase Aβ clearance include, but are not limited to, RAGE inhibitors and Aβ immunotherapy (Tables 1 and 2). The first clinical trial of Aβ immunotherapy

was with AN1792. The trial was suspended due to detection of meningoencephalitis in 6% of the patients treated with the Aβ vaccine [110]. Despite this adverse reaction, there were signs of efficacy as suggested by reduction in CSF tau (normally elevated in AD subjects) and improvement in the neuropsychological test battery [111, 112]. Passive immunization in animal models with cerebral amyloid angiopathy has been shown to cause cerebral hemorrhage [113]. A balance of efficacy and safety against autoimmunity and cerebral hemorrhage will be the challenge for the future of Aβ immunotherapy.

The mechanisms by which Aβ causes neuronal toxicities are not completely understood. It has been suggested that monomeric Aβ, however, is largely innocuous while oligomeric Aβ (dimers, trimers and dodecamers) are the toxic species. Aβ oligomers, not monomers, have been shown to trigger progressive loss of synapses in organotypic hippocampal culture [114] and impair cognition in animal models [115, 116]. It should be noted that Aβ oligomerization appears to be distinct from Aβ fibrillization. The latter could be beneficial in shunting Aβ away from forming the toxic oligomeric species [117]. It may, therefore, be important for therapeutic agents that inhibit oligomerization not to block fibrillization. The most advanced compound of this type, tramiprosate (Alzhemed), is in Phase III trial (Table 1). Preclinical studies show that tramiprosate maintains Aβ in the non-fibrillar form, reduces Aβ-induced cell death and lowers amyloid deposition in transgenic mouse brains [118]. Unfortunately, as of the writing of this article, results from the Phase III trial in North America have been deemed inconclusive due to the high variability among trial sites. Other compounds currently in clinical trial that inhibit Aβ oligomerization/aggregation include PBT-2 (Phase II) (see Table 2 and Chapter 4) and cyclohexanehexol (Phase I) [119].

5.2.3 Targeting Tau Pathologies

As discussed earlier, tau hyperphosphorylation is a key pathology in AD. In the cerebrospinal fluid of AD patients phosphorylated and total tau levels are elevated. Unlike APP transgenic mice, transgenic mice expressing either wild type tau [120] or FTDP-17 tau [121, 122] have been shown to display clear loss of neurons. Removing proline-directed phosphorylation sites on tau abolishes tau toxicities in Drosophila [22]. Inhibition of tau kinases has been shown to ameliorate certain tau pathologies [123, 124] and improves behavioral functions in tau transgenic mice [125]. Significant drug discovery efforts have thus targeted tau kinases. Leading tau therapeutics in Phase I clinical trials are glycogen synthase kinase 3 inhibitors (see Chapter 5). Other tau kinases of interest include cyclin-dependent kinase 5 (Cdk5), extracellular signal-regulated kinases (ERK), microtubule-affinity regulating kinase (MARK), casein kinase-1 (CK-1) and p38 [126]. One of the challenges in developing tau kinase inhibitors is to select the right kinase to target among a number of candi-

dates. On the other hand, there is evidence that activities of some tau kinases (e.g. GSK3β) may prefer priming by other kinases [127, 128] – thus inhibition of one kinase may preclude phosphorylation by others. A recent study suggests that tau pathologies and behavioral deficits in tau transgenic mice can be rescued by tau immunotherapy [129]. Stabilization of microtubules may offset lost tau function associated with neurodegenerative tauopathies [130]. Tau oligomerization [131], cleavage [132] and degradation [133] are other therapeutic opportunities under preclinical investigation. Given the significance of tau pathologies in AD and the sparse tau therapeutics in clinical development, this field remains a largely untapped opportunity.

5.2.4 Targeting Microgliosis

Activation of microglia surrounding amyloid plaques in AD brains could be a double-edged sword. On one hand, activated microglia can release harmful inflammatory substances. On the other hand, activated microglia can scavenge toxic Aβ. Microglial activation can be inhibited by non-steroidal anti-inflammatory drugs (NSAIDs). Epidemiological studies show that NSAID usage is associated with a lower risk of AD [134]. However, clinical trials of NSAIDs in AD patients have been largely disappointing [134]. The lack of efficacy of NSAIDs can be ascribed to its potential use only for prevention but not treatment, the inappropriate doses or drugs chosen in the trials or a faulty hypothesis. In contrast to NSAIDs, Aβ immunotherapy may depend at least in part on the activation of microglia [34]. Aβ immunotherapy is currently being investigated in clinical trials (Tables 1 and 2). It has been proposed that there may be multiple activation states of microglia: some could be associated with release of toxic inflammatory substances while others with the beneficial removal of Aβ (Morgan 2006). A more precise characterization of these different states of activation may lead to more precise and efficacious treatments targeting microglial activation.

5.2.5 Targeting Multiple Functional and Pathological Deficits

Although the above discussion of different therapeutic approaches was structured according to their effects on particular AD deficits, some of them may have a wide range of effects across symptoms and pathologies. For example, amyloid immunotherapy has been shown to provide acute cognitive improvement [135] and reduction in both amyloid and tau pathologies [136] in animal models. The peroxisome proliferator-activated receptor-γ (PPARγ) is a ligand-activated transcription factor. It regulates expression of a wide variety of genes including those involved in lipid and glucose metabolism and inflammatory responses. Activation of PPARγ also suppresses BACE1 ex-

pression [137, 138]. PPARγ agonists may thus address the amyloid pathology, neuroinflammatory responses and risk factors (e.g. hypercholesterolemia, obesity, and diabetes) of AD (see Chapter 3). An FDA approved drug on the market for the treatment of diabetes, Avandia, has been shown to provide benefits for AD patients who do not carry the apoE4 gene [139]. A phase III clinical trial is ongoing to test its efficacy in AD patients (Table 1).

Five different mechanisms of actions for the treatment of AD are discussed separately in the following chapters. In the opening chapter Drs. Jeffrey Kao and George Grossberg review the use of cholinesterase inhibitors currently on the market as symptomatic treatments for AD. The next four chapters discuss potential disease modifying therapeutic opportunnities that have entered clinical studies. In Chapter 2 Drs. Holly Soares and Larry Sparks review the pleiotropic effects of HMG-CoA reductase inhibitors (statins) that may affect AD pathogenesis. In Chapter 3 Drs. Qingguang Jiang, Shweta Mandrekar and Gary Landreth discuss how PPAR-γ agonists can be used to regulate metabolism of lipids and glucose and inflammatory responses to ameliorate deficits in AD patients. Both mechanisms discussed in Chapters 2 and 3 have benefited from the availability of marketed drugs for a different indication and have been fast tracked to Phase III clinical trials for the treatment of AD. In Chapter 4 Drs. Anthony White and Ashley Bush review metal chelating agents as a mechanism to reduce Aβ oligomerization and neurotoxicities. Small clinical trials have provided encouraging results and new metal chelating agents have been advanced to Phase II trials. Finally, in Chapter 5 Drs. Ratan V. Bhat, Stefan Berg, Jeremy Burrows, and Johanna Lindquist summarize the role of glycogen synthase kinase 3 (GSK-3) in AD and the potential benefits of GSK3 inhibitors, a couple of which have recently entered Phase I clinical trials.

6 Outlook

Current treatments for AD are largely symptomatic therapy (see Chapter 1). Due to the progressive nature of AD, symptomatic treatments, especially those with modest efficacy, could be limited. For instance, patients may experience an initial improvement in symptoms only to find that their symptoms continue to worsen over time even though they may still be better than placebo controls (Fig. 4). This medical need, despite the presence of several current symptomatic treatments, drives the search for disease modifying therapies that may slow, halt or even reverse the progression of AD. With the exception of therapies that actually reverse and cure the disease, therapies that only slow or halt disease progression, although still considered to be highly beneficial, will not by themselves improve symptoms of the patients but simply reduce the rate of their functional decline (Fig. 4). From the patient's perspective it produces "silent" efficacy that can only be appre-

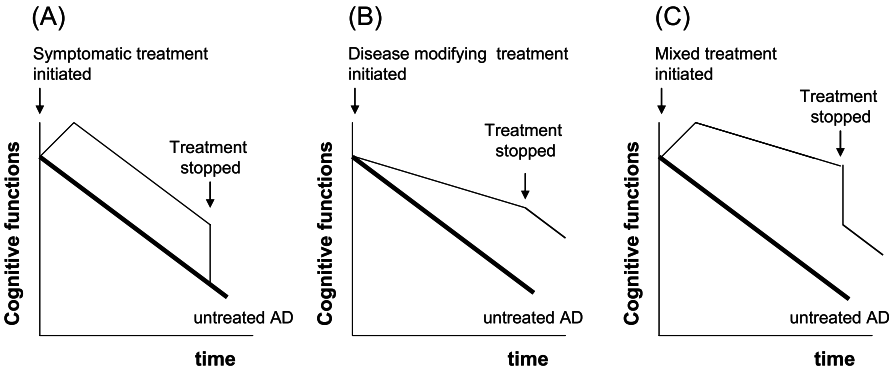

Fig. 4 Possible scenarios of symptomatic, disease modifying and mixed treatment on cognitive functions of AD patients. **A** Symptomatic treatment provides a rapid boost to cognitive functions. Upon withdrawal of treatment, cognitive functions return to the same level of the untreated group. **B** Disease modifying treatments can be divided into those slowing, halting and reversing disease progression. The figure shows a disease slowing progression which does not provide any immediate improvement in cognitive functions but slows the rate of cognitive decline. Upon withdrawal of treatment, cognitive functions are still higher than the untreated group. **C** Mixed symptomatic and disease modifying treatment provides both a rapid boost to cognitive functions as well as a long lasting effect even upon treatment withdrawal. Mixed treatment may be achieved by combining symptomatic and disease modifying treatments while certain target mechanisms alone may produce mixed treatment effects

ciated over the course of the disease. It becomes a philosophical question whether or not patients and their families would or should choose to use such pure disease modifying treatments, maintaining the patient in a prolonged state of compromised mental abilities. This underscores the importance of a continued search for more efficacious symptomatic treatments to be used in combination with disease modifying therapies. There is evidence that some therapeutic approaches can be mixed, utilizing both symptomatic and disease modifying therapies. They are not only expected to alter the course of the disease but also provide immediate improvement in symptoms (Fig. 4). For example, chronic treatment with Aβ antibodies provides disease modifying effects in APP transgenic mice [34] while acute treatment has been shown to improve cognitive functions without any effects on amyloid plaque deposition in the same model [140]. If replicated in clinical trials, these mixed symptomatic and disease modifying treatments will be highly desirable.

In order for patients and their families to be able to appreciate the "silent" efficacy from therapies that slow disease progression and increase compliance, a disease biomarker will be important. This is analogous to measuring blood cholesterol for the control of cardiovascular diseases. Patients on cholesterol lowering agents may not be able to perceive any benefits but will continue to comply with taking the medicine knowing that their blood

cholesterol level is under control and that their long term risk of developing cardiovascular diseases is minimized. On a lager scale biomarkers can be used to select specific patient populations appropriate for a specific clinical trial and to monitor the efficacy of a therapeutic agent. In order to demonstrate that a treatment can slow or halt the progression of AD, clinical trials often require a long duration (12–18 months) in order to be able to discern a difference between the control and treatment groups. In fact, unexpected stability in control groups (which may still be under symptomatic treatments for ethical reasons) over a short time frame may jeopardize the ability to demonstrate efficacy of a potential treatment under investigation. The long duration needed is translated into time consuming and costly clinical trials. A disease biomarker closely associated with the course of the disease whose change can be detected early by a therapeutic agent will be highly valuable in making early decisions for the fate of a clinical trial. Although a number of candidates have been suggested to be associated with AD, there is currently not a widely accepted disease biomarker for AD that is equivalent to cholesterol for cardiovascular diseases. In an effort to efficiently advance research in biomarkers for AD, the National Institute of Health, the Alzheimer's Association and the pharmaceutical industry are sponsoring a $60 million plus initiative known as the Alzheimer's Disease Neuroimaging Initiative (ADNI) [141]. Data from this study will be made available to the public. Similar efforts are planned in Europe, Australia and Japan.

AD is a complicated disease that is impending on our aging population increasingly exposed to a number of risk factors. Our improved understanding of the disease and its pathologies has generated a myriad of potential therapeutic approaches. Some of these are being investigated in clinical trials while others are being tested in preclinical studies. Both symptomatic and disease modifying approaches will be important in improving the overall well being of AD patients. It should be noted that symptomatic treatments for AD should not be limited to cognitive enhancement but should also include therapies for behavioral changes (e.g. agitation, hallucination, depression and delusion) that could be very difficult for families caring for these patients. Different disease modifying treatments with different mechanisms of action may be combined in controlling the progression of AD. Prevention is always better than cure. Reducing risk factors (e.g. hypercholesterolemia, hypertension, diabetes and obesity) and increasing resistance (e.g. engaging in physical, mental and social activities) may ward off the likelihood of contracting AD. Our continued commitment in searching for efficacious treatments for AD together with a healthier lifestyle will be important in fighting against the growing threat of this deteriorating disease.

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Cholinesterase Inhibitors

Jeffrey Kao (✉) · George Grossberg (✉)

Department of Neurology & Psychiatry, St. Louis University School of Medicine,
1438 South Grand Boulevard, St. Louis, MO 63104, USA
Kaoj@slu.edu, grossbgt@slu.edu

Abstract Alzheimer's disease is an illness that affects not only the patients themselves but also those around them. Traditionally, cholinesterase inhibitors have been the first-line medications used in treating Alzheimer's disease. There are currently four approved cholinesterase inhibitors: tacrine, donepezil, rivastigmine, and galantamine. Each of these medications has a unique pharmacokinetic profile and mechanism of action. Researchers in the past 5–10 years have accumulated much data on the use of cholinesterase inhibitors. The use of cholinesterase inhibitors may preserve activities of daily living, slow progression of memory loss, and improve behavioral and cognitive symptoms associated with Alzheimer's and other related dementias. There is also some evidence to suggest that the efficacy in some cases may last more than a few years. In addition, there is some evidence that the use of cholinesterase inhibitors may be associated with reduction of caretaker stress. The use of cholinesterase inhibitor, however, does not drastically improve

or reverse Alzheimer's disease and their efficacy is not always of clinical significance. This view was reflected by a recent guideline put forth by the National Institute of Clinical Excellence in Great Britain. However, for the millions of patients who are already on cholinesterase inhibitors and have benefited from them, cholinesterase inhibitors remain to be the drug of choice in combating dementia.

Keywords Acetylcholinesterase inhibitors · Donepezil · Rivastigmine · Galantamine · Tacrine

Abbreviations

ADAS-cog	Alzheimer's disease assessment scale, cognitive subscale
ADL	Activities of daily living
Bid	Twice a day
CIBIC	Clinician's interview-based assessment of change
CYP	Cytochrome P
FDA	Food and Drug Administration
MMSE	Mini mental status exam
NPI	Neuropsychiatric inventory
PET	Positron emission tomography
PO	By mouth
Qday	Each day
Qhs	At night
QID	Four times a day
SIB	Severe impairment battery

1 Overview and Pathogenesis of Alzheimer's Disease

Alzheimer's disease is a devastating illness. It occurs mainly in the elderly population. Once afflicted, Alzheimer's disease produces progressive and unrelenting damage to the human brain. The average lifespan after being diagnosed with this illness is about 8–10 years [1]. Patients steadily lose cognitive functions including memory, executive functioning, and the ability to care for themselves. In addition, behavioral symptoms of agitation, depression, and psychosis are often co-morbid with Alzheimer's disease. This devastating illness affects not only the patients but also the families and anyone that provides care for them. In the United States, the staggering financial cost of the disease accounts for nearly $100 billion per year in medical and custodial expenses, with the average patient requiring about $27 000 per year for medical and nursing care. Furthermore, 80% of caregivers of patients with Alzheimer's disease report stress, and about 50% report depression [2].

There are currently no laboratory tests that can confirm Alzheimer's disease. It is a diagnosis based on clinical assessment. Despite this shortcoming,

the current clinical diagnostic criteria are quite sensitive and specific in diagnosing Alzheimer's disease. For example, the sensitivity and specificity of the Diagnostic and Statistical Manual of Mental Disorders IV criteria (Table 1) in the diagnosis of Alzheimer's disease are 76% and 80% respectively [3]. Of course, the gold standard for diagnosing Alzheimer's disease is a postmortem autopsy of the patient's brain. In a typical gross brain afflicted with Alzheimer's disease, one immediately will note diffuse atrophy, enlarged ventricles, and flattened sulci. There are two main microscopic changes that occur in the brain. They are senile amyloid plaques and neurofibrillary tangles. Senile plaques occur in between neurons, whereas the neurofibrillary tangles occur within neurons. Neurofibrillary tangles are formed when tau proteins, which help maintain the exoskeleton of neurons, become abnormally phosphorylated. Neurofibrillary tangles usually first sprout out in the entorrhinal and hippocampal cortex and as the disease progresses. Other areas of the brain are affected as well. Amyloid plaques are from deposits of poorly soluble amyloid-beta proteins that are abnormally cleaved from amyloid precursor protein. The amyloid plaques cause inflammation around neighboring neurons and eventually destroy neighboring neurons through oxidative damage and other inflammatory processes. The extent of amyloid plaque deposition correlates with the severity of the disease. The neuronal tracts impacting the amyloid plaques and neurofibrillary tangles are primarily cholinergic [4].

The nucleus basalis of Meynert, located in the basal forebrain, is the main location of the cholinergic neurons. The cholinergic neurons project to the amygdala, hippocampus and other parts of the neocortex. Additional cholinergic neurons in the reticular system also project to the cerebral cortex, the limbic system, the hypothalamus, and the thalamus. Acetylcholine is

Table 1 Diagnostic criteria for dementia of the Alzheimer's type (adapted from DSM IV-TR)

Development of cognitive deficits manifested by both
1. Memory impairment – difficulty in learning new memory and/or recall
2. At least one of the following cognitive deficits
a. agnosia
b. aphasia
c. apraxia
d. disturbance in executive functioning
3. Cognitive impairments cause significant social and occupational impairments and are significant decline from previous baseline functioning
4. Cognitive deficits are not due to other neurological, psychiatric, or other systemic illnesses
5. Cognitive deficits do not occur exclusively in the setting of delirium

synthesized in the cholinergic axon terminal from acetyl coenzyme A and choline by the enzyme choline acetyltransferase [4]. Once made, acetylcholine is packaged into storage vesicles for release when triggered by an action potential. There are two cholinesterase enzymes capable of metabolizing acetylcholine once it is released into the synaptic cleft. They are called acetylcholinesterase and butyrylcholinesterase, formerly known as pseudocholinesterase. When acetylcholine is metabolized in the synaptic cleft, it is broken down into choline. The resulting choline is taken back up into the presynaptic neuron and is recycled to make new acetylcholine molecules. There is some evidence that as Alzheimer's disease progresses, the level of acetylcholinesterase decreases and butylcholinesterase increases [5]. Researchers have shown that a deficiency in cholinergic functioning is related to impairment in memory, in particular short-term memory. Acetylcholine may also be involved in mood and sleep. In normal people, anticholinergic agents impair learning and memory [4]. Thus drugs that would promote cholinergic functioning in neurons by inhibiting the cholinesterases would seem logical in preventing worsening of memory as seen in Alzheimer's disease.

2
Basics of Cholinesterase Inhibitors

There are currently four cholinesterase inhibitors approved by the US Food and Drug Administration for the treatment of Alzheimer's disease. They are tacrine, donepezil, rivastigmine, and galantamine. Although each of the cholinesterase inhibitor behaves uniquely, the main mechanism of action shared by all the cholinesterase inhibitors as a class is reversible inhibition of acetylcholinesterase and with some, butyrylcholinesterase. Tacrine is nonselective for all forms of acetylcholinesterases. Both rivastigmine and galantamine are selectively active in the central nervous system, with rivastigmine probably most centrally selective, with minimal activity in periphery. Donepezil has more peripheral action [6]. All cholinesterase inhibitors are readily absorbed across the gastrointestinal tract. Tacrine was the first acetylcholinesterase inhibitors to be approved by the FDA in 1993. However, hepatotoxicity and the need for frequent dosing limited the usage of this drug. Donepezil was the next drug approved, in 1997. It has long half-life of 70 h in elderly with a simple once-a-day dosing regimen. In 2000, rivastigmine and in 2001, galantamine were approved by the FDA, each with a good safety profile. Rivastigmine and galantamine each reach peak concentration in about 1 h after oral administration. The effectiveness of each of the acetylcholinesterase inhibitors appears to be similar as a class. In the following sections, each cholinesterase inhibitor will be discussed separately and in more detail (Table 2).

Table 2 Properties of available cholinesterase inhibitors

Drug	Tacrine	Donepezil	Rivastigmine	Galantamine
Mechanism of action	inhibits both AChE[a] and BChE[a], reversible	inhibits mainly AChE, minimal peripheral anti-AChE activities, reversible	inhibits both AChE and BChE, reversible	inhibits reversibly AChE and modulates nicotinic acetylcholine receptors
Metabolism	CYP1A2	CYP2D6 and CYP3A4	plasma, AChE-mediated hydrolysis	CYP2D6 and CYP3A4
Time-to-Peak (CNS)	1–2 h	3–4 h	1 h	1–2.5 h
Half-life (plasma)	2–4 h	70–80 h	2 h	7 h
Protein Binding	high	high	intermediate	low
Elimination	mainly hepatic	mainly hepatic	renal	renal and hepatic
Dosing	20–40 mg qid	5-10 mg qd	6–12 mg bid	16–24 mg bid
Common Side Effects	hepatotoxity GI side effects	GI side effects, better tolerated	GI side effects, need to take with food	GI side effects, may need to take with food

[a] acetylcholinesterase
[b] butylcholinesterase

2.1 Tacrine

Tacrine is the first acetylcholinesterase inhibitor approved for treatment of Alzheimer's disease. Tacrine is short-acting, and is a reversible inhibitor of both acetylcholinesterase and butyrylcholinesterase [7, 8]. Tacrine is highly protein bound, rapidly absorbed orally, however absorption is reduced with food intake. Due to the side effect of hepatotoxicity, tacrine is no longer a popular drug. It increases serum glutamic-oxaloacetic transaminase (SGOT), and serum glutamic-pyruvic transaminase (SGPT) in 25–30% of patients. Patients with signs of jaundice confirmed by elevated total bilirubin should not be on tacrine. Liver enzymes should be monitored weekly for at least the first 18 weeks, then, they should be monitored once every 3 months. Liver enzymes tend to increase during the first 6–12 weeks [9, 10].

2.2 Donepezil

Donepezil was the second cholinesterase inhibitor approved in the US [6]. It is currently the only acetylcholinesterase approved by the FDA for the treatment of severe Alzheimer's disease. Donepezil is a reversible, noncompetitive cholinesterase inhibitor. It has a high binding specificity for brain acetylcholinesterase, with little to no affinity for butylcholinesterase. Compared with the other cholinesterase inhibitors in this class, donepezil may be better tolerated, with less gastrointestinal side effects. The long half-life of this medication allows once a day dosing (See Table 3).

Table 3 Donepezil

Pharmacokinetics [11]:

Highly protein bound and has a very high oral bioavailability. It is metabolized by CYP 450 2D6 and 3A4. Elimination half-life is approximately 70 h. The peak plasma concentration is reached in 3–4 h while steady-state is reached in about 2 weeks [6].

Dosing [11]:

1. The drug comes in 5-mg or 10-mg tablets or orally disintegrating form.
2. Medication is initiated at 5 mg po qhs, and may be increased after 4–6 weeks to 10 mg po qhs.

Side effects [11]:

1. Prolonged treatment appears safe, common side effects, mainly cholinergic, include nausea, vomiting, diarrhea, muscle cramps, nightmares.
2. Side effects are generally mild and transient, occur in about 20% of patients and usually resolve within 3 weeks.
3. Side effects more common with 10-mg than the 5-mg dose.
4. Other side effects may include weight loss, bradycardia, syncope, insomnia, nocturia.

Drug-drug interactions [11]:

1. Highly protein bound, may displace other protein bound drugs such as digoxin or coumadin.
2. CYP 2D6 inhibitors such as fluoxetine, cimeditine can raise level of donepezil.
3. CYP 3A4 inhibitors such as grapefruit juice, amiodarone, ketoconzaole can raise level of donepezil.
4. Antipsychotic agents: acetylcholinesterase inhibitors may increase the risk of antipsychotic-related extrapyramidal symptoms.
5. Watch for excessive cholinergic stimulation with other cholinergic agents.
6. St. John's Wort may decrease donepezil levels.
7. Concurrent use with beta-blockers may cause bradycardia.

2.3 Rivastigmine

Rivastigmine became the third acetylcholinesterase inhibitor to be approved by the FDA in 2000 for the treatment of Alzheimer's disease [6]. It is a pseudo-irreversible agent, with prolonged interaction at acetylcholinesterase receptors. Similar to tacrine, rivastigmine also inhibits both acetylcholinesterase and butyrylcholinesterase to a significant degree. The drug is very rapidly absorbed into the central nervous system after oral intake. This rapid rise in CNS level stimulates the chemoreceptor trigger zone and may cause nausea and vomiting. One case of esophageal rupture due to severe vomiting has

Table 4 Rivastigmine

Pharmacokinetics [11]:

One unique property of this medication is that it is not metabolized by the CYP450 enzyme system, thus there are no drug-drug interactions reported. Absorption of this drug is fast and rapid and is complete within one hour of administration. About 40% protein bound. It is extensively metabolized by cholinesterase-mediated hydrolysis in the brain. It has an elimination half life of 1.5 h. 97% of the metabolite is excreted in the urine.

Dosing [11]:

1. Initiate therapy at 1.5 mg po bid with titration every four weeks, as tolerated, up to a dose of 6 mg po bid.
2. If treatment is interrupted for longer than several days, it should be restarted at the lowest daily dose and then titrated again.
3. Administration should be administered with full meals.
4. Dosing change is not recommended in renal or hepatic impairment; titrate to individual tolerance.

Side effects [11]:

1. Symptoms are more common at dosage above 6 mg a day.
2. Watch mainly for gastrointestinal side effects, somnolence, fatigue, and may cause weight loss.
3. Other side effects may include dizziness, headache, fatigue, insomnia.

Drug interactions [11]:

1. Anticholinergics: effects may be reduced with rivastigmine
2. Antipsychotic agents: cholinesterase inhibitors may increase the risk of antipsychotic related EPS, monitor
3. Cigarette use increases the clearance of rivastigmine by 23%
4. Neuromuscular blockers: depolarizing neuromuscular blocking agents effects may be increased with rivastigmine
5. NSAIDS: patients may be at increased risk for peptic ulcers or GI bleeding with concomitant use.

been reported; thus initiating this medication requires slow titration [12]. In order to reduce the gastrointestinal side effects, it is advisable to take this medication after a full meal. Rivastigmine is dosed twice daily (see Table 4). Of note, a 24-h rivastigmine transdermal patch is pending FDA approval.

2.4 Galantamine

Galantamine was the last of the acetylcholinesterase inhibitor to be approved by the FDA [6]. It is indicated for the treatment of mild to moderate dementia of the Alzheimer's type. Galantamine enhances cholinergic transmission by competitively and reversibly inhibiting acetylcholinesterase. Unique to this acetylcholinesterase is that it also modulates allosterically the nicotinic acetylcholine receptors, thus potentially amplifying cholinergic activity. The half-life of galantamine is about 7 h, and is dosed twice a day (see Table 5). Most common side effects are gastrointestinal symptoms, which can be eased by taking with food. The use of galantamine has been associated with increased mortality in patients with mild cognitive impairment [13–15]. Increased mortality, however, has not been observed in patients treated for Alzheimer's dementia and other types of dementia. Recently, galantamine extended release capsules were approved for treatment of Alzheimer's disease.

Table 5 Galantamine

Pharmacokinetics [11]:

It is not highly protein binding. Absorption is rapid with distribution of 2–3 times higher in the brain than in plasma. It is metabolized by CYP2D6 and CYP3A4. Bioavailability is high with plasma half-life of 7 h. Time to peak is about 1 hour and 2.5 h with food for immediate release.

Dosing [11]:

1. Immediate release: initiate with 4 mg po bid with morning and evening meals, after 4 weeks, if well tolerated, the dose should be increased to 8 mg po bid, a further increase to 12mg po bid should only be attempted after another 4 weeks. The recommended dose range is 16–24 mg/day.
2. Extended release: initiate with 8 mg po qam, after 4 weeks, if well tolerated, the dose should be increased to 16 mg po qam, a further increase to 24 mg po qam should only be attempted after another 4 weeks.
3. If therapy is interrupted for greater than 3 days, restart at the lowest dose and increase to current dose to prevent side effects.
4. Renal impairment – maximum dose is 16 mg/day. Use is not recommended in severe renal dysfunction (ClCR < 9 ml/min).
5. Hepatic impairment: In moderate liver dysfunction (Child–Pugh score 7–9), maximum dose is 16 mg/day. Use is not recommended in severe liver dysfunction (Child–Pugh score 10–15).

Table 5 (continued)

Side effects [11]:

1. Adverse effects become more frequent at 24 mg po q day.
2. Most common side effects include gastrointestinal (nausea, vomiting, diarrhea).
3. Other side effects can include syncope which is dose-related, headache, dizziness, insomnia, tremor similar to other cholinesterase inhibitors

Drug–drug interactions [11]:

1. Drugs that are inhibitors of CYP2D6 and CYP3A4 such as paxil, cimeditine, ketoconazole, and erythromycin can increase galantamine level.
2. Concurrent use with beta-blockers, calcium blockers, amiodarone may lead to bradycardia.
3. Synergistic effects with cholinergic agents, leading to worsening of cholinergic side effects.
4. Concurrent use with NSAIDS may lead to gastric ulcers.
5. Antipsychotics may increase the risk of antipsychotic-related extrapyramidal symptoms.
6. St. John's wort may decrease galantamine serum levels

The extended release capsules display similar efficacy and bioequivalence when compared with the regular immediate release capsules. One advantage of the extended release capsules is the once daily dosing [16].

3 Overview of Cholinesterase Inhibitors in the Treatment of Alzheimer's Disease

The use of cholinesterase inhibitors may preserve activities of daily living, slow progression of memory loss and improve behavioral and cognitive symptoms associated with Alzheimer's and related dementias. Treating Alzheimer's disease often takes great patience on the part of physicians as well as patients and caregivers. Response to drug may take weeks, sometimes months before caregivers may notice a difference in symptoms, if at all. The efficacy produced by cholinesterase inhibitors is not always clear-cut. For example, there is some evidence that donepezil may benefit moderate to severe Alzheimer's dementia in outpatients, however, the results have been conflicting in the nursing home population [17]. In addition, both donepezil and galantamine have not yet shown consistent results in improving behavioral and psychotic symptoms in patients with Alzheimer's disease. Improvement in psychosis is seen with rivastigmine which has shown improvements in behavioral symptoms in mild to moderate Alzheimer's dementia and in Lewy Body Dementia [17].

A recent meta-analysis by Trinh and colleagues looked at the efficacy of cholinesterase inhibitors relative to behavioral symptoms. In this study, they reviewed published literature from 1966–2001. A total of 29 parallel-group or crossover randomized, double-blind, placebo-controlled trials of outpatients with mild to moderate Alzheimer's disease were included in the study. Patients were treated for at least 1 month with a cholinesterase inhibitor. Among the assessment tools used during this study were the Neuropsychiatric Inventory, the Alzheimer Disease Assessment Scale, Activities of Daily Living and the Instrumental Activities of Daily Living. The study showed a small but significant improvement with use of cholinesterase inhibitors. There was no difference among the cholinesterase inhibitors as a class [18].

In a similar study, Lanctot and colleagues looked at efficacy and safety of donepezil, rivastigmine, and galantamine in a recent meta-analysis of randomized, double-blind, placebo-controlled, parallel-group trials. They concluded that there were significant therapeutic effects with the cholinesterase inhibitors although they often were accompanied by significant side effects [19]. Several large, multi-centered, double-blind, placebo-controlled studies have generally supported the use of cholinesterase inhibitors in improving cognition. Efficacy has been shown to last up to 1 year and beyond [20, 21].

On the other hand, a recent analysis by Kaduszkiewicz and colleagues, looking at the efficacy of donepezil, rivastigmine, and galantamine in Alzheimer's disease, reached a different conclusion. This study included literature search of all published double-blind, randomized-controlled trials. The researchers concluded that there were methodological errors, which included having incomplete data, failing to correct for differences among comparisons, and using inappropriate methods in measuring outcomes which may have led to overestimation of efficacy in these trials. In addition, the modest benefits may have been overshadowed by the cholinergic adverse side effects of the cholinesterase inhibitors [22].

To this date, there has not yet been a randomized, double-blind, placebo-controlled trial comparing the efficacy of all of the cholinesterase inhibitors. The few studies available compared the efficacy between donepezil and rivastigmine [23], and two studies compared donepezil and galantamine [24, 25]. Each of the studies is sponsored by pharmaceutical companies with each sponsor favoring their own product. The methodological limitations in these trials are significant, including lack of adequate blinding, not having a long enough efficacy study, having small sample sizes, and not dosing the medications appropriately to their maximum daily dosage [26]. Due to the lack of evidence in comparison of efficacy among the cholinesterase inhibitors, there is still much debate on which cholinesterase inhibitor is most efficacious. Deciding which cholinesterase to use often depends on the side effect profile of the medications, pharmacokinetics, dosing schedule, patient tolerability, individual clinicians' personal preference, and which drug has been studied in

which dementia. For example, rivastigmine recently won FDA approval for treating Parkinson's dementia.

3.1 Use of Donepezil in the Treatment of Alzheimer's Disease

The efficacy of donepezil in Alzheimer's dementia has been demonstrated in several trials. In a study by Rogers and colleagues, the efficacy of donepezil in treating patients with mild to moderate Alzheimer's disease was investigated in a randomized, double-blind, placebo-controlled, fashion. Patients were followed for 24 weeks followed by a 6-week placebo washout period. The primary efficacy was measured by the Alzheimer's Disease Assessment Scale, cognitive subscale (ADAS-cog) and the Clinician's Interview Based Assessment of Change-Plus (CIBIC plus). There was statistically significant improvement in cognition in patients who were on donepezil versus placebo at the end of 24 weeks. After the 6-week washout period with placebo, there was no difference in the scores from both groups. This suggests that donepezil improved cognition in patients while they were taking the medication, but if stopped, the improvement in cognition might be reduced to minimal. Patients in this study tolerated donepezil well with most cholinergic side effects being mild in severity and transient [27]. Other trials have been able to produce similar results in cognitive improvement of patients with Alzheimer's Disease [28, 29]. In a randomized, placebo-controlled, double-blind study by Greenberg et al., using ADAS-cog as primary measure, they were able to show cognitive effects in patient treated with donepezil in as little as 6 weeks. There was however, no difference in caregiver-rated global impressions at the end of the study. In addition, this study also was limited by having a small sample size [30].

In the AD 2000 study, 566 patients were referred to memory clinics in the U.K. with mild to moderate Alzheimer's dementia. This was a randomized, double-blind study with the average age of the patients at 75 ranging from 46–93 years old. The results of the trial showed that there was a 0.8 point benefit in the Mini Mental Status Exam (MMSE) score in the patients taking donepezil versus placebo. Moreover, there was a small but significant difference in activities of daily living in favor of drug. Despite the small improvement in cognition, the study did not show efficacy in delaying progression of disability or into institutional care. The study also failed to find significance in reduction of caregiver and patient stress. The behavioral problems associated with Alzheimer's dementia were also not shown to be reduced [31].

Although the AD 2000 study did not find significant differences in delaying time to institutionalization in patients with Alzheimer's disease, there have been some open label studies which shown otherwise. A retrospective, open-label study which followed Alzheimer's patients over 3 years who were

enrolled in a memory clinic, 96% of whom were on donepezil, found significant differences in delaying institutional care. These results showed that there was an initial cognitive difference which decreased the rate at which MMSE declined over the years. The study found that 40% of the untreated patients ended up in institutional care during the follow-up period, versus 6% who were being treated [32]. In an observational study, Geldmacher and colleagues concluded that patients who were taking donepezil 5 or 10 mg/day for at least 9–12 months had a 21.4-month mean delay in time to institutionalization when compared with patients who were not taking donepezil [33].

The efficacy of donepezil in moderate to severe Alzheimer's disease has been documented in a few studies. One particular study investigated the use of donepezil in patients with moderate to severe Alzheimer's disease with mini mental status exam score of 5 to 17. This randomized, double-blind, placebo-controlled trial followed 290 subjects over a 24-week period. At the end of the study, it was found that patients who were on donepezil had significant improvements in cognition, behavior, and in activities of daily living [34]. In a recent study looking at donepezil in moderate to severe Alzheimer's dementia, residents of nursing homes in Sweden were randomized to receive donepezil in a double-blind study [35]. Subjects were at least 50 years old (average age of 85) with average mini mental status exam score of 6. In the donepezil group, subjects started with 5 mg of the medication which would be increased to 10 mg after 4 weeks if the drug was well-tolerated. All in all, 128 subjects were in the donepezil arm, while 120 subjects were in the placebo arm. The investigators were able to show statistically significant improvement in the donepezil arm with regards to the Severe Impairment Battery (assessment of cognitive function in the area of memory, language, attention, orientation, social interaction etc.), the MMSE, and the ADAS-cog. However, there was no significant difference in the caretakers' ratings of improvement. In addition, this trial demonstrated the tolerability of donepezil, as 91% of the subjects in the donepezil arm received the 10 mg dosage at the end of the trial [35].

Although caretaker's ratings of improvement in patients with Alzheimer's disease are often conflicting, there is some evidence that donepezil may be associated with reduced caretaker's stress [36]. This was assessed in a randomized, double-blind, placebo-controlled study investigating the efficacy of donepezil treatment in patients with moderate to severe Alzheimer's disease and the benefits of treatment on caretaker's stress level. In all, 290 subjects with MMSE score ranging from 5–17 participated and were randomized to take donepezil or placebo. The efficacy of donepezil on the patients with Alzheimer's disease was measured using the Disability Assessment for Dementia, which mainly assesses activities of daily living. The caretaker's stress level was measured using the Caregiver Stress Scale. At the end of 24 weeks, the investigators found that patients on donepezil showed statistically significant slower decline in activities of daily living compared with placebo.

Moreover, the donepezil group also demonstrated less caregiving time spent and lower levels of stress compared with the placebo group [36].

There is some evidence that donepezil may have benefits in patients with early stage Alzheimer's disease whose symptoms no longer qualifies them for mild cognitive impairment [37]. A study by Seltzer and colleagues enrolled 153 patients with baseline MMSE scores of 21–26 with mild impairment of activities of daily living. In this double-blind, randomized, placebo-controlled study, subjects were followed over 24 weeks and were assessed with the ADAS-cog and the MMSE as primary measures. The trial results showed a statistical significance in both measures, although the improvements were modest with a 2.3 point improvement in the ADAS-cog and a 1.8 point improvement in the MMSE at the end of 24 weeks compared with placebo [37].

3.2 Use of Rivastigmine in the Treatment of Alzheimer's Disease

Rivastigmine's approval by the FDA in 2000 was supported by several trials. One such trial was by Corey-Bloom and colleagues [38]. In this randomized, placebo-controlled trial, 699 patients with mild to moderately severe AD were enrolled. Patients were randomized to either high dose rivastigmine (6–12 mg/day), low dose rivastigmine(1–4 mg/day), or placebo. Subjects were then followed over a 52-week period. At the end of the study, subjects originally treated with high dose rivastigmine of 6–12 mg/day had significantly better cognitive function than patients originally treated with placebo [38]. This study however was limited by the fact that from week 26–52, the study became an open-label study. In a different study, Birks and colleagues compiled data of randomized, double-blind, placebo-controlled trials from the Cochrane database. In all, there were seven trials, which included 3370 subjects. The researchers found that after 26 weeks, high dose of rivastigmine in the range of 6–12 mg per day was associated with improved score on the ADAS-cog. Fewer patients progressed to severe dementia, 55% of patients on rivastigmine versus 59% of patients on placebo, a modest but statistical significant result. While on the high dose 6–12 mg per day, patients however, also had significantly more cholinergic side effects such as nausea and vomiting [39].

In one of the first studies that showed compelling evidence for rivastigmine in treatment of Alzheimer's disease, Rosler and colleagues investigated 725 subjects with mild to moderate Alzheimer's disease across 45 centers in Europe and North America [40]. This was a prospective, randomized, double-blind, placebo-controlled trial. Subjects were followed over a 26-week period with dose adjustment occurring over the first 12 weeks. Subjects were either on high-dose rivastigmine, (6–12 mg/day), low dose rivastigmine (1–4 mg/day) or placebo. Efficacy was measured by the ADAS-cog, CIBIC, and the Progressive Deterioration scale. Result at the end of the study showed significant

improvement across all measures in patients taking high dose rivastigmine (6–12 mg/day) compared with the placebo group, and that the improvement was dose-dependent. There was no difference in cognition in subjects on low dose rivastigmine (1–4 mg/day) compared with placebo. In addition, there were no major adverse effects reported during the study. Rivastigmine was tolerated well with most side effects being transient, relating to gastrointestinal symptoms such as nausea, vomiting, anorexia, and diarrhea [40].

The tolerability of rivastigmine was also demonstrated by Bilikiewicz et al. in an open-label study looking at the safety, tolerability, and efficacy of rivastigmine in mild to moderate Alzheimer's disease [41]. Sixty-two patients living in community setting were enrolled. Rivastigmine was started at 1.5 mg po bid and increased every 2 weeks up to maximum dose of 6 mg po bid if tolerated by patient. MMSE and ADAS-cog were used to assess the efficacy of the medication. At the end of the 26-week trial, 89% of the patients completed the trial with 72% of the patients who completed the trial receiving the maximum 12 mg per day dose. The researchers did not find decline in the MMSE and ADAS-cog from baseline, suggesting that rivastigmine may have slowed the progression of cognitive decline in patients with Alzheimer's disease. In addition, the study showed that rivastigmine was tolerated well and there were no serious adverse effects reported. This trial was limited by the small sample size and its open-label study design [41].

In addition to improving cognition in mild-moderate dementia, rivastigmine may be efficacious in improving cognition in patients with moderate to severe Alzheimer's disease. Doraiswamy et al. evaluated long-term efficacy of rivastigmine in Alzheimer's disease in a post-hoc investigation [42]. They found that after 52 weeks, the rivastigmine group was associated with a significantly smaller decline in ADAS-cog score. Subjects who had rivastigmine initiated earlier also had statistically less decline compared to subjects who had a 6-month delay in starting rivastigmine. In addition, the data seems more robust in the moderate to severe dementia group when compared with the mild-moderate dementia group [42]. Currently, rivastigmine is not indicated for severe Alzheimer's disease. However, with the approval of donepezil for the treatment of severe Alzheimer's disease, other drugs in this class of cognitive enhancers may soon follow.

Besides improvement in cognition, rivastigmine has also been reported to benefit behavioral symptoms associated with Alzheimer's disease. Cummings et al. investigated the efficacy of rivastigmine in behavioral disturbances of nursing home residents with moderate to severe Alzheimer's disease in a 26-week study. This study was a multi-center study involving 29 nursing homes and 173 subjects. Efficacy was measured using the Neuropsychiatric Inventory-Nursing Home scale, MMSE, and ADAS-cog [43]. At the end of the 26-week study, patients who had received rivastigmine showed improvement in several neuropsychiatric categories including delusions, hallucinations, agitation, irritability, nighttime disturbance, and appetite changes [43].

This study however, was limited by its open-labeled study design. The effect of rivastigmine on neuropsychiatric symptoms and behavioral disturbance related to Alzheimer's disease was looked at by Grossberg using literature searches published on MEDLINE and EMBASE. The result of the searches suggest that there seems to be some evidence that rivastigmine can also help with neuropsychiatric and behavioral symptoms associated with Alzheimer's disease [44].

The efficacy in behavioral symptoms, however, was not duplicated in a study comparing the efficacy of rivastigmine versus quetiapine or placebo in controlling agitation of institutionalized patients with Alzheimer's disease. In this small study, 93 subjects were enrolled during which 31 subjects were randomized to receive rivastigmine, quetiapine or placebo. The subjects were followed over a 26-week period [45]. At the end of the study, the researchers found that there was no difference in cognition among the three groups. In fact, the quetiapine group was associated with worsening cognition compared with the placebo group [45]. It must be noted that in addition to not having a large sample size, this study included many subjects with severe dementia who had significant behavioral disturbance that would probably be harder to treat at baseline.

There have been few studies looking at the long-term effects of rivastigmine, as defined by effect beyond a 6-month period. The preliminary data seems to show some promise. Grossberg et al. looked at data from four 6-month, placebo-controlled, randomized trials and two open-label extension studies over a -2 year period. The data suggest that there was less cognitive decline in patients on rivastigmine and that the benefit might extend up to 2 years [46]. In an observational study by Farlow et al., 37 patients, a subgroup of an earlier study by Corey-Bloom et al. [37], who had been on rivastigmine for 5 years were followed. At the end of the study, the results suggest that there was benefit in the level of cognition, global functioning and activities of daily living in patients who took rivastigmine beyond the 6-month period. The medication was well-tolerated and relatively safe, no major adverse effects were reported [47]. This study however was limited by its open-label, retrospective design. In addition, the sample size was very small.

The benefit of rivastigmine may extend beyond improving cognition and behavioral symptoms in patients who are taking the medication. Farlow and colleagues investigated the effect of discontinuing rivastigmine [48]. They contacted subjects who were previously enrolled in randomized, rivastigmine-placebo trials, who had during the trial, dropped out of the study for whatever reason. The subjects were taking rivastigmine in the range of 6–12 mg/day at one point in the previous trials. These subjects were compared with the placebo group. The researchers found a statistically significant fewer decline in levels of cognitive function, as measured by the MMSE and the ADAS-cog in patients on rivastigmine, compared with the placebo group. Suggesting perhaps that rivastigmine may have a disease modifying effect

on the course of Alzheimer's disease in patients who were previously on this medication [48].

3.3 Use of Galantamine in the Treatment of Alzheimer's Disease

Galantamine is the last acetylcholinesterase inhibitor to be approved for the treatment of mild to moderate Alzheimer's disease. It is claimed that the nicotinic modulating ability of galantamine enhances cholinergic neurotransmission and thus makes it a more potent cholinesterae inhibitor. Whether or not the mechanism of allosteric modulation of presynaptic nicotinic receptors enhances cholinergic neurotransmission leading to greater efficacy, this is a unique mechanism not shared by the other cholinesterase inhibitors. Similar to the other cholinesterase inhibitors, studies of galantamine so far have shown modest efficacy in the areas of cognition, activities of daily living, and behavioral disturbance.

In one of the earlier studies investigating the efficacy of galantamine in mild to moderate Alzheimer's patients, Tariot and colleagues enrolled 978 patients in a randomized, placebo-controlled, double-blind trial. Patients were randomized to receive either placebo, or galantamine doses of 8, 16, or 24 mg per day at the end of 8 weeks. Among the scales used to measure efficacy were the ADAS-cog, the Clinician's Interview-Based Impression of Change plus Caregiver Input, Activities of Daily Living, and the NPI. After following the patients for 5-months, the investigators noted modest but statistically significant efficacy across all outcome measures in patients who were receiving 16 mg or 24 mg/day compared with placebo. The 8 mg/day group did not separate from placebo. The 24 mg/day group did slightly better than the 16 mg/day, however, the difference was not statistically significant. In this study, galantamine was also well-tolerated, there was no difference in the discontinuation rate between all groups [49].

In another earlier study assessing the efficacy of galantamine in patients with mild-moderate Alzheimer's disease, Rockwood and colleagues enrolled 386 patients with an MMSE of 11–24. This was a short study, efficacy was assessed at the end of 12 weeks. Subjects were randomized to receive either placebo or 24 mg/day or 32 mg/day of galantamine at the end of 4 weeks. Primary outcomes measures were assessed using the ADAS-cog and the Clinician's Interview Based Impression of Change plus caregiver input. The investigators found statistically significant efficacy across all measures in patients taking galantamine compared with those that were on placebo, except for the behavior scale. One limitation of this trial was its short duration since the efficacy of cholinesterase inhibitors often is not detected until several months later. However, given the short time course and the rapid escalation of dosage, 82% of subjects who were on high-dose galantamine completed the trial, implying that the medication was well tolerated. There was no difference in

efficacy between those that were taking 24 mg or 32 mg/day of galantamine. Lack of difference on the behavior scale may be attributed to the short duration of the trial. However, this study showed that galantamine is well-tolerated even with rapid titration, and that efficacy could be assessed in as short as a 3-month period [50].

In investigating whether galantamine can maintain activities of daily living in patients with mild to moderate Alzheimer's disease, Galasko et al. evaluated 659 patients in a randomized, placebo-controlled trial. The subjects were on maintenance dose of 16 or 24 mg of galantamine for 5 months. Using the Alzheimer's dementia Cooperative Study ADL Inventory, the investigators were able to show that patients who were on galantamine performed better in activities of daily living, which was strongly correlated with cognition. Efficacy seemed to be more pronounced in patients with moderate versus mild dementia [51].

The effect of galantamine on behavioral disturbance in patients with mild to moderate Alzheimer's disease was investigated by Cummings and colleagues. A total of 978 patients were randomized to placebo, 8 mg, 16 mg, or 24 mg/day of galantamine. They were followed for 21 weeks. Behavioral disturbances were assessed via the Neuropsychiatric Inventory. The study showed that patients who were on 16 mg or 24 mg/day of galantamine had statistically significant better total scores on the Neuropsychiatric Inventory when compared with placebo and the 8 mg/day group at the end of the 5-month study. There were no statistically significant difference between the placebo and 8 mg/day group and between the 16 mg and 24 mg/day group. Furthermore, the study showed that at high doses of galantamine, 24 mg/day, there was reduced caregiver stress as assessed by the Neuropsychiatric Inventory distress scale. The reduced caregiver stress was likely associated with less behavioral disturbance, thus the potential benefit of the medication may extend beyond benefiting just the patients [52].

Olin and Schneider performed an analysis using the Cochrane database of randomized, double-blind, placebo-controlled trials comparing galantamine and placebo. Measures of outcomes in these trials included the ADAS-cog, the Clinical Global Impression of Change, the Activities of Daily Living Scale, the Disability Assessment for Dementia scale, and the NPI. There were consistent benefits in activities of daily living, behavior and cognition in patients who were on galantamine when compared with placebo. The study did not show a consistent dose-dependent benefit of galantamine. There were, however, consistently significant benefits at doses above 8 mg when compared with placebo. There was also a high rate of withdrawing galantamine due to adverse side effects, most commonly related to gastrointestinal side effects. The study concluded that at slow titrations, a dose of 16 mg/day seems to be best tolerated [53].

Despite some evidence that galantamine is well tolerated in rapid titration [50], other studies have shown that rapid titration and high maintenance

dose often leads to many unwanted cholinergic side effects with galantamine. Wilcock and colleagues evaluated the efficacy and safety of galantamine in 653 patients with mild to moderate Alzheimer's disease across 86 clinics in Europe and Canada. Patients were randomized to receive placebo or a maintenance dose of 24 or 32 mg after a period of 4 weeks, in a rapid titration schedule. At 6-month evaluation, in addition to showing more efficacy in the areas of cognition, activities of daily living and behavioral disturbance, the galantamine group also had a higher discontinuation rate due to side effects when compared with the placebo group. The discontinuation largely occurred during the titration phase. In terms of efficacy, there was no difference in the 24 mg versus the 32 mg group, with the 32 mg group having a higher discontinuation rate [54]. When titrated at a slower pace, the rate of discontinuation on galantamine of 24 mg/day had been low when compared with placebo [49]. One additional finding in this study was that there was no difference in the efficacy of galantamine, whether or not the patients had possessed the apolipoprotein E4 genotype. This finding is contrary to an earlier study that the apolipoprotein E4 genotype may reduce the efficacy of galantamine in patients with Alzheimer's disease [55]. The impact of apolipoprotein E4 genotype on Alzheimer's disease is still not fully elucidated and this remains an active area of research.

In a novel study using computerized neuropsychological tests as an outcome measure, Caramelli and colleagues in Brazil looked at 33 subjects with probable Alzheimer's disease in a prospective, open-label study. The computerized neuropsychological tests included reaction time tests that evaluate attention and memory. One such test was the face recognition test. Subjects were shown familiar and unfamiliar faces and would press buttons based on whether or not they could recognize the familiar face. Efficacy would be measured by the reaction time to pushing the correct button. Subjects were tested at baseline and at the end of the 3-month trial when they were either on 24 mg/day or 16 mg/day of galantamine depending on tolerability. The researchers were able to show statistically significant reductions in reaction times at the end of 3 months when compared with baseline [56]. This study however had very few subjects and the reduction in reaction time could have been due to the familiarity of subjects with using the computerized tests the second time around. There was also no placebo comparator.

There is some evidence that galantamine may be effective in more advanced Alzheimer's disease. Wilkinson et al. performed a post-hoc analysis of patients with baseline MMSE scores less than or equal to 12. Over a 6-month period, galantamine showed statistically significant improvement in cognition and functional activities when compared with placebo [57].

In a study looking at long-term efficacy of galantamine in patients with mild-moderate Alzheimer's disease, Ranskind et al. looked at 194 patients who had received open-label galantamine for 36 months. A total of 75 patients dropped out of the study during the 36 months. The rate of cognitive

decline in patients who dropped out of the study was compared with patients who were still taking galantamine. Using the ADAS-Cog assessment scale, the researchers found that patients who were taking galantamine at the end of the 36-month study had significantly less cognitive decline compared with those that were not taking galantamine. The results of the study imply that galantamine may be able to slow cognitive decline in patients with mild to moderate Alzheimer's disease [58].

4
Brain Anatomical Changes Associated with the Use of Cholinesterase Inhibitors

With the ever-increasing sophistication of neuroimaging, it is now possible to detect changes in the brain that were not possible to detect in the past. By taking advantage of this new technology, researchers have been able to show molecular and structural changes in the brain associated with the use of cholinesterase inhibitors. However, research using neuroimaging to detect changes induced by cholinesterase inhibitors is still in its infancy. There have only been a few studies on this topic and most of the studies lack appropriate sample size and rigorous study design. Despite the short-comings, the studies that are currently available hold promise that future research is bound to lead to new discoveries.

Using positron emission tomography, or PET scanning, Nordberg and colleagues were able to show higher cholinergic activity in the brains of patients afflicted with Alzheimer's disease who were taking tacrine [59]. In another study, using PET, Mega and colleagues looked at brain metabolism in patients with Alzheimer's disease who were taking galantamine. Their data show that patients who had improved in the areas of cognition and behavior also showed significant activation in the striatalthalamofrontal regions on PET scanning [60]. However, this study had several limitations. First of all, this was a small prospective, open-label study involving only 19 patients. Furthermore, it may be difficult to attribute the changes on PET solely due to the administration of galantamine, as patients who did not exhibit improvement in cognition but were also receiving galantamine did not show this change on PET.

In a similar study using PET technology to assess the impact of cholinesterase inhibition in the cortical brain, Bohnen and colleagues enrolled 14 subjects with Alzheimer's disease who were administered donepezil for 12 weeks. Their data showed a 19% donepezil induced acetylcholinesterase inhibition with most of the inhibition occurring at the anterior cingulate cortex [61]. The degree of acetylcholinesterase inhibition was limited in the cortex when compared with the 70–90% acetylcholinesterase inhibition normally found in peripheral red blood cells [62]. This implies that the cholinesterase in-

hibitor may have some difficulty targeting receptors in the brain. The Bohnen study also revealed some correlation between cholinesterase inhibition and attention and executive function but failed to reveal correlation in memory functions [61]. This study, however, was limited by its open-label treatment design, small sample size, and relatively short duration of treatment.

With the greater details that can be rendered by magnetic resonance imaging, Hashimoto and colleagues incorporated this technology to investigate hippocampal volume in 54 patients who were on donepezil and 93 control patients. Subjects in the donepezil group were on 5 mg/day, which was the maximum dose approved in Japan at the time. Using the MRI-based volumetric technique, subjects were followed prospectively for one year and their hippocampal volume loss during this time was compared with the control group. The study showed that subjects who were on donepezil had less hippocampal volume loss compared with the control group [63]. However, this study was limited by its open-label study design as the subjects were not randomized. In addition, the control group was a cohort group from a previous follow-up study which introduces another confound into the study. It must be noted however, that the investigator who was reading the MRI was blinded.

In a separate study but with more rigorous experimental design investigating hippocampal volume loss, Krishnan and colleagues assessed 67 patients with Alzheimer's disease who were treated with donepezil [64]. In this randomized, double-blind, placebo-controlled study, patients received donepezil titrated up to maintenance dose of 10 mg/day. At the end of 24 weeks, patients' hippocampal volumes were assessed with MRI and cognition was assessed using the ADAS-cog. In addition, investigators also incorporated proton magnetic resonance spectroscopy to assess levels of *N*-acetylaspartate. Previous studies have shown *N*-acetylaspartate to be a marker of neuronal structure integrity. It may also be a nonspecific marker of neuronal loss in a variety of brain disease including Alzheimer's disease [65, 66]. One would expect to find decreased levels of *N*-acetylaspartate in a diseased brain. The results of the study showed that compared with placebo, the donepezil group had smaller decrease in hippocampal volume at the end of 24 weeks and also had better ADAS-cog score. However, the increase in the level of *N*-acetylaspartate was only seen between weeks 6-18 in the donepezil group. This difference was not seen at the end of the study [64].

5
Combination Treatment of Cholinesterase Inhibitors with Memantine

Besides the cholinesterase inhibitors, one of the current treatments available for the treatment of Alzheimer's disease is memantine. Since this chapter is devoted to cholinesterase inhibitors, discussion of memantine will only be in the context of its combination treatment with cholinesterase inhibitors.

Memantine has a distinct mechanism of action. It is a noncompetitive *N*-methyl-D-aspartate receptor antagonist. As more data become available from recent research, it is now known that other neurotransmitters, besides acetylcholine, are involved in Alzheimer's disease. In the glutamate hypothesis, excessive activation of NMDA by glutamate is implicated in neurotoxicity and neuronal ischemia. Normally, NMDA receptors are activated by glutamate, which is an excitatory neurotransmitter in the brain. Thus, by blocking this excessive activation, memantine may have a neuroprotective effect [67]. Currently in the United States, memantine is approved for treatment in moderate to severe Alzheimer's disease. Since memantine works via a different mechanism than cholinesterase inhibitors, combining the two medications may enhance efficacy of treatment in Alzheimer's disease [68]. In a drug interaction study, adding memantine to galantamine appears to be safe. There was no significant change in the levels of acetylcholinesterase inhibition or in galantamine's pharmacokinetic profile with the addition of memantine. Furthermore, this combination treatment was tolerated well by the participants [69]. Currently, trials looking at combination therapy of memantine and cholinesterase inhibitors are scarce. The safety data demonstrated in preliminary studies coupled with the logical rational behind a combination therapy will certainly lead more researchers to conduct well-designed clinical studies.

One such study was performed by Tariot and colleagues who followed 404 patients with moderate to severe Alzheimer's disease and MMSE from 5–14 [70]. This was a randomized, double-blind, placebo-controlled study. All the patients were already on a stable dose of donepezil when memantine or placebo was added. Patients who were receiving memantine eventually were taking 20 mg/day. At the end of 24 weeks, the investigators found that the group that was receiving donepezil and memantine had better scores in the areas of cognition, activities of daily living, and behavior when compared with the group that was receiving donepezil and placebo. Memantine was well tolerated in this study. One interesting finding is that the placebo group actually had a higher drop-out rate when compared with the memantine group due to adverse reactions [70].

6
Use of Cholinesterase Inhibitors in Other Dementia-Related Illnesses

Besides their use in Alzheimer's disease, cholinesterase inhibitors have been extended to treat other dementias and related illnesses, which may include vascular dementia, dementia with Lewy Bodies, dementia associated with Parkinson's, mild cognitive impairment, and mixed dementia. Research is particularly important in this area since it is not uncommon for Alzheimer's disease to be co-morbid with other types of dementia. Therefore, it is important to devote a section briefly discussing recent findings and the use of

cholinesterase inhibitors in the treatment of dementias other than Alzheimer's disease.

With the prevalence of cardiovascular diseases such as hyperlipidemia, diabetes, hypertension, which may over time lead to cerebrovacular disease, there is a direct correlate between the presence of vascular disease and dementia. Studies have shown that there is also a cholinergic deficit in vascular dementia due to ischemia to neurons in the basal forebrain and the cholinergic pathways. Thus it makes sense to treat vascular dementia with cholinesterase inhibitors. In the studies thus far, patients with vascular dementia, who were treated with either donepezil, rivastigmine, or galantamine have shown improvement in cognition, behavior, and activities of daily living [71]. Black and colleagues studied 603 patients with vascular dementia in a randomized, double-blind, placebo-controlled study. Their result shows that donepezil was well tolerated and that patients who were on donepezil had significant improvement in the areas of cognition and activities of daily living [72].

Studies have also shown benefits of cholinesterase inhibitors in the treatment of mixed dementia which has features of both Alzheimer's disease and vascular dementia [73]. Erkinjuntti et al., in a randomized, double-blind, placebo-controlled study followed 592 patients with both vascular dementia and mixed dementia [74]. When compared with placebo, patients who were on galantamine had statistically significant improvement in all measures of cognition and global functioning. Kumar et al. found similar efficacy when they assessed the use of rivastigmine in patients with Alzheimer's disease who also had vascular risk factors. Over 600 subjects were followed in their study. Results showed efficacy in cognition and activities of daily functions in patients who were on high doses (6–12 mg/day) of rivastigmine [75]. It is important to note that the cholinesterase inhibitors are not FDA approved for the treatment of vascular or mixed dementia.

In the past few years, dementia with Lewy Bodies has become increasingly recognized and may now be the second most common cause of progressive dementia, after Alzheimer's disease. In a multicenter, international study, McKeith and colleagues studied 120 patients with dementia with Lewy Bodies in a randomized, double-blind, placebo-controlled fashion. At the end of the 20-week trial, the researchers noted improvement in areas of behavior including anxiety, hallucinations, attention, and memory in patients who were on a high dose of rivastigmine (6–12 mg/day) [76, 77]. A small, open-label study by Shea et al. showed that donepezil may have efficacy in cognition and behavioral disturbance in patients with dementia with Lewy Bodies [78]. Case reports have also described possible efficacy of cholinesterase inhibitors in improving neuropsychiatric symptoms associated with dementia with Lewy Bodies, in particular hallucinations and agitation [79, 80]. No drug is currently FDA approved for the treatment of Lewy Body Dementia.

Parkinson's disease is a common neurodegenerative disease occurring usually in the elderly. Often comorbid with Parkinson's disease is dementia,

which is associated with cholinergic deficits and occurs in 30–50% of patients with Parkinson's disease [81]. Emre and colleagues randomized 541 patients who had Parkinson's disease with dementia to either rivastigmine or placebo. Their result showed that patients who were on rivastigmine had statistically significant improvement in the areas of cognition and behavioral symptoms, however many patients dropped out due to side effects of the medication. Importantly, rivastigmine did not exacerbate extrapyramidal symptoms [82]. In a small double-blind, randomized, crossover trial comparing donepezil and placebo, Ravina and colleagues only found modest efficacy in a few outcome measures including the MMSE [83]. In other small studies, cholinesterase inhibitors have all been associated with improvement in general global, cognitive and behavioral measures [84–87]. These studies also show that the cholinesterase inhibitors did not exacerbate the symptoms of Parkinson's disease. Recently, the FDA approved rivastigmine for the treatment of Parkinson's dementia.

Another condition that has been increasingly recognized is mild cognitive impairment which may be a precursor to and risk factor for Alzheimer's disease. Investigators have looked at whether administering cholinesterase inhibitors to patients with mild cognitive impairment could delay or prevent Alzheimer's disease. In a study by Peterson and colleagues, vitamin E and donepezil were evaluated for treatment of patients with mild cognitive impairment. In all, 769 patients were enrolled in this double-blind, randomized, placebo-controlled study. The results from the study show that there was no difference in the rate of progression to Alzheimer's disease in the vitamin E and placebo group. The donepezil group had lower rates of progression during the first year, however this effect was negligible after 3 years [88]. In another randomized, double-blind, placebo-controlled study evaluating efficacy of donepezil in mild cognitive impairment, Salloway and colleagues found only modest efficacy in the secondary outcomes. No efficacy was seen in the primary outcome measure which was time to conversion to Alzheimer's disease [89]. The manufacturer of galantamine recently reported on two trials involving 2048 subjects with mild cognitive impairment on galantamine. The death rate, from vascular causes such as stroke or MI, was statistically significantly higher in patients who were on galantamine when compared with placebo [90]. At the present time, no drug is FDA approved for the treatment of mild cognitive impairment.

7
Recent Recommendations by the National Institute of Clinical Excellence

Recently, the National Institute of Clinical Excellence (NICE), which is an independent organization in Britain that provides guidelines on promoting good health and treating illness, revised their recommendations on the use

of cholinesterase inhibitors in the treatment of dementia. In their most recent recommendation from January 2007, NICE states that cholinesterase inhibitors should only be used in patients with moderate dementia, those with MMSE of 10–20 [91]. In those with mild or severe dementia, the panel did not think that the medications made enough of a difference. This has affected hundreds of thousands of patients with Alzheimer's disease in Great Britain. Opponents of this recommendation are worried that patients with Alzheimer's disease will be discriminated against by the health care system [92]. Not only will the insurance companies stop reimbursing for cholinesterase inhibitors in patients with mild or severe Alzheimer's disease, patients might not be as forthcoming about their illness or seek treatment since now it would be harder to have access to one of the few treatments currently available for Alzheimer's disease. In addition, opponents of this study are also quick to point out that NICE did not take into consideration improvements in the quality of life of the caretakers of patients with Alzheimer's disease [92]. Although the current evidence does not show that cholinesterase inhibitors can drastically improve Alzheimer's disease or reverse the illness, there is enough evidence to show that patients who are taking cholinesterase inhibitors may perform cognitively better and have less behavioral disturbances than patients who are not on the medications.

8
The Future of Treating Alzheimer's Disease

The mechanisms underlying the progression of Alzheimer's disease are complicated. With the current therapy of cholinesterase inhibitors, we have just scratched the surface in treating this complicated illness. In addition to enhancing cholinergic neurotransmission, the latest medications used to treat Alzheimer's disease involve blocking neurotransmission by glutamate thus protecting the brain from neurotoxicity. Knowing the pathogenesis of this complicated disease is essential to developing new therapies. Researchers have already begun researching other ways to slow or prevent the progression of this disease process. Future medications may have mechanism of actions that prevent or reduce the amyloid plaques by inhibiting enzymes that produce the plaques, interfering with formation of the amyloid sheets, or helping to speed the clearance of the amyloid plaques once they have developed [93]. With an expanding population of ever-increasing longevity, new medications that can modify the course of Alzheimer's disease and possibly reverse the progression of the disease are desperately needed. In short, we are moving from symptomatic to disease-modifying therapies in Alzheimer's disease.

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Beyond Cholesterol: Statin Benefits in Alzheimer's Disease

Holly D. Soares[1] (✉) · D. Larry Sparks[2]

[1]Translational Medicine,
Pfizer Global Research and Development,
One Eastern Point Rd, Groton, CT 06340, USA
Holly.D.Soares@pfizer.com

[2]Roberts Lab of Neurodegenerative Research, Sun Health Research Institute,
10515 W. Santa Fe Drive, Sun City, AZ 85351, USA

Abstract Early epidemiological studies first implicated that cholesterol lowering treatments, such as statins, may be preventative in the development of dementia. While early studies raised significant hopes, more recent epidemiological studies suggest a more complex association between cholesterol, statin use and AD. Despite the controversy, prospective randomized placebo control studies in AD do support cognitive benefit from statin treatment. The management of cholesterol within the brain differs significantly from peripheral compartments and recent data support statin effects on brain cholesterol intracellular trafficking and plasma membrane redistribution. Thus, statin-induced cognitive alterations may occur through subtle changes in cholesterol endocytic transport mechanisms as well as through mechanisms completely independent of cholesterol modification. The current treatise examines the convergence of cholesterol and APP processing and proposes a model to highlight how cholesterol and statin modulation might impact generation of amyloidogenic Aβ peptide fragments in the brain. In addition, the present treatise reviews effects of atorvastatin on non-cholesterol, inflammatory endpoints including IL-3, IL-13 and serum amyloid P. Results suggest that IL-3, IL-13 and serum

amyloid P endpoints alter following atorvastatin treatment suggesting statins may have mechanistic utility beyond cholesterol lowering.

Keywords 24S-hydroxycholesterol · Alzheimer's disease · APP · Cholesterol · Low density lipoprotein receptor · Serum amyloid P · Statins

Abbreviations

AD	Alzheimer's Disease
ADAM	A Disintegrin And Metalloprotease
ADAS-Cog	Alzheimer's Disease Assessment Scale – Cognitive Subscale
ADCLT	Alzheimer's Disease Cholesterol Lowering Trial
APH1	Anterior Pharynx Defective 1 Homologue
APP	Amyloid precursor protein
Aβ	Amyloid beta
BBB	Blood brain barrier
βCTF	Beta C terminal Fragment
CSF	Cerebral Spinal Fluid
CGIC	Clinical Global Impression of Change
GCMS	Gas chromatography mass spectrometry
GDS	Global Deterioration Scale
GM-CSF	Granulocyte Monocyte Colony Stimulating Factor
GSKβ	Glycogen Synthase Kinase 3 Beta
HMG-CoA	3-Hydroxy-3-Methylglutaryl Coenzyme A
iNOS	Inducible Nitric Oxide Synthase
IL	Interleukin
LDL	Low density lipoprotein
LDLR	Low density lipoprotein receptor
LRP	Low-density lipoprotein receptor-related protein-1
LTP	Long term potentiation
LXR	Liver X receptor
MMSE	Mini Mental State Exam
NMDA	N-methyl-D-aspartate
NPI	Neuropsychiatric Inventory
PSEN	Presenilin
SorLA	Sorting protein-related receptor containing LDLR class A repeats

1 Introduction

A recent review of demographic trends suggests that by 2040, over 81 million people worldwide will suffer from some form of dementia [1], making dementia a serious healthcare concern for the twenty-first century. Currently, Alzheimer's disease (AD) is the most prevalent form of dementia accounting for approximately 50–60% of all cases and estimates suggest that by 2050, over 13 million people will have AD in the US alone [2]. AD manifests it-

self as debilitating cognitive deterioration in the elderly with devastating consequences for affected individuals and care-givers. Unfortunately, current treatment options remain limited despite considerable efforts to identify effective disease modifying drugs.

Prevailing neuropathological and genetic evidence support the involvement of at least three major mechanisms in the underlying etiology of AD. These include aberrant processing of the amyloid precursor protein (APP), the hyperphosphorylation of tau [3] and excessive stimulation of inflammatory pathways [4]. Regarding APP processing, much of the scientific investigation has focused upon the abnormal accumulation of amyloidogenic peptides Aβ40 and Aβ42. Aβ40 and Aβ42 are derived from the enzymatic cleavage of APP by β- and γ-secretase complex enzymes and are the major Aβ peptide constituents of plaques [3]. Overproduction of Aβ40 and Aβ42 is a common phenotypic characteristic of the known mutations of early onset AD. Known mutations include those identified in APP on chromosome 21 [5], in presenilin 1 (PSEN1) on chromosome 14 [6] and in presenilin 2 (PSEN2) on chromosome 1 [7, 8]. The ratio of Aβ42/40 is currently considered a risk factor for AD and dementia [9, 10] and reductions in CSF Aβ42 is one of the most reproducible findings in the literature [11]. As a result, the measurement of Aβ and soluble APP peptides have become something of a common denominator in the assessment of potential AD treatment strategies and attempts to understand Aβ modulation are important components of current AD drug development programs.

Although the study of familial or early onset AD has lent great insight into the underlying etiology of AD pathology, cases of familial or early onset AD constitute less than 1% of all AD cases [12]. Interestingly, one of the most robust genetic markers for late onset AD is not an APP or presenilin mutation, but rather a polymorphism in a protein known to modulate cholesterol, ApoE [13–16]. ApoE is a 34 kDa secreted glycoprotein that occurs in humans as three isoforms. The ApoE3 isoform (Cys112, Arg158) is the most common followed by ApoE4 (Arg112, Arg158) and ApoE2 (Cys112, Cys158). Several studies have shown that ApoE4 is the single most important risk factor in late onset AD [13–16]. ApoE isoforms can modulate lipid panels in AD subjects [17] and lipid raft composition differs depending upon the ApoE isoform [18]. In addition, recent studies suggest that various isoforms can impact cholesterol trafficking within the cell with the ApoE4 variant favoring late endosomal pathways [19, 20]. Even though decades of research have focused on the involvement of ApoE in AD, the relations between the ApoE4 allele, cholesterol and Aβ processing are only now slowly emerging. Literature reports suggests that cholesterol may, in fact, be an important regulator of amyloid load within the brain and several epidemiological studies have lent credence to the idea that cholesterol lowering agents such as statins may have some efficacy in treating AD. The current treatise reviews evidence supporting statin use in AD and the mechanisms of cholesterol-Aβ interactions. Data are also presented demonstrating potential benefit beyond cholesterol lowering.

2
Epidemiological Studies of Cholesterol and AD

Initial interest in cholesterol and Alzheimer's disease stemmed from a lengthy historical backdrop. One of the first studies linking cholesterol to AD stemmed from an observation that senile plaques were quite prevalent in the brains of non-demented patients who had died from coronary heart disease [21], a population known to possess elevated cholesterol levels. Additional studies soon followed demonstrating that the ApoE4 allele of the ApoE cholesterol transporter was a major risk factor for AD [13–16]. Is there a clear association between cholesterol and Alzheimer's disease?

Although a few studies have observed an association between elevated cholesterol, dementia and AD [22, 23], the majority of cross-sectional studies typically report no association or lower levels of cholesterol in patients with AD compared to controls or VaD patients [24–30]. In two longitudinal population-based studies, higher midlife cholesterol levels were reported to be associated with an increased risk of AD 20–30 years later in life [31, 32]. Other population-based studies have reported no association between elevated cholesterol levels and AD [33–37]. Interestingly, recent reports suggest that mild mid-life hypercholesterolemia followed by sustained decreases in cholesterol levels can precede cognitive decline [33, 38]. A similar finding was noted in cross-sectional studies where high levels of cholesterol in autopsy brains obtained from 40–55 yr subjects were associated with increased brain amyloid load [39], again suggesting mid-life exposure to hypercholesterolemia may be key to the subsequent increased risk of dementia. Interpretation of population-based findings can be complicated by the fact that patients with cardiovascular disease die younger (survivorship bias), AD diagnosis may not have been autopsy confirmed and cholesterol modulation is highly dependent upon individual genetic backdrop. Furthermore, cholesterol itself is a relatively insensitive measure and more recent studies support the utility of various apolipoprotein constituents as more meaningful measures of cholesterol status. The fact that individual genotypes can also significantly influence the biological response to cholesterol exposure makes it difficult to accurately access the relationship of cholesterol to AD based solely on epidemiological evidence.

3
Epidemiological Studies of AD and Statin Use

Although the association between elevated cholesterol and increased risk of AD remains unclear, pharmacologically based epidemiological studies examining statin use have provided informative insights. Statins are a class of drugs known as 3-hydroxy-3-methylglutaryl coenzyme A (HMG–CoA) reductase

inhibitors [40]. HMG–CoA reductase is the first enzyme in the cholesterol synthesis pathway and inhibition essentially blocks de novo synthesis of cholesterol [40]. As expected, the first HMG–CoA reductase inhibitors were shown to be efficient inhibitors of cholesterol [41, 42]. Today, statins are one of the most prescribed medications for lowering blood cholesterol. In 2000, two seminal epidemiological studies were published raising hopes that statins may have some utility in the prevention of dementia presumably through cholesterol lowering mechanisms. These studies utilized a case-control and a nested case-control analysis to examine the relationship between statin use and the prevalence of dementia. One study utilized hospital patient databases in Illinois and Arizona to examine prevalence of AD in over 57 000 patients 60 yrs or older. Initial results suggested that the prevalence of probable AD in lovastatin and pravastatin (but not simvastatin) users was significantly lower compared to the total population [43]. Similarly, cross-sectional analysis of data from the UK General Practise Research Database provided corroborative evidence supporting protective effects of statins in dementia [44]. The studies were subsequently criticized for "indication" or "prescription" bias stemming from practitioner reluctance to prescribe statins to demented patients. More often than not, such patients were already considerably ill and benefits remained unclear resulting in insufficient scientific merit to justify additional costs.

Since the publication of these two early studies, a number of studies have adopted a more prospective cohort approach to the examination of statin use in patients who develop dementia and AD over the course of a set period (usually between 5–10 yrs). While many of the subsequent studies described seemingly protective effects in cross-sectional case-control analysis [36, 45–51], the majority of prospective cohort analysis failed to identify a reduced risk of dementia in incident cases of dementia or AD [36, 46, 47, 51]. One notable exception comes from a study that examined a population identified by common AD risk factors and co-morbidities (e.g., first degree relatives) [52]. In a high AD risk population, statin use was associated with a lowered risk of AD [52]. A summary of the epidemiological findings for statin use in AD are shown in Table 1.

Again, co-morbidities (e.g., diabetes, cerebrovascular disease, head trauma), genetic backdrop (e.g., ApoE4 load), diagnostic uncertainties and lipid status can confound interpretation. Furthermore, statins are not all created equal and vary quite significantly in the degree of lipophilicity [53]. As a result, statins differ in the ability to impact brain cholesterol and apolipoproteins modulation [54, 55]. In addition, statins have been reported to differentially alter intracellular cholesterol trafficking [56, 57]. Thus, conflicting results may also be partially attributed to diversity in statin pharmacodynamic activity. Nevertheless, in the paucity of epidemiological consensus, it is unlikely that statin megatrials for AD prevention will be initiated any time soon. Failed results from hormone [58–61] and modest findings from antioxidant studies [62, 63] have also positioned the field into a more cautious stance towards

Table 1 Summary of risk of AD and dementia associated with statin use

Population	Conclusion	Refs.
753 Dementia or probable AD out of 56,790	Positive: Case-control analysis reporting lower prevalence of probable AD in patients on lovastatin and pravastatin.	[43]
284 Dementia; 888 Controls	Positive: Nested case-control analysis reporting lower risk of dementia in statin users.	[44]
492 Dementia (326 AD); 823 Controls	Positive: Case-control analysis reporting that statins and lipid lowering agents reduced risk of AD in patients less that 80 yrs of age.	[48]
229 Dementia out of 655	Positive: Case-Control reporting lower prevalence of dementia in statin users.	[45]
170 Dementia (147 probable or possible AD) out of total 845	Positive: Demented individuals less likely than non-demented counterparts to be taking lipid lowering drugs.	[49]
309 (AD); 3,088 Non-AD controls	Positive: Nested Case-control reporting lower risk of AD in statin relative to non-statin users.	[50]
244 Prevalent AD, 119 Prevalent VaD, 2,226 Controls 119 Incident AD; 54 Incident VaD; 995 Controls	Negative: Cross-sectional analysis noted use of LLA agents was associated with lower risk of AD. Prospective cohort study reported no association of lipid lowering agents and AD.	[36]
312 Incident dementia (168 Probable AD); 1,496 Controls	Negative: Cross-section case control analysis noted statin use associated with lower incidence of AD. Prospective cohort study reported no association of statins with AD.	[46]
355 prevalent dementia (200AD) 4540 non-demented 185 Incident dementia (104 AD) out of 3308	Negative: In prevalent cases, statin use was inversely associated with dementia. In incident cases, no association was noted between statin use and AD.	[51]
480 Incident dementia (245 AD) out of 2798	Negative: Case-control study noted protective effects of statins in dementia. In prospective cohort analysis, statin use was not associated with lower risk or dementia or AD.	[47]
895 AD; 1,483 non-demented relatives of AD	Positive: Statin use associated with lowered risk of AD in high risk population (e.g. relatives of AD).	[52]

large prevention trials. Despite recent ambiguity in epidemiological findings, there have been some recent studies to suggest statins may have beneficial effects on cognitive decline.

4
Statins and Cognition

Preliminary enthusiasm in statins and cognition had been tempered by negative findings from two large cardiovascular studies examining the effects of statins on mortality and cardiovascular events where cognition was assessed as a secondary outcome. In 2002, the Foundation Heart Protection study evaluated effectiveness of 40 mg simvastatin in 20,536 elderly adults, including 5,806 between the age of 70–80 years. A telephone interview for cognitive status was used to assess cognition over the 5-year study period, and no differences in cognition was observed between placebo vs. statin treated groups [64]. The second large, Prospective Study of Pravastatin in the Elderly (PROSPER) evaluated pravastatin use in 5804 70–80 year-old cardiovascular patients at risk for dementia for 3.2 years. A number of cognitive tests were assessed including mini mental state exam (MMSE), Stroop and word learning digit recall memory tests. Again, no effect on cognition was noted [65]. The prior results are in contrast to a third cardiovascular health study that utilized a modified mini mental state exam to assess cognitive function in 3,334 cardiovascular elderly patients 65 years or older. The CHS study reported the rate of cognitive decline was less in those taking statins compared to untreated group [66]. Finally, sporadic reports have suggested that statins may impede cognition in the elderly [67]; however, subsequent meta-analysis does not support these findings [68], and statin use is considered to be safe in neurological populations [69].

The Heart Protection and PROSPER results do draw into question preventative effects of statins on dementia. On the other hand, the CHS study raises the possibility that statins may beneficially impact the rate of cognitive changes. Cardiovascular-based studies only indirectly measure the impact on dementia since most cardiovascular studies exclude patients with dementia and cardiovascular trials employ cognitive tests that are relatively insensitive for populations that are not already demented. Furthermore, assessment of cognitive decline in patients already suffering from cognitive deficits remains a critical component to the effective treatment of AD.

Two observational studies do suggest that statin use may slow cognitive decline in AD patients. In a 34.8 month study, the effects of lipid lowering agents (LLA) were assessed in 342 AD patients. Patients treated with LLAs were reported to exhibit a slower decline on the MMSE compared to untreated patients [70]. A second study examining decline in the global deterioration scale over a 12-month period in 224 AD patients taking statins and other medications commonly prescribed for AD reported a lower risk of cognitive deterioration in AD subjects on statins [71]. Although these studies serve only as indirect measures of efficacy in cognition, they provide supportive evidence to suggest statins may have symptomatic benefit in AD. Placebo-controlled treatment trials will ultimately provide definitive data.

To date, two randomized, placebo-controlled treatment trials using AD specific endpoints for cognition and functional improvement have been published describing statin efficacy on AD. In a double-blind, placebo-controlled study of 44 AD patients, a significant effect was noted on MMSE, but not on the Alzheimer's disease assessment scale – cognitive subscale (ADAS-Cog) following a 26-week assessment period [72]. A second double-blind, placebo-controlled intent-to-treat study in 67 AD subjects reported significant improvement on the ADAS-cog at 26 weeks and a positive trend in improvement in ADAS-Cog, NPI and CGIC, but not on the ADL or the MMSE, following treatment with atorvastatin for 12 months [73]. A significant beneficial effect was noted on the geriatric depression scale (GDS) scale [73]. These studies provide some of the first evidence to suggest statins may have beneficial utility on cognition in AD. Unfortunately, the studies were small and preliminary positive findings require confirmation in larger confirmatory studies.

Two large multi-center trials are ongoing to examine the efficacy of atorvastatin and simvastatin in the treatment of AD. The cholesterol-lowering agent to slow progression (CLASP) study is an 18-month study designed to examine effects of simvastatin (20 mg/day titrated to 40 mg/day) in 400 AD subjects (http://www.clinicaltrials.gov). The lipitor enhancement of aricept in Alzheimer's disease study (LEADe) is a 20-month study to examine effects of atorvastatin (80 mg/day) in 600 AD patients. Both studies will complete by late 2007–2008 and will provide more data regarding statin effects on cognitive and functional endpoints in AD.

5 Statins and Aβ

Cholesterol can impact Aβ synthesis through both direct and indirect mechanisms and it has been hypothesized that statins, as modulators of cholesterol, should decrease Aβ levels. Indeed, statin induced reductions in Aβ have been supported by cell culture studies. Early in vitro models showed 4uM treatment of lovastatin or simvastatin could reduce beta amyloid secretion of cultured cells [74–76]. Results from in vivo models have been mixed. In guinea pigs, high doses of simvastatin decreased brain Aβ [75]. Atorvastatin decreased brain Aβ peptide levels in PSAPP mice [77] and pravastatin and lovastatin reduced brain Aβ in TgCRND8 transgenics [78]. In non-transgenic mice, simvastatin, lovastatin, and atorvastatin all reduced Aβ peptide levels [56]. However, statin effects in transgenic models are not uniform across the board, and other labs have reported increased brain Aβ in Tg2576 females following lovastatin treatment [79].

Reports describing effects on alpha and beta APP enzymatic cleavage products, including sAPPalpha and sAPPbeta, are mixed as well. In vitro, rosuvastatin increased alpha secretase activity [80]. In vivo, pravastatin and

lovastatin elevated sAPPalpha in TgCRNDA mice [78], while simvastatin, lovastatin, and atorvastatin appeared to have little effect on alpha C terminal fragments [56]. Simvastatin, lovastatin and atorvastatin all reduced beta cleaved C terminal fragments [56], again supporting the notion that statins nudge biological systems into favoring non-amyloidogenic over amyloidogenic peptide pathways. Interestingly, Burns et al. reported simvastatin, lovastatin and atorvastatin all induced translocation of cholesterol from cytofacial to exofacial layers [56], suggesting subtle intracellular effects on plasma membrane redistribution.

In summary, statins can lower Aβ peptide levels in guinea pigs and some transgenic murine lines. Although reports are mixed, statins may cause cells to favor non-amyloidogenic (e.g., alpha cleavage) over amyloidogenic (e.g., beta cleavage) pathways. Interestingly, most of the statins do not appear to alter total brain cholesterol, but may influence cholesterol membrane distribution and cholesterol trafficking. Subtle effects on cholesterol trafficking and APP processing may explain reports that statins ameliorate learning and memory deficits in Tg2576 mice and improve cognition in non-transgenic lines [81].

The clinical picture regarding statin modulation of peripheral Aβ peptide levels in humans is largely negative. In 2001, statin effects on plasma Aβ were examined in 22 hypercholesterolemic Japanese patients following 5 mg simvastatin treatment [82]. Although the study reported no change in plasma Aβ levels, patients also exhibited no lowering of plasma cholesterol raising questions regarding the effectiveness of the dose used in the study. Subsequent studies with larger hypercholesterolemic patient populations again reported no changes in plasma Aβ levels at doses that were effective in lowering peripheral cholesterol [83–86]. To date, only one study has reported decreases in Aβ levels following 12 weeks of 20 and 40 mg lovastatin treatment in hypercholesterolemic subjects [87]. Thus, statins do not appear to modulate blood Aβ levels in hypercholesterolemic patients.

Aβ levels in hypercholesterolemic patients are known to differ from AD patients and a number of studies have been conducted in AD patients examining statin effects on CSF and plasma Aβ levels. Studies in AD patients treated with simvastatin or atorvastatin showed significant lowering of cholesterol and LDL, but no significant changes in CSF or plasma Aβ at any dose or at any time tested [72, 84, 88, 89]. In the clinic, and not unlike observations from transgenic studies, statin effects on sAPPalpha and sAPPbeta levels have been mixed. Sjogren et al. [88] reported significant decreases in both CSF sAPPbeta and sAPP alpha levels while Hoglund et al., demonstrated increases in sAPPalpha [90] with no effect on sAPPbeta. In summary, the majority of reports seem to show that in both hypercholesterolemic and AD patients, statins do not alter plasma and CSF Aβ peptides. Although there is no consensus yet on statin modulation of CSF soluble alpha and beta APP cleavage products, current data supports some modulation on APP processing.

Do statins modulate APP processing? In vitro studies seem to suggest statins can decrease Aβ peptide levels. However, in vitro studies come with caveats, including the fact that prodrug forms of statins require metabolism for activation and that high statin doses are required to produce Aβ lowering, implying a non-specific effect. In rodent models, statin modulation of alpha and beta APP cleavage and down-regulation of Aβ peptide levels largely showed decreases in brain amyloid load with one notable exception. Contradictory results may be partially attributed to profoundly divergent phenotypic characteristics of AD transgenic models [91]. In addition, statins differ in pharmacodynamic and lipophillic properties perhaps explaining variability in effects on Aβ load. Despite mixed results in both rodent and clinical models, statins do appear to influence APP cleavage patterns in CSF compartments. In the clinic, it is not yet known whether statins directly impact brain amyloid plaque load or soluble Aβ peptide generation. Additional clinical studies are required to clarify the mechanistic effects of statins on APP modulation in AD brain.

6 Cholesterol and Aβ

Statins have been reported to improve cognitive endpoints in rodents, and inhibit cognitive decline in the clinic. Interestingly, the majority of rodent studies have failed to describe consistent decreases in total brain cholesterol levels following statin treatment. Although statins possess activities independent of cholesterol lowering, subtle modulation of brain cholesterol trafficking may partially explain effects on neurological function. A growing body of literature report significant overlaps between amyloid and cholesterol trafficking. Indeed, brain cholesterol turnover and APP processing appear to converge to a common endocytic trafficking route within the cell. Based upon recent data implicating statin modulation in cholesterol trafficking within intraneuronal compartments, it is quite possible that statins may exert effects through both cholesterol and APP intracellular trafficking rather than through effects on total brain cholesterol. A brief review of both cholesterol and amyloid trafficking will serve to highlight the significant overlap between the two pathways.

The brain is one of the most cholesterol rich organs of the body and contains 25% of total body cholesterol [92]. While the body manages cholesterol metabolism primarily through the liver, the brain cholesterol compartment is essentially isolated from body cholesterol pools by the blood brain barrier (BBB). During early CNS development, the rate of cholesterol synthesis is quite high due to extensive myelination. As the brain matures, cholesterol synthesis and turnover slow dramatically with an estimated total turnover rate ranging from 4–6 months [93] in adult rat brain. The turnover rate is even slower in humans than in rodents (0.03% per day vs. 0.4% in rodent) [94].

Nevertheless, neurons have extensive membrane turnover requirements, especially in brain sub-domains where synaptogenesis is extensive such as regions involved in learning and memory. Within cells, cholesterol is unevenly distributed between the cytofacial and exofacial bilayers of the plasma membrane with 70% of brain cholesterol localized to the cytofacial domains [95, 96]. Increased levels of cholesterol within exofacial layers occur with aging [18, 97] and cholesterol is continuously released from the exofacial layers into the surrounding interstitial fluid. Interestingly, statins have been reported to alter intermembrane cholesterol localization and trafficking [18, 56, 57, 98, 99]. Specifically, pravastatin, simvastatin, lovastatin and atorvastatin all stimulate changes in cholesterol distribution between cytofacial and exofacial plasma membrane regions [56, 57]. Changes in membrane cholesterol distribution can significantly alter cellular signaling and function suggesting statins could have very profound effects via modulation of cholesterol trafficking. Curiously, cholesterol itself does not appear to be transported to any great extent across the BBB and, in fact, most of the brain cholesterol trafficking occurs within the confines of the BBB [92, 100]. In addition, neuronal cholesterol has been reported to exist primarily in an un-esterified form [101]. Thus, cholesterol rapid recycling pathways are thought to be heavily utilized within the CNS [94]. Any excess brain cholesterol is thought to be primarily removed by passive diffusion of hydroxylated cholesterol metabolites [92] and, to a lesser extent, by highly regulated apolipoprotein-dependent efflux systems [94]. The long half-life of brain cholesterol, in combination with extensive use of rapid cholesterol recycling pathways, may partially explain the lack of total brain cholesterol lowering in rodent models where the observational period is less than brain cholesterol half-life.

Re-uptake and efflux of brain cholesterol is still poorly understood. In peripheral organs, endocytosis of cholesterol occurs through clathrin coated mechanisms where cholesterol endosomes are believed to traffic through a specialized recycling system including late endosomal-lysosomal compartments [94]. It is within these compartments that cholesterol acidification and hydrolysis of esters occurs. A number of cholesterol transporters utilized in the periphery have also been identified in the brain including the 10-member low density lipoprotein receptor (LDLR) family [102], the ATP binding cassette transporter family (ABCA/ABCG) [103] and the Neimann–Pick type C proteins [93]. LDLR family members primarily mediate cholesterol endocytic pathways. Recent literature also suggests that the LDLR receptors are important mediators of Aβ clearance [104]. ABCA/ABCG have been implicated in reverse cholesterol transport mechanisms [101, 103, 105]. Transport of cholesterol through late endosomal and trans-Golgi networks depend largely on NPC protein [93]. In the brain, it is believed that high density lipid (HDL), rather than low density lipid (LDL), constitutes the primary lipid particle. Furthermore, astrocytes seem to be the major provider of new cholesterol to neurons.

Figure 1 summarizes a hypothetical model of brain cholesterol turnover based upon emerging reports from the literature. When required, astrocytes provide a significant portion of cholesterol to neurons possibly through export from ABCA receptors [101, 105, 106]. ApoE binds HDL-cholesterol particles and enter neuronal synapses via one of the LDL receptor family members. Interestingly, different cell types express different LDLR family members and binding can elicit quite diverse phenotypes. For example, binding to LRP1B is believed to induce association of APP with α-secretases favoring generation of non-amyloidogenic fragments [107]. Binding to LRP via interaction with FE65 elicits APP trafficking to late endosomes [108] resulting in increased amyloidogenic peptide formation [102]. Finally, ApoE binding to ApoE2 recep-

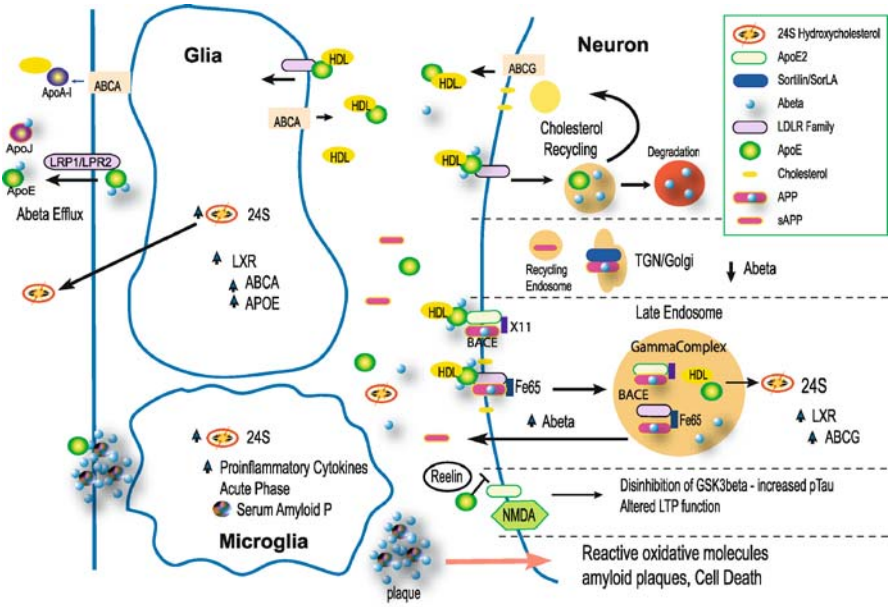


Fig. 1 Link between cholesterol and Aβ modulation. Under normal conditions, lipid particles containing apolipoproteins, cholesterol and Aβ undergo endocytosis via LDLR pathways. Cholesterol is recycled to membrane while Aβ segregates to degradative pathways. Excess amyloidogenic Aβ synthesis could occur as follows: 1) Sortilin/Sorla receptor mutations or decreases in Sortilin/Sorla expression may allow more amyloidogenic processing of APP. 2) Excess cholesterol may stimulate ApoE2-X11-APP, APP-Fe65 and BACE targeting to late endosomal compartments stimulating more amyloidogenic peptide synthesis. Additional AD related pathology resulting from excess cholesterol could occur as a result of ApoE2 inhibition of Reelin-GSK3β pathways resulting in increased tau phosphorylation. The cholesterol metabolite, 24S hydroxycholesterol stimulates liver x receptor (LXR) expression and increased synthesis of ApoE, the ABCG and ABCA transporters which are implicated in Aβ transport. 24S hydroxycholesterol also stimulates inflammatory sequelae and release of acute phase proteins. The acute phase protein serum amyloid P can stabilize Aβ peptides promoting plaque formation and complement activation

tors, in association with X11α/β, target β-secretase APP cleavage products to γ-secretase containing late endosomal compartments again stimulating amyloidogenic peptide formation [109]. Of note, the ApoE4 protein variant is more effective in increasing Aβ production via the ApoE2-X11 pathway providing a plausible explanation for why the ApoE4 allele poses a higher risk for AD [109].

In the hypothetical model, neuronal cholesterol that is no longer targeted for rapid membrane recycling transits to late endosomal compartments and is metabolized by CYP46A1 to 24S-hydroxycholesterol. Within neurons and astrocytes, 24S-hydroxycholesterol, also known as cerebrosterol, can stimulate liver X receptor (LXR) expression [110]. LXR can stimulate ABCA/G and ApoE up-regulation in astrocytes which in turn favors cholesterol export [105, 106]. While 24S-hydroxycholesterol beneficially stimulates cholesterol efflux, excessive 24S-hydroxycholesterol levels may adversely stimulate proinflammatory cytokine release [111] and possibly acute phase proteins such as serum amyloid P (Fig. 1). Serum amyloid P is highly resistant to degradation and binding to Aβ inhibits subsequent proteolysis favoring plaque seeding [112]. Serum amyloid P and other pentraxin members are also well known for an ability to activate complement [113].

APP itself occurs in three alternatively spliced isoforms with APP695 present primarily in neurons and APP770/751 present in peripheral and glial compartments. APP is a type I membrane glycoprotein that is trafficked through the constitutive Golgi secretary pathway. APP travels to the plasma membrane where it can be cleaved by α-secretase (the non-amyloidogenic cleavage at residues Lys16 Leu17 of Aβ peptide). A number of zinc metalloproteinases can cleave at the α-site and include TACE/ADAM17, ADAM9, ADAM10 and MDC-9 and an aspartyl protease BACE2 [114]. Interestingly, APP only transiently stays at the plasma cell surface. Both cleaved and unprocessed APP is rapidly internalized via an internalization (YENPTY) motif. BACE1 is the major β-secretase [115]. BACE resides primarily within trans-Golgi and Golgi cellular compartments [116] and the precise localization of actual BACE cleavage remains controversial. It is believed that BACE cleaves APP in late endosomal compartments [116]; however, others have proposed that cleavage can occur at plasma membrane surfaces [109]. The multimeric γ-secretase complex consisting of presenilin, nicastrin, presenilin enhancer 2 homolog (PEN2) and anterior pharynx defective 1 homolog (APH1) cleaves β-C-terminal fragments (βCTFs) within the late endosomal compartments to generate amyloidogenic Aβ (4 kDa) peptide fragments which are released into the extracellular space. Once in the extracellular space, the fate of Aβ peptides remains unclear. Quite possibly, soluble and perhaps fibrillary forms of Aβ peptide can bind ApoE and re-enter the endocytic pathway through LRP-mediated pathways [117]. Presumably, endocytosed Aβ is targeted for degradation. However, it is possible that excess of fibrillary forms may interfere with vesicular trafficking function resulting in intracellular accumulation and cell death.

How might cholesterol pathways and amyloidogenic Aβ synthesis converge in a pathogenic pathway? A plausible point of convergence between cholesterol and amyloid endocytosis may occur via ApoE-lipid receptor endocytic mechanisms where LDLR family members largely dictate processing endpoints for both cholesterol and APP (see Fig. 1). The model is supported by data that report increases in Aβ as well as increases in extracellular cholesterol result in increased vesicular trafficking [118]. Under normal conditions, Aβ and cholesterol may traffic through similar recycling compartments where intact cholesterol is rapidly recycled to the plasma membrane while Aβ fragments enter compartments targeted for degradation. Under conditions of excess cholesterol, rapid recycling pathways may be bypassed in favor of late endosomal trafficking which results in hydrolysis of excess cholesterol. However, trafficking to late endosomal compartments also favors formation of amyloidogenic peptide fragments and excess cholesterol may induce an intracellular switch to late endosomal trafficking patterns which in turn stimulate increased Aβ peptide formation. There is evidence to suggest that accumulation of cholesterol within late endosomal compartments, as a result of a mutation in a late endosomal cholesterol transport protein NPC, can indeed stimulate Aβ formation [119]. Furthermore, insoluble Aβ accumulates in late endosomes along with cholesterol in cells that express a mutant form of NPC [120].

The model outlined in Fig. 1 is also consistent with relatively recent developments linking the LDLR-related receptor SorLA/SOR1/LR11 APP sequestration in Golgi compartments [102]. It has been hypothesized that SorLA pathways may regulate APP entry into vesicles required for subsequent cleavage. Indeed, loss of SorLA results in aberrant increases in Aβ production and SorLA expression has bee reported to be lost in AD brain [102]. A genetic association between SORL1 and AD has recently been reported [121], emphasizing the importance of intracellular trafficking in amyloid processing (Fig. 1).

Finally, reports in the literature suggest ABCA/ABCG transporters have significant impact on APP trafficking and Aβ clearance. Increased ABCG1 expression has been reported to increase APP availability for cleavage as evidenced by increases in sAPPα, sAPPβ and Aβ peptides [122]. Furthermore, expression of ABCG1 is upregulated in Down's syndrome brains [122]. Thus, cholesterol transport pathways greatly influence APP modulation.

In addition to cholesterol modulation, ApoE-LDLR interactions can stimulate non-Aβ pathogenic pathways associated with AD. For example, blockage of Reelin signaling by ApoE-ApoER2 mechanisms can result in reductions in long term potentiation (LTP) via inhibition of the NMDA receptor [123]. In addition, inhibition of Reelin signaling can produce disinhibition of GSKβ yielding increased tau phosphorylation [123]. Thus, excess cholesterol may stimulate ApoE-ApoER2 blockage of Reelin signaling yielding non-Aβ pathopysiological events associated with AD.

In summary, the cholesterol-APP endocytosis model is consistent with observations that high dietary cholesterol can increase Aβ immunoreactivity in

the brain of New Zealand white rabbits [124] and in transgenic mice [125–127]. Indeed, a recent survey of AD-related genetic polymorphisms reflect an overwhelming preponderance of studies clearly implicating the importance of lipid homeostasis in AD [128]. However, cholesterol induced brain Aβ changes may not be reflected in CSF or plasma. Epidemiological studies linking total blood cholesterol with AD have not produced a clear picture. Again, these findings may be attributed to the insensitivity of total blood cholesterol as an indicator of brain cholesterol homeostasis. Recent studies suggest use of apolipoprotein profiles may provide a more accurate measure of risk assessment and, in the case of AD, CSF apolipoprotein profiles may provide a more accurate depiction of brain cholesterol homeostasis. In brain, statins do not appear to lower total cholesterol, but may exert quite subtle effects on cholesterol trafficking. In the periphery, statins do not appear to alter blood or CSF Aβ peptide levels, but effects on human brain Aβ levels remain unclear. Finally, statins have been reported to alter inflammatory pathways via modulation of isoprenylation pathways and it is possible that statins may be impacting neuronal function via alternative mechanisms. In order to determine whether statins might be altering additional pathways in AD, plasma samples from AD patients treated with 80mg atorvastatin for one year were analyzed for changes in cerebrosterol, apolipoproteins, interleukins and serum amyloid P.

7
Plasma Biomarkers for Atorvastatin Treatment in AD

7.1
24S-Hydroxycholesterol (Cerebrosterol)

Previously, a prospective placebo-controlled, randomized intent-to-treat study had been run to test atorvastatin for benefit in the treatment of mild-moderate AD patients. Preliminary results from the Alzheimer's disease cholesterol lowering trial (ADCLT) has been described in detail [73]. In brief, 67 AD subjects were randomized to either 80 mg atorvastatin or placebo and treated for one year. Blood for biomarker analysis was collected at baseline, 3, 6, 9 and 12 months post-dosing. To determine whether atorvastatin might impact brain cholesterol, a subset of samples was analyzed by GCMS for changes in 24S-hydroxycholesterol (also known as cerebrosterol). Statistical analysis utilizing a repeated measures approached identified no statistical differences in 24S-hydroxycholesterol concentration over time between atorvastatin and placebo treated groups. However, 24S-hydroxycholesterol levels were slightly increased at 9 and 12 months in the placebo group and were unchanged at the same time points in the atorvastatin treated group (Fig. 2).

24S-hydroxycholesterol, a CYP46 enzyme derived metabolite, crosses the BBB [100, 129] and levels have been reported to be elevated in early AD [130–

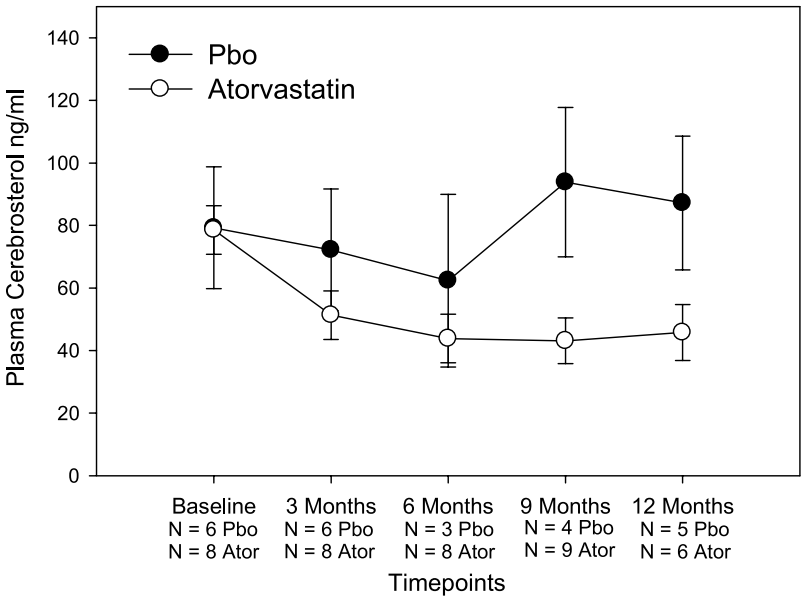


Fig. 2 Effects of atorvastatin treatment on plasma cerebrosterol levels

134]. Some studies have also suggested that CYP46A1 polymorphisms may be linked to AD [135–145]. As discussed previously, 24S-hydroxycholesterol is an important regulator of ApoE -mediated cholesterol transfer from astrocytes to glia and excess 24S-hydroxycholesterol may be neurotoxic and pro-inflammatory [100, 111, 146–148]. Reports regarding statin effects on 24S-hydroxycholesterol have been mixed. Some studies demonstrated decreases in 24S-hydroxycholesterol levels following statin treatment [90, 149, 150] and one study suggested a potential to reverse pro-inflammatory events associated with increased 24S-hydroxycholesterol levels [111]. Other reports suggest statin effects on 24S-hydroxycholesterol may not be robust [147, 149–151]. Modest decreases in 24S-hydroxycholesterol may have benefit on brain function by reducing ApoE levels which have been reported to potentially impede LTP and stimulate the phosphorylation of tau.

7.2 Apolipoproteins

Prior studies have indeed shown that atorvastatin treatment in AD significantly lowers plasma ApoE levels [152]. This is in agreement with other reports demonstrating statin lowering of ApoE levels [153, 154]. Decreases in ApoE might, upon first glance, appear to impede Aβ clearance to the bloodstream as recent reports show Aβ40 can be cleared through LRP1-ApoE interactions and Aβ 42 through LRP2-ApoJ [104]. However, excess ApoE

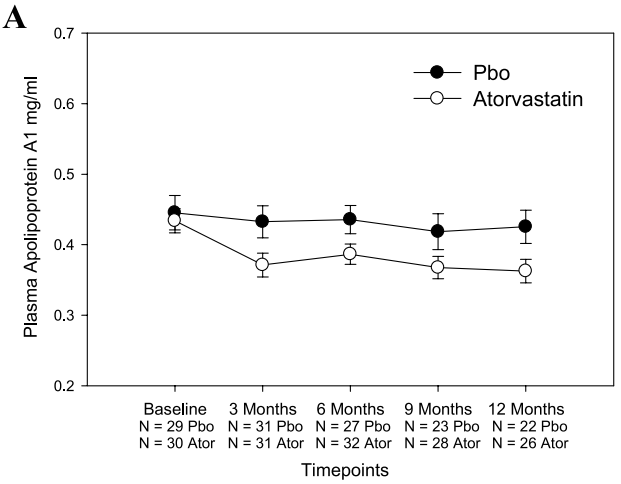
has also been hypothesized to impede LTP and stimulate tau phosphorylation [123, 155]. In addition, ApoE4 variants may actually stimulate Aβ peptide production [109]. Excess levels of ApoE, in combination with excess cholesterol, might force neurons into favoring late endosomal trafficking pathways, which in turn, favor amyloidogenic peptide formation. In order to further the understanding of statin effects on apolipoprotein species, plasma samples from the ADCLT studies were also assessed for changes in apolipoprotein CIII, Apolipoprotein A1 and Apolipoprotein H. There were no significant effects on Apolipoprotein A1 nor on Apolipoprotein H (Fig. 3a and b). In contrast, significant decreases in apolipoprotein CIII were noted in atorvastatin patients compared to placebo controls (Fig. 3c).

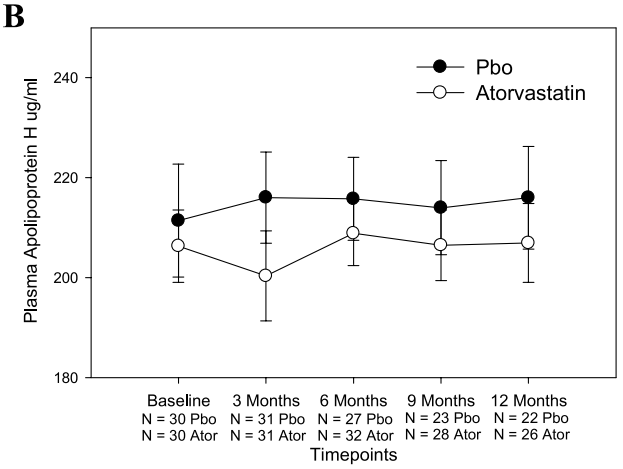
Differential effects of statins on apolipoproteins are well known [156, 157]. Statins can reduce apolipoprotein B with little effects on ApoA-I. Indeed, some suggest the ratio of ApoB:ApoA-I may be better predictors of cardiovascular risk factors than cholesterol:LDL ratios [156] and assessment of lipoproteins in AD may prove to be a better approach to understanding dementia risk. Apolipoprotein CIII is believed to delay lipolysis of apoB lipoproteins and can cause hypertriglyceridemia. Apo C-III induces monocyte adherence to vascular endothelium, an early essential event in atherosclerosis [158]. Excessive apolipoprotein CIII, is a known risk factor for coronary heart disease [159] and a weak genetic association between apolipoprotein CIII and Alzheimer's has been reported [160]. Sporadic genetic associations on proteins involved in lipid metabolism appear to be a consistent theme in the literature and lend credence to the hypothesis that aberrant lipid metabolism contributes to AD neuropathology [128]. In summary, reduction of apolipoprotein CIII may have beneficial effects on improving blood flow through the reduction of monocyte adherence and possibly on triglyceride levels.

7.3 Interleukins

Much of the prior discussion has focused upon statin effects on cholesterol pathways. However, recent data suggest statin benefit in AD may occur via mechanisms completely independent of cholesterol lowering. For example, samples are known to modulate isoprenylation pathways and have potentially very potent anti-inflammatory activities [161]. Indeed, inhibition of proteins that are dependent upon isoprenylation, such as the Ras GTPase superfamily, has been suggested to be an import step in the inhibition of iNOS stimulated cytokine release [161]. In order to better understand

Fig. 3 Effects of atorvastatin on **A** apolipoprotein A1, **B** apolipoprotein H, and **C** apolipoprotein CIII. Atorvastatin had no effect on apolipoprotein A1 and H. Treatment significant decreased apolipoprotein CIII in AD patients throughout the duration of treatment ►


A
Plasma Apolipoprotein A1 mg/ml
0.7
0.6
0.5
0.4
0.3
0.2
Pbo
Atorvastatin
Baseline N = 29 Pbo N = 30 Ator
3 Months N = 31 Pbo N = 31 Ator
6 Months N = 27 Pbo N = 32 Ator
9 Months N = 23 Pbo N = 28 Ator
12 Months N = 22 Pbo N = 26 Ator
Timepoints


B
Plasma Apolipoprotein H ug/ml
240
220
200
180
Pbo
Atorvastatin
Baseline N = 30 Pbo N = 30 Ator
3 Months N = 31 Pbo N = 31 Ator
6 Months N = 27 Pbo N = 32 Ator
9 Months N = 23 Pbo N = 28 Ator
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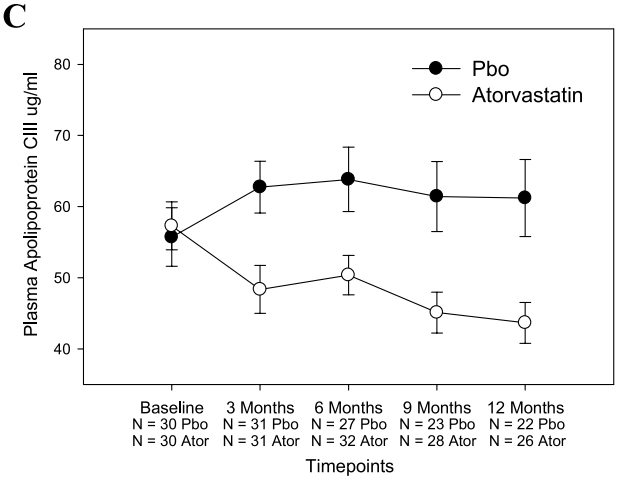
Timepoints


C
Plasma Apolipoprotein CIII ug/ml
80
70
60
50
40
Pbo
Atorvastatin
Baseline N = 30 Pbo N = 30 Ator
3 Months N = 31 Pbo N = 31 Ator
6 Months N = 27 Pbo N = 32 Ator
9 Months N = 23 Pbo N = 28 Ator
12 Months N = 22 Pbo N = 26 Ator
Timepoints

statins' anti-inflammatory effects in AD, plasma from AD subjects treated with atorvastatin was examined for changes in a subset of cytokine interleukins (See Table 2).

In most cases, atorvastatin had no effect on interleukin levels following one year of treatment in AD subjects. Use of a repeated measures statistical analysis approach suggested that IL-3 and IL-13 were significantly increased in the atorvastatin treated AD subjects compared to placebo controls. IL-13 was first described as a T-cell antigen with anti-inflammatory activities that inhibit type-1 dominated cell-mediated immune responses [162]. IL-13 is functionally related to IL-4 with some distinct activities that have been reviewed in detail. Studies have shown that IL-13 has antitumor and

Table 2 Effect on Interleukin subset

Analytes	Baseline Mean + SEM		1 yr mean + SEM	
IL-10	P	10.5 ± 0.6	P	10.9 ± 0.8
	S	9.8 ± 0.7	S	11.7 ± 0.6
IL-12p40	P	0.52 ± 0.07	P	0.46 ± 0.05
	S	0.43 ± 0.02	S	0.54 ± 0.05
IL12-p70	P	58.9 ± 16.7	P	56.1 ± 14.3
	S	40.8 ± 4.7	S	36.6 ± 4.5
IL-13[a]	P	20.0 ± 2.3	P	21.7 ± 2.4
	S	16.0 ± 1.7	S	34.1 ± 3.6
IL-16	P	782.2 ± 55.0	P	740.7 ± 59.1
	S	778.6 ± 64.8	S	855.5 ± 44.2
IL-18	P	293.1 ± 36.1	P	295.5 ± 21.6
	S	284.3 ± 25.6	S	313.5 ± 25.7
IL-1alpha	P	0.22 ± 0.01	P	0.22 ± 0.01
	S	0.20 ± 0.01	S	0.26 ± 0.01
IL-3[a]	P	0.24 ± 0.02	P	0.24 ± 0.02
	S	0.19 ± 0.02	S	0.38 ± 0.03
IL-4	P	24.3 ± 1.1	P	24.2 ± 1.6
	S	24.3 ± 2.9	S	25.9 ± 2.6
IL-5	P	14.1 ± 0.9	P	14.1 ± 0.9
	S	12.6 ± 1.0	S	14.3 ± 1.1
IL-7	P	50.5 ± 5.1	P	45.1 ± 2.8
	S	46.2 ± 4.0	S	50.9 ± 4.4
IL-8[b]	P	15.9 ± 0.9	P	15.7 ± 1.1
	S	18.9 ± 1.6	S	18.8 ± 1.5

[a] $p < 0.002$
[b] Significant difference at baseline

antiproliferative activities, but T_H1 cell-mediated host defenses are also important to mediate tumor rejection in vivo [162]. Thus, IL-13 effects are likely to be complex.

IL-3, along with IL-5 and GM-CSF are believed to be important for the differentiation and function of myeloid cells [163]. IL-3 is also important for modulating T_H1 and T_H2 immune responses and dendritic cells derived from IL-3 induction in vitro preferentially exhibit T_H2 responses. Increases in a T_H2 response may partially explain increases in IL-13 release. Thus, the effects of atorvastatin treatment on cytokines is complex and does not follow a simplistic relationship in the lowering of inflammatory endpoints. Atorvastatin appears to alter cytokines that possess both anti-inflammatory and inflammatory effects and the clinical significance is not yet obvious. A modest stimulation of inflammatory pathways may actually have some benefit by augmenting pathways involved in Aβ clearance and may serve to resolve some of the pathophysiology associated with plaque deposition.

7.4 Pentraxins

Finally, atorvastatin effects were also examined on a class of proteins known to be involved in plaque generation and complement activation, the pentraxins. The pentraxins are acute phase proteins best exemplified by the short variants, C reactive protein and serum amyloid P [113, 164]. Both are known to be closely associated with senile plaques in the brain of AD patients [113, 164]. Pentraxin expression is stimulated by local inflammatory events and pentraxins are known to activate the classical complement cascade through an antibody independent mechanism [113]. Expression analysis suggest pyramidal neurons can produce amyloid P [164]. Amyloid P is highly resistant to proteolysis and binding of serum amyloid P to proteins, like Aβ, can protect peptides from subsequent degradation. Indeed, it is possible that serum amyloid P provides the seed for plaque formation.

Samples from the ADLCT study were examined for changes in C reactive protein and serum amyloid P. There were no statistically different changes in CRP levels between the atorvastatin treated population and the placebo control group (Fig. 4a). However, CRP levels were sequentially lower with longer time on atorvastatin compared to placebo. Atorvastatin effects on serum amyloid P were quite dramatic. Serum amyloid P levels were significantly lower in atorvastatin treated group compared to placebo controls (Fig. 4b) and essentially every atorvastatin treated patient showed a decrease in amyloid P levels (data not shown), although not all atorvastatin treated patients showed clinical benefit.

Thus, atorvastatin may have effects on inflammatory endpoints independent of cholesterol lowering. Activity on serum amyloid P could result in the lowering of plaque formation and inhibition of complement activation.

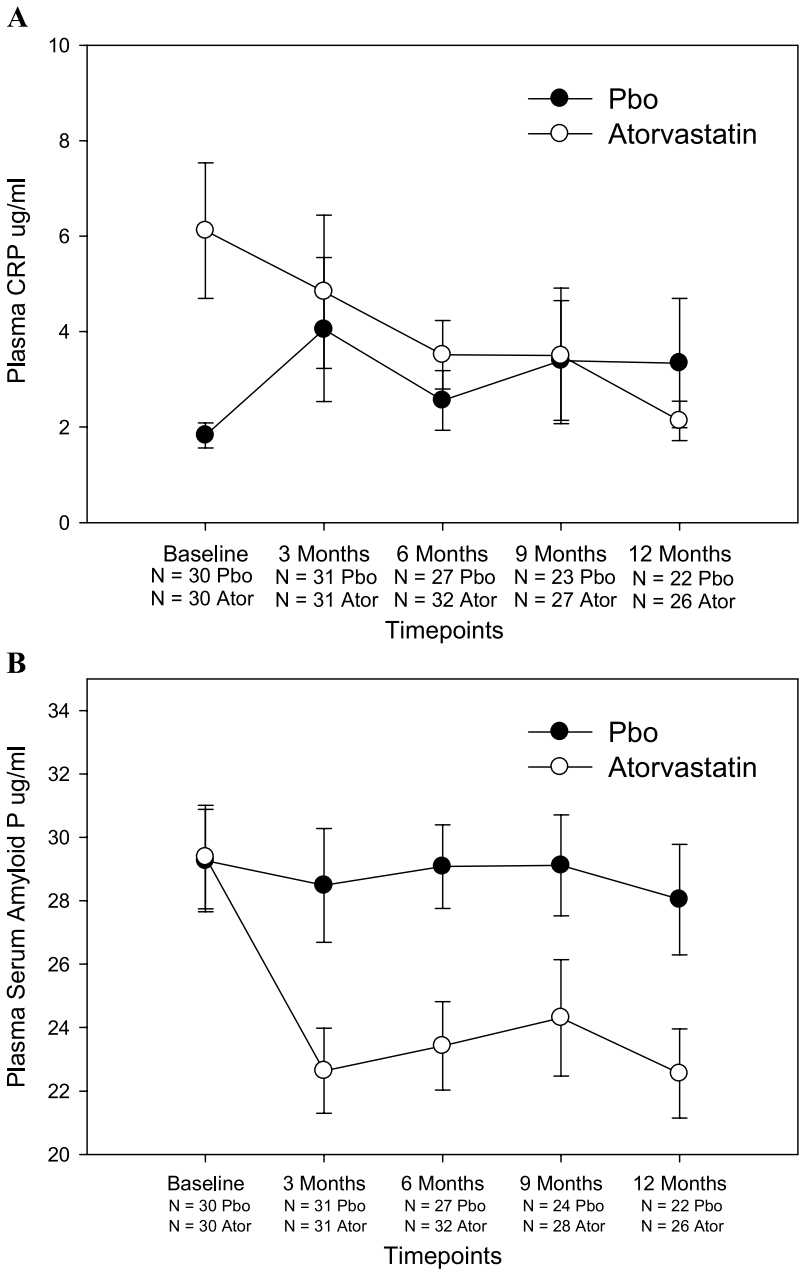


Fig. 4 Effect of atorvastatin on the pentraxins C-reactive protein (**A**) and serum amyloid P (**B**) in AD subjects. **A** There were significant baseline differences between placebo and atorvastatin treated groups and no significant treatment effects were noted. However, CRP levels did trend downwards in atorvastatin treated group. **B** Atorvastatin significantly decreased serum amyloid P levels throughout the duration of treatment

8 Summary

Despite the lack of a clear association between blood cholesterol and the risk of AD, there is a preponderance of literature to support the role of cholesterol in APP processing within the brain. Brain cholesterol metabolism differs significantly from peripheral compartments with notable use of cholesterol recycling mechanisms to account for the long half-life of brain cholesterol. Lipid particle makeup in central compartments also differ slightly from peripheral, and it is possible that use of central apolipoprotein profiles may prove to be a more sensitive measure of risk assessment in AD. A review of statin utility in the prevention of AD failed to delineate a consensus position. These findings may be due to the pleiotropic nature of statins themselves and to a significant impact of genetic backdrop. Statins do appear to be modulating cognition in at least one placebo-controlled prospective clinical study and a plausible explanation for impact on neuronal function does exist through an examination of the convergence of APP and cholesterol endocytic trafficking. Finally, biomarker studies of plasma from AD patients treated with atorvastatin show significant effects of atorvastatin on factors that are not directly related to cholesterol lowering benefit including a subset of interleukins and serum amyloid P. Additional studies are required to fully understand the range of statin-mediated effects on AD neuropathology.

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PPARγ Agonists for the Treatment of Alzheimer's Disease

Qingguang Jiang · Shweta Mandrekar · Gary Landreth (✉)

Alzheimer Research Laboratory, Department of Neurosciences,
Case Western Reserve University School of Medicine,
10900 Euclid Ave., Cleveland, OH 44106, USA
gel2@case.edu

Abstract Alzheimer's disease (AD) is a complex neurodegenerative disorder with aging, genetic and environmental factors contributing to the development and progression of the disease. The complexity of this disease presents substantial challenges for the development of new therapeutic agents. AD is typified by pathological depositions of β-amyloid peptides (Aβ) and neurofibrillary tangles within the diseased brain. AD has also been demonstrated to be associated with a significant microglia-mediated inflammatory component, dysregulated lipid homeostasis as well as regional deficits in glucose metabolism within the brain. The peroxisome proliferator-activated receptor-γ (PPARγ) is a prototypical ligand-activated nuclear receptor which coordinates lipid, glucose and energy metabolism and is found at elevated levels in the AD brain. A recently appreciated physiological function of this type of receptor is its ability to modulate inflammatory responses. Thus, PPARγ may act to modulate multiple pathophysiologic mechanisms that contribute to the disease and represents an attractive therapeutic target for the treatment of AD.

Keywords AD · Amyloid · ApoE · LXR · PPARγ

Abbreviations

ABCA1	ABC cassette transporter A1
ABCG1	ABC cassette transporter G1
AD	Alzheimer's disease
ApoE	Apolipoprotein E, ApoE
Aβ	beta amyloid
APP	β-amyloid precursor protein
BBB	blood brain barrier
CNS	central nervous system
CSF	cerebrospinal fluid
fMRI	functional magnetic resonance imaging
IDE	insulin degrading enzyme
LOAD	late onset Alzheimer's disease
LXR	liver X Receptor
LRP	low density lipoprotein receptor-like protein
NSAIDs	nonsteroidal anti-inflammatory drugs
PPAR	peroxisome proliferator-activated receptor
PGC-1	peroxisome proliferator-activated receptor gamma coactivator 1
TZD	thiazolidinedione

1 Introduction

It has now been 100 years since Dr. Alois Alzheimer's first description of a patient with presenile dementia, which we now recognize as Alzheimer's disease (AD). AD is the leading cause of dementia in the elderly with approximately 4.5 million individuals in the United States and 15 million worldwide afflicted by this disease. As a consequence of demographic trends in industrialized societies, the increasing prevalence and expanding toll of the disease present a number of significant challenges. In the intervening period we have arrived at some understanding of disease causation and pathogenesis in familial forms of AD. However, despite substantial effort, it remains unclear what causes the most common, late onset, sporadic forms of Alzheimer's disease (LOAD). More importantly, there are currently no effective therapies for either the treatment or prevention of the disease. Thus, there is some urgency in the effort to develop new therapies for AD.

In the recent past it has been recognized that the risk for AD is influenced by peripheral metabolism. For example, diabetes confers a significantly greater risk for AD, and this finding is of particular concern owing to the ongoing epidemic of type II diabetes in the developed world. Moreover, there is accumulating evidence that obesity, diet, exercise and general activity levels influence susceptibility to AD. The manner through which whole body

metabolism interacts with the central nervous system (CNS) to modulate AD risk is unclear. One of the next challenges in understanding disease pathogenesis is to establish the mechanisms through which diet and lifestyle impacts brain health and Aβ homeostasis.

This review addresses a number of controversies arising from the use of agonists of the peroxisome proliferator-activated receptor gamma (PPARγ) as therapeutic agents for treatment of AD. PPARγ is a ligand activated transcription factor. Little is known about PPARγ actions in the brain. However, in the past few years agonists of PPARγ have been shown to have salutary actions in a number of CNS disease models [1] and this has spurred investigation of the underlying biological actions of these receptors in the brain. The suggestion that PPARγ might be of utility in treatment for AD arose from consideration of its effects on insulin action, inflammation and lipid metabolism. This discussion is particularly timely as the outcomes of the first clinical trials of these drugs are being reported [2]. The mechanisms subserving their actions in AD are presently unclear. Thus, an active debate of these issues is needed as these agents are being advanced into phase III clinical trials for the treatment of AD. Several potential mechanisms have been advanced as rationales for the therapeutic use of PPARγ agonists in AD. Although each of the individual mechanisms seems plausible, there is no consensus on what is the dominant mode of PPARγ action, and it is probable that PPARγ agonists act through multiple parallel pathways to affect disease pathophysiology in humans and in animal models.

2 PPARγ

The PPARs comprise a family of ligand-activated transcription factors (type II) that belong to the nuclear receptor superfamily. The physiological roles of the PPAR subfamily of nuclear receptors are to act as lipid sensors, allowing the coupling of intake of dietary lipids to induction of genes that are responsible for their subsequent metabolism [3]. PPARγ is expressed at highest levels in fat, but operates similarly in other tissues to regulate insulin sensitivity and modulate serum glucose levels, lipid storage and metabolism [4]. The actions of this receptor on insulin sensitivity and energy metabolism in peripheral tissues are well described. The biology of these receptors in the brain is less well understood.

The natural ligands of PPARγ are long chain fatty acids, eicosanoids, oxidized lipoproteins and lipids, corresponding to its function in regulating the metabolic response to dietary lipid intake [5]. PPARγ is of particular importance as this nuclear receptor also acts to regulate both lipid and carbohydrate metabolism and participates in the regulation of serum glucose levels. Owing to these actions, PPARγ has been targeted for drug development for the treat-

ment of type II diabetes. Two thiazolidinedione (TZD) agonists of PPARγ, Actos™, (pioglitazone) and Avandia™ (rosiglitazone) are FDA approved and widely prescribed for this indication. In addition, PPARγ activation results in potent anti-inflammatory actions.

2.1 Mechanisms of PPARγ Transcriptional Regulation

PPARγ is a ligand-activated transcription factor which binds to sequence specific promoter elements of its target genes and directly regulates their

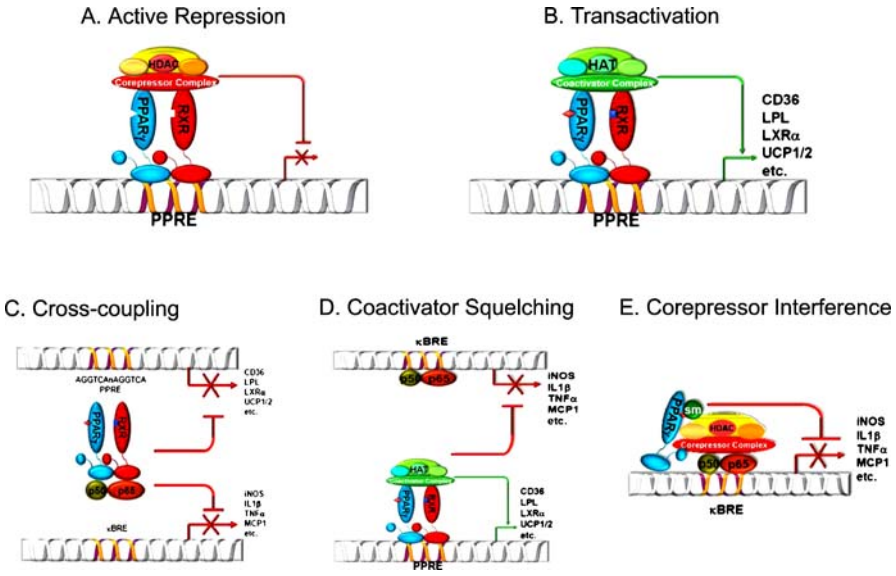

Fig. 1 Mechanisms of PPARγ transactivation and transrepression. **A** Active repression. In the absence of agonists, PPARγ/RXR heterodimer binds the PPARγ response element (PPRE, DR-1) in association with a corepressor complex and actively represses the expression of PPARγ target genes. **B** *Ligand-dependent transactivation*. In the presence of ligands, the receptor heterodimer undergoes a conformational change that results in an exchange of the corepressor complex for the coactivator complex. The coactivator complex contains multiple enzymatic activities including histone acetyltransferase (HAT) and chromatin remodeling activities. PPARγ has been postulated to inhibit the expression of inflammatory genes through several transrepression mechanisms. **C** *Cross-coupling*. A direct physical interaction between PPARγ and NFκB transcription factors mutually inhibits PPARγ and NFκB-dependent gene expression. **D** *Coactivator-squelching*. Ligand-activated PPARγ competes with NFκB transcription factors for limited amounts of coactivator complexes and thereby prevents transcriptional initiation of proinflammatory genes. **E** *Corepressor-interference*. Upon ligand-binding, PPARγ becomes sumoylated and recruited to NFκB-corepressor complex. This interaction prevents the stimulus-induced dismissal of corepressor complex from the promoters of proinflammatory genes and thereby maintains these genes in a repressed state

expression [6]. The PPAR subfamily of nuclear receptors act as dominant regulators of lipid metabolism [3]. These receptors immediately transcriptionally transactivate gene expression in response to ligands that are directly obtained from the diet or generated through normal metabolic processes, inducing genes of lipid metabolism, thus allowing the integration and coordinated regulation of whole body metabolism. In addition, they potently suppress the innate inflammatory response through a transcriptional regulatory process known as transrepression in myeloid lineage cells, such as microglia and macrophages, and in vascular cells [7, 8]. The mechanisms of PPARγ transcription regulation are summarized in Fig. 1.

2.1.1 Transactivation Mechanism

Like other type II nuclear receptors, PPARγ forms an obligate heterodimer with the retinoid X receptor. The receptor complex binds to the PPAR responsive element (DR1, AGGTCAnAGGTCA) within the promoters of its target genes [9, 10]. In the absence of ligand, the transcriptional activity of PPARγ is suppressed through its constitutive association with a nuclear corepressor complex, comprised of NCoR/SMRT and histone deacetylases. The corepressor complex maintains chromatin in a condensed state and prevents recruitment of coactivator complexes and transcriptional initiation. Upon binding of ligand to the receptor, the corepressor complex is dismissed and the transcriptional coactivator complex recruited, initiating the induction of target gene expression [11]. The coactivators, such as CBP and p300, act to drive gene expression due to their intrinsic histone acetyl transferase activity. Histone acetylation results in decondensation of chromatin and formation of a larger transcriptional complex which subsequently recruits the basal transcriptional apparatus, facilitating gene expression.

2.1.2 Transrepression Mechanisms

PPARγ agonists robustly inhibit proinflammatory gene expression and this effect underlies many of the salutary actions of these drugs. It is generally believed that the principal action of PPARγ in suppressing the inflammatory response is to functionally interfere with NFκB actions (and to a lesser extent those of AP1 and STATs) on the promoters of inflammatory genes. PPARγ inhibits proinflammatory gene expression through mechanisms that do not involve its DNA binding domain, but acts to regulate the assembly of transcriptional complexes on the promoters of these genes [7].

There have been a number of mechanisms proposed to account for the transcriptional transrepression by PPARs. PPARγ has been reported to directly interact with NFκB, forming inactive transcriptional complexes. This

mechanism, termed *cross-coupling*, results in the mutual inhibition on NFκB-dependent inflammatory gene expression, as well as the expression of PPARγ target genes. PPARγ has been shown to inhibit iNOS and COX-2 expression through a process termed *coactivator squelching*. Upon agonist binding to PPARγ, the receptor associates with transcriptional coactivators, acting to sequester the limited pools of coactivators, preventing their association with NFκB-driven promoters, thus inhibiting inflammatory gene expression [12, 13]. While each of these mechanisms remains plausible they provide an incomplete explanation for PPARγ-mediated transrepression.

Very recently, a novel mechanism, *corepressor interference*, by which PPARγ could exert its anti-inflammatory activity, was proposed. NFκB-regulated inflammatory genes are maintained in a repressed state through their association with nuclear corepressor (NCoR) complexes and upon exposure to proinflammatory stimuli this complex is dissociated and gene expression initiated. Glass and colleagues reported that the PPARγ ligand-binding domain is sumoylated upon ligand-binding. This modified receptor then binds to NCoR containing complexes that are resident on the promoters of NFκB-regulated genes. This interaction prevents the dismissal of the corepressor complex and thus suppresses NFκB-dependent inflammatory gene expression [14].

3 Alzheimer's Disease and PPARγ

3.1 Pathology

Alzheimer first documented the presence of extracellular senile plaques throughout the cortex and hippocampus of the AD brain and this is the most prominent pathological feature of the disease. He also described intracellular neurofibrillary tangles. The succeeding century of work has been centered on establishing the pathophysiology leading to development of these characteristic anatomic hallmarks of the disease.

3.1.1 Amyloid Pathology

Amyloid plaques are extracellular deposits of Aβ peptides which can be most readily visualized in autopsy sections stained with silver impregnation techniques or upon staining with the lipophilic dye Congo Red or thioflavine-S. Similar staining can often be observed in the vessel walls of capillaries or larger blood vessels, a pathological feature known as cerebral amyloid angiopathy that accompanies AD. Plaques are generally found throughout the

cerebral cortex and the hippocampus. In the mid-1980s, β-amyloid (Aβ), a 4 kD peptide, was identified as the main constituent of cerebrovascular amyloid in both AD and Down's syndrome. These studies led to subsequent successful cloning of the gene encoding the β-amyloid precursor protein (APP). As predicted, the APP gene was mapped to chromosome 21 and encodes a type I transmembrane glycoprotein which has a large ectodomain, a single transmembrane domain and a short cytoplasmic tail. The main components of senile plaques, Aβ peptides (either 40 or 42 amino acids long) are generated from APP by sequential proteolytic cleavage by β-secretase and γ-secretase. The Aβ peptides accumulate, form fibrils and are then deposited within the brain. Genetic studies of familial forms of the disease led to the discovery of mutations within the amyloid precursor protein (APP) and the presenilin genes that are linked to AD [15]. It is now clear that the majority of mutations found in the APP gene that are linked to inherited forms of AD are clustered in the vicinity of the cleavage sites of β- and γ-secretases. The most common mutations leading to AD are in the presenilin genes, which are essential components of the γ-secretase complex. Although the exact process leading to the eventual neuronal loss remains unknown, the genetic evidence supports that dysregulated Aβ homeostasis is the principal cause of AD.

PPARγ involvement in ameliorating AD-related pathology has been the focus of a number of recent studies directed at dissecting the mechanisms through which PPARγ regulates Aβ production and metabolism. These studies have led to contradictory results. One mechanism through which PPARγ is able to accomplish this is by regulating Aβ production. When neuroblastoma cells (stably transfected with amyloid precursor protein) were stimulated with inflammatory cytokines, they activated the APP processing enzyme BACE1. This resulted in an increase in the secretion of Aβ, which was inhibited following activation of PPARγ and its suppression of BACE1 transcription [16]. Upon inspection, a PPARγ response element was discovered in the promoter region of the BACE1 gene, and binding of PPARγ to this response element suppresses its transcription and subsequently results in reduced BACE1 levels and a subsequent inhibition of Aβ production [17].

In addition, a study by d'Abramo reported that the activation of this nuclear receptor was able to inhibit the expression of APP. They demonstrated that overexpression of PPARγ in cultured cells decreased Aβ secretion by promoting APP degradation. They demonstrated that PPARγ activation resulted in increased ubiquitination of APP, leading to its subsequent degradation [18].

Camacho et al. have reported an alternative mechanism by which PPARs may be affecting Aβ homeostasis [19]. They have shown that activation of endogenous PPARγ or overexpression of the receptor led to the dramatically enhanced clearance of Aβ from the media of both neuronal and non-neuronal cells. The mechanism subserving this effect is unknown.

3.1.2 Neurofibrillary Tangles

Neurofibrillary tangles are found within neurons of the cerebral cortex and hippocampus and consist of insoluble intracellular fibrils that are comprised of hyperphosphorylated forms of *tau*, a microtubule-associated protein [20, 21]. The microtubules are essential for axonal transport and the structural stability of neuronal processes. Therefore, it is believed that impaired axonal transport contributes to neuronal degeneration that typifies the disease. Neurofibrillary tangles are not unique to Alzheimer's disease and can be found in a variety of other neurologic disorders.

A recent study reported by d'Abramo and colleagues has shown that the PPARγ agonist troglitazone, was able to significantly reduce the phosphorylation of *tau* [22]. However, these effects of this PPARγ ligand were independent of the transcriptional activity of the receptor and represent an off-target effect of this TZD.

3.2 Pathophysiology

Clinical symptoms of AD include cognitive impairment, memory loss and disorientation. Increasing evidence suggests that risk for AD can be influenced by life style choices such as diet and exercise that directly affect metabolism. Moreover, concurrent metabolic diseases such as diabetes, metabolic syndrome, atherosclerosis and other types of cardiovascular disease are associated with an increased risk for AD [23, 24]. Thus, the physiological status of peripheral organ systems impacts the central nervous system and affects biological processes that are critical to the pathogenesis of AD. The relationships between insulin resistance, inflammation, cholesterol homeostasis and PPARγ in AD are summarized in Fig. 2.

3.2.1 Dyslipidemia

Genetic studies and epidemiological observations strongly suggest a relationship between dyslipidemia and AD. Mid-life high serum cholesterol levels have been reported to correlate with an increased incidence for AD [25, 26]. A series of epidemiological studies have demonstrated that patients receiving a class of lipid-lowering agents, 3-hydroxy-3-methyglutaryl coenzyme A reductase inhibitors (also known as statins), for treatment of hyperlipidemia have a greatly reduced risk ($\sim$70%) for developing AD [27]. Several genes regulating cholesterol homeostasis have been reported to be associated with AD, including *Apoe, Lrp1, Abca1, Lxrβ, Cyp46, Acat* and *Cetp* [28, 29].

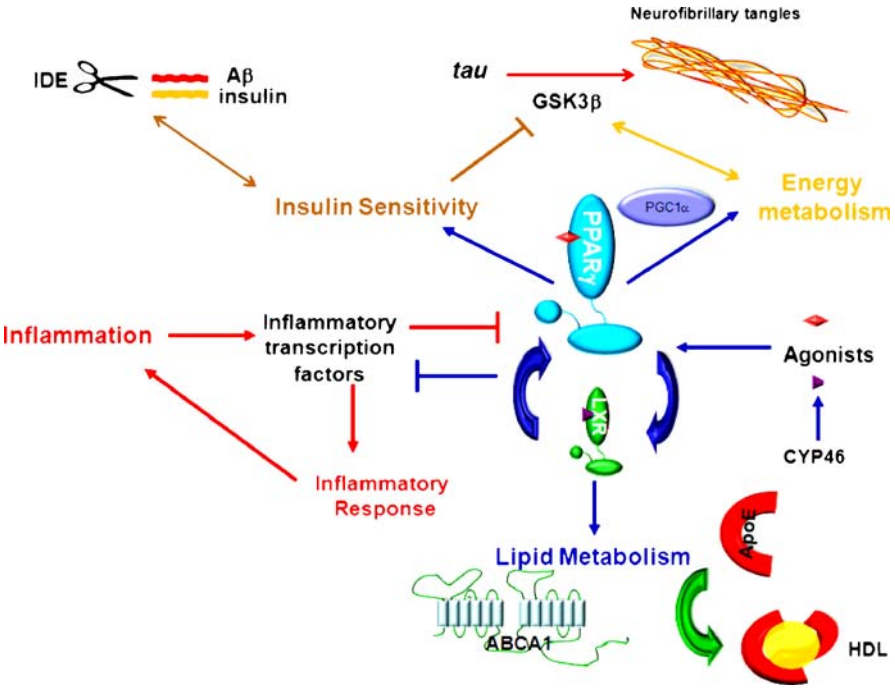

Fig. 2 PPARγ as an integrator of metabolism and immunity and its implication in AD

The *Apoe* gene is the best known *bona fide* risk factor for sporadic AD [28]. ApoE plays an important role in lipid transport throughout the body. It participates in plasma lipoprotein metabolism, cholesterol homeostasis and local lipid transport processes. ApoE is produced by various cell types, including liver, kidney, fat cells and macrophages. In the brain, it is primarily synthesized and secreted within HDL particles by astrocytes and mediates the efficient transport and recycling of cholesterol within the central nervous system [30–32]. In humans, three naturally occurring variants of *Apoe*, E2 (Cys_{112}/Cys_{158}), E3 (Cys_{112}/Arg_{158}) and E4 (Arg_{112}/Arg_{158}) have been demonstrated to modify the risk or age of onset of LOAD. The *apoE4* allele dose-dependently increases the risk while E2 allele reduces the risk. *Apoe3* is the most common form, representing ~78% of the population, whereas *Apoe4* represents 15% and *Apoe2* represents 7%. The increased risk attributable to *Apoe4* is estimated to be about 20–30%. Although the precise mechanism by which *Apoe4* modifies the risk remains unknown, it appears that *Apoe4* exhibits a similar effect in elevating Aβ levels, favoring the deposition of Aβ peptides within the brain.

These genetic linkages in early onset AD have not provided critical insight into the biological basis of LOAD. The only accepted risk factor for LOAD is possession of an E4 allele of the *Apoe* gene [15]. The available evidence

suggests that ApoE plays important roles in the ability of the brain to clear Aβ peptides. There is no clear understanding of the mechanisms that underlie the increased risk of AD associated with the expression of *Apoe4* allele, but it is likely to involve its lipid transport function. Indeed, there are newly recognized linkages of AD pathogenesis and risk to lipid metabolism of the brain [28].

Neurons rely upon a ready supply of cholesterol for maintaining a broad array of physiological functions such as membrane synthesis, myelin maintenance, electrical signal transduction, synaptic transmission, and plasticity. Cholesterol metabolism in the CNS is unique compared with the rest of the body. Because of the existence of the blood-brain barrier (BBB), almost all the sterol required for new membranes comes from de novo synthesis within the CNS [33]. In addition, the brain has evolved highly efficient mechanisms to maximize the utilization of cholesterol. Unlike other membrane lipid components, cholesterol cannot be synthesized at neuronal terminals. Therefore, synaptic function depends largely on cholesterol supplied from either axonal transport from the cell body and or uptake of lipidated ApoE produced by astroglia via neuronal lipoprotein receptors.

Neurons take up ApoE-containing HDL particles largely via the ApoE receptor, low-density lipoprotein receptor-related protein 1 (LRP1). LRP is highly expressed in the CNS. Internalization of ApoE-containing lipoprotein particles via LRP affects neuronal membrane remodeling. *Lrp1* maps to chromosome 12q13, a region that has been reported to show association with LOAD [34–36], although this remains controversial [37, 38]. LRP has been shown to mediate the clearance of soluble Aβ through a receptor-mediated uptake mechanism [39]. Loss of receptor-associated protein (RAP) exacerbates the development of amyloid deposits in APP transgenic mice [40]. Deane et al. recently reported that LRP mediated the clearance of soluble Aβ across the brain capillary endothelium into the peripheral circulation through transcytosis [41].

The lipidation of ApoE is carried out primarily by the ABC cassette transporter ABCA1. ABCA1 mediates loading of ApoE with phospholipid and cholesterol. The apolipoprotein acts as a structural scaffold for the formation of HDL particles. Mutations in the *Abca1* gene cause Tangier's disease, a rare autosomal recessive disease characterized by severe HDL deficiency and a defect in cellular cholesterol efflux. ABCA1 acts to regulate ApoE function in the CNS [30, 31]. Recently, three independent groups reported that, in four different animal models of AD, inactivation of the *Abca1* gene facilitates Aβ fibrogenesis and deposition within the brain [42–44]. Importantly, the expression of the *Abca1* gene is directly regulated by the nuclear receptors, LXRs, PPARγ and RXR. The *Lxr*β gene has recently been reported to be genetically associated with AD [45].

Because the rate of cholesterol synthesis exceeds the actual rate of consumption, the brain has evolved a unique mechanism to export excess

cholesterol which requires *Cyp46*. *Cyp46* encodes the brain specific cholesterol 24-hydroxylase and catalyzes the conversion of cholesterol into 24-S-hydroxycholesterol, a more hydrophilic molecule which is readily capable of crossing the BBB through diffusion [33]. *Cyp46* is exclusively expressed in neurons, particularly in a subset of large, metabolically active neurons such as cortical pyramidal cells and Purkinje cells in the cerebellum. Importantly, serum and cerebrospinal fluid (CSF) 24S-hydroxycholesterol are increased in early phases of AD [46], possibly reflecting increased brain cholesterol synthesis or turnover during neurodegeneration. An intronic *Cyp46* polymorphism has been reported by several independent groups to be associated with increased risk of AD and shows synergism with *Apoe4* allele [47], although this linkage is controversial [48, 49]. Intriguingly, Johansson et al. reported that *Cyp46A1* variants may interact with ApoE to influence Aβ_{42} levels in AD [50]. Importantly, the product of cholesterol 24-hydroxylase, 24S-hydroxycholesterol, can activate LXRs and induce the expression of LXR target genes, including *Apoe* and *Abca1* [51].

Other lines of evidence also support the notion that deregulated cholesterol homeostasis may contribute to the AD pathogenesis. AD patients have been reported to develop intracellular Aβ accumulation in the late endosomes and lysosomes. Similar pathological features, including swollen late endosomes and Aβ accumulation, have also been reported in Niemann–Pick type C disease patients [52, 53], *Npc1* deficient mice as well as in mouse models of AD [54, 55]. The *Npc1* gene product is essential for the mobilization of cellular cholesterol. Excess cholesterol can be transported into endoplasmic reticulum and esterified by acyl coenzyme A : cholesterol acyltransferase (ACAT) and stored in lipid droplets. Inhibition of ACAT activity has been reported to reduce Aβ levels in vitro and plaque pathology in animal models of AD [56, 57].

PPARγ agonists have been shown to lower circulating levels of triglycerides, cholesterol and nonesterified fatty acids in human and animal models of dyslipidemia [3]. Individuals with dominant negative mutations of *Pparγ* gene exhibit hypertriglyceridemia and low HDL cholesterol [58]. In adipocytes and macrophages, PPARγ activation leads to induction of a large number of genes, most prominently those associated with lipid metabolism, including CD36, lipoprotein lipase and liver X receptor α (LXRα), among others [9, 59]. Almost nothing is known about PPARγ regulation of CNS lipid metabolism.

A significant subset of the actions of PPARγ activation arises from its ability to induce the expression of a related nuclear receptor, Liver X receptor α (LXRα). LXRs regulate genes that are involved in lipid metabolism and reverse cholesterol transport, including *Apoe* and *Abca1*, among others. PPARγ agonists have been shown to increase both ABCA1 and ApoE mRNA and protein levels and this effect is believed to be secondary to its induction of LXRα expression [60–62]. Significantly, it has recently been reported that rosiglita-

zone treatment of mice expressing the human *Apoe4* allele induces a two-fold increase in brain ApoE4 mRNA [63].

It has been demonstrated that there is a reciprocal positive regulation of both receptors which comprises a feed forward mechanism to orchestrate the expression of both classes of receptors. PPARγ activation stimulates LXRα expression and visa versa. Recent evidence suggests that this results in synergistic induction of both PPARγ and LXR target gene expression [64]. Significantly, genetic variants of *Abca1, Nr1h2* (LXRβ) and *Cyp46* (24S-cholesterol hydroxylase) have been proposed to be associated with an increased risk for AD [45, 47, 65, 66].

In the brain, ABCA1 is responsible for the cellular efflux of phospholipids and cholesterol by transferring these lipid species to ApoE [30, 31]. The lipidation status of ApoE is an important determinant in governing AD pathogenesis as evidenced by the observations that knockout of the *Abca1* gene results in higher plaque burden and elevated brain Aβ peptide levels [42–44]. It was reported that certain genetic variants of *Abca1* modify the risk or onset of AD [29, 65], although other studies failed to identify the same association [67].

3.2.2 Inflammation

Neuroinflammation has been postulated to play a role in AD pathogenesis [68]. A number of inflammatory cytokines, chemokines and activated glial markers have been found at elevated levels in the AD brain [69–73]. The formation of Aβ fibrils and their deposition in the parenchyma of the brain elicit a response from microglia, the brain's tissue macrophages. The focal deposits of Aβ are closely associated with a significant microglial-mediated inflammatory response [74]. Indeed, persistent activation of abundant plaque-associated microglia typifies the human disease and its murine models. Microglia bind Aβ fibrils through a complex of cell surface receptors which serves to activate tyrosine-kinase based intracellular signaling cascades [75]. The activation of Aβ-stimulated signaling pathways mediates the acquisition of a reactive, proinflammatory phenotype accompanied by the elaboration of a wide range of proinflammatory cytokines, chemokines, acute phase proteins, as well as reactive nitrogen and oxygen species. The chronic activation of microglia and their attendant production of proinflammatory molecules is postulated to exacerbate and accelerate the disease progress, culminating in neuronal death. In addition to microglia, several other cell types in the brain are also responsive to Aβ, including astrocytes [76, 77] and endothelial cells [78].

Inflammation has been proposed to accelerate amyloid deposition and disease progression, whereas anti-inflammatory therapies inhibit Aβ generation and slow disease progress. A number of retrospective epidemiological studies revealed that long-term use of nonsteroidal anti-inflammatory drugs

(NSAIDs) decreased the incidence of AD [79]. Several recent prospective NSAIDs clinical trials have failed to demonstrate a salutary effect of these drugs and have been interpreted as evidence that a generic anti-inflammatory strategy is not effective. However, these studies have been criticized on the basis that the epidemiological data support a trial design that involved sustained treatment during the prodromal stages of the disease. Alternatively, it has been argued that a subset of NSAIDs may be effective in slowing the disease progress through mechanisms unrelated to their anti-inflammatory action [80–83]. The effect of NSAID on AD risk are particularly relevant owing to the observation that some NSAIDs bind to and activate PPARγ [84]. Indeed, these findings provided the rationale that led to the initial studies of PPARγ action in in vitro AD models [85] and in animals overexpressing APP (see below) [86, 87]. From a more contemporary perspective, it is not clear whether NSAIDs are present in the brain at sufficient concentrations to activate endogenous PPARγ at levels that are biologically meaningful.

The role of PPARγ in regulating the microglial inflammatory responses has recently been expertly reviewed by Bernardo and Minghetti [88]. The anti-inflammatory actions of PPARγ agonists are likely to account for their positive effects in a number of animal models of CNS disease [1]. There is extensive and compelling evidence that PPARγ agonists robustly suppress proinflammatory gene expression. There are now over two dozen studies showing that microglia activation in response to inflammatory stimuli, including fibrillar Aβ, is sensitive to receptor agonists. In many of these studies these effects have been shown to be dependent on PPARγ. This is significant as some anti-inflammatory effects of the synthetic ligands might have been mediated through off-target effects of the drugs [89]. PPARγ agonists have been reported to inhibit the expression of inflammatory cytokines, chemokines, MMPs, COX-2, iNOS, each of which is reliant upon NFκB-dependent transcriptional effects [7].

Significantly, inflammation and lipid metabolism exhibit close functional interrelationships and are subject to coordinate, reciprocal regulation. PPARγ and LXRs have been reported to reciprocally regulate genes involved in both immunity and lipid metabolism [6, 90]. While the primary focus of the action of PPARγ in inflammation has focused on receptor-mediated inhibition of inflammatory gene expression, there is a reciprocal effect of inflammation on nuclear hormone expression. Feingold and colleagues have extensively examined the inflammation-mediated suppression of PPARγ and RXR expression [91].

3.2.3 Energy Metabolism

The advent of new imaging technologies has allowed the analysis of brain energy metabolism. A number of studies have now documented that glucose

utilization is impaired in brain regions involved in memory and cognition in AD patients. Significantly, familial AD patients exhibited impaired cerebral glucose metabolism in advance of symptomatic onset and in the absence of detectable structural changes in the brain [92]. It is now possible to employ positron emission tomography (PET) and functional magnetic resonance imaging (fMRI) to evaluate glucose metabolism as an early biomarker for AD. Additionally, several independent studies have shown a link between the *Apoe4* allele and glucose utilization in the brain. These studies detect an *Apoe4* dose-dependent impairment in glucose utilization in brain regions affected by the disease [93–97]. Bookheimer and colleagues have found, using fMRI, that memory recall tasks stimulated glucose utilization in regions affected by Alzheimer's disease and this effect was more pronounced in *ApoeE4* carriers in comparison to those who expressed the *Apoe3* allele [98].

PPARγ plays critical roles in energy metabolism due to its direct effects on mitochondrial function and ultimately ATP production. Mitochondria may be key players in cerebral hypometabolism observed in AD, as this organelle plays critical roles in both energy metabolism as well as neuronal apoptosis. In the diseased brain, the numbers of neuronal mitochondria are greatly reduced and those remaining have very distinct morphological changes in their size and the number or cristae they contain. These morphological changes are seen mainly in neurons that have lost their dendritic arborization [99]. Therefore, therapeutic strategies that aim at maintaining mitochondrial integrity are of importance.

PPARγ activation by its agonist pioglitazone resulted in a significant increase in mitochondrial DNA copy number as well as the expression of genes that are involved in mitochondrial biogenesis in fat tissue [100]. A recent study has found analogous changes within the brain in response to oral rosiglitazone treatment [63]. Indeed, PPARγ activation stimulated brain mitochondrial biogenesis and this stimulation was dependent on the ApoE isoform [63]. PPARγ may elicit these changes through the peroxisome proliferator-activated receptor gamma coactivator 1 (PGC-1) family of proteins, these coactivators positively regulate mitochondrial function and oxidative metabolism [101]. PPARγ has been reported to stimulate the expression of PGC-1α [102], which in turn, induces expression of the uncoupling proteins which stimulate mitochondrial biogenesis and respiration in muscle cells [103–105]. PGC-1 also stimulates the expression of a variety of genes that are vital for the oxidative phosphorylation pathway as well as duplication of mitochondrial DNA content [106, 107].

Significantly, PGC-1α knockout mice also show lesions in the brain regions affected in Alzheimer's disease [106]. It should be noted that TZD agonists of PPARγ have a number of effects on mitochondrial metabolism, many of which are receptor-independent actions of the drugs [108]. Roses et al. have postulated that the PPAR agonists act to improve mitochondrial function and

this may be the basis of their beneficial effects on memory and cognition in AD patients [109].

3.2.4 Insulin Sensitivity

A number of studies have suggested that perturbation of insulin metabolism is associated with AD [24, 110–113]. In the past decade, large studies have found that individuals with Type 2 diabetes are twice as likely to develop AD when compared to gender matched healthy subjects [114–119]. Insulin resistance and hyperinsulinemia, two characteristics of type II diabetes, have been shown to have a high correlation with memory impairment and risk for AD. There is substantial evidence establishing a role of insulin in cognition. Insulin can pass the blood-brain barrier and enter the CNS via a receptor-mediated transport process. Insulin receptors in the mammalian brain have a very specific pattern of expression and are localized to the hippocampus and medial temporal cortex, both are areas associated with memory. In addition, brain insulin receptor signaling has been reported to be significantly reduced in AD, an indication of insulin resistance [120, 121]. Furthermore, an acute increase in peripheral insulin concentrations results in the rapid elevation of CSF and brain insulin levels [122]. Craft and colleagues have found that insulin administration improves memory [123] and this is accompanied by increases in plasma and brain levels of Aβ peptides and inflammatory markers [124]. Fehm and colleagues have shown that insulin administered intranasally has been shown to be transported to the hippocampus and hypothalamus and results in an increase in memory performance in humans and rodents. Ho et al. reported that diet-induced insulin resistance enhanced the production of both Aβ_{40} and Aβ_{42} and plaque formation by increasing the activity of γ-secretase and decreasing the activity of insulin-degrading enzyme (IDE) [125]. It should be noted that hyperinsulinemia is also associated with elevated levels of inflammation in the brain [126].

It has been postulated that hyperinsulinemia in the brain may contribute to amyloid build up due to inhibition of Aβ degradation by IDE. IDE degrades both insulin and Aβ peptides [127, 128]. IDE levels have been shown to be reduced in the brains of AD patients and are further reduced by 50% in brains of AD patients homozygous for the *Apoe4* allele [129]. Farris et al. have shown that a normally occurring polymorphism in IDE that can induce diabetes is sufficient to alter Aβ degradation [130]. Genetic ablation or partial loss of *Ide* by either mutations or alternative splicing leads to hyperinsulinemia and elevated Aβ levels simultaneously [130–132]. In addition, Aβ degradation by IDE was competitively inhibited by insulin. Therefore, impaired IDE functions or elevated basal insulin levels may hinder Aβ clearance and/or glucose metabolism, thereby initiating or accelerating the disease process. Recently,

rosiglitazone has been shown to increase brain IDE levels in an animal model of AD [133]. Thus, *Ide* could potentially be responsible for the association between hyperininsulinemia and AD. However, whether this relationship is physiologically relevant remains controversial owing to low levels of insulin in the brain.

It has been argued that one action of insulin in the brain is to influence the activity of the protein kinase, glycogen synthase kinase 3β (GSK3β). GSK3β is the principal protein kinase that phosphorylates *tau*, resulting in the formation of neurofibrillary tangles. Its activity is also essential for efficient glucose utilization in response to insulin. Significantly, neuronal insulin resistance resulting from inactivation of the insulin receptor is associated with *tau* hyperphosphorylation. It has been argued that hyperinsulinemia or decreased insulin sensitivity could cause elevation of GSK3β activity and the subsequent hyperphosphorylation of *tau*.

PPARγ agonists have been widely prescribed for the treatment of Type II diabetes mellitus. Although extensive body of evidence suggests a critical role for PPARγ in the regulation of insulin sensitivity, the mechanisms underlying this action remain elusive. The ability to activate PPARγ is closely correlated with the antidiabetic actions of TZDs, suggesting that receptor-dependent activation of PPARγ target genes may underlie the insulin sensitizing effect of this class of drugs. Resistance of peripheral tissues to insulin is the primary characteristic of type II diabetes and occurs fairly early in the disease progression. TZDs ameliorate this pathology by lowering circulating levels of triglycerides as well as free fatty acids. It does this through the induction of genes that promote the storage of fatty acids in adipocytes while repressing those that aid in their release [134, 135]. One target gene that is central to improving muscle glucose disposal is the GLUT4 insulin-dependent glucose transporter. GLUT4 is expressed in the CNS and analogous actions may occur in the brain [136–138]. GLUT4 mediates insulin-stimulated glucose uptake in these tissues following translocation from intracellular storage sites to the plasma membrane. PPARγ activation also influences insulin signalling upstream of GLUT4. PPARγ activation upregulates the expression of the insulin signaling intermediates IRS-1 and -2, facilitating transduction of insulin action [139, 140].

Adipocytes have been shown to be a source of adipokines, especially, adiponectin and resistin. Adiponectin lowers serum glucose and enhances glucose utilization in skeletal muscle and thereby prevents insulin resistance [141]. Resistin has been shown to induce glucose intolerance. Importantly, both adiponectin and resistin have been shown to be regulated by PPARγ [142, 143]. PPARγ agonists increase adiponectin gene expression and circulating adiponectin levels while individuals with dominant negative mutations of PPARγ exhibit dramatically lower circulating adiponectin levels [144, 145]. PPARγ agonist administration to mice resulted in a modest elevation of brain adiponectin levels [63].

4
PPARγ Agonist Therapy in AD

4.1
Preclinical Studies of PPARγ Agonists in Animal Models of AD

There have been four studies investigating the effects of PPARγ agonists in murine models of AD. The first of these studies investigated the effects of pioglitazone in Tg2576 mice that overexpress a mutant form of APP [86]. This study employed 12-month-old Tg2576 mice with established plaque pathology which were then treated with the drug for 4 months. These animals were treated with oral pioglitazone at a dosage of 120 ppm (20 mg/kg/day) in standard chow. Pioglitazone was used in these studies due to its ability to pass the blood-brain barrier, although it does so poorly [146]. A second group of animals was treated with the NSAID ibuprofen as a positive control, since Lim and colleagues had shown plaque reduction in this animal model following oral ibuprofen therapy [147]. Indeed, ibuprofen was shown to reduce plaque burden by 60% which was largely due to a decrease in plaque number. Pioglitazone-treated animals did not exhibit a statistically significant change in plaque pathology. Both ibuprofen and pioglitazone treatment resulted in a small, but significant, reduction in soluble Aβ_{40} peptide levels. Ibuprofen treated animals exhibited a dramatic reduction in soluble Aβ_{42} levels. While soluble Aβ_{42} levels in pioglitazone-treated animals were lower, they did not reach statistical significance. Both ibuprofen and pioglitazone have anti-inflammatory actions and their effects on markers of microglial activation were evaluated. Ibuprofen treatment resulted in an approximate 50% decrease in the numbers of CD45 and CD11b positive, activated microglia. Pioglitazone had no effect on the expression of these markers. This experiment served to show that pioglitazone exhibited effects on soluble Aβ levels in the brain. However, this drug did not significantly affect AD related pathology or microglia-mediated inflammation. These findings were interpreted as evidence that the poor penetrance of pioglitazone into the brain as a result of its poor BBB permeability limited its efficacy.

This report was followed by a study of similar design but using a higher dose of pioglitazone (240 ppm; 40 mg/kg/day) in younger animals overexpressing the APP V717I mutation at 10 months of age. These animals were just beginning to exhibit plaque pathology. In these experiments the mice received an acute drug treatment for 7 days. Ibuprofen-treated animals comprised the controls for this study. Heneka and colleagues observed that treatment with the higher dosage of pioglitazone or ibuprofen resulted in reduced Aβ plaque burden. Pioglitazone treatment resulted in a 20% reduction in Aβ_{42} levels (but not Aβ_{40}) in the brain [148]. The brains of these animals exhibited reduced numbers of activated microglia as reflected in lower levels of several inflammatory markers. This study provided a clear demonstration of

a positive effect of pioglitazone on AD pathology. Moreover, this study is particularly valuable as it illustrated the limitations imposed by the poor BBB permeability of pioglitazone. Importantly, PPARγ agonists were shown to act to suppress the generation of AD-related pathophysiology and can do so over short intervals.

The effect of rosiglitazone in the Tg2576 model of AD was investigated by Pedersen and colleagues. These authors had previously demonstrated that Tg2576 mice exhibit several metabolic abnormalities, including increased fasting serum glucocorticoid levels and insulin insensitivity. Rosiglitazone treatment for 6 weeks restored insulin responsiveness in these mice [149] and lowered glucocorticoid levels. They found that insulin levels in 8-month-old Tg2576 mice were lower than normal, however, as the animals aged insulin levels increased and they became hyperinsulinemic by 13 months of age. This latter effect was corrected with rosiglitazone therapy. The basis for these transgene-related peripheral physiologic effects is unexplained. These authors extended these studies to examine whether rosiglitazone therapy would affect age-related behavioral impairment in the Tg2576 mice [150]. They found that 4 months of treatment with oral rosiglitazone reversed the impairment in spatial and reference memory tasks observed in the transgenic mice. In these tasks the mice are fasted overnight and exhibit elevated glucocorticoid levels at the time of testing. The glucocorticoids have well-described negative effects on learning and memory. Indeed, high corticosterone levels have previously been shown to have an effect on cognition and memory and glucocorticoids have been shown to have a role in insulin receptor desensitization [151–153]. These authors hypothesized that the impaired behavioral performance could be attributed to the elevated serum corticosterone levels. Indeed, treatment of the mice with metyrapone, an inhibitor of glucocorticoid production also improved their behavior. Rosiglitazone treatment was found to normalize serum glucocorticoid levels and they postulated that this was the basis of the better behavioral performance. This study presents a novel perspective on the action of this drug because one of the major concerns of PPARγ agonists is their site of drug action and these experiments suggest that rosiglitazone could elicit its effects by acting in the periphery, and not within the CNS.

The treatment of 9-month-old Tg2576 mice with rosiglitazone for 7 months did not result in any changes in Aβ plaque pathology. However, drug treatment was associated with a selective reduction in brain Aβ_{42} levels, but Aβ_{40} levels were unchanged. The authors reported that Tg2576 mice exhibit a selective decrease of IDE mRNA levels in the hippocampus. However, this was not correlated with any change in enzyme activity. Conversely, despite normal IDE RNA levels in the frontal cortex, they found lower IDE activity. Rosiglitazone treatment normalized IDE activity only in the frontal cortex and the authors argue that restoration of IDE activity may account for the lower Aβ_{42} levels. Glucocorticoid administration has also been shown to increase cellular

APP levels as well as BACE expression, augmenting Aβ formation as well as accelerating the formation of neurofibrillary tangles [154]. AD patients also exhibit elevated levels of plasma cortisol. Furthermore, a study carried out in macaques demonstrated that year-long administration of cortisol was correlated with a reduction in IDE protein and mRNA levels in the frontal cortex and hippocampus and a selective increase in the levels of Aβ_{42} levels without affecting overall plaque burden suggesting a correlation between high glucocorticoid levels and IDE and AD pathophysiology [155]. The hypothesis that rosiglitazone's actions in the Tg2576 mice arise from its effects on peripheral glucocorticoid levels is compelling. PPARγ agonists are not reported to have analogous effects on glucocorticoids in humans and thus the relevance of this mechanism to AD therapy is unclear.

In summary, the animal studies of PPARγ agonists provide clear evidence that these drugs can affect AD-related changes in Aβ homeostasis and ameliorate the behavioral impairment observed in these animals. There are significant differences in the Aβ species that are subject to regulation by these drugs. However, the data demonstrate that PPARγ agonists can act both within the brain and in the periphery to affect processes related to disease pathogenesis.

4.2 Clinical Trials

The availability of FDA-approved PPARγ agonists has allowed the rapid translation of their use into clinical studies with AD patients. The outcomes of two pilot clinical trials have recently been reported. Watson et al. (2005) have reported the results of a small clinical study examining the effects of rosiglitazone in patients with mild AD. They found that 6 months of drug treatment resulted in enhanced memory and cognitive function compared to placebo-treated control patients [156]. Geldmacher and colleagues have recently completed a small study of the actions of pioglitazone in mild to moderate AD cases [157]. This study, designed primarily as a safety study, demonstrated a small, but statistically insignificant, improvement in memory. The clinical usage of PPARγ agonists is associated with increased adiposity, edema, and other less frequent side effects. These drugs are generally well tolerated and safe in the elderly [158].

A large phase II clinical trial of rosiglitazone was conducted by GlaxoSmithKline involving over 500 patients [2]. This placebo-controlled study treated patients with mild to moderate AD with rosiglitazone for 6 months. Drug therapy was associated with an enhancement of attention and memory. Importantly, the efficacy of drug treatment was related to *Apoe* genotype. Individuals with an *Apoe4* allele have significantly increased risk for AD. These patients did not respond to the drug, whereas those patients possessing only *Apoe2* or *Apoe3* alleles demonstrated significant functional improve-

ment. It is presently unclear why *Apoe4*-positive patients failed to respond to the drug. However, the outcome of this clinical trial is consistent with previous findings with respect to the influence of the *Apoe4* genotype on insulin action [112, 159, 160].The authors postulate that rosiglitazone acts on brain mitochondria [2]. A large phase III trial of rosiglitazone in AD patients is currently underway.

5 Conclusions

There is considerable excitement surrounding the initial success of clinical trials of PPARγ agonists in treatment of AD. The complex biology of this receptor and our poor understanding of their actions in the brain present a number of challenges in understanding its modes of action. It seems probable that PPARγ works through multiple mechanisms to elicit its effects. Remarkably, there is almost nothing known about PPARγ action in neurons and this is an area that needs to be investigated. The capacity of this receptor to act to coordinate lipid and carbohydrate metabolism suggests mechanistic linkages to peripheral organ systems. The association of diabetes, dyslipidemia and metabolic syndrome with increased risk for AD underscores the potential roles PPARγ might play in treatment of AD and emphasizes the importance of our understanding of AD within the larger context of metabolism.

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Metal Complexing Agents for the Treatment of Alzheimer's Disease

Anthony R. White[1] (✉) · Ashley I. Bush[2]

[1]Department of Pathology, The University of Melbourne, 3010 Victoria, Australia
arwhite@unimelb.edu.au

[2]The Mental Health Research Institute, 155 Oak Street, 3052 Parkville, Victoria, Australia

Abstract Alzheimer's disease (AD) is a progressive neurodegenerative disorder characterized by neuronal dysfunction, reactive gliosis, and the formation of amyloid plaques

and neurofibrillary tangles in the brain. There is a growing body of evidence to support a central role for biometals such as copper (Cu), zinc (Zn) and iron (Fe) in many critical aspects of AD and other neurodegenerative diseases. The amyloid beta (Aβ) peptide and its parental molecule, the amyloid precursor protein (APP) both modulate homeostasis of Cu and Zn in the brain. Perturbations to biometal metabolism in AD lead to fundamental changes in Aβ and APP expression as well as peptide aggregation and free radical production. These changes can subsequently promote neuronal oxidative stress and cell toxicity. Modulation of metal bioavailability in the brain has been proposed as a potential therapeutic strategy for treatment of AD patients. The lipid soluble metal ligand, clioquinol (CQ) has shown promising results in animal models and small clinical trials involving AD patients and a new generation of metal-ligand based therapeutics is currently under development. Further research will be necessary to fully understand the complex and interdependent pathways of biometal homeostasis and amyloid metabolism in AD. This information will be critical for developing efficacious metal-based pharmaceuticals for treatment of AD while limiting side-effects from disruption of normal metal-dependent metabolic activities.

Keywords Alzheimer's disease · Amyloid · Copper · Clioquinol · MPAC

Abbreviations

AD	Alzheimer's disease
ADAS-Cog	Alzheimer's disease assessment scale-cognitive subscale
Aβ	Amyloid beta
APP	Amyloid precursor protein
BACE	Beta-site cleaving enzyme
CCS	Copper chaperone of SOD
CNS	Central nervous system
CQ	Clioquinol
CSF	Cerebrospinal fluid
CuBD	Copper-binding domain
DFO	Deferoxamine
DMT1	Divalent metal transporter 1
DP-109	1,2-Bis(2-aminophenyloxy)ethane-*N*,*N*,*N*′,*N*′-bis(2-octadecyloxyethyl)ester,*N*,*N*′-disodium salt
DS	Down's syndrome
EPR	Electron paramagnetic resonance
ICP-MS	Inductively coupled plasma-mass spectrometry
IRE	Iron responsive element
MMP	Matrix metalloprotease
MPAC	Metal-protein attenuating compound
MRI	Magnetic resonance imaging
MT	Metallothionein
NFT	Neurofibrillary tangles
NMR	Nuclear magnetic resonance
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
SOD1	Superoxide dismutase 1

1 Alzheimer's disease

Alzheimer's disease (AD) is the most common form of age-related dementia. The disease manifests in the form of progressive cognitive decline with early memory loss and subsequent deficits in intellectual functions [1]. The neuropathology of the disease is characterized by extracellular deposition of aggregated amyloid beta (Aβ) protein and intraneuronal formation of neurofibrillary tangles [2, 3].

Aβ is the main constituent of senile plaques and cerebrovascular amyloid deposits [2, 3]. Aβ is a 39–43 amino acid peptide derived from the larger amyloid beta (A4) precursor protein (APP) by proteolytic cleavage [4–6]. APP is cleaved by the beta-site cleaving enzyme (BACE) and gamma secretase to generate Aβ peptides with carboxyl-terminal heterogeneity [7]. Alternatively, alpha secretase cleaves APP within the Aβ sequence, precluding formation of the Aβ peptide. In both cases, soluble, secreted APP molecules are released from the surface of the cell. While Aβ1–40 is the major form of Aβ, Aβ1–42, has a higher propensity to aggregate and is greatly enriched in amyloid deposits [8].

Pathological mutations close to, or within, the Aβ domain of APP cause familial forms of AD (FAD) (www.alzforum.org/res/com/gen/alzgene/default.asp). FAD mutations are also found in genes associated with Aβ processing, including presenilin 1 and 2 [9, 10], while risk factors for late onset AD include apolipoprotein E4 (Apo-E4) and polymorphisms of alpha-2 macroglobulin (α2m). Although several genetic lesions associated with FAD result in elevated Aβ1–42 levels, this alone does not explain the aetiopathogenesis of AD onset. Over-expression of Aβ1–42 occurs from birth in FAD and people with Down's syndrome (DS), however, amyloid deposition does not appear in childhood [11]. In all forms of AD, age-related metabolic or environmental changes are responsible for the slow but progressive onset of amyloid deposition and subsequent cognitive failure in early (FAD or DS) or late adulthood (sporadic AD). The neurochemical factors responsible for this age-related pathological process are still poorly characterized, however, growing evidence supports an important role for biometals such as copper (Cu), iron (Fe) and zinc (Zn) in Aβ accumulation and neuronal degeneration.

1.1 Aging and metals

Aging is accompanied by increases in the redox active metals iron (Fe) and copper (Cu). The significance of this is that the great majority of chemical radical and reactive oxygen species/reactive nitrogen species (ROS/RNS) are generated by a reaction catalyzed by these transition metals [12]. Damage by radical attack and ROS/RNS is abundantly apparent in AD-affected

brain tissue [13–17]. Pathological radical and ROS/RNS generation is catalyzed by these metal ions when in a free ionic state or when in a bound form where a chemical coordination site to a biological substrate or to O_2 becomes available [15, 16, 18]. Hence, the body goes to great lengths to carefully handle these metals with specialized transport systems and binding proteins. In health, Fe and Cu are catalytic centers of several essential enzymes, like superoxide dismutase 1 (SOD1), which exploits the reactivity of Cu to carefully allow it to react with one substrate (O_2^-) by forming a tertiary structure around the Cu that will only admit the substrate [19].

In the cell, the free ionic pool of Cu and Fe is very small. If this pool increases it will foster radical and ROS/RNS generation, which damages multiple biochemical targets. This may contribute to the aging process itself. An elevated Cu/Fe pool may also increase adventitious binding to proteins, withdrawing these metals from safe transport or quarantine, and making them available for catalytic chemistry on the side chains of proteins. This can lead to protein oxidation, crosslinking, loss of function, polymerization, and amyloid formation [20].

Age-dependent changes in these metal species, as well as changes in metal transport and storage proteins, have been observed in both animals and man. In rats, levels of manganese (Mn), Fe, Cu and Zn measured by inductively coupled plasma mass spectrometry (ICP-MS) in 18 different brain regions, exhibit a region-specific increase from postnatal day one to day 147 [21]. Other studies in normal mice (such as BL6/SJL) have also demonstrated significant age-related increases in Cu (46% increase from 2.8 to 18 months), Fe (51% increase from 2.8 to 18 months) and cobalt (Co) (66% increase from 2.8 to 18 months) levels in whole brain (without olfactory bulb, cerebellum or brain stem). Other metals such as Zn and Mn, however, did not change [22–24]. Increases in Fe over the first 28 weeks of postnatal life are also associated with region-specific alterations in the proton-coupled metal-ion transporter, DMT1, that contains an IRE in the 3′-UTR. The increase in Fe with aging in the rat brain is independent of ferritin [25].

Plasma Cu levels in rats are low at birth and increase sharply after postnatal day 10, concomitantly with the Cu storage protein ceruloplasmin. In contrast, plasma Zn levels were highest at birth and decreased slowly to adult values [26]. Older rats (20–22 months) have also been shown to have lower total plasma Fe content than middle aged rats (8–10 months), in addition to lower levels of the Fe-containing protein, hemoglobin [27].

In humans, studies of healthy men (8–89 years, $n = 408$) have demonstrated that plasma Cu concentrations increase steadily, whereas Zn levels tend to remain constant throughout life (until the age of 75). In subjects greater than 75 years of age, there are increases in serum Cu and decreases in Zn [28]. This is consistent with the bulk of the literature that reports that aging is characterized by elevated plasma Cu levels [29–34] and decreased plasma Zn concentrations [29, 35–39]. These changes may be associated with

concomitant alterations in the levels of metal ions within the human brain, possibly reflected in an age-related increase in ceruloplasmin in the superior temporal gyrus of normal individuals [40]. Furthermore, electron paramagnetic imaging has demonstrated an age-related increase in clusters of Cu and Fe ions within the brain [41]. Fe is reported to be elevated with age in several brain regions, and is particularly implicated in the onset of Parkinson's disease and Huntington's disease [42–44]. The elevated Fe is detectable with MRI procedures [45–53]. Cu is paramagnetic and therefore potentially detectable by MRI, however, there are no published studies on MRI of Cu in humans.

The age-related increases in blood and brain Fe and Cu are not associated with exposure. Unlike the aluminum (Al) theory of AD, which was popular in the 1980s, the corruption of Aβ as a metalloprotein with aging has nothing to do with toxicological exposure. According to the metal theory of AD, age-dependent redistribution of endogenous redox active metals are the main biochemical denominator linking the aging process with AD.

There is evidence of an increase in the redox-active fraction of plasma Cu in human aging [54]. This arises because of the apparent increase in the proportion of an oxidized form of ceruloplasmin, an antioxidant ferroxidase that carries > 90% of total plasma Cu. The damaged ceruloplasmin allows the Cu it binds (6 moles per protein unit) to become EPR-detectable [54]. This is an example of how damage to a cuproprotein may unleash abnormal redox chemistry, a principle that we believe underlies the pathology of AD.

2
The role of Cu in Alzheimer's disease

2.1
Cu homeostasis in Alzheimer's disease brain

Cu is normally found at relatively high levels in the brain (100–150 μM) with substantial variations at the cellular and subcellular level [55–57]. Ionic Cu is compartmentalized into a post-synaptic vesicle and released upon activation of the NMDA-R but not AMPA/kainate-type glutamate receptors [58]. The Menkes Cu7aATPase is the vesicular membrane Cu transporter, and upon NMDA-R activation, it traffics rapidly and reversibly to neuronal processes, independent of the intracellular Cu concentration [58]. Cu ions function to suppress NMDA activation and prevent excitotoxicity by catalyzing S-nitrosylation of specific cysteine residues on the extracellular domain of the NR1 and NR2A subunits of the NMDA receptor [58]. The concentrations of Cu in the synaptic cleft can reach approximately 15 μM. Subsequently, Cu is cleared by uptake mechanisms from the synaptic cleft. Several studies have shown that Cu levels increase with age in the brains of mice [22–24].

Although similar studies have not yet been performed on humans, Fe homeostasis is similar between mice and humans suggesting that Cu levels may also be elevated with age in humans [42, 43, 49]. Abnormal levels of Cu have been observed in sub-cortical regions of the brain, such as the hippocampus, amygdala and olfactory bulb and in the neocortex [59–61]. Microparticle-induced x-ray emission (micro-PIXE) analysis of cortical and accessory basal nuclei of the amygdala revealed elevated Cu levels (3–5 fold) in the neuropil of AD brain when compared to control tissue [57]. Cu was highly concentrated in regions of the brain most affected by AD pathology. Cu homeostasis is also altered in the cerebrospinal fluid (CSF) where Cu levels reach 2.2 fold higher than controls. This is accompanied by increased ceruloplasmin levels in the CSF of AD patients [40, 62].

Interestingly, despite the gross increases in cerebral Cu, sub-cellular Cu levels appear to be deficient in AD brain [63]. This is supported by the fact that several cuproenzymes reveal decreased activity in AD brain tissue. Cytochrome c oxidase (COX) and peptidylglycine alpha amidating monooxygenase have significantly reduced activities in brain and CSF respectively [64–66]. Deficiencies in COX levels and activity may be responsible for the deficit in energy metabolism characteristic of AD brain [67, 68]. Low ceruloplasmin levels have also been reported in AD brain [40] despite being elevated in CSF. The antioxidant, Cu/Zn superoxide dismutase (SOD1), likewise, shows reduced activity in both AD brain and transgenic animal models of AD despite increased protein expression. The reduced activity likely results from a deficiency in active site Cu [69, 70]. This is supported by the restoration of SOD1 activity by dietary Cu supplementation [70].

2.2
Impaired Cu homeostasis and oxidative stress

The ability of Cu to readily alternate between the Cu(II) and Cu(I) transition states is critical for cuproenzyme activity. However, this means that aberrant Cu metabolism can result in neurotoxic free radical production and associated increases in oxidative stress. Upon chemical or enzymatic reduction to the Cu(I) state, Cu can interact with available oxygen to form the highly reactive and toxic OH [71]. Cells have many overlapping mechanisms to prevent or repair OH damage including Cu chaperones, antioxidant molecules such as glutathione and ascorbate and antioxidant enzymes (the glutathione pathway, catalase and SOD1) [71]. In fact, under normal circumstances, cells may have little bioavailable Cu [72], thus preventing Cu(I) mediated oxidative damage. In addition, the brain has relatively high oxygen consumption but low antioxidant capacity compared to many other tissues. This fact is probably critical to our understanding of AD as an age-related disorder. Due to the oxygen metabolism/antioxidant imbalance, free radical damage may accumulate after lengthy periods of normal metabolic activity. In the brain, this would be fur-

ther exacerbated by aberrant metal homeostasis, lower energy metabolism and abnormal antioxidant levels.

Indeed, histological studies reveal that the AD brain shows excessive levels of many markers of oxidative damage associated with amyloid plaques and neurofibrillary tangles [73–75]. These include 4-hydroxynonenal (lipid peroxidation marker) [14], nitrotyrosine [76], AGE-modified tau [77], redox active metals [15], protein carbonyls (oxidized protein) [78] and increased nucleic acid damage [79]. Importantly, a strong correlation exists between the AD brain regions with highest Aβ load (and associated metal deposits) and markers of oxidative damage. Antioxidant levels are also altered in AD brain including decreased SOD1 activity [80, 81], increased catalase, and altered ferritin and hemeoxygenase-1 [82].

2.3
Pathological Cu interactions with Aβ peptide

Pathological interactions between Cu and Aβ may have a key role in the elevated oxidative stress levels characteristic of AD brain. Recent biophysical studies on synthetic Aβ have culminated in a proposed model of Aβ-Cu interaction (Fig. 1). Cu ions bind to Aβ monomer via 3 histidine residues and also via a bridging histidine molecule in aggregated Aβ [83]. Cu has been shown to induce significant Aβ aggregation at mildly acidic conditions (pH 6.6), which reflects the likely micro-environmental conditions in AD neuropil [84, 85]. Aβ has high and low affinity-binding sites for Cu (K_{app} 10 and K_{app} 7 respectively [85], while the affinity of Aβ1–42 for Cu is even higher (K_{app} 17.8 and K_{app} 8 [85]) (Table 1). In fact, the affinity of the Cu-binding site of Aβ1–42 approaches that of SOD1 and is therefore highly likely to be occupied in vivo [86]. This has been supported by Ramon spectroscopic studies of Aβ1–42 enriched senile plaques, demonstrating Cu co-ordinated to histidine residues [87]. Moreover, in vitro studies have shown that Aβ1–42 will rapidly aggregate in the presence of 1 μM Cu or less, a concentration of Cu well within the range of estimated extracellular levels in the brain.

Interactions between Cu and Aβ result in free radical generation in vitro and may contribute to the neuropathology of AD. (Fig. 2). Synthetic Aβ is capable of reducing Cu(II) to Cu(I) and this reaction produces hydrogen peroxide (H_2O_2) as a by-product [88] (Table 1). Tyrosine 10 has a pivotal role in H_2O_2 generation by Aβ with tyrosyl radicals facilitating electron transfer to drive the reaction [12]. This was supported by mutation studies that demonstrated a loss of H_2O_2 production, peptide cross-linking and neurotoxicity upon substitution of the key tyrosine residue in Aβ. Interestingly, the redox potentials for different species of Aβ are Aβ42>Aβ40≫rodent Aβ which accurately reflects the role of the respective peptides in amyloid pathology (rodents do not form amyloid plaques in the brain) [89]. Generation of H_2O_2 by Aβ-Cu can result in oxidative damage by diffusion of

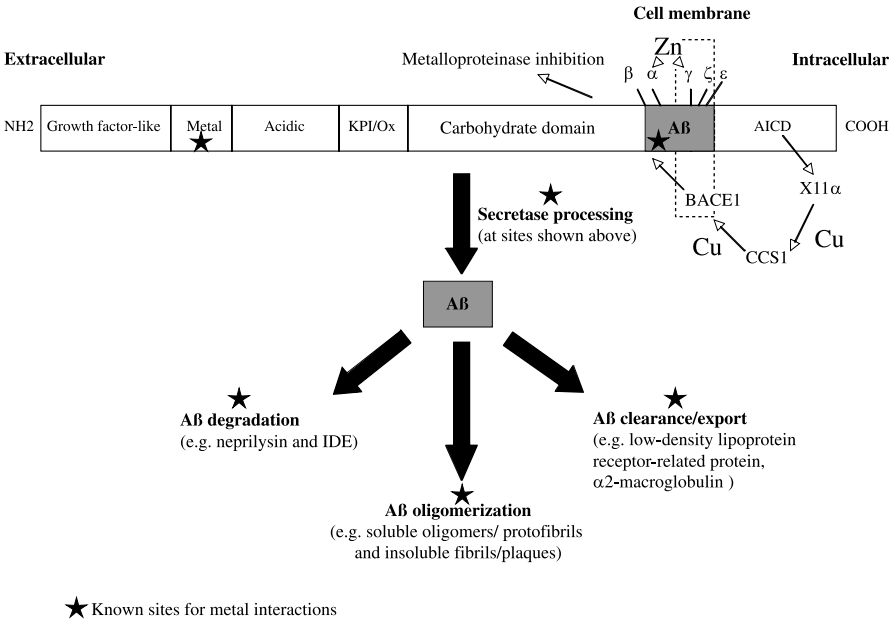

Fig. 1 Overview of APP and Aβ-associated interactions with metals. Metals such as Cu, Zn, and Fe interact with multiple binding sites and modulate processing of APP and Aβ (∗). Metals have important effects on Aβ processing (cleavage), oligomerization, degradation and clearance. These metal-dependent effects contribute significantly to AD neuropathology. The intracellular carboxyl terminus of APP interacts with X11α (MINT), which in turn interacts with the Copper Chaperone of SOD1 (CCS1) directing copper away from SOD1 [173]. CCS1 interacts with a Cu^{+}-binding site on the intracellular carboxyl terminus of BACE1 [174], although it is not yet clear whether this influences BACE activity. A metalloproteinase inhibitory domain has been identified in the portion of APP immediately upstream from the Aβ domain [175]. Abbreviations: AICD: APP intracellular domain, Aβ: amyloid beta, BACE: Beta-site APP cleaving enzyme, CCS1: copper chaperone for superoxide dismutase 1 (SOD1), IDE: insulin degrading enzyme, KPI/Ox: Kunitz protease inhibitor/MRC OX-2 domain

H_2O_2 through the cell membrane and subsequent oxidation of lipids and intracellular proteins. However, greater oxidative damage can be mediated through interaction of H_2O_2 with Aβ-bound Cu(I) resulting in OH [89]. OH will react with lipids, proteins or nucleic acids resulting in extensive modifications that are often irreversible and impede normal cellular turnover of these components. Interestingly, OH can also attack Aβ itself, further promoting aggregation and cross-linking of peptides. Oxidative damage to Aβ can result in di-tyrosine-mediated cross-linking between Aβ peptides and subsequent covalent oligomerization [12, 90, 91]. The AD brain in fact, contains a high di-tyrosine content [91]. The altered oxidative environment can also promote covalent interactions between Aβ and other proteins, result-

Table 1 Key evidence demonstrating interactions between Cu and amyloid or APP

Model system	Interaction
Cell-free	• Cu binds to Aβ at two sites. • Cu binds to the N-terminus of APP inducing lipid peroxidation. • Aβ-Cu interactions generate free radicals.
Cell culture	• Aβ-Cu promotes neurotoxicity. • APP CuBD peptides modulate Cu neurotoxicity. • APP/APLP2o/o neurons have highly elevated Cu.
Animal models	• APPo/o mice reveal elevated Cu. • APP transgenic mice reveal decreased Cu. • Increasing brain Cu in mice reduces amyloid deposition. • Treatment of APP transgenic with metal chelators reduces amyloid deposition.
Human studies	• High Cu levels are found in amyloid plaques in AD brain. • Treatment with metal chelators (CQ or DFO) slows cognitive decline in AD patients.

CQ: clioquinol, DFO: desferroxamine. See Maynard et al. [63] for detailed review of the evidence outlined in the table

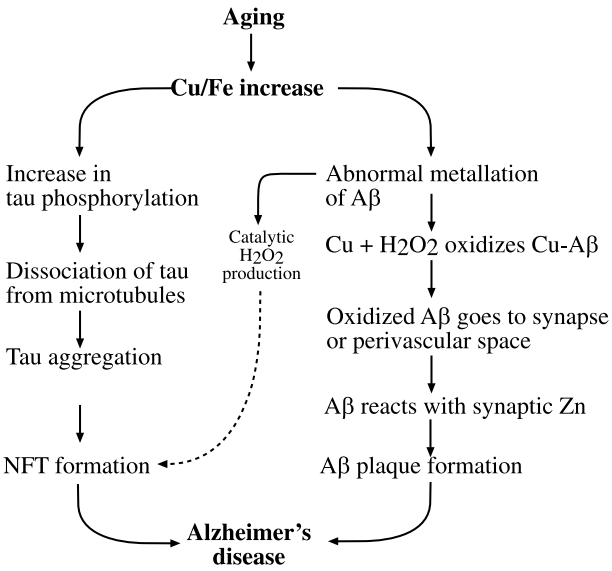

Fig. 2 Proposed model for the neuropathology of AD based upon abnormal metal interactions. During aging, Cu and Fe levels increase in the CNS with increased metallation of Aβ peptide. Cu binding to Aβ results in production of reactive oxygen species and auto-oxidation of Aβ peptide. Oxidized Aβ contributes to synaptic pathology and plaque formation. Metals may also promote phosphorylation of tau and enhance NFT formation, further contributing to AD neuropathology

ing in accumulation of multi-protein aggregates with greater resistance to clearance [92–94]. Furthermore, several recent studies have shown that Aβ neurotoxicity is closely associated with its ability to interact with cell membranes. Modification of the peptide structure and metal-binding activity can reduce or increase Aβ-membrane interactions with substantial changes to peptide toxicity through lipid peroxidation [95, 96]. For example, substitution of the Methionine-35 residue in Aβ enhances interaction with neuronal membranes resulting in elevated toxicity [97].

Interaction with Cu promotes Aβ neurotoxicity in cell culture and further supports a role for Cu in AD pathology (Table 1). Aβ oligomers are toxic to cultured neurons and Cu enhances this effect through increased peptide aggregation and free radical production [89, 98]. The sub-cellular localization of Aβ-Cu toxicity is not known, however, recent studies have shown that Aβ1–42 can interact with Cu in neuronal mitochondria resulting in specific inhibition of the terminal complex COX [99]. This effect could explain the reduced energy metabolism of AD brain. Neurons depleted of essential antioxidants such as glutathione are also more susceptible to Cu-Aβ mediated toxicity [100] and this mimics the altered antioxidant profile observed in brains of AD patients. Moreover, Aβ-Cu neurotoxicity can be further exacerbated by reducing agents found at high concentrations in the brain. Plasma and brain homocysteine levels are often elevated in the elderly and have been associated with old-age dementia. Data from recent studies strongly suggests that increased homocysteine levels may be a risk factor for AD [101] [102–104]. Homocysteine levels similar to those observed in the brains of patients with hyperhomocysteinemia were found to induce neurotoxicity from trace levels of Cu (as low as 0.4 nM Cu) in cell culture and this toxic activity was further exacerbated by interaction between Cu and Aβ [105].

One of the consequences of Aβ-Cu mediated free radical generation is likely to lead to oxidation of membrane cholesterol resulting in oxysterols including the highly toxic species known as 4-cholesten-3-one [106, 107]. Other reductants including ascorbate and catecholamines can also enhance Aβ-Cu toxicity through promotion of Cu(II) reduction [98, 108, 109]. The increased toxicity of Aβ in the presence of Cu and reductants is mediated through more efficient generation of H_2O_2 and subsequent OH production from H_2O_2 and Cu(I) interaction [98]. Again, the efficiency of this cytotoxic activity is greater for Aβ1–42 than Aβ1–40, reflecting the prominent role of the former in AD pathology.

2.4
The APP N-terminal Cu-binding domain

APP belongs to a multigene family containing the two known homologues amyloid precursor like proteins 1 and 2 (APLP1 and APLP2). Only APP contains the Aβ region, however, there is considerable sequence homology

between APP and APLP2 and this extends to the APP N-terminal Cu-binding domain (CuBD) [110–113] (Table 1). Indeed, the CuBD is well conserved across a large number of species [110, 112, 114–117]. The degree of conservation in this CuBD suggests an important evolutionary role for APP and its homologues in Cu homeostasis, a supposition supported by cell culture and animal studies [118]. The APP CuBD is situated between residues 135–166 and binds Cu(II) with a K_d of 10 nM [119]. Studies on short peptide fragments of the APP CuBD revealed that histidine residues 147, 149, and 151 were critical for Cu(II)-binding and co-ordination while cysteine 144 and 158 promoted Cu(I) formation with high efficiency [118]. At physiological pH, co-ordination of Cu(I) occurred via 5 nitrogen donors, including three side-chain imidazoles and two adjacent amide nitrogens [120]. Subsequent NMR studies on recombinant APP124–189 revealed that the CuBD contained an alpha-helix (147–159) and triple-stranded beta sheets (133–139, 162–167 and 181–188), joined by a disulphide bridge (cysteine 137 and 187) [121]. Cu(II) co-ordination in this case was mediated through two histidine residues with the Cu contained within a cysteine-rich site close to the protein surface [121]. Notably, despite differences in Cu co-ordination between short (135–166) and long (124–189) sequences of APP, phenotypic effects in vitro were identical [118, 121].

2.5
APP-Cu mediated neurotoxicity

Reduction of Cu(II) to Cu(I) by the CuBD results in free radical formation and neurotoxicity in cell culture [118]. The APP CuBD peptide, as well as the full-length protein, induce lipid peroxidation in the presence of Cu(II) and this is dependent on the APP CuBD-mediated Cu(I) formation [118, 122] (Table 1). That this toxic effect was not restricted to addition of exogenous, recombinant APP was shown by increased Cu toxicity in primary murine neurons when compared to APP-deficient neurons (APPo/o) [123]. Again, this toxic effect was Cu(I)-dependent as the Cu(I) selective chelator, bathocuproine disulphonate (BCS) could inhibit the increased Cu-mediated toxicity in wild-type neurons [122]. Whether APP-Cu directly or indirectly (through increased lipoprotein oxidation) contributes to AD pathology is uncertain, however, increased levels of APP (and Cu) are associated with senile plaques in AD brain.

APP homologues can also modulate Cu-dependent lipid peroxidation and neurotoxicity [122, 124]. Interestingly, the resultant effects on Cu metabolism in vitro are defined by the level of amino acid conservation in the CuBD. The CuBD of higher-order animals including human (APP and APLP2), *F. rubripes* (pufferfish FuguAPP) and *X.laevis* (toad xAPP) revealed increased lipid peroxidation and neurotoxicity in the presence of Cu(II), while *N.japonica* (electric ray elAPP) had little effect on Cu toxicity [118]. In con-

trast, *C. elegans* (nematode APL-1) and to a lesser extent, *D. melanogaster* (fruit fly APPL) CuBDs substantially reduced lipid peroxidation and Cu neurotoxicity in vitro, suggesting an early evolutionary role for the CuBD in Cu homeostasis. In this case, APL-1 CuBD may have simply afforded protection in a high Cu environment while further along the evolutionary path, APP CuBD has developed into a more complex Cu-regulatory protein with Cu(I) formation likely to be a key facet in modulating Cu-binding and trafficking. The protective or toxic effects of the respective CuBDs correlated with conservation of the central histidine residues, 147 and 151 (toxic) or substitution with tyrosine and lysine, respectively, in APL-1 (protective) [118]. The presence of histidine 147 and 151 was associated with high reductive potential for Cu(II) and decreased affinity for Cu(I) while APL-1 CuBD exhibited the opposite traits [118]. Interestingly, both the human and *C. elegans* CuBDs afforded neuroprotection against Cu(II) when APP and APL CuBD peptides were injected into mouse hippocampus. The reason for the disparity with cell culture studies is uncertain but could be related to increased de-toxification of Cu after reduction to Cu(I) by the peptides [125].

2.6
APP modulation of Cu homeostasis

APP and APLP2 are likely to have important roles in Cu homeostasis in animals [123]. This is supported by the finding that APPo/o and APLP2-deficient (APLP2o/o) mice revealed 40 and 16% increased Cu levels, respectively, in the cerebral cortex of adult animals [123] (Table 1). Moreover, as expected, transgenic mice expressing human APP (Tg2576) revealed decreased Cu levels compared to non-transgenic littermates [24]. The relative contributions of the APP N-terminal CuBD and Aβ CuBD to these effects on Cu levels are uncertain. APLP2 does not contain the Aβ region and APLP2o/o mice revealed increased Cu compared to wild-type animals, albeit, less than that observed in the APPo/o. This suggests that both the N-terminal CuBD and C-terminal Aβ CuBD contribute to Cu homeostasis. Further confirmation of this was shown by Maynard et al. [24] who demonstrated decreased Cu levels in mice over-expressing the C-terminal 100 residues of APP, including the Aβ CuBD. True delineation of these CuBDs and their effects on Cu homeostasis awaits the generation of a transgenic animal expressing the N-terminal region of APP and without the Aβ CuBD.

Recently, the overlapping effects of APP and APLP2 CuBDs on Cu homeostasis have been partially delineated through examination of double knockout animals. Although APP/APLP2 double knockout mice reveal embryonic lethality, cultured embryonic neurons and fibroblasts from these mice revealed even greater differences in Cu dysregulation than single APP or APLP2 knockouts. Primary cortical neurons from APP/APLP2o/o embryos revealed a 60% increase in Cu while fibroblasts revealed 300% increased Cu compared

to wild-type controls [126]. Clearly, this establishes APP and to a lesser extent, APLP2 as crucial Cu homeostatic proteins. Given the aberrant metabolism of both APP and Cu in AD brain, an important role for APP-Cu dysregulation in AD pathology is highly likely.

How APP (and APLP2) modulate Cu in vivo is not known, although the Cu reductase activity of APP (and Aβ) is consistent with the activity of well-characterized Cu transporters such as Fre1 in yeast [127]. The APP N-terminal CuBD has a 3D structure very similar to Cu chaperones, supporting a role for APP in cellular Cu trafficking. APP molecules may also be released from the cell surface by α-secretase, thus removing excess Cu from the cell. This is supported by the report that liver contained an 80% increase in Cu in APPo/o mice compared to controls [123]. The liver is an important site of Cu homeostasis in mammals. Further evidence for an APP Cu-detoxification model comes from studies on APP over-expressing cells. Borchardt et al. [128] found that exposure of these cells to increased Cu switched APP metabolism from the Aβ secretory pathway to α-scretase mediated release of APP. The increased secretion of APP may have increased the cell's capacity to off-load excess Cu although this has yet to be confirmed. The decreased Aβ production may have important consequences in terms of therapeutic control of Aβ deposition. Recently, two studies reported a decrease in cerebral amyloid load in transgenic APP mice with elevated Cu levels [70, 129] (Table 1). Although the mechanism involved is not yet clear, these findings are consistent with the hypothesis that neurons in AD brain may be deficient in Cu and that this contributes to elevated Aβ deposition. Correction of this Cu imbalance could help normalize Aβ turnover in the brain.

3
The role of Zn in Alzheimer's disease

3.1
Zn homeostasis in Alzheimer's disease brain

The overall Zn level in the brain has been estimated as approximately 150 μM [130]. Although the normal intracellular concentration of unbound Zn^{2+} is probably sub-nanomolar, the extracellular level may be in order of 500 nM [131]. However, extraordinarily high levels of Zn occur in the synaptic cleft. Ionic Zn is compartmentalized into the same presynaptic vesicles as glutamate itself [132], possibly as a counter-ion since its osmotic concentration in the synapse is similar to glutamate (reviewed in Frederickson et al. [133]). Glutamatergic Zn levels are modulated by the ZnT3 transmembrane Zn transporter, which is only expressed in the CNS [133]. As with Cu, Zn ions also modulate NMDA-R activity and inhibit excitotoxicity. This is achieved through saturable binding of Zn to a specific site on the NMDA-R.

Concentrations of Zn are approximately 300 μM and this puts Zn at one to two orders of magnitude higher than synaptic Cu. This highlights the fact that Zn is not a trace element but a major ionic regulator of synaptic transmission and other neuronal processes. As with Cu, abnormally high levels of Zn have been found associated with amyloid plaques in AD brain [57, 87, 134] and APP transgenic mice (Tg2576) [135]. Similarly, spectroscopic studies have shown Zn binding to histidine residues in amyloid cores in AD brains [87]. The highest concentrations of Zn have also been associated with brain regions most affected in AD pathology, including the hippocampus, neocortex and amygdala [136]. As Zn re-uptake after synaptic release is an energy-dependent process, the energy depletion associated with AD brain may contribute further to elevated extracellular Zn levels [137]. Loss of M1 receptors may also cause Zn to pool extracellularly [138]. Total Zn levels in the brain rise late in AD, in association with the Aβ burden [139]. This may be caused by lipid peroxidation preventing the normal export of Zn [140].

3.2
Zn interactions with Aβ

Aβ1–40 binds Zn at high and low affinities (K_d 107 nM and K_d 5.2 μM) [141]. Binding of Zn is mediated via histidine residues and is thus abolished at acid pH [84]. Histidine 13 in particular is believed to play a central role in coordination of Zn and subsequent Aβ oligomer assembly [142] (Fig. 1). Zn levels as low as 300 nM can rapidly precipitate synthetic Aβ1–40 [141, 143]. The potential role for Zn as a mediator of Aβ aggregation in vivo is highlighted by the effect of Zn depletion on cerebral amyloid deposition. Zn-transporter 3 (ZnT3) has a central role in maintenance of synaptic Zn concentrations. ZnT3-deficient mice have been crossed with the Tg2576 AD mice resulting in a 50% reduction in amyloid burden in the central nervous system compared with normal Tg2576 littermates [144]. ZnT3 activity may also promote the cerebral amyloid angiopathy, which commonly occurs in AD brain [145]. Additionally, when post-mortem samples from AD-affected brain were treated with zinc-selective chelators, Aß precipitates were dissolved and this was accompanied by the release of zinc from the pellet into the soluble phase [144].

Although Zn appears to contribute to amyloid aggregation and deposition in vivo, there is evidence that Zn may also act to inhibit the toxic action of Aβ. Cell culture data has revealed that Zn may quench peroxide production of synthetic Aβ thus inhibiting Aβ neurotoxicity [86, 146]. In this case, Zn-induced aggregation may be beneficial and is consistent with the growing body of evidence demonstrating a lack of correlation between synaptic pathology and amyloid deposition [147]. Deposition of Aβ may reflect a protective response to reduce the potent toxicity of soluble oligomeric forms of Aβ [148]. However, it appears that this process is not fully effective as oxidative damage is still associated with Aβ plaques.

3.3
The APP N-terminal Zn-binding domain

Zn binds to APP in the N-terminal region upstream from the CuBD (APP181–200) with a K_a of 750 nM [149]. Zn binding by APP is primarily structural and promotes heparin affinity. The role of Zn–APP interactions in vivo are not known although adult APPo/o mice revealed a non-significant increase in Zn levels in the brain. Conversely, Zn levels were slightly decreased in Tg2576 mice over-expressing APP [24]. These findings suggest that APP may have a similar effect on cerebral Zn levels as it does for Cu, albeit with a diminished end result due to the higher CNS Zn load [63].

3.4
Interaction between Zn and metalloproteins in Alzheimer's disease

Alpha 2 macroglobulin (α2m) is a Zn-binding inhibitor of matrix metalloproteases (MMPs) [150]. α2m also binds Aβ and enhances Aβ uptake and degradation through the low density lipoprotein receptor-related protein (LRP) [151]. Binding of Zn to α2m enhances the Aβ clearance activity. However, during ageing, α2m-mediated clearance becomes less efficient resulting in accumulation of α2m and Aβ in senile plaques [152]. Due to its high affinity for Zn, α2m may act as a Zn 'sink', perturbing normal Zn metabolism in the extracellular matrix. Accumulated α2m could also impair MMP-mediated degradation of Aβ.

Metallothionein (MT) is an important intracellular Zn-binding protein with several high affinity-binding sites per molecule. MTs are induced via metal response elements in response to stress or increased heavy metal levels. Studies have shown that MT expression is altered in AD in a complex manner. While reduced expression of MT-III has been observed in neurons of AD brain [153, 154], MT-I and MT-II isoforms may be elevated in astrocytes and microcapillaries [155]. Together with changes to other metal-regulatory protein metabolism, including APP, ceruloplasmin, ferritin and others, these findings further highlight the central role of altered metal homeostasis in AD pathology.

3.5
Zn metalloproteases and Alzheimer's disease

Several Zn metalloproteases play an important role in Aβ turnover in the CNS [156]. Insulin degrading enzyme (IDE), neprilysin (NEP), endothelin-converting enzyme, angiotensin-converting enzyme, thimet oligopeptidase and MMPs have all demonstrated Aβ cleavage activity in vitro and/or in vivo [156]. Numerous studies have examined in great detail how Zn modulates the protease activity of these enzymes [157]. Unfortunately, little is known

about how metal homeostasis affects Aβ-degrading metalloprotease activity in the brain. As reviewed here, there are substantive changes to Zn metabolism both intracellular and extracellular in AD brain tissue. It is intuitive to believe that such changes may also affect activities of the Zn metalloproteases involved in Aβ catabolism. In fact, our research has shown that altered intracellular levels of Zn (and Cu) induced by cell permeable metal-ligand complexes can result in up-regulation of Aβ degrading MMP activity. This novel effect occurs through activation of a phosphoinositol-3-kinase mediated pathway [158]. However, considerable research is still needed to address the gap in our knowledge of Zn metalloprotease-mediated degradation of Aβ.

4
The role of Fe in Alzheimer's disease

4.1
Fe homeostasis in Alzheimer's disease brain

Fe is essential for many cellular processes in the brain including oxygen transport, electron donation, DNA synthesis and synthesis of neurotransmitters. Fe is also crucial for myelin synthesis [159–162]. Cellular Fe movement is strictly controlled to prevent aberrant Fe-mediated free radical generation. Fe accumulation in the brain increases until about middle age where it remains steady [49]. As with Cu and Zn, abnormal Fe accumulation in certain CNS compartments occurs in AD [163]. Fe levels are highly elevated in senile plaques (up to 1 mM) and NFTs [16, 57]. Increased parenchymal Fe levels correlate with brain regions of greater risk for AD pathology [164]. Fe accumulation in the AD brain has been linked to increased oxidative damage to lipids, proteins and nucleic acids [15]. Cellular Cu deficiency in AD may be responsible for diminishing ceruloplasmin (Cp) ferroxidase activity that in turn may contribute to cellular Fe accumulation in AD, since Cp deficiency leads to Fe accumulation in the CNS [165]. Fe-binding proteins reveal aberrant metabolism in AD. Heme oxygenase levels are greatly increased in neurons and astrocytes of the hippocampus and cortex in AD brain [166]. Furthermore, there is increased localization of transferrin to glial cells, and ferritin-containing microglia and plaques are prominent in AD brain [167, 168]. However, unlike Cu and Zn, there is no evidence yet for a direct-binding interaction between Fe and Aβ in vivo.

4.2
Interactions between Fe and Aβ

Aβ binds Fe resulting in aggregation of the peptide *in vitro* [169]. As with Cu-Aβ interactions, binding of Fe results in reduction of Fe(III) to Fe(II) and

concurrent generation of H_2O_2 [98, 170]. Subsequent interaction between Aβ-Fe(II) and H_2O_2 results in OH formation via Fenton chemistry and increased propensity to oxidize lipids and proteins in close vicinity to the radical [98]. Fe-reducing capacity of Aβ1–42 is greater than for Aβ1–40 [98].

4.3
Fe and APP

There is further evidence linking aberrant Fe metabolism to AD pathology. Rogers et al. [171] identified a 'type II' Fe-responsive element (IRE) in the 5′ untranslated region of the transcript for APP. Increases in cellular Fe levels may increase APP protein translation through binding of Fe regulatory proteins to the APP IRE [171]. Conversely, depleted cellular Fe down-regulates APP expression as confirmed by Fe chelation experiments [171]. The normal metabolic function of this process is unknown although there is a close association between Fe and Cu metabolism via APP.

5
Metal interactions with tau and other AD neurochemistry

In addition to the metal interactions with APP and Aβ that may directly affect the generation of Aβ and its aggregation and toxicity, biometals have been found to interact with many of the proteins and activities that surround APP. These interactions are likely to subserve physiological purposes, and may reflect a role for APP metabolism in metal homeostasis. Zn has been shown to interact with and inhibit the gamma-secretase complex [172]. The intracellular carboxyl terminus of APP interacts with X11α (MINT), which in turn interacts with the Cu Chaperone of SOD1 (CCS1) directing Cu away from SOD1 [173]. CCS1 interacts with a Cu(I)-binding site on the intracellular carboxyl terminus of BACE1 [174], although it is not yet clear whether this influences BACE activity. This may be part of the mechanism by which Cu added to cell culture increases Aβ release into the culture medium (unpublished data). A metalloproteinase inhibitory domain has been identified in the portion of APP immediately upstream from the Aβ domain [175].

Tau binds Cu [176], which is enriched in NFTs [16, 79]. Recent data indicates that oxidative stress both in cell culture and in SOD2 knockout mice induces tau hyperphosphorylation (Fig. 2). A model emerges where corruption and accumulation of Aβ decorated with Cu^{2+} generates hydrogen peroxide and oxidative stress leading to the hyperphosphorylation of tau, and subsequent NFT formation. Recent reports have indicated that both Zn and Fe(III) induce aggregation of hyperphosphorylated tau [177] while reduction of Fe (III) to Fe (II) reverses tau aggregation [178].

6
Metal ligands as inhibitors of Aβ aggregation and neurotoxicity

6.1
In vitro dissolution of amyloid

Current attempts to inhibit the progression of AD pathogenesis are largely centered on achieving a reduction in Aβ aggregation and deposition in the brain. A number of potential therapeutic compounds are being developed and tested with the aim of inhibiting Aβ generating secretases, promoting clearance of aggregated or monomeric Aβ or dissolution of aggregated Aβ. In the latter category, metal–protein attenuating compounds (MPACs) have been successfully trialled in AD animal models and small groups of AD patients [179–181]. The basis of MPAC action is either the removal of metals from their respective binding sites on Aβ or interfering with metal–protein interactions resulting in abrogation of peptide aggregation. Future MPACs are likely to be developed that specifically target sites on Aβ necessary for metal–protein binding and metal-dependent protein–protein assembly.

The process of metal chelation involves the interaction between at least two donor groups on a ligand and an ionic substrate resulting in a 'ring' structure [86]. The potential use of a metal ligand as a therapeutic is affected by many properties such as size, charge, hydrophobicity and density. The latter is a measure of how many available groups can bind to the ionic substrate, i.e., bidentate has two groups whereas hexadentate has six [86]. The selection of optimal metal ligands for therapeutics may depend on a trade-off between several characteristics. For example, a hexadentate ligand may have a higher specificity for a particular metal, however, its increased size can reduce its ability to cross the blood–brain barrier. 1,10 phenanthroline has a high affinity for Cu, Zn and Fe through its nitrogen groups, however, binding of these metals results in a positive charge, which reduces its ability to cross membranes. Alternatively, 8-hydroxyquinolines chelate through both nitrogen and oxygen groups and tend to have better membrane penetration properties through formation of neutral complexes [182]. The development of therapeutic metal ligands will depend on the localization and target of the metal, i.e., whether the metal is intracellular or extracellular, easily bioavailable or tightly bound.

Unlike the hydrophobic mechanism that forms Aβ fibrils, metal-induced Aβ precipitation proceeds through two pathways- 1. reversible, ionically-mediated oligomerization, 2. Cu-mediated Aβ oxidation and cross-linking. High affinity chelators both inhibit and reverse Aβ precipitation induced by metal ions, and dissolve deposits from post-mortem human brain tissue [143, 183] (Table 2). Aβ also recruits the contaminating metal ions in ordinary laboratory buffers to form the micronuclei that seed the precipitation of peptide solutions into fibrils. Therefore, even the formation of fibrils in the

absence of exogenously added metals is abolished by the addition of chelators [91]. Incubation of Aβ with high affinity chelators like bathocuproine, bathophenanthroline or DTPA also abolishes Aβ redox activity when the peptide is complexed with Fe(III) or Cu(II) [91] (Table 2). Importantly, clioquinol (CQ), the prototypic MPAC, has a relatively low (nanomolar) affinity for Cu(II) and Zn(II) yet is able to abolish the aggregation and redox consequences of Aβ-metal interactions at least as efficiently as traditional higher affinity chelators [12, 107, 179]. CQ complexes Cu(II) and Zn(II) with 2:1 stoichiometry [184]. NMR and studies of radiolabeled CQ have shown no direct binding interaction of the MPAC to Aβ [98, 179]. Therefore, the physicochemical properties evidenced by CQ confer some advantages in accessing the metal binding sites over the traditional chelators. These properties may include low MW, low charge, hydrophobicity (since the Aβ-metal complexes are amphipathic) and small surface area (using SPARTAN 04, these are calculated as follows: CQ: volume= 0.1895 nm^3; area= 2.051 nm^2; CQ-Cu complex (2:1): volume= 0.3756 nm^3; area= 3.837 nm^2).

Cu(II) induces the radicalization of Aβ, and the consequent formation of soluble, cross-linked oligomers of Aβ that include dityrosine-bridged species. Evidence suggests that the toxicity of Aβ is linked to these oxidative reactions [12, 96]. CQ potently inhibits these reactions [12, 107].

6.2
Treatment of neuronal cell culture models with metal ligands

Aβ toxicity in cell culture has been linked to the ability of the peptide to recruit Cu(II) from the medium, which consequently fosters the redox reactivity of the Aβ [89]. This explains why the toxicity of Aβ variants correlates with the affinity of the peptide for Cu(II) and its subsequent redox activity (Aβ42>Aβ40> rat/mouse Aβ) [89, 98]. Modifications that disrupt the coordination of Cu(II) to Aβ inhibit toxicity [96, 185], correlate precisely with the generation of cholesterol oxidation products [107]. CQ and other Cu(II) chelators block these redox reactions, and correspondingly inhibit the toxicity of Aβ in cell culture [107, 186].

6.3
Treatment of Alzheimer's disease animal models with metal ligands

Clioquinol (CQ) was initially tested on post-mortem AD brain for its Aβ solubilizing activity. These studies revealed that CQ doubled the amount of soluble Aβ in brain extracts [179]. CQ was subsequently tested in APP transgenic mice (Tg2576) in a 12-week blind, controlled study [179]. An amount of 20 mg/kg daily of CQ significantly reduced the level of insoluble Aβ in brain extracts. A further 9-week study (30 mg/kg daily) was performed on older mice (21 months versus 12 months in the earlier study). In this case, in-

Table 2 Evidence supporting the use of metal ligands to treat Alzheimer's disease

Milestone	Key findings	Refs.
Metal interactions with Aβ	• Demonstration that Cu and Zn bind to synthetic Aβ and induce peptide aggregation. • Aβ-Cu interactions generate free radicals. • Aβ-Cu induces free radical mediated neurotoxicity.	[89, 141]
Association between metal homeostasis and Aβ/APP	• Elevated Cu, Zn and Fe levels associated with amyloid plaques. • Cu metabolism altered in APPo/o and APP overexpressing cells and animals.	[24, 57] [122, 123]
Dissection of toxic Aβ-Cu interactions.	• Cu-mediated di-tyrosine cross-linking of Aβ. • Aβ-Cu induced lipid oxidation.	[83, 91]
In vitro solubilization of aggregated synthetic Aβ peptide.	• EDTA and CDTA inhibited formation of Aβ 'seeds' and solubilized aggregated Aβ.	[91, 143]
Aβ toxicity is mediated by Cu/Fe interaction.	• BPS, DTPA and CQ abolished Aβ redox activity. • BCS and CQ inhibited Aβ neurotoxicity.	[98, 186] [197]
In vitro solubilization of amyloid.	• TPEN, BCS and CQ dissolved Aβ from AD brain samples. • TETA and BCA solubilized Aβ from extracts of APP transgenic mouse brain.	[179, 183]
Treatment of animal models of AD.	• CQ increased solubility of Aβ in brains of APP transgenic mice treated for 12 weeks (20 mg/kg/day). • Insoluble Aβ was decreased by 49% and amyloid deposits were reduced in APP transgenic mice treated with CQ for 9 weeks (30 mg/kg/day). • Substantial reduction in amyloid load observed in APP transgenic mice treated with DP-109 for 3 months (5 mg/kg/day)	[179, 187]
Treatment of AD patients.	• Pilot phase-2 trial involving 36 AD patients. CQ given at escalating dose (125–375 mg/twice daily) over 36 weeks. CQ significantly slowed cognitive decline in suffers of moderate AD. Plasma Aβ1–42 levels significantly reduced at 500 mg CQ/day. • PBT-2 (second generation metal-ligand) in phase-2 clinical trials.	[180]

EDTA	ethylenediamine tetra-acetic acid
CDTA	cyclohexane-trans-1,2-diamine tetra-acetic acid
BPS	bathophenanthroline disulphonate
DTPA	diethylenetriamine penta-acetic acid
BCS	bathocuproine disulphonate
TPEN	tetrakis-(2-pyridylmethyl)ethylenediamine
TETA	1,4,7,11-tetraazacyclote-tradecane-*N*,*N*9,*N*0,*N*-tetraacetic acid
BCA	bicinchoninic acid
CQ	clioquinol
DP-109	1,2-bis(2-aminophenyloxy)ethane-*N*,*N*,*N*′,*N*′tetra-acetic acid

soluble (sedimentable) Aβ was reduced by 49% [179] (Table 2). Importantly, immunohistochemical analysis of brain sections revealed a dramatic decrease in the appearance of amyloid deposits and serum levels of Aβ were also significantly decreased [179]. Interestingly, there were significant increases in cerebral Cu (19%) and Zn (14%), which may have resulted from increases in bioavailable metals released from Aβ deposits [179]. Encouragingly, assessment of animal behavior revealed a significant improvement in overall health with no obvious signs of toxicity [179].

A more recent animal study supported the clinical potential of metal ligands to treat AD. Lee et al. [187] treated Tg2576 mice using the metal ligand [1,2-bis(2-aminophenyloxy)ethane-*N*,*N*,*N*′, *N*′-bis(2-octadecyloxyethyl)ester, *N*,*N*′-disodium salt] DP-109 at 5 mg/kg daily for 3 months (Table 2). Analysis of mice revealed a substantial reduction in the burden of amyloid plaques compared to controls as well as a reduction in cerebral amyloid angiopathy. Consistent with the CQ study, DP-109 also increased soluble levels of Aβ in the brain [187]. Interestingly, several additional compounds have shown to inhibit Aβ accumulation in vivo and are known to exhibit metal-binding properties. Cyclohexanehexol treatment of transgenic AD mice inhibited Aβ aggregation and plaque formation [188] and it is known that this and other inositol compounds are metal ligands [189].

6.4
Treatment of Alzheimer's disease patients with CQ

Regland et al. [190] treated moderately affected AD subjects ($N = 18$, open label) with CQ at 20 or 80 mg/kg/day for 21 days. No adverse effects were reported. The patients exhibited an improvement in performance in ADAS-Cog of 2.7 points ($p < 0.07$). Of the ADAS-Cog subtests, the naming, instructions and comprehension scales showed significant improvement ($p < 0.05$). In the high-dose group, significant improvement was seen in comprehension ($p < 0.05$). In the low-dose group, there were no significant differences. CSF Aβ1–42 and tau levels at the completion of the study showed no differences compared to baseline, but there was a transient rise in CSF tau at day 7.

This study was followed by a larger double-blind placebo-controlled trial involving 36 patients also with mild to moderate AD treated over 36 weeks [180]. CQ doses were 125 mg twice daily (first 12 weeks), 250 mg twice daily (weeks 13–24) and 375 mg twice daily (weeks 25–36). Subsequent analysis of patients revealed that CQ significantly arrested the rate of cognitive decline (on ADAS-cog) at week 24 (and a tendency to significance at week 36) in the patients suffering moderate dementia [180]. The patients with mild dementia did not deteriorate in ADAS-cog scores during the study period, making it impossible to appreciate a change in deterioration rate over the study in this subgroup. CQ significantly lowered plasma Aβ1–42 levels, while plasma Zn levels were elevated and there was no change in plasma Cu [180].

The decrease in plasma Aβ is congruent with the lowering of plasma Aβ in CQ-treated transgenic mice as the treatment debulks the brain Aβ burden (there was a significant correlation between brain Aβ and plasma Aβ levels) [179]. The increase in plasma Zn normalized the levels from the abnormally low baseline levels, which is typical of levels in older subjects. This effect is compatible with the debulking of cerebral amyloid burden upon treatment with CQ, so preventing Zn from being trapped in plaques and CAA. 15% of plasma Zn is in communication with Zn released from synaptic activity [140]. The elevation (and normalization) of plasma Zn in CQ-treated AD patients underscores that the mechanism of CQ activity in AD does not involve systemic chelation.

In 2005, a case report was published of two patients with early onset of AD (one with the "London" APP mutation) who received treatment with CQ 250 mg bid for 9 and 14 months [191]. Both cases exhibited focally augmented cerebral glucose metabolism with arrested clinical deterioration over this period and neither developed signs of neurotoxicity. In the familial AD case (the only one who consented to lumbar puncture) CSF-tau levels rose at 4 months and fell to below baseline at 9 months of treatment, and the Aβ42/40 ratio also rose at 4 months and fell to below baseline at 9 months of treatment [191].

Although there were clearly promising signs for the potential clinical utility of CQ based on these small studies, further clinical studies have been postponed due to purification issues with large-scale manufacture (http://www.pranabio.com/downloads/PBT_AR_2005.pdf).

6.5
Treatment of Alzheimer's disease patients with traditional hydrophilic chelators

Desferrioxamine (DFO) is a hexadentate Fe-selective chelator used in a 2-year single-blind trial for treatment of AD (Table 2). The basis for this trial at the time was the potential removal of Aluminium (Al), which once had been considered as possibly linked to AD aetio-pathology [192]. The association between Al and AD has not been supported by the weight of recent studies [193], however, in the DFO trial 48 patients were treated with either 125 mg DFO twice daily, oral placebo or no treatment. DFO treatment resulted in a significant reduction in rate of decline of daily living activities. These findings are interesting as DFO does not penetrate the BBB and therefore its access to the brain would be limited to sites of putative BBB disruption [182]. Although the target in this case was Al, it is likely that the beneficial effect of DFO may have resulted from chelation of Fe, Cu or Zn. Further clinical research into DFO has diminished due to difficulty of administration (painful intramuscular injection) and anaemia resulting from Fe-depletion.

D-penicillamine has also been trialed in AD patients. Subjects were enrolled in a small 6-month, double-blind, placebo-controlled trial [194]. Nine

patients for each group completed the trial. Oxidative stress, trace metals and clinical parameters were evaluated. At the start of the study total peroxides and Cu serum content of AD patients were significantly higher and antioxidants were significantly lower than in healthy controls. Cu and peroxides were significantly correlated in the AD population. After treatment with d-penicillamine, oxidative stress markers were significantly decreased, but neither the placebo control group nor the treatment group declined on cognitive performance during the period of the study.

7
Future directions in Alzheimer's disease metallochemistry and therapy

Our understanding of how metal homeostasis is altered in AD is still very limited. In the past 10 years, there has been a steady increase in the number of publications describing metal–amyloid interactions, particularly at the biophysical level. Future research in this area must adequately address our lack of knowledge about biometal metabolism in the aging brain. Of fundamental importance is determining whether loss of biometal homeostasis drives aberrant amyloid metabolism, aggregation, deposition and toxicity or if other causes of amyloidogenesis result in perturbations to metal homeostasis. In reality, it is likely that both mechanisms will have important roles to play in progression of AD pathology. While these studies are in progress, animal trials should continue to evaluate the potential of novel metal ligands for treatment of AD and other neurodegenerative disorders. These trials may be able to build upon the early successes of CQ and DP-109 and soon find their way to clinical trials. Three metal-coordinating compounds have thus far been tested in AD patients (DFO, d-penicillamine and CQ). Each of these agents showed promising results from their limited trials, providing encouragement that the future will bring more exciting results in this field from novel drug designs.

Future approaches are being sought to overcome some of the problems inherent to metal ligand-based therapy. In particular, increasing access to drugs through the BBB is of prime concern, not only for metal ligands, but for many potential CNS drugs. Some of these approaches include the use of proligands where the ligand only becomes active upon entering the brain [195], and nano-particles coated with specific ligands such as penicillamine that may enter the brain through normal uptake mechanisms such as the LDL receptor [195, 196]. Linking metal ligands or other drugs to 'universal' membrane penetrating peptides such as HIV-TAT peptide may also improve CNS delivery.

Future design of metal ligands for AD treatment is likely to be centered on molecules that specifically bind to metal-binding sites on Aβ and/or APP rather than broad spectrum metal chelators that can potentially strip metals

from many sites within the body. Such molecules (MPACs) are being designed to fit into the known or anticipated metal-binding sites on monomeric or aggregated Aβ, thus resulting in reduced cytotoxicity. The principle of targeting metal-binding sites on proteins is well established in pharmacology with many drugs binding active site metal with relative specificity (i.e., metalloprotease inhibitors). It may also be beneficial to target-related aspects of Aβ metabolism and turnover. For example, targeting of the 5′ untranslated region IRE on APP mRNA may result in overall reductions in both Aβ-Cu/Fe and APP-Cu-related neurotoxicity [171]. Additionally, we have found that modulating cellular metal levels using CQ can increase extracellular Aβ degradation through up-regulation of matrix metalloproteases [158]. Metal targeting drugs could possibly be designed to have multiple beneficial effects in vivo, leading to down-regulation of APP translation, dissolution of Aβ and increased Aβ degradation. Such pharmaceuticals may prove particularly beneficial when used in conjunction with other potential treatments including secretase inhibitors and Aβ immunotherapy.

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GSK-3 Inhibitors for the Treatment of Alzheimer's Disease

Ratan V. Bhat (✉) · Stefan Berg · Jeremy Burrows · Johanna Lindquist

AstraZeneca R&D Södertälje, B212, 501 E1, 15185 Södertälje, Sweden
Ratan.Bhat@AstraZeneca.com

Abstract Glycogen synthase kinase 3 (GSK-3) has emerged as a prominent therapeutic target for intervention in several diseases including non-insulin-dependent diabetes mellitus, Alzheimer's disease, stroke, bipolar disorder and affective disorders. In the present review we briefly summarise the properties of GSK-3, focusing primarily on the role of GSK-3 in Alzheimer's disease. Furthermore, we discuss the potential for therapeutic benefit of GSK-3 inhibitors.

Keywords Glycogen synthase kinase 3 · Alzheimer's disease · Tau · Beta amyloid

1 Introduction

Alzheimer's disease is a chronic neurodegenerative disorder and the most common cause of dementia in elderly people. The onset of disease is insidious. Loss of memory and depression are an early indicator of the disease. As the disease progresses, there is an impairment of learning abilities and object recognition, disorientation and decline in language function. Within a few years of onset, quality of life begins to rapidly deteriorate. At later stages clinical symptoms include motor dysfunction, hallucinations and psychoses. The pathological hallmarks of Alzheimer's disease include extracellular amyloid plaques and intraneuronal neurofibrillary tangles (NFTs). Synaptic loss and neurodegeneration is also evident early within the limbic regions of the brain, resulting in an increased rate of brain atrophy. The molecular entities that have been suggested to contribute to the neuropathological changes in Alzheimer's disease have been diverse. More recently there has been compelling pre-clinical evidence that some of these changes might be associated with an increase in the protein kinase glycogen synthase kinase 3 (GSK-3) activity. GSK-3 has been shown to co-localise with neuropathological lesions in Alzheimer's disease, and recent findings in cells and animal models support the position that GSK-3 inhibitors could be therapeutically beneficial in slowing down the progression of the disease. In this article, we summarise the role of GSK-3 as a key point of intervention in Alzheimer's disease and discuss the action of the GSK-3 inhibitors in pre-clinical models.

2 GSK-3 in Alzheimer's Disease

GSK-3 is highly conserved between different eukaryotic species, displaying close DNA sequence homology. Similarly, high amino acid sequence homology indicates that GSK-3 has conserved structure and function in different species. GSK-3 exists in two isoforms encoded by different genes in mammals, GSK-3α and GSK-3β [1]. Both isoforms display a ubiquitous tissue expression pattern [2], but although structurally similar, they are not functionally redundant. The differential expression of GSK-3α and GSK-3β indicates that they may display different roles in cellular functions and tissues.

2.1 Expression of GSK-3 in Brain

GSK-3 is developmentally regulated displaying high levels during early postnatal period in the rat, but also expressed at lower levels in adult brain [3–5]. GSK-3β is the comparatively dominant isoform expressed in neuronal tis-

sue [6]. In situ hybridisation demonstrates that GSK-3α and GSK-3β mRNAs are localised in diverse regions of the brain and prominent expression is observed in the hippocampus, cerebral cortex and the cerebellar Purkinje neurons [5].

GSK-3β deficiency in genetically modified mice is embryonic lethal, supporting a potentially important function during embryonic development where GSK-3 affects the Wnt and NFκB signalling pathways [7]. These findings also suggest that GSK-3α cannot compensate for the function of GSK-3β, further supporting distinct functions for the two isoforms. Selective overexpression of GSK-3β in the central nervous system (CNS) causes a reduction in brain and spinal cord volume in conjunction with reduced dendritic mass in the spinal cord. The reduction in brain volume is accompanied by an increased neuronal density, but no apparent loss of neurons [8].

2.2 Regulation of GSK-3 Activity

GSK-3 was originally described for its role in metabolic pathways as a kinase phosphorylating and inactivating glycogen synthase (GS) in skeletal muscle [9]. Today, it is accepted that GSK-3 function is not restricted to glycogen metabolism, and GSK-3 has been shown to exert pleiotropic functions as the kinase phosphorylates numerous substrates in diverse cell types.

Unlike most kinases, GSK-3 is constitutively active in cells. Activity can, however, be dynamically modulated by the phosphorylation status of the two isoforms. GSK-3β activity is regulated by inhibitory phosphorylation of Ser9 (Ser21 in GSK-3α) and stimulatory phosphorylation of Tyr216 (Tyr279 in GSK-3α) [10, 11]. The Ser9 phosphorylation on GSK-3β induces a conformational change of the N-terminal part of the protein, thus preventing substrates from interacting with GSK-3 [12]. Several kinases have been shown to phosphorylate and thereby inactivate GSK-3 on the inhibitory site. For example, p90/RSK and PKB/Akt phosphorylate GSK-3 in response to insulin and growth factors, thus resulting in lowered GSK activity [10, 13, 14]. In *Dictyostelium*, the tyrosine kinase ZAK1 phosphorylates Tyr216, which augments GSK-3β activity. The mammalian counterpart of ZAK1 has not been identified and it has been suggested that stimulatory phosphorylation of Tyr216 occurs via autophosphorylation [15]. Gene silencing of either GSK-3α or GSK-3β by RNA interference, resulting in 70–80% knockdown of their respective proteins, reduces Tyr216 phosphorylation on GSK-3β and increased inhibitory Ser phosphorylation of the remaining isoform. Thus, gene silencing of one of the two isoforms decreases overall GSK-3 activity and suggests cross talk and autoregulation between two GSK-3 isoforms [16]. These results further support the notion that phosphorylation of the inhibitory Ser may also be regulated by autophosphorylation.

Most GSK-3 substrates require a priming phosphorylation by another kinase at Ser/Thr four residues upstream of the Ser residue, which is phosphorylated by GSK-3, in order to enable GSK-3 action. This unusual substrate specificity provides a potential for tight regulation of substrate specificity dictated by specific cellular conditions. In addition to the requirement of a priming phosphorylation, the subcellular localisation and the formation of substrate-specific GSK-3 protein complexes enables tight regulation of GSK-3 activity and contributes to tight substrate specificity. Substrates phosphorylated by GSK-3 include structural (tau, Synapsin 1, MAP1B, MAP2, NCAM, CRMP), signalling (PKA, MARK, PP1, PPI2, β-catenin, Axin, PDH, IRS-1) and nuclear (CREB, c-jun, c-myc, p53, NFκB, eIF2B, HSF-1) proteins [17].

2.3 GSK-3 in Alzheimer's Disease Brain

The levels of active GSK-3 are increased in the frontal cortex in Alzheimer's disease patients. Studies of the cellular distribution of active GSK-3 shows that GSK-3 co-localises with several phospho-tau epitopes in the somatodendritic compartment, an early event preceding the formation of NFTs [18]. Active GSK-3, measured as Tyr216 phosphorylation, co-localises with pretangle and tangle bearing hippocampal and cortical neurons in Alzheimer's disease brain [4]. It should be noted, however, that phosphorylation and dephosphorylation are often very rapid dynamic events and as a consequence, the post-mortem stability of phosphorylated GSK-3 in these samples is likely to be very short [19]. Elevated levels of GSK-3 protein in Alzheimer's disease brains are observed in some studies, whereas others fail to show similar results. A recent study on a limited number of subjects shows that total GSK-3 levels were not increased in Alzheimer's disease patients [4]. However, the limited number of patients, heterogeneity in the population and differences in disease progression are important factors contributing to the difficulty in interpreting these results.

2.4 Tau

Among the structural proteins which are subject to GSK-3 regulation, the microtubule-associated protein tau is a prime target in Alzheimer's disease pathology. Tau binds to tubulin and promotes microtubule assembly and stability in a phosphorylation-dependent manner. The phosphorylation status of tau is balanced by antagonistic kinase and phosphate activities. Inappropriate hyperphosphorylation of tau is a key event in contributing to cytoskeletal abnormalities and tau pathology in Alzheimer's disease. When hyperphosphorylated, tau's affinity for the microtubule is reduced and as a consequence tau dissociates from the microtubules. This leads to abnormal accumulation

of tau within the somatodendritic compartment of neurons, leading to loss of microtubule function, and consequently the cytoskeletal architecture deteriorates, leading to neuritic dystrophy. Abnormally phosphorylated tau forms filaments in cultured neuronal cells [21]. Two types of tau filaments have been described: straight filaments (SFs) and paired helical filaments (PHFs), which are two twisted helical strands. Post-translational modifications of tau, such as glycosylation, glycation, phosphorylation and ubiquitination, are necessary for the formation of insoluble tau filaments and incorporation as SF and PHF tau, which precede the NFTs. The NFTs are neuropathological hallmarks of Alzheimer's disease.

A spatial and temporal relation between increased GSK-3β activity and the progression of NFT and neurodegeneration has been demonstrated. Also, the density of NFTs is suggested to be closely correlated with cognitive decline in Alzheimer's disease [22, 23]. This correlation is supported to a higher degree in the literature compared to what is seen for amyloid plaques. Tau hyperphosphorylation disrupts cellular processes such as axonal transport, a deficiency thought to contribute to the early stages of Alzheimer's disease pathogenesis [24]. In tauopathies, GSK-3 is identified as a major kinase mediating aberrant tau phosphorylation at sites AT8 (Ser202/Thr205), AT100 (Ser214 and/or Ser212), TG3 (Thr231 and/or Ser235) and PHF-1 (Ser396/Ser404) [25–27]. Genetically modified mice, where GSK-3β can be overexpressed in adult brain, show elevated GSK-3 activity in cortex and hippocampus in conjunction with hyperphosphorylation of tau in somatodendritic compartments, neurodegeneration-associated changes and gliosis [28]. These GSK-3β inducible mice develop pre-tangle-like structures in the hippocampus; however, NFTs were not observed suggesting that additional modifications of tau and possibly prolonged phosphorylation effects may be necessary for complete neurofibrillary pathology. Furthermore, these mice also display spatial memory deficits [29], suggesting that abnormal GSK-3 activity in adult brain can lead to pathological and cognitive deficits. Recently, inhibition of GSK-3 has also been implicated in the induction of long-term potentiation (LTP). LTP has been suggested to be dependent on phosphorylation-induced increases in synaptic transmission or putatively changes in the synaptic spine morphology [30]. It is feasible that GSK-3 may contribute to both these processes. Moreover, in mice overexpressing GSK-3, LTP was abolished [31]. A recent report provides evidence for a role of GSK-3 inhibition in the maintaining of synaptic plasticity by regulating the interplay between LTP and LTD (long-term depression). In rat hippocampal slices, GSK-3 inhibition was shown to prevent the induction of LTD. Suggestively, LTP activates the *N*-methyl-D-aspartate (NMDA) receptor leading to an enhanced activity of GSK-3 via PP1 and the PI3K/Akt pathway and prevention of LTD as a consequence. Thus, results suggest that GSK-3 activity is essential for NMDA receptor-dependent LTD [32]. Collectively, these findings provide further insight into a potential molecular role of GSK-3 in the synaptic

mechanisms contributing to the cognitive decline seen in Alzheimer's disease patients.

2.5 GSK-3 and Neuronal Death

Given the association of GSK-3 with neurodegenerative disorders, the function of GSK-3 in processes governing cell survival and cell death has been extensively studied. Increased GSK-3 activity induces cell death in most cellular models. However, GSK-3 has been shown to promote both cell survival and cell death in different cellular systems and in response to diverse stimuli. Several reports have implicated GSK-3 in promotion of neuronal cell death in response to a variety of conditions including growth factor withdrawal [33], mitochondria toxins [34], beta amyloid [35] and oxidative stress [36]. In rat cortical neurones, gene silencing of either GSK-3α or GSK-3β indicates that inhibition of either isoform is able to rescue neurones from glutamate-induced cell death [16]. Although GSK-3 inhibition has been implicated in cellular survival, the relevant downstream pathways and targets regulating neuronal survival remain to be elucidated. It has been suggested that GSK-3 is upstream of the pro-apoptotic protease caspase-3 [37]. GSK-3 plays an important role in several signal transduction systems which influence cell proliferation and survival, such as an inhibitory component of the wnt signalling pathway through β-catenin phosphorylation, the PI3K pathway and NFκB. Clearly, GSK-3 can have multiple roles in cell survival, both facilitating and inhibiting apoptosis. Moreover, these findings support the complexity of regulation of GSK-3 activity in various tissues during different developmental stages.

2.6 GSK-3 and Amyloidosis

Gene-silencing studies using small interfering RNA against the GSK-3α and GSK-3β isoforms suggest that GSK-3 inhibition per se decreases beta amyloid production in cells and in animal models of amyloidosis [38, 39]. Surprisingly, RNA interference-induced depletion of either GSK-3 isoform seems sufficient to block amyloid production. Recent studies have reported that GSK-3 can indirectly affect the processing of amyloid precursor protein (APP). For example, overexpression of dominant negative GSK-3β in HEK-SwAPP751 cells decreases beta amyloid secretion [40]. When mice overexpressing inactive GSK-3 (under Thy1 promoter) were crossed with transgenic mice harbouring the APP Swedish mutation, a significant decrease was observed in APP phosphorylation (Tyr668) and maturation and beta amyloid load. Furthermore, these mice performed better in spatial learning tasks compared to the APPsw amyloidosis mouse [41]. Conversely, GSK-3β^{S9A} tg mice which

overexpress active GSK-3β in the CNS have increased levels of beta amyloid [40]. It is believed that proteolytic processing of APP occurs by the sequential activities of β- and γ-secretases, which results in the release of the beta amyloid peptide. In earlier studies, the role of GSK-3 in APP processing was attributed to an interaction with the γ-secretase complex activity and presenilin-1 (PS1), a component of the γ-secretase complex. PS1 was shown to directly bind GSK-3β and tau in co-immunoprecipitation experiments from human brain samples [42]. A different mechanism has been suggested by Akiyama et al., who propose that GSK-3 acts on the β-secretase pathway via blocking of Pin1 interaction with phosphorylated C99Thr668, which leads to decreased turnover of C99, a product of β-secretase cleavage of APP [43]. Thus, it is tempting to speculate that GSK-3 may be the key intersection point at which Alzheimer's disease-related tau hyperphosphorylation and beta amyloid formation converge.

3
Glycogen Synthase Kinase: the Protein

GSK-3β is part of the CMGC (containing CDK, MAPK, GSK3 and CLK) family of protein kinases. The closest kinases are its isoform GSK-3α (which is 76% identical across the entire sequence and 91% identical in the catalytic domain: pairwise alignment of amino acids 119 to 403 in GSK-3α to corresponding amino acids in GSK-3β), its splice variants GSK-3β2 (which has a 13 amino acid insert in the catalytic domain) and a further splice variant (containing a 33 amino acid deletion) [44, 45]. Aside from GSK-3α and the GSK-3β splice variants, the next closest within the kinome based on pairwise alignments across the entire protein sequence (in-house data) are all within the CMGC family: CDK3 (35% identity, 59% similarity), MAK (34.4% identity, 58.2% similarity), CDK7 (37.7% identity, 54.3% similarity), CDK2 (34% identity, 57.7% similarity), ICK (33% identity, 58% similarity), CDK5 (35.2% identity, 54.5% similarity) and ERK5 (34.3% identity, 55.1% similarity). When comparing within the ATP binding site the closest CMGC kinase usually referred to outside the GSK family is CDK2, though when specifically comparing pairwise alignments of the gatekeeper and hinge region (Leu132 to Pro136), the closest kinases (across the kinome) are GSK-3α (80% identity, 100% similarity), SGK2 (80% identity, 80% similarity), MSK2 (80% identity, 80% similarity) and PRKX/Y (60% identity, 100% similarity).

GSK-3β binds ATP and the majority of those compounds that are reported to inhibit the kinase have been shown experimentally, or are predicted based on structure, to mimic and compete with ATP. The measured K_m for ATP for GSK-3β has been shown to range between 20 and 50 μM in different studies [46, 58]. With the cytosol ATP concentration somewhere in the region of 2 mM (though some reports on neurons put this figure slightly lower, even be-

low millimolar levels [47], possibly as a result of the high energy consumption of the cells), the expected "drop-off" in potency from enzyme to cell based on ATP competition (for a typical ATP-competitive inhibitor) would be approximately 40–100-fold, in the absence of unusual kinetics, non-selectivity or alternative mechanisms of inhibition.

The kinase ATP binding site has been described extensively (e.g. [48]). It has a hinge region consisting of Asp133, Tyr134 and Val135 which provide the hydrogen bond acceptors and donors to the ligands, and it is clear from X-ray crystal structures that different ligands can have different hydrogen bonding patterns and to different atoms within the hinge region. The gatekeeper residue is Leu132 and the rear part of the pocket is defined by two residues forming a salt bridge (Lys85 and Glu97). The main amino acids stretching from the "roof" of the kinase ("roof" defined as being the region above ATP in the orientation in which the ATP-hinge binding, when viewed in the plane of the ligand, appears on the left side) involve Ala83, Val70 and Ile62, with Tyr134 and Phe67 approaching from either side. Val110, Leu188, Cys199, Thr138, Asp200, Asn186 and Gln185 line the base of the kinase below the ligand. The mouth of the ATP site leading to solvent is marked by Ile62, Glu137, Thr138, Arg141, Gln185, Lys183 and Asn186, with Arg144, Tyr140, Lys183 and Asp181 positioned further out.

Much has been written concerning strategies to optimise selectivity particularly against CDK2 (the closest kinase, based on the ATP binding site, outside the GSK-3 family) [49]. The most dramatic differences between the two kinases, and those features which can be exploited to achieve selectivity, are the gatekeeper residue (Leu132 in GSK-3β and Phe80 in CDK2) and the GSK-3β salt bridge (Lys85:Glu97), which is positioned very differently to the CDK2 salt bridge (Lys83:Asp145) as a result of Glu97 (GSK-3β) being replaced by Leu148 (CDK2), as well as the numerous changes around the mouth of the ATP pocket (e.g. Asp86 in CDK2 vs Thr138 in GSK-3β). X-ray structures with a variety of different structures have also demonstrated that GSK-3β is a much more forgiving protein in accommodating volume within the pocket. Several structures (results not shown) show increased selectivity between GSK-3β and CDK2 as a result of the higher tolerated binding groove in GSK-3β which is not tolerated in CDK2.

The differences between GSK-3β and GSK-3α are very small and equate to only one amino acid difference in the ATP binding site. As a consequence it is common for inhibitors of GSK-3β, even those selective against numerous other protein kinases, to have poor selectivity over GSK-3α.

Of course there are other binding sites possible through which GSK-3β could be inhibited other than the ATP site. One such low-affinity cation binding site is discussed below with respect to inhibition by the lithium ion. It has recently been suggested that thiadiazolidinone structures may be binding in an oxy-anion hole close to the activation loop (discussed in detail below). Furthermore, inhibition can be achieved by inhibition of substrate binding (such

as the GSK-3β binding proteins) though of course this is a particularly challenging mechanism for a small molecule. Alternatively, a small molecule that locked the kinase in its inactive state or blocked activating phosphorylation events could also provide a theoretical inhibition strategy.

The optimal kinase selectivity profile of a GSK-3β inhibitor is not yet fully understood: perhaps a truly selective inhibitor would be the most safe and efficacious—certainly it would be the most useful in unambiguously linking effects to a single mechanism. Alternatively there may be additional efficacy benefits to inhibiting other key kinases that either phosphorylate tau directly or are indirectly involved as priming kinases. What is clear, however, is that GSK-3β is a constitutively active kinase and phosphorylation at serine-9 reduces its activity through releasing the N-terminal domain to act as a pseudo-substrate, blocking access to the catalytic domain. Therefore, compounds which inhibit those kinases that facilitate this inactivating phosphorylation event would be undesirable due to their inhibition of a key inactivating pathway. This suggests that kinases such as p70 S6 kinase, p90Rsk, cyclic AMP-dependent protein kinase (protein kinase A, PKA), Akt (protein kinase B, PKB) and protein kinase C (PKC) isoforms (along with any respective upstream kinases in these pathways, e.g. PI3K or mTOR) are anti-targets over which it would be worthwhile to achieve selectivity.

Phosphorylation of a tyrosine residue on the activation loop of GSK-3β (Y216) appears to be important for substrate phosphorylation, either because the inactive unphosphorylated conformation of the tyrosine directly impedes access to the substrate binding groove [12] or because phosphorylation is a prerequisite to achieving the active conformation [50]. Inhibition of this process through inhibition of GSK-3β itself (which of course is the target anyway!), assuming autophosphorylation, or possibly Fyn or Pyk2 may be beneficial.

When comparing kinase selectivity, it is preferable to compare cellular selectivity (because of the differing K_m for ATP between kinases) but often this is not feasible; thus, comparison of the K_i values, taking into account the differing K_m values, can be a useful measure instead. Within the context of this review, IC_{50} and K_i values are both used and, although not ideal, selectivity is compared on that basis.

4 Inhibitors of GSK-3

Inhibitors of GSK-3β: structure, physical chemistry, mechanism of action, potency and selectivity

There are a number of small-molecule inhibitors that are claimed to have inhibitory properties versus GSK-3β. At the time of writing over 330 PCT international patent applications alone mention GSK-3β or tau kinase. Many of

these, however, refer to GSK-3β in passing, as one kinase amongst a host of others either as a target kinase or to be used as a selectivity measure; fewer specifically appear to focus on predominantly GSK-3β alone.

Although a significant proportion of those articles actually focused on describing GSK-3β inhibitors claim their use in Alzheimer's disease as well as in other indications (principally type II diabetes mellitus), there are relatively few examples where Alzheimer's disease appears to be the principal focus. For example, a simple cross-referencing of the 330 PCT international patent applications with the term "tau" reveals only 58 filings.

In this review we focus on the discussion of those small molecules which are described not only to inhibit GSK-3β in a primary kinase assay, but also where there is published data on how the compounds influence the level of tau phosphorylation in either a cellular or in vivo system (see [55] for a broader review). This latter criterion narrows down the compounds for discussion considerably, to only nine distinct chemical sub-types, where two such series from AstraZeneca—3-aminopyrazinyl-2-carboxamides and oxindolequinazolines—are reported for the first time. In addition to these there are two other inhibitor classes described in the literature which will not be discussed in the course of this review: sodium valproate [51], where there is conflicting data [52], and large molecules such as GSK-3β binding proteins (e.g. amongst others GSKIP or FRAT-1 [50, 53]), which are beyond the scope of this focus on small molecules.

The nine broad chemotypes of GSK-3β inhibitors where inhibitory effects on tau phosphorylation have been disclosed include:

1. Lithium chloride [54]
2. Hymenialdisine—a marine natural product [55]
3. Paullones [84]
4. Indirubins [57]
5. Nitrothiazole urea [58]
6. 3-Aminopyrazinyl-2-carboxamides [59]
7. Oxindolequinazoline [60]
8. Bisarylmaleimides and anilino-arylmaleimides [61]
9. Thiadiazolidinones [62]

For each of the above classes of compound the following areas will be discussed and critiqued based on published experimental data as well as calculated data (for such areas as physical chemistry and permeability—where other data are not available):

- Potency vs kinase (inhibition of phosphorylation of peptide substrate)
- Mechanism of action
- Selectivity vs other kinase inhibitors or off target effects (if reported)
- Calculated physical properties
- Predicted permeability—particularly with respect to the blood–brain barrier (BBB)

4.1 Lithium Chloride

Lithium has been used to treat bipolar disorders based on its mood stabilising effects for several decades. Considerable effort has been put into understanding the mechanism by which lithium operates. The recent discovery that lithium inhibits GSK-3 at therapeutic concentrations has introduced the possibility that GSK-3 represents a key target of lithium's action in the brain.

The lithium cation (Li^+) has been shown to inhibit GSK-3β with a potency around 2 mM IC_{50} (3.3 mM K_i) and, although weak, this concentration is achievable clinically, thus offering the potential as a useful therapeutic. Other than GSK-3β, Li^+ has been shown to also inhibit polyphosphate 1-phosphatase, inositol monophosphatase, casein kinase-II (CKII), MAP kinase-activated protein kinase-2 (MAPKAP-K2) and p38-regulated/activated kinase (PRAK) as well as activating, in cells, PI3-kinase/PKB and c-jun N-terminal kinase (JNK) [63–66]. Naturally this polypharmacology complicates the interpretation between target and function of Li^+.

A very elegant study [67] from Harwood and Ryves demonstrated that Li^+ is non-competitive with respect to peptide substrate or ATP and instead inhibits GSK-3β by competing with Mg^{2+} ions. Li^+ showed a similar mode of inhibition with both GSK-3α and GskA (the *Dictyostelium* GSK-3 protein homologue) which differs from GSK-3β in the catalytic core by 29%.

As a consequence of establishing this mechanism the authors noted that any such species which interfered with Mg^{2+} concentrations such as metal chelators (including, of course, ATP) should also have an effect. Indeed, when ATP concentrations rise above the cellular Mg^{2+} concentration it starts to become inhibitory. The precise mechanism of inhibition cannot be displacement of Mg^{2+} from the ATP–Mg^{2+} complex by Li^+ because Li^+ is not a general kinase inhibitor, is non-competitive with respect to ATP and also the K_a of the ATP–Mg^{2+} complex is 250 times greater than that for the ATP–Li^+ complex [68]. Li^+ and Mg^{2+} have similar ionic radii (74 and 72 pm, respectively, for a coordination number of 6), which Harwood and Ryves suggest leads to a replacement of Mg^{2+} by Li^+ in a low-affinity binding site (currently at an unknown position) on GSK-3β resulting in disruption of catalytic function. Interestingly, no other Group I metal ion has shown comparable inhibitory properties, consistent with their increased ionic radii (e.g. Na^+ 102 pm for a coordination number of 6). The explanation would clearly explain the similar profile observed vs GSK-3α and GskA (and the similarity between these proteins may offer some hints as to the possible binding sites).

The total cellular concentration of Mg^{2+} lies between 5 and 10 mM, though the free and available concentration of the ion is only 0.6–1.2 mM. Harwood's study indicates that at this lower concentration of Mg^{2+}, the potency of Li^+ may be improved in vivo due to its concentration dependence on Mg^{2+} ions.

As a metal ion, Li^+ is clearly able to cross through biological membranes and access the interior of the cell in all tissues in the body and lithium chloride is extremely soluble in water (55 g dissolves in 100 mL of water). It is an interesting therapeutic—on the one hand extremely weak in potency, yet on the other hand achieving sufficient exposure within the neuronal cytosol, based on clinical exposure, to show potency. Given Li^+'s unique mode of action there are, perhaps, opportunities for it to be combined with small-molecule ATP inhibitors, though the success of this approach would depend entirely on the, as yet unclarified, mode of action.

4.1.1 Efficacy of Lithium on Tau and Beta Amyloid

Lithium reduces the phosphorylation of tau in primary neurons [69–71]. Studies performed in Sf9 cells overexpressing FTDP-17 tau, a tau mutation resulting in tau aggregate formation resembling PHFs, display a reduction in the amount of polymer in response to lithium treatment. This suggests that phosphorylation of FTDP-17 tau by GSK-3 induces a conformational change favouring the formation of fibrillar polymers [72]. Thus, this finding implies that inhibition of GSK-3 not only regulates the phosphorylation status of tau but also tau assembly into filaments. As described above, an abnormal phosphorylation of tau is believed to have an important role in the destabilisation of microtubules, which contributes to the disruption of microtubule structures in tangle-bearing neurons. Lithium has the ability to restore the stability of microtubules in 3T3 cells co-transfected with tau and GSK-3β [73]. Thus, inhibition of GSK-3 not only inhibits fibril formation but also can recover the microtubule stability. In *Drosophila* overexpressing wt human tau 0N3R isoform, lithium treatment reverses axonal transport and locomotor deficits induced by tau abnormalities [74]. In addition, several studies have reported that lithium can inhibit tau phosphorylation and formation of tau aggregates in rodents on epitopes reported to be hyperphosphorylated in Alzheimer's disease brain [75–78]. In aged p25 tg mice overexpressing the CDK5 activator p25, chronic treatment with lithium leads to a reduction of an age-dependent increase in tau hyperphosphorylation [79]. In these mice, an increased phosphorylation at the AT8 and PHF-1 sites on tau and increased GSK-3 activity was observed. This suggests cross talk between CDK5 and GSK-3, and that CDK5 may indirectly affect tau phosphorylation via regulation of GSK-3 activity.

Several reports suggest that lithium is able to inhibit beta amyloid production in different cellular models, including cultured neurons [38–40, 80]. An opposing result was reported by Feyt et al., who showed that lithium increases amyloid production in CHO cells and cultured rat neurons expressing human APP [81]. This effect was attributed to an increased γ-secretase activity as the level of βCTF (C-terminal fragment), a cleavage fragment produced by γ-secretase processing of APP, was also elevated. Furthermore, a combined

treatment of primary neurons with lithium and the small-molecule GSK-3 inhibitor SB-415286 resulted in a less pronounced increase in beta amyloid production. Since amyloid production could be attenuated by selective GSK-3 inhibition by SB-15286, it was concluded that the lithium-mediated increase in beta amyloid production was by a different mechanism and not via GSK-3 inhibition [81]. Acute oral administration of lithium significantly reduces beta amyloid production in the brains of PDAPP (APPV717F) mice, an animal model for amyloidosis [39]. This effect was seen after 3 h of stimulation, suggesting that the effect of lithium may be direct and not dependent on transcriptional or translational regulation of downstream targets. In a more chronic setting, PDAPP mice fed with lithium for 7 months, starting at 1 month of age, show decreased beta amyloid levels in hippocampus and decreased total plaque area [40]. In an amyloid mouse model heterozygous for the APP Swedish transgene (Tg2576) and the PS1P264L mice, a reduction of beta amyloid was accomplished following oral administration of lithium [38].

Collectively, inhibition of GSK-3 by lithium has been reported to decrease amyloid production and amyloid plaque burden in different in vivo models of amyloidosis. Chronic lithium treatment not only reduces beta amyloid production but also amyloid plaque load.

4.1.2
Lithium and Long-Term Potentiation

As previously discussed, an association between neuronal plasticity and LTP has been suggested where increased GSK-3 activity may impair synaptic plasticity. Overexpression of GSK-3 impairs LTP in transgenic mice conditionally overexpressing GSK-3β, whereas in non-transgenic littermates lithium had no effect on LTP. In the GSK-3 transgenic mice, LTP could be restored by a chronic pre-treatment with lithium, thus suggesting that GSK-3 inhibition may facilitate the initiation or maintenance of LTP [31].

In conclusion, lithium is a moderate inhibitor of GSK-3, which in preclinical studies has been shown to influence pathophysiological mechanisms of Alzheimer's disease, i.e. both decreasing the hyperphosphorylation of tau and reducing the metabolism of beta amyloid. However, the action of lithium is complicated by the diverse actions associated with this compound. Due to the toxic effects associated with long-term use of lithium and therapeutically higher concentrations to decrease GSK-3 activity, it may not be considered as a prime candidate for treatment of Alzheimer's disease.

4.2
Hymenialdisine

Hymenialdisine (HD), Fig. 1, is a marine sponge extract which was isolated in 1997 along with a series of related metabolites. The compound was dis-

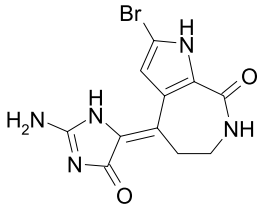

Fig. 1 Hymenialdisine—a marine natural product

covered as a kinase inhibitor via the screening of an extract library against CDK1/cyclin B and subsequently found to inhibit other kinases in the CDK family, GSK-3β and CKI (casein kinase I) all below 100 nM (Table 1).

The superior potency vs GSK-3β led to the exploration of effects on tau phosphorylation, both in a natural substrate kinase assay and in Sf9 cells. In the former, HD inhibited tau phosphorylation in a dose-dependent manner with an IC_{50} of 33 nM. In Sf9 cells expressing human tau23, HD was shown to inhibit tau phosphorylation at the AT8, AT100, AT180 and PHF1 site [55]. However, since these sites are not restricted to GSK-3 phosphorylation and the fact that these experiments were done with a single very high dose (50 μM), a concentration at which HD inhibits several other kinases besides GSK-3, the physiological relevance of this observation, in the context of GSK-3 inhibition, needs to be validated.

HD is known to inhibit CDK1/cyclin B in an ATP competitive fashion and the protein–ligand structure was solved in CDK2—demonstrating, unequivocally, the binding to the ATP binding site. On the basis of this it is reasonable to assume that HD inhibits GSK-3β in a similar ATP competitive manner and binds in the ATP binding site. A *docking* representation of the binding of HD in GSK-3β using knowledge of the CDK2 structure is shown in Fig. 2. The Br group points towards the solvent exposed opening of the pocket, with the key hinge binding interactions between the carbonyl of Asp133 and the NH of the seven-membered lactam and the NH of Val135 with the carbonyl

Table 1 Kinase inhibition selectivity of hymenialdisine

Kinase inhibition (IC_{50} nM)	GSK-3β	CDK1/ cyclin B	CDK2/ cyclin A	CDK2/ cyclin E	CDK3/ cyclin E	CDK4/ cyclin D1
Hymenialdisine	10	22	70	40	100	600
Kinase inhibition (IC_{50} nM)	CDK5/ p25	CDK6/ cyclin D2	Erk1	PKCα	PKCγ	CKI
Hymenialdisine	28	700	470	700	500	35

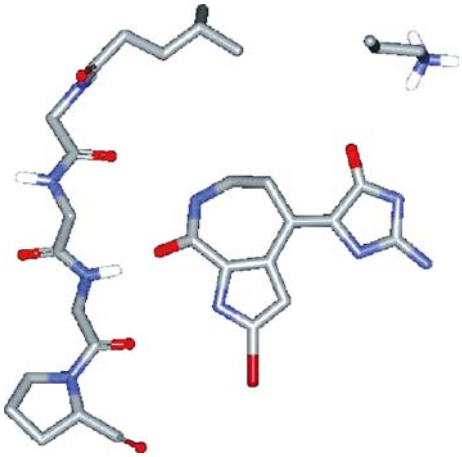

Fig. 2 Schematic showing a predicted docking pose of HD in GSK-3β. This is not an X-ray structure

oxygen of the lactam. This then places the imidazolinone in a position to interact favourably with the salt bridge yet minimises steric clashes with the Leu gatekeeper.

A summary of properties for HD is given in Table 2. These are calculated values using Advanced Chemistry Development (ACD/Labs) Software version 8.14 for Solaris (©1994–2007 ACD/Labs). The molecule is kinked by virtue of the seven-membered ring and the low log*D* contributes to a good predicted solubility. It is extremely rich in heteroatoms resulting in a large number of solvating groups and high polar surface area (PSA); on the basis of the H-bond donor count alone, the absorption through the gastrointestinal membrane, let alone the blood–brain barrier (BBB), might be expected to lead to a limited exposure in the CNS. Interestingly, the predicted brain–plasma ratio [82] is 0.0 (likely range 0.0–0.1), which reflects the likely low permeability and high free fraction of the compound. Clearly, HD inhibits kinases within the CMGC family and the desirability or otherwise of this is unclear; in addition, although some impressive selectivities are observed vs several groups of kinases, it is likely that there is significant inhibition within the kinome. However, tau phosphorylation is inhibited at a high concentra-

Table 2 Predicted properties for HD. ACD/Labs version 8.14

Log*D* (pH 7)	No. H-bond acceptors	No. H-bond donors	Solubility (μM) (pH 7, 25 °C)	MWt	Polar surface area (Å^2)
1.2	7	5	160	324	112

tion of HD, though without a concentration response no further conclusions can be made. Thus HD's potential as an attractive, potent small-molecule inhibitor of GSK-3β for use in animal models of Alzheimer's disease is likely to be limited due to CNS exposure although, even if sufficient, any interpretation of effect may be complicated by the lack of specificity for GSK-3β.

4.3 Paullones

Paullones represent a family of benzazepinones which were first identified and synthesised as CDK inhibitors. Due to the homology between the CDK family and GSK-3β, alsterpaullone (Fig. 3), one of the most active members of the chemical family vs CDK2, was tested and shown to also be a potent inhibitor of GSK-3β (Table 3).

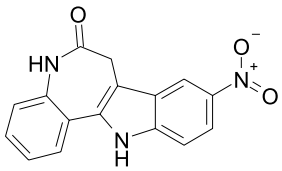

Fig. 3 Alsterpaullone

Table 3 Kinase inhibition selectivity of alsterpaullone

Kinase inhibition (IC_{50} nM)	GSK-3β	CDK1/ cyclin B	CDK2/ cyclin A	CDK2/ cyclin E	CDK4/ cyclin D1
Alsterpaullone	4	35	15	200	> 10 000
Kinase inhibition (IC_{50} nM)	CDK5/ p35	Erk2	PKCα	PKCγ	CK1
Alsterpaullone	40	4500	> 100 000	> 100 000	> 100 000

Out of 35 kinases tested, inhibition sub-100 nM was only achieved within the GSK-3 and CDK families (CDK1, 2 and 5). Impressive selectivites were observed over the PKC, CK and Erk families, though there is a significant part of the kinome against which the selectivity is unknown.

Alsterpaullone is an ATP competitive inhibitor of GSK-3β and the crystal structure of the protein–ligand complex has been solved, demonstrating

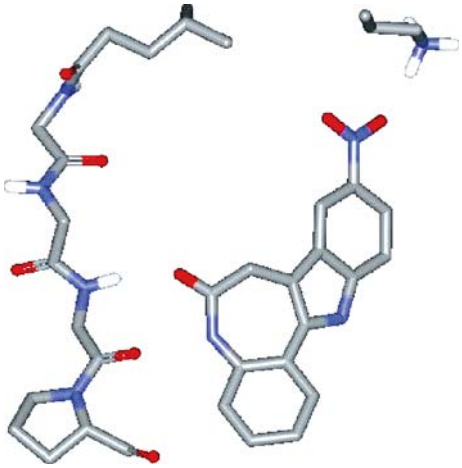

Fig. 4 Crystal structure of alsterpaullone bound to GSK-3β. (Crystal structure PDB entry 1Q3W)

binding to the ATP binding site (Fig. 4). The hinge–ligand interactions are between the lactam in alsterpaullone and the NH and carbonyl of Val133. This places the nitro group in a favourable position to interact with the salt bridge, and the lack of functionality close to the gatekeeper residue contributes to the non-selectivity vs CDK2.

A summary of properties for alsterpaullone is given in Table 4. The molecule is slightly twisted by virtue of the kink in the seven-membered lactam ring and has a moderately high log*D*, which would suggest low solubility. The H-bonding groups contribute to a moderate PSA (91 $Å^2$) and that coupled with the moderately high lipophilicity would suggest good absorption and BBB permeability. The predicted brain–plasma ratio [82] is 0.3 (likely range 0.1–1.1), which reflects the likely good permeability and protein/tissue binding predictions of the compound. Inhibition of tau phosphorylation in a natural substrate binding assay is very high (IC_{50} 33 nM) and at a single concentration of 20 μM phosphorylation of the PHF-1 epitope Ser396 and Ser404 in Sf9 cells is clearly inhibited. Alsterpaullone is a potent small-molecule inhibitor of GSK-3β with demonstrated activity vs tau phosphorylation and good predictions of permeability. Studies in vivo will help to

Table 4 Predicted properties for alsterpaullone. ACD/Labs version 8.14

Log*D* (pH 7)	No. H-bond acceptors	No. H-bond donors	Solubility (μM)MWt (pH 7, 25 °C)		Polar surface area ($Å^2$)
3.6	6	2	6	293	91

assess the advantage or otherwise of non-selectivity within the CDK family. As a nitro group containing compound concerns would exist that bioactivation to a genotoxic species may be an issue; however, it is clear that other paullones in the series (e.g. kenpaullone) can show good potency along with replacement of the nitro group.

In Sf9 cells expressing human tau23, alsterpaullone was shown to inhibit tau phosphorylation at the AT8, AT100, AT180 and PHF1 sites [84]. However, these experiments were done with a single very high dose (20 μM), a concentration at which alsterpaullone may also inhibit Erk2 and PKA. Thus, the physiological relevance of these findings needs to be followed up in a physiologically more relevant setting. In cultured rat cortical neurons, alsterpaullone has been shown to prevent calcineurin inhibitor-induced apoptosis associated with caspase-3 activation [85].

4.4 Indirubins

Indirubins have been reported to cross the BBB [86]. Indirubin-3-monoxime is also an effective inhibitor of the CDKs. Indirubin-3-monoxime has been shown to inhibit tau phosphorylation at the AT8, AT100, AT180 and PHF1 site in Sf9 cells expressing human tau23. In cerebellar granular neurones, indirubin-3-monoxime inhibits tau phosphorylation at Ser199 [87, 88]. However, the concentrations used in these experiments (10–20 μM) are well above the reported IC_{50} values for CDK1 and CDK5; thus, these findings need to be confirmed in a physiologically relevant setting. In cerebellar granular neurones, indirubin-3-monoxime has also been shown to protect neurones against cell death induced by growth factor deprivation [87].

The bis-indole indirubins are active ingredients of traditional Chinese medicines that were first identified as CDK inhibitors and then subsequently screened vs GSK-3β. Indirubin-3′-monoxime (Fig. 5) is one of the more potent GSK-3β inhibitors with some selectivity within the CDK family (Table 5).

Indirubin-3′-monoxime has been demonstrated to be ATP competitive and to bind in the ATP binding site (X-ray crystal structure of protein–ligand complex solved). The crystal structure is shown in Fig. 6. The key hinge region binding interestingly involves all three of the key H-bonding elements:

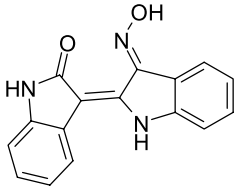

Fig. 5 Indirubin-3′-monoxime

Table 5 Kinase inhibition selectivity of indirubin-3′-monoxime

Kinase inhibition (IC_{50} nM)	GSK-3β	CDK1/ cyclin B	CDK5/ P25
Indirubin-3′-monoxime	22	180	100

Fig. 6 Crystal structure of indirubin-3′-monoxime in GSK-3β. (Crystal structure PDB entry 1Q4I)

the carbonyl of Asp133 binding to the NH of the indole, then the NH of Val135 donating to the oxygen acceptor, and then the proton stabilised by H-bonds both to the carbonyl of Val135 as well as internally to the indole nitrogen.

A summary of properties for indirubin-3′-monoxime is given in Table 6. The molecule is flat, has a moderate log*D* and acidic character which leads to acceptable predicted solubility. Although the number of H-bond donor groups is quite large ($n = 3$), it is likely that a tautomeric form in which the hydroxy indole is intramolecularly bonded to the indole nitrogen would be favoured and, if so, this would lead to improved permeability and less desolvation penalty. The PSA is moderate (74 $Å^2$) and the predicted brain–plasma ratio [82] is 0.3 (likely

Table 6 Predicted properties for indirubin-3′-monoxime. ACD/Labs version 8.14

Log*D* (pH 7)	No. H-bond acceptors	No. H-bond donors	Solubility (μM)MWt (pH 7, 25 °C)		Polar surface area ($Å^2$)
2.2	5	3	120	277	74

range 0.1–1.0), so since the compound is not particularly lipophilic it is likely that CNS penetration would be acceptable. Inhibition of tau phosphorylation in a natural substrate binding assay is good (IC_{50} 100 nM) and at a single concentration of 20 μM phosphorylation of Thr212 (recognised by AT100) in Sf9 cells is clearly inhibited. Indirubin-3′-monoxime is a potent small-molecule inhibitor of GSK-3β with some selectivity (eightfold) within the CDK family and acceptable predictions of permeability.

4.5 Nitrothiazole Urea

AR-A014418 (Fig. 7) is a potent small-molecule GSK-3 inhibitor belonging to the thiazole chemical class that has been reported by AstraZeneca [58]. AR-A014418 inhibits GSK-3 by competing with ATP binding with a K_i of 38 nM. In addition, this inhibitor is highly specific for GSK-3, as it does not significantly inhibit 26 other kinases tested, including CDK2 and CDK5 (Table 7). AR-A014418 is unique in this sense because most reported GSK-3 inhibitors are also powerful inhibitors of the closely related cyclin-dependent kinase (CDK) family of protein kinases. In cells overexpressing human four-repeat tau AR-A014418 inhibits tau phosphorylation at a GSK-3-specific site (Ser396) in a dose-dependent fashion, exhibiting an IC_{50} of 2.7 μM. Similar to the effect described for lithium, AR-A014418 has also been shown to decrease the assembly of phosphorylated tau into tau aggregates resembling PHFs in cells [72]. AR-A014418 has also been demonstrated to inhibit beta-amyloid-induced neuronal cell death. The inhibition of cell death by AR-A014418 correlates with inhibition of GSK-3 activity. Furthermore, AR-A014418 inhibits neuronal death induced by reduced PI3K pathway activity [58]. These results indicate that GSK-3 inhibition can be neuroprotective.

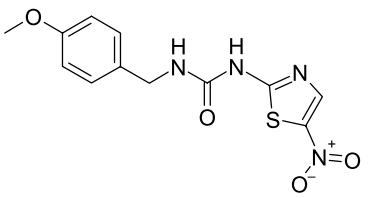

Fig. 7 AR-A014418

Table 7 Kinase inhibition selectivity of AR-A014418

Kinase inhibition (K_i nM)	GSK-3β	CDK2/ cyclin E	CDK5	pTau 3T3 cells EC_{50}
AR-A014418	38	> 100 000	> 100 000	2700

The crystal structure of AR-A014418 in GSK-3β has been solved (shown in Fig. 8) showing H-bonds from one NH of the urea to the carbonyl of Val135 as well as the thiazole nitrogen interacting with the NH of Val135. Furthermore, a possible atypical CH H-bond to the carbonyl of Asp133 may exist. The close fit of the nitro group to the gatekeeper residue is thought to contribute to the high selectivity [49].

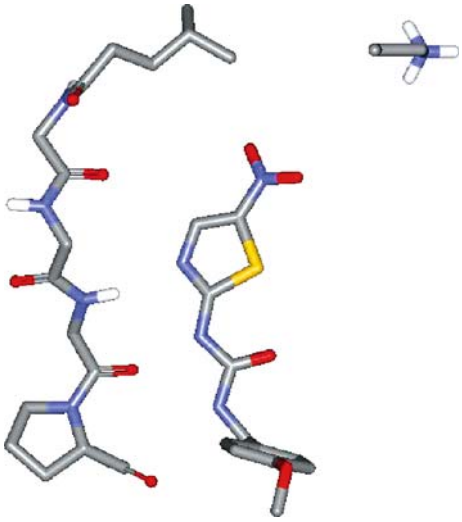

Fig. 8 Crystal structure of AR-A014418 bound to the hinge region of GSK-3β

A summary of predicted properties for AR-A014418 is shown in Table 8. AR-A014418 is a small, relatively hydrophilic and highly selective molecule. The break in planarity afforded by the benzyl group and low log*D* is likely to have contributed to the good solubility prediction. Although the PSA is high, coupled with the presence of a urea (high Δlog*P*) and considerable H-bonding (incurring likely desolvation penalties), the brain penetration, though on the low side, is sufficient for efficacy (as detailed below). The presence of a nitro group highlights the generic risk of potential generation of mutagenic metabolites via bioactivation. Nevertheless, the favourable properties of AR-A014418 make it an excellent tool as has been demonstrated.

Table 8 Predicted properties for AR-A014418. ACD/Labs version 8.14

Log*D* (pH 7)	No. H-bond acceptors	No. H-bond donors	Solubility (μM)MWt (pH 7, 25 °C)		Polar surface area (Å^2)
1.4	8	2	310	308	109

4.5.1 In Vivo Efficacy of AR-A014418

The efficacy of AR-A014418 has been studied in different in vivo models of tauopathy. AR-A014418 has demonstrated a reduction in both the phosphorylation of soluble tau and the formation of insoluble tau (PHF tau), and a reversal of microtubule dysfunction in human tau tg mice and JNPL3 mice (expressing tau mis-sense mutation P301L) [75]. Overexpression of the wt human Tau 0N3R isoform in *Drosophila* motor neurons leads to disrupted axonal transport resulting in vesicle aggregation and loss of locomotor function accompanied by neuronal cell death. Co-expression of constitutively active GSK-3 and Tau 0N3R in *Drosophila* motor neurons further enhances axon transport and locomotor phenotypes [73]. Administration of AR-A014418 in *Drosophila* reverses both the axonal transport and locomotor deficits, suggesting that this phenotype is GSK-3 dependent. AR-A014418 induces reduced immobility time in forced swim tests and inhibited amphetamine-induced activity in rats [90]. These behavioural changes are consistent with the effects of current antidepressant therapies, thus suggesting that small-molecule GSK-3 inhibitors may be useful in the treatment of bipolar disorder and depression.

4.5.2 Tau Splicing

Apart from its role in regulating the phosphorylation status of tau, GSK-3 has been attributed a novel role in the regulation of tau splicing. Cortical neurons treated with AR-A014418 display an increased proportion of the tau four-repeat isoform as a consequence of alternative exon 10 splicing, supporting a novel role for GSK-3 in alternative tau exon 10 splicing [91]. The suggested mechanism by which GSK-3 modulates tau mRNA splicing involves phosphorylation of the splice factor SC35, shown to co-localise with GSK-3 in nuclear speckles. These findings raise a possible novel role for GSK-3 in tauopathies, regulating tau mRNA splicing; however, the relevance of these findings in relation to disease remains to be determined.

4.5.3 Pharmacokinetic Properties of AR-A014418

The pharmacokinetic properties of AR-A014418 have been studied in Sprague–Dawley rats. After per oral dosing of 1 μmol/kg of AR-A014418, the maximal total concentration (C_{max}) in plasma was 3.75 μM with an area under curve (AUC) of 22.4 μM h and the half-life was determined to be 8.7 h with a T_{max} of 0.26 h. After an i.v. bolus dose of 3 μmol/kg, followed by an infusion of 3 μmol/kg/h over 2 h to obtain a steady state between plasma and

brain levels, the brain concentration was observed to be 0.13 μM. In the majority of the in vivo experiments discussed above, 30 μmol/kg was used, thus achieving total brain concentrations well above the concentration expected to inhibit GSK-3 [92]. Interestingly, AR-A014418 has been radiolabelled with ^{11}C in order to investigate the compound for PET studies [83], and was shown to be an unsuitable ligand due to the low uptake values of radioactivity at 5 and 30 min (0.04 and 0.06% ID/g of wet tissue).

In summary, AR-A014418 is a potent and specific GSK-3 inhibitor capable of intervening with both tau phosphorylation and beta-amyloid-induced toxicity. AR-A014418 is also the only published specific GSK-3 inhibitor with documented in vivo efficacy consistent with an Alzheimer's disease modifying mode of action. Thus, AR-A014418 represents an important research tool to study the therapeutic potential of GSK-3 inhibition in neurological disease.

4.6
3-Aminopyrazinyl-2-carboxamides

AZ11125357 (Fig. 9) is a novel small-molecule GSK-3 inhibitor identified as a result of a high-throughput biochemical screen using purified recombinant human GSK-3. AZ11125357 belongs to the pyrazine chemical class of GSK-3 inhibitors that has been developed by AstraZeneca. AZ11125357 inhibits GSK-3β by competing with ATP binding with a K_i of 8 nM. AZ11125357 has been tested for selectivity against CDK2 and found to inhibit CDK2 with a K_i of 85 nM, thus displaying 11-fold selectivity versus GSK-3β (based on a comparison of K_i values).

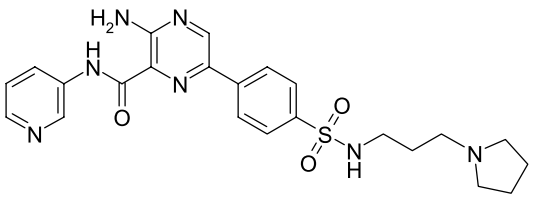

Fig. 9 AZ11125357

3T3 fibroblasts stably expressing human four-repeat tau protein and endogenous levels of GSK-3 were used to evaluate whether AZ11125357 inhibited GSK-3 mediated tau phosphorylation in cells. The effect on tau phosphorylation was determined by Western blotting. In 3T3 cells stably expressing human tau, AZ11125357 inhibited phosphorylation on tau at a site specifically phosphorylated by GSK-3β (Ser396), with an IC_{50} of 207 nM. These data are summarised in Table 9.

Table 9 Kinase inhibition selectivity of AZ11125357

Kinase inhibition (K_i nM)	GSK-3β	CDK2/ cyclin E	pTau 3T3 cells EC_{50}
AZ11125357	8	85	207

AZ11125357 is one member of a series of pyrazines that have been extensively explored at AstraZeneca. Although an X-ray crystal structure does not exist for this compound, other solved structures within the series allow docking of the structure with a high degree of confidence. Figure 10 shows a docking of the pyrazine in GSK-3β. The key interactions include the NH_2 of the pyrazine to the carbonyl of Asp133, the pyrazine nitrogen to the NH of Val135 and the pyridine nitrogen interacting with the salt bridge.

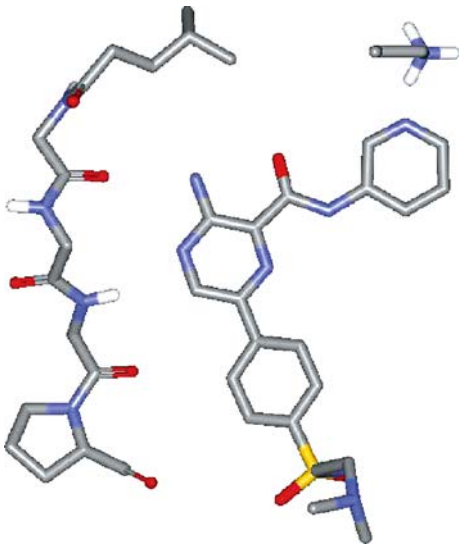

Fig. 10 Schematic showing a predicted docking pose of AZ11125357 to the hinge region of GSK-3β. This is not an X-ray structure

A summary of predicted properties of AZ1112537 is shown in Table 10. AZ11125357 is predicted to be soluble as a result of its low log*D* and basic side chain even though some planarity through the structure exists. The high PSA combined with extensive H-bonding would suggest that permeability for this particular compound (despite intramolecular H-bonding between the amide carbonyl and NH_2) would be restricted and indeed the predicted brain–plasma ratio [82] is 0.0 (likely range 0.0–0.2). Nevertheless the com-

Table 10 Predicted properties for AZ11125357. ACD/Labs version 8.14

Log*D* (pH 7)	No. H-bond acceptors	No. H-bond donors	Solubility (μM)MWt (pH 7, 25 °C)		Polar surface area (Å^2)
0.7	10	4	50	482	112

pound exemplifies many attractive characteristics which, with optimisation, could yield highly improved examples.

4.7 Oxindolequinazolines

AZ10316813 (Fig. 11) is a novel small-molecule GSK-3 inhibitor belonging to the oxindolequinazoline chemical class which has been developed by AstraZeneca. AZ10316813 inhibits GSK-3β by competing with ATP binding at a K_i of 39 nM and demonstrates selectivity against more than ten other kinases tested including IGF1R, ZAP70, PTK2, SRC, JAK3, Abl, p38, JNK1, PKA, MAP2K1 and CHK1. AZ10316813 is non-selective versus CDK2 (K_i: 21 nM) and in 3T3 cells stably expressing human tau, AZ10316813 inhibited phosphorylation on tau Ser396, with an EC_{50} of 130 nM, as shown in Table 11.

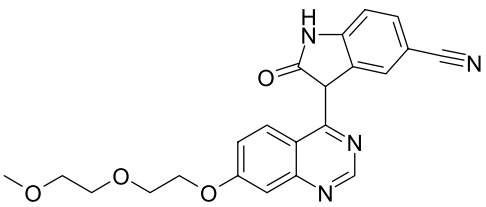

Fig. 11 AZ10316813

Table 11 Kinase inhibition selectivity of AZ10316813

Kinase inhibition (K_i nM)	GSK-3β	CDK2/ cyclin E	pTau 3T3 EC_{50}
AZ10316813	39	21	130

The X-ray crystal structure of AZ10316813 has been solved in GSK-3β and is shown in Fig. 12. The key hinge region binding involves all three of the key H-bonding elements: the carbonyl of Asp133 binding to the NH of the indole,

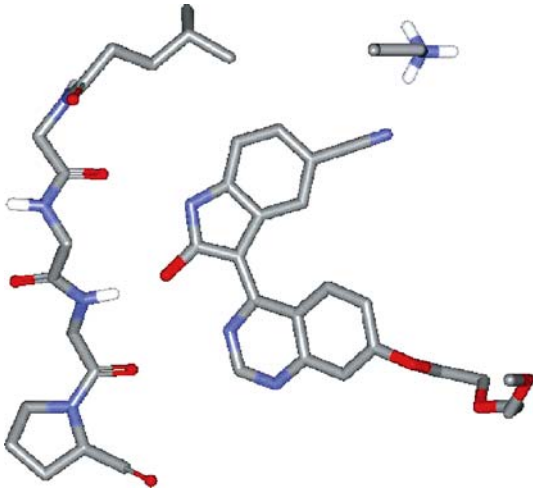

Fig. 12 Crystal structure showing the hinge binding of AZ10316813 to GSK-3β

then the NH of Val135 donating to the oxygen acceptor and then the proton stabilised by H-bonds both to the carbonyl of Val135 as well as internally to the quinazoline nitrogen.

A summary of the predicted properties of AZ10316813 is shown in Table 12. The moderate lipophilicity coupled with the branching alkoxy side chain is predicted to give acceptable solubility. The high PSA and H-bonding is offset somewhat by the internal H-bond of the tautomerised indolone with the *ortho*-quinazoline nitrogen, but despite this the predicted brain–plasma ratio for the compound is 0.1 (likely range 0.0–0.2). Clearly the in vivo efficacy of such a compound will depend greatly on the experimental permeability and pharmacokinetic parameters.

Table 12 Predicted properties for AZ10316813. ACD/Labs version 8.14

Log*D* (pH 7)	No. H-bond acceptors	No. H-bond donors	Solubility (μM) (pH 7, 25 °C)	MWt	pK_a (acid)	Polar surface area (Å^2)
1.6	8	2	40	404	8.1	113

4.8 Bisarylmaleimides and Anilino-arylmaleimides

A variety of bisarylmaleimides have been reported as potent GSK-3 inhibitors (Table 13). These compounds have been shown to inhibit tau phosphorylation

Table 13 Kinase inhibition selectivity of maleimides

Kinase inhibition (K_i nM)	GSK-3β	CDK2	CDK4	PKCβ	pTau EC_{50} nM
SB-415286	180	–	–	–	
SB-216763	35	–	–	–	
UICGUMC-22	2.3	–	–	–	
Lilly-41	0.7[a]	434[a]	929[a]	914[a]	0.3[b]

[a] Figures quoted are IC_{50} not K_i values
[b] Analysis of Ser396 phosphorylation of tau in SY5Y cells

in a neuronal cell line, SHSY5Y, measuring inhibition of Ser396 phosphorylation [93]. Some of the reported bisarylmaleimides display cellular IC_{50} values less than 1 nM, thus making them the most potent GSK-3 inhibitors reported in a functional cellular assay.

SB-216763 and SB-415286 are maleimide ATP-competitive inhibitors developed by SmithKline Beecham Pharmaceuticals that inhibit GSK-3 with K_i values of 9 and 31 nM, respectively [61]. In primary cerebellar granule neurones, an inhibition of Thr181 and Ser202 tau phosphorylation was shown with both these compounds. In HEK293 cells overexpressing recombinant tau and GSK-3β, SB-216763 and SB-415286 treatment led to a reduction in tau phosphorylation at Ser202 [13]. SB-216763 and SB-415286 have been shown to cause a decrease in inhibitory Ser9 phosphorylation of GSK-3β. This effect is possibly via activation of an upstream kinase p90rsk, previously shown to phosphorylate GSK-3 on Ser9 [94, 95]. It has been reported that SB-216763 and SB-415286 prevent neuronal cell death in cerebellar granule neuronal cultures and chicken DRGs after NGF withdrawal or inhibition of the PI3 kinase pathway [61]. In addition, SB-216763 was shown to prevent prostaglandin E2 (PGE2)-induced cell death in cultured rat cortical neurons, suggestively by inhibition of caspase-3 activation [96].

Maleimides from Illinois University have been shown to inhibit tau phosphorylation at the GSK-3-specific site Ser396. In a cellular model of Parkinson's disease, changes in the level of phosphorylated tau were shown to be associated with increased α-synuclein expression and a decrease in cell viability. In these cells, maleimides 18 and 22 protect against neuronal cell death. Thus, it was suggested that GSK-3 inhibition might protect neuronal cells against cell death by decreasing the level of α-synuclein [97].

The maleimides constitute the single largest chemotype of structures shown to inhibit GSK-3β. They originate from the early identification of bisindolylmaleimides and similar sub-structures as kinase inhibitors (cf. staurosporine). SB-415286 and SB-216763 were some of the first from SmithKlineBeecham, their structures differing in that one is a phenyl

indolyl-maleimide (SB-216763) and the other a phenyl anilino-maleimide (SB-415286). UICGUMC-22 (University of Illinois at Chicago, Georgetown University Medical Center—compound 22) [98] is an indolyl azaindolyl-maleimide, and finally Lilly's compound 41 is a highly functionalised indolyl imidazocinnolinyl-maleimide (see Fig. 13).

Potency in this series can be extremely good (Table 13) with good to excellent selectivities obtainable depending on the substitution around the aryls connected to the maleimide. SB-216763 and SB-415286 are reported to show little activity vs a panel of 24 kinases (including PKB), though specific and more exhaustive data are unavailable. Although selectivity for UICGUMC-22 is unknown, Lilly-41 has marvellous selectivity over kinases such as PKCβII, which is all the more impressive given that their start point appears to have been a potent PKCβII inhibitor. Lilly authors noted, in fact, that due to a tight binding situation the true level of potency of several of their inhibitors could only be truly assessed by titrating down the concentration of enzyme in their assay; thus, Lilly-41 may be even more potent than reported.

Examples from the maleimide series have been demonstrated to be competitive with ATP and they are grouped together because their ligand binding to the hinge region is usually similar, involving the NH of the maleimide binding to the carbonyl of Asp133 and one of the carbonyls of the maleimide binding to the NH of Val135. The precise orientation of the two aryl or anilino

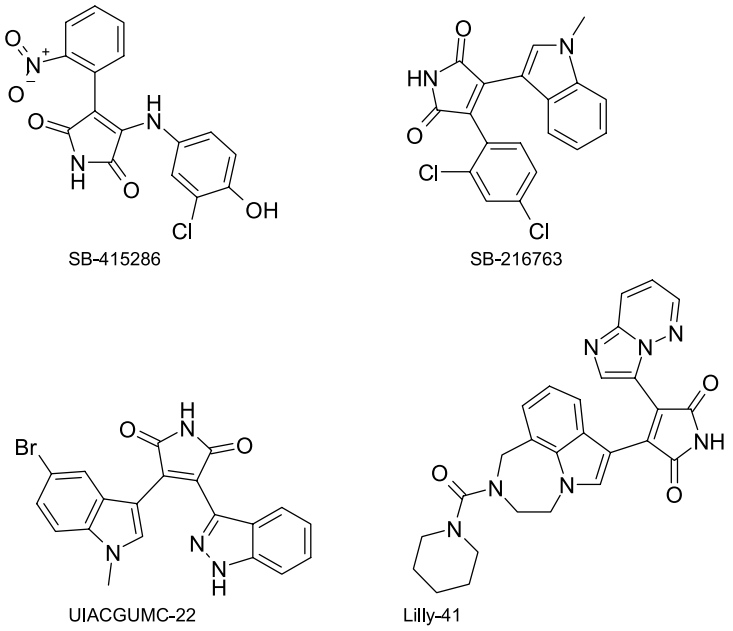

Fig. 13 Four examples from within the maleimide series

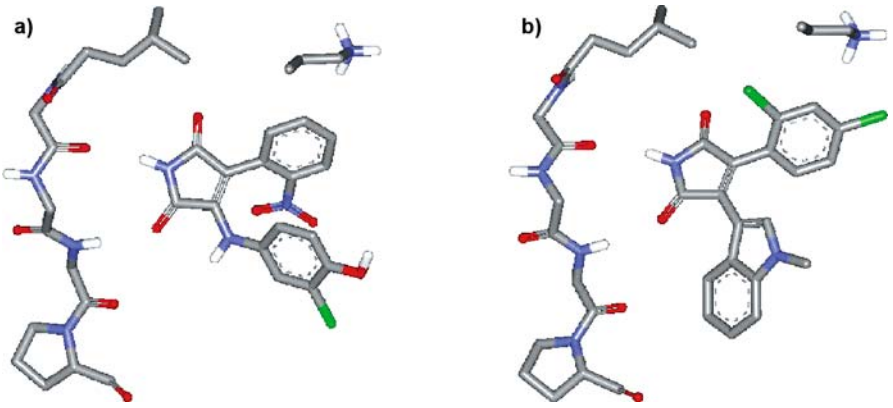

Fig. 14 **a** Schematic showing predicted docking pose of SB-415286 to the hinge region of GSK-3β. These are not X-ray structures. **b** Schematic showing predicted docking pose of SB-216763 to the hinge region of GSK-3β. These are not X-ray structures

groups depends on the substitution pattern since there is a space constraint close to the gatekeeper and salt bridge. Dockings of SB-415286 and SB-216763 in GSK-3β are shown in Fig. 14a and b, respectively. Since these are dockings and several alternative poses were acceptable, the precise mode and conformation would only be able to be unambiguously determined by X-ray crystallography.

A summary of properties for the maleimides is given in Table 14. The molecules are usually twisted as a result of steric clashes between the *ortho* positions of each aryl group. The predicted solubility of these four examples is low except for SB-415286, which is significantly less lipophilic.

Predicted permeabilities based on H-bonding groups and PSA would suggest that SB-415286 would have the least and at the other end of the spectrum SB-216763 would have extremely good permeability (low PSA, high lipophilicity and few solvating atoms). The exquisitely potent Lilly-41 has a lot of H-bond acceptors, one of which is a urea such that the $\Delta \log P$ of the com-

Table 14 Predicted properties for maleimides. ACD/Labs version 8.14

Name	Log*D* (pH 7)	No. H-bond acceptors	No. H-bond donors	Solubility (μM) (pH 7, 25 °C)	MWt	Polar surface area (Å^2)
SB-415286	1.7	11	3	150	360	124
SB-216763	4.6	4	1	1	371	46
UICGUMC-22	3.9	6	2	2	421	80
Lilly-41	2.1	10	1	3	496	105

pound is likely to be high and this would be expected to negatively impact on permeability. The predicted brain–plasma ratios [82] for the four compounds are shown in Table 15.

Table 15 Predicted brain–plasma ratios

Name	Brain–plasma ratio	Likely range
SB-415286	0.1	0.0–0.6
SB-216763	0.9	0.2–3.7
UICGUMC-22	0.3	0.1–1.2
Lilly-41	0.1	0.0–0.4

Additional selectivity profiling and pharmacokinetics of the maleimides are ideally required to assess their attractiveness and importance as in vivo tools. Nevertheless, exciting cellular inhibition of tau, in conjunction with varied degrees of brain exposure, suggests that these compounds could prove to be extremely interesting tools.

4.9 Thiadiazolidinones

The heterocyclic thiadiazolidinones (TDZDs) are reported to be non-ATP-competitive inhibitors of GSK-3β with activity in the micromolar range [62]. The thiadiazolidinones represent the first non-ATP-competitive GSK inhibitors. In vitro kinase assays demonstrate inhibition of GSK-3β with TDZD-8 (Fig. 15) with an IC_{50} of 2 μM. TDZD-8 has been reported to be selective versus CDK1, PKA, PKA and casein kinase II (CK2); however, selectivity versus the closest homology kinase CDK2 has not been reported. Given that the thiadiazolidinones do not compete with ATP for binding, it is likely that achieving kinase selectivity is less of an issue. Also, the cellular potency drop-off, seen with ATP-competitive inhibitors, is potentially considerably smaller for these compounds, acting via a non-ATP-competitive mode of action. However, when tested in primary neuronal cultures, no detectable inhibition of GSK-3β in response to TDZD-8 was achievable [99]. The second generation of the thiadiazolidinones with increased potency will provide further insight into the significance of the non-ATP-competitive approach for GSK-3 inhibition. Also, if the thiadiazolidinones turn out to bind irreversibly to GSK-3, the future therapeutic potential of these compounds could be questionable.

Thiadiazolidinones, exemplified by TDZD-8, are a fascinating series of non-ATP-competitive GSK-3β inhibitors identified by Neuropharma. The structures contain, at their core, a five-membered thiadiazolidinone heterocycle including a weak N–S bond. The reported inhibition potency of TDZD-8 vs GSK-3β is

moderate and the selectivity vs other kinases is excellent, with no inhibition observed up to 100 μM, a truly astonishingly level of specificity (Table 16).

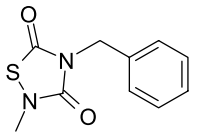

Fig. 15 TDZD-8

Table 16 Kinase inhibition selectivity of TDZD-8

Kinase inhibition (IC_{50} nM)	GSK-3β	CDK1/ cyclin B	CKII	PKA	PKC
TDZD-8	2000	> 100 000	> 100 000	> 100 000	> 100 000

Although the mechanism of inhibition has been demonstrated to be independent of ATP, it is still unclear precisely how the compounds inhibit the kinase. Several suggestions have been considered by Neuropharma scientists: one of these (Fig. 16) includes the hypothesis that the compound binds in an oxy-anion hole in the activation loop; the second possibility reported in 2005 [89] suggests an extended binding conformation in the ATP binding site such that the active site Cys199 could potentially react with the sulphur atom in TDZD-8. This would, indeed, explain the non-ATP-competitive nature of the inhibition and also explains the SAR requirements for sulphur in the ring. The rarity of an active site Cys at such a location in other kinases could presumably explain the selectivity. Heterocycles possessing weak S–N bonds are prone to fragmentation and covalent bond formation with sulphur nucleophiles, so the irreversible binding of compounds in this class to the kinase, acting as so-called suicide inhibitors, is not inconceivable.

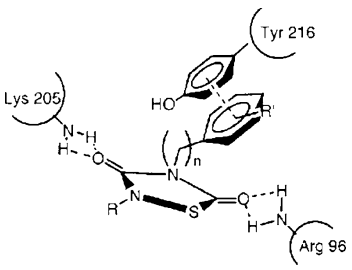

Fig. 16 Proposed binding of TDZD-8 to activation loop of GSK-3β. (Figure transposed from Fig. 3 of [62])

Table 17 Predicted properties for HD. ACD/Labs version 8.14

Log*D* (pH 7)	No. H-bond acceptors	No. H-bond donors	Solubility (μM) (pH 7, 25 °C)	MWt	Polar surface area (Å^2)
0.3	4	0	14 000	222	66

TDZD-8 is a very small molecule with many ideal properties: high solubility, low PSA and low lipophilicity as a result of its low molecular weight rather than abundance of heteroatoms. The predicted permeability across a biological membrane is expected to be high and the predicted brain–plasma ratio [82] is 0.6 (likely range 0.1–2.4) (Table 17). This coupled with the amazing selectivity profile makes the compound a fascinating tool for in vivo study. The moderate potency could be overcome by sufficiently high free compound levels in plasma and brain on high enough oral dosing. Of course, if the inhibition is irreversible and thus time-dependent, then an atypical pharmacokinetic–pharmacodynamic relationship will operate.

5
Therapeutic Potential of GSK-3 Inhibitors

The current therapeutics for Alzheimer's disease are limited to drugs that provide only marginal symptomatic benefit in the clinic. This is achieved by attenuating cognitive deficits through inhibiting acetylcholinesterase and increasing the levels of the neurotransmitter ACh or by antagonists for the NMDA receptor. However, this type of therapy does not affect the underlying pathology or halt the progressive neuritic dystrophy and neuronal damage. In addition, over time these therapies become ineffective. Therefore, there is an urgent medical need to develop agents which delay or reverse the progression of Alzheimer's disease.

The doses of lithium given in bipolar patients will at best cause about 25% inhibition of total GSK-3 activity [71]. Given the observation that lithium reduces GSK-3 activity and tau phosphorylation at therapeutically relevant concentrations, lithium has been tested for its ability to slow down the progression of Alzheimer's disease. In a retrospective study including controls, patients currently on lithium and patients with a history of lithium prescribed, cognition and memory was assessed using the Mini-Mental State Examination (MMSE) score. No significant difference in MMSE scores between control and patients currently on lithium was found. However, the patient group with a history of lithium showed significantly better MMSE scores compared to the control group [100].

In a prospective study designed to investigate the pharmacodynamic effects of lithium treatment on GSK-3 activity in patients with mild Alzheimer's disease, 71 patients received either lithium or a placebo for 10 weeks. Patients where assessed for cerebrospinal fluid (CSF) phospho-tau levels and cognitive function was assessed by Alzheimer's disease assessment scale (ADAS-Cog). No effect of lithium on CSF phosphorylated tau levels could be shown. However, a clinically significant effect on cognition was measured by ADAS-Cog [101]. It is feasible that a 10-week lithium treatment might not be sufficient to see an effect on phosphorylated tau or that GSK-3 inhibition will not be reflected in the CSF phospho-tau or total tau levels. CSF turnover is quite rapid (about 6 h) and a more predictive readout might be the tau/beta amyloid ratio that has been shown to be more sensitive [102].

The length of lithium treatment, the number of subjects and the level of GSK-3 inhibition achievable with lithium might be borderline to measure a cognitive improvement in these studies. However, these findings indicate that GSK-3 inhibition may slow down the cognitive decline of Alzheimer's disease patients and warrants further studies using more potent GSK-3 inhibitors.

The link between GSK-3 and Alzheimer's disease is strongly supported by both pre-clinical in vivo models for tauopathy and localisation in human Alzheimer's disease brain. Therefore, in initial clinical trials it will be critical to test the concept of GSK-3 inhibition in Alzheimer's disease, first by assessing safety and tolerability, and later on efficacy in disease.

Studies in transgenic mice have suggested that tau pathology is reversible only if intervention occurs early in the disease process [103, 104]. Thus, for a drug treatment to be successful, it is feasible that it has to be administered early in disease.

Taking into account the diverse roles and substrates described for GSK-3, therapeutic intervention by GSK-3 inhibitors must be performed with some caution. From a clinical perspective, any potential side effects via inhibition of GSK-3 must be linked to the disease population and the length of time it takes for the effect to be observed in humans.

Concerns relating to the inhibition of kinases known to phosphorylate tau relate to degree of inhibition. For any given kinase a balance is necessary. Thus, full inhibition of GSK-3 is probably not required to affect disease progression. Also, it is still unclear whether transient inhibition or sustained inhibition of GSK-3 is required to attenuate tau phosphorylation for an extended period of time. A detailed evaluation of the pharmacokinetic and pharmacodynamic relationship of a GSK-3 inhibitor coupled with biomarker endpoints could help contribute to overcoming similar issues.

Cognitive tests and neuroimaging, such as functional MRI or PET, provide helpful but elaborate tools in the diagnoses of Alzheimer's disease. However, whether these methods will be sensitive enough to assess a halted disease progression in response to drug treatment remains to be shown.

Disease modifying treatment strategies for Alzheimer's disease have led to a need for diagnostic tools to assess the efficacy of drugs in clinical trials and to identify the disease at an early stage. The diagnostic tool of biochemical biomarkers in CSF, such as total tau and phospho-tau, has been evaluated in several studies for Alzheimer's disease patients. In a > 4 year follow-up study in 137 patients with mild cognitive impairment (MCI), an association between CSF concentrations of total tau and phospho-tau were found. CSF concentrations of total tau and phospho-tau significantly increased in patients that developed Alzheimer's disease. This association was found to be independent of other described risk factors such as age, sex and ApoE genotype. Also, the combination of CSF total tau and Abeta42/phospho-tau181 ratio was the most sensitive method to detect if patients with MCI would later develop Alzheimer's disease. Thus, it was concluded that the concentrations of total tau, phospho-tau181 and the tau/Abeta ratio in CSF are associated with future development of Alzheimer's disease in patients with MCI [104]. These types of biomarkers may be useful to distinguish MCI patients who will progress to Alzheimer's disease patients before clinical trials. It is unclear, though, whether these biomarkers can be used to monitor disease progression after/during treatment. In relation to the use of biomarkers in GSK-3 clinical trials, several questions still remain. How much of the phospho-tau in CSF is present as a result of GSK-3 activity in the brain? Will inhibition of brain GSK-3 be reflected in the CSF phospho-tau or total tau levels? How much change do we expect to see after GSK-3 inhibition over time? Equally important questions include the relation between CSF production and CSF elimination, and the relation between phospho-tau production and the clearance of phospho-tau in CSF.

GSK-3 phosphorylates the majority of PHF phosphorylation sites and co-localises with pre-tangle and tangle bearing neurons in Alzheimer's disease brain. The above summarised studies with various GSK-3 inhibitors suggest GSK-3 as a prime target for therapeutic intervention in neurodegenerative tauopathies including Alzheimer's disease. Ideally, a therapeutic approach for Alzheimer's disease would target both tau and amyloid pathologies. GSK-3 appears to be a molecular link between tau abnormalities and amyloidosis. GSK-3 inhibitors currently in the clinic will hopefully provide some guidance as to whether therapeutic intervention by GSK-3 inhibition is efficacious in Alzheimer's disease.

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