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Therapeutic Perspectives of Drugs Targeting Toll-Like Receptors Based on Immune Physiopathology Theory of Alzheimer's Disease

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Abstract

Alzheimer's disease (AD) is a neurodegenerative disease associated with the development of dementia. It has been established that the pathological hallmarks of neurofibrillary tau protein tangles and senile β -amyloid protein plaques lead to degeneration of neurons *via* inflammatory pathways. The progressive death of neurons, primarily cholinergic, results in a gradual and fatal decline of cognitive abilities and memory. By targeting these pathological hallmarks and their associated pathways, AD drug therapy can potentially attenuate the disease state. In this review article, we focus on newly proposed and experimental AD drug treatment. We discuss three characteristic areas of AD treatment: prevention of neurotoxic β -amyloid protein plaque formation, stability of neuronal tau proteins, and increase in neuronal growth and function. The primary drug therapy methods and patents discussed include the use of neurotrophic factors and targeting of the amyloid precursor protein cleavage pathway as prevention of β -amyloid formation and tau aggregation.

Keywords

Alzheimer's disease; β -amyloid; neurotrophic factors; colivelin

1. INTRODUCTION

Alzheimer's disease (AD) is a neurodegenerative disease associated with progressive development of dementia. Although the mechanism of AD development is not well understood, age is the primary risk factor [1]. About 95% of cases are considered simply sporadic, while the remaining 5% are attributed to inherited familial genetic mutations [2]. The familial genetic mutations linked to AD involve the cleavage of a protein known as amyloid precursor protein (APP) [3]. AD is associated with two major pathological hallmarks, neurofibrillary tau protein tangles and a product of APP cleavage known as senile

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CONFLICT OF INTEREST

The authors confirm that this article content has no conflict of interest.

plaques, both of which lead to the degeneration of neurons *via* an inflammatory pathway. The aggregation of these protein plaques in the brain signals an immune response involving nitric oxide, reactive oxygen species, prostaglandins, and pro-inflammatory cytokines, including tumor necrosis factor α (TNF α), interleukin-1 β (IL-1 β), and interleukin-6 (IL-6) [4, 5]. Whether the inflammation is the root of the disease or a secondary result is unknown [2]. This inflammation leads to the death of neurons, primarily cholinergic, and results in a gradual and debilitating loss of cognitive function and memory [6]. Current clinical drug treatments for AD, including acetylcholinesterase inhibitors, antioxidants, and glutamate antagonists, increase neuronal activity but fail to address the pathological hallmarks that appear to provoke the neurological damage [7-9].

We focused this review on therapeutic drugs that target the pathological hallmarks of AD directly, in an attempt to not only slow the progression of AD but also to potentially reverse neurodegeneration. Newly proposed and experimental AD treatment drugs focus primarily on three characteristic areas of AD development: prevention of neurotoxic β -amyloid protein plaque formation, stability of neuronal tau proteins, and increase in neuronal growth and function. We summarize the potential drugs targeting β -amyloid protein plaque formation, tau protein aggregation, and loss of neuronal growth and function for the treatment of AD (Fig. 1). These drug targets are aimed to slow or attenuate the progression of AD. The purpose of this review article is to explore potential drug therapy targets in inflammatory AD pathways; and to discuss recent therapeutic compounds and neurotrophic factors targeting these pathways for the treatment of AD.

2. PROTEOLYTIC PROCESSING OF β -AMYLOID PEPTIDES IN AD

The primary hallmark of AD, and thus a major focus of drug design, is the neuronal amyloid protein aggregation known as the β -amyloid (A β). To fully understand current AD anti-amyloid drug designs, it is important to understand the process of A β formation (Fig. 2). The two most prevalent β -amyloid peptides found aggregated in the brains of AD patients are A β 40 and A β 42 [10]. Both are variants of the A β 4 polypeptide and contain 40 and 42 amino acids, respectively. A β 4 (both 40 and 42) is derived from the proteolysis of amyloid peptide β -amyloid protein plaque (β APP), a large type-I membrane protein that has a relatively unknown function [11] (Fig. 2). Cleavage is achieved first *via* one of two primary enzymes, α -secretase or β -secretase, followed by a secondary cleavage by an enzyme known as γ -secretase [12] (Fig. 2). Processing *via* the enzyme known as α -secretase cleaves the APP within a central region known as γ -secretase, known as p3 [11]. However, processing of APP by an enzyme known as β -secretase, which cleaves outside the designated A β domain, leads to the formation of the aforementioned amyloid-prone A β 40 and A β 42 [12]. APP is first cleaved by β -secretase at the amino terminus into two parts, sAPP β and C99, and then the C99 half is cleaved by γ -secretase into one of several amyloid-prone A β 4 forms [12]. Genetic mutations in the regions that encode for formation γ and cleavage of these peptides are highly associated with the early development of AD and therefore are regarded as a critical step in the pathogenesis of AD [11]. It is on chromosome 21 specifically that β APP processing sites are encoded. Interestingly, Down's syndrome (DS) patients, who have an extra chromosome 21, express early onset AD symptoms [13]. The extra chromosome 21 means an extra copy of the APP gene, and therefore marked increases in A β 42 [13].

Mutations of the γ -secretase complex have also been associated with the development of early onset AD. Specifically, mutations in the γ AD. presenilin 1 and presenilin 2 components of this complex are suggested to be a major cause of early onset AD. Furthermore, presenilin 1 and 2 are catalytic aspartyl protease subunits within the γ -secretase and their mutation results in progression of the AD amyloid pathway *via* increased A β 40/42 production from γ APP cleavage [14].

Ischemic episodes may also increase A β formation [15]. A recent study verified the association between brain ischemia and β - and γ -secretase upregulation [15]. Ischemic oxidative stress γ - was indicated to increase β - and γ -secretase regulation, thereby directly increasing β -amyloid levels. The study also indicates that increased γ -secretase activity enhances β -secretase upregulation and function. This process is mediated by the increased presence of β -amyloid and therefore can be considered a positive feedback loop [15].

These β APP processing sites are often the consequences of genetic mutations, which result in improper cleavage [16]. If the genetic mutations are located next to the A β cleavage domain, those are known as β -secretase mutations [16]. Mutations occurring within the actual A β cleavage domain are known as α -secretase mutations [16]. Both α and β mutations appear to enhance the pathogenesis of AD [11]. Mutations that enhance β -secretase directly enhance the amyloid-forming pathway, as β secretase products are cleaved by γ -secretase, an imprecise enzyme that may result in the aggregate-prone A β 40 and A β 42 [17]. Mutations of the α -secretase increase the levels of available APP substrate accessible to the amyloid forming β -secretase, thereby indirectly increasing amyloid production [11]. Examples include a mutation at amino acids 670/671 of APP, known as “the Swedish” mutation, which exhibits an increased rate of β -secretase cleavage as well as a mutation known as the “arctic mutation”, further discussed here [18].

3. NEUROTROPHIC FACTORS IN AD

Prevention of cell death has been the main focus for the treatment of AD in animal models as well as in humans. Most efforts are focused in the use of neurotrophic factors to protect cholinergic neurons and, in turn, maintain their normal function. A previous study has shown that a deficit of exogenous supply of trophic factors may be linked to neurodegeneration of cholinergic neurons [19]. We discuss here several types of neurotrophic factors that might be candidates for AD therapy.

3.1. Nerve Growth Factor in AD

Neurotrophic factors in AD are tested to ameliorate cholinergic functions [20]. Among these neurotrophic factors, nerve growth factor (NGF) was found to have positive restorative effects in AD. Studies conducted in aged rats demonstrated that NGF reversed age-associated declines in basal forebrain cholinergic neurons and supported recovery from deficiencies in spatial memory [21]. The ability of NGF to prevent degeneration of cholinergic neurons is also demonstrated in primates [19, 22, 23]. NGF is considered to be effective for the survival of cholinergic neurons that are affected in AD (Reviewed by [24]).

Although the mechanism through which NGF acts is unknown, studies have shown that NGF stimulates protein kinase c-coupled M1/M3 muscarinic receptors. This can lead to an increase of the non-amyloidogenic secretory pathway of amyloid precursor protein (APP) signaling [reviewed by [25]]. Neurotrophic and neuroprotective actions might be induced by the cleavage of the APP by the α -secretase within the β -amyloid sequence, which generates non-amyloidogenic C-terminal APP fragments and soluble APPs alpha, a concept that is explored in greater detail within following sections. Therapeutic actions may be focused on stimulation of cholinergic function with increases in non-amyloidogenic APP without elevating the expression of APP.

3.2. Fibroblast Growth Factor in AD

Fibroblast growth factor (FGF) is involved in neuroprotection of selective neuronal populations against neurotoxic effects leading to AD [26, 27]. FGF possesses wide mitogenic and cell survival activities and is involved in diverse biological effects [reviewed by [28]]. An *in vitro* study has shown that FGF protected cultured hippocampal neurons against β -amyloid peptide toxicity [29]. It is noteworthy that FGF interacts with β -amyloid peptide and induces neuroprotection in the AD model [30, 31]. The neuroprotective mechanism of FGF may occur at the level of synapses; the loss of synapses is one of the characteristics of AD, which is promoted by β -amyloid peptide [32]. Furthermore, it is postulated that polymorphism in the FGF gene increases the risk of AD in patients suffering from this disease [33]. Since most of these studies have investigated the protective effects of FGF in the *in vitro* AD model, it is possibly warranted to explore these effects *in vivo* in the AD model with the use of efficient methods of delivery.

3.3. Brain Derived Neurotrophic Factor in AD

Brain derived neurotrophic factor (BDNF) is considered as another potential neurotrophic factor for treatment of AD. BDNF mediates synaptic plasticity and cognitive function [34]. Anatomically, a loss of synaptic contacts leads to the neuropathology of the disease, including deficits in cognition in AD [34]. In humans suffering from AD, BDNF mRNA and protein were found decreased in cholinergic neurons of the cortex and hippocampus [35]. Basal forebrain cholinergic neurons in AD patients show a reduced supply of target-derived BDNF [9]. A deficit in pro-BDNF protein also was found in the parietal cortex in AD [9]. It is clear that the reduction of BDNF levels in AD models, particularly in cholinergic neurons, indicates that this neurotrophic factor is a key factor in AD [35]. While other neurotrophic factors stimulate neuronal growth and protection, BDNF primarily enhances the cholinergic functions of neurons [35]. Considering the current prevalence and success of acetylcholinesterase inhibitors as AD drug therapy, BDNF therapy is certainly promising.

Although the mechanism of actions of BDNF is not clear, it might be mediated through the stimulation of postsynaptic TrkB receptors, which lead to depolarization *via* activation of the sodium ion conductance [36]. Through the depolarization, BDNF may be involved in long-term potentiation and cognition, which is characteristic of AD [37]. The molecular mechanism involved in the downregulation of BDNF in AD is unknown. Moreover, in regards to the BDNF gene, evidence has suggested that polymorphism of the BDNF gene is associated with late-onset AD [38]. Thus, BDNF polymorphism could be a predisposing

factor for AD [38]. This suggests that BDNF gene variants are considered as risk factors for AD [39, 40].

The activation of TrkB by BDNF initiates a downstream signal that is crucial for plasticity and memory development [41-44]. It has been demonstrated that activation of TrkB increased synapse density and therefore is associated with improved cognition that is independent of the amyloid or tau pathways [45, 46]. The TrkB pathway is particularly relevant to AD because many AD patients have significant damage and loss of neurons by the time symptomology appears. Activation of TrkB specifically promoted the restoration of synaptic contacts, thereby improving cognitive function in later stage in AD patients [47]. One particular TrkB agonist, 7,8-dihydroxyflavone, has demonstrated neuroregenerative effects in AD. When 7,8-dihydroxyflavone is administered to lesioned mice, it increased the density of dendritic spines and reaffirmed the possibility of cognitive improvement in even severely damaged neuronal networks [47].

Interestingly, studies have identified that BDNF may be signaled through serotonin (5-HT) (reviewed by [48]). Evidence suggests that BDNF and 5-HT signaling play an important role in the pathogenesis in AD. The co-regulation between BDNF and 5-HT is characterized by activation of 5-HT receptors coupled to cAMP production and cAMP response-element binding protein activation, which induces BDNF gene transcription (reviewed by [49]). In turn, BDNF may stimulate the growth and sprouting of 5-HT neurons and possibly other types of neurons [50]. 5-HT and BDNF activate several genes that are a part of neural plasticity and survival, which might be a key factor in neurodegenerative diseases, including AD [49]. The interactions between BDNF and 5-HT play a role in neurogenesis, particularly with neuronal stem cells located in the hippocampus (reviewed by [48]). Together, these suggest that BDNF is a key factor in maintaining cell division, cell survival and cell function to lead to neuroprotection.

While the role of BDNF in neuroprotection is well established, it has been suggested and debated that BDNF polymorphisms and mutations may be directly mechanistically related to AD development, potentially as promoters of tau and amyloid aggregation [33, 51-53]. A recent study on BDNF knockout mice, however, indicates the possibility that reduced BDNF is simply a consequence of the AD pathology and does not play a significant individual role in AD prevention [54]. The study reaffirmed the therapeutic value of BDNF as beneficial to synaptic and cognitive function in AD patients but was unable to determine any potential for BDNF to act directly as an agent in AD pathology [55].

3.4. ADNF and ADNP Derived Peptides and Colivelin in AD

The use of derived peptides from activity-dependent neurotrophic factor (ADNF) and activity-dependent neuroprotective protein (ADNP) has proven successful in the attenuation of neurodegeneration in AD. Both ADNF and ADNP are synthesized and released from astroglia [56, 57]. The release of these trophic factors is regulated by vasoactive intestinal peptide [58]. The protective effects of ADNF and ADNP are based on nine amino acids (SALLRSIPA) and eight amino acids (NAPVSIQ), respectively [Reviewed by [59]]. Thus, the derived peptides from ADNF and ADNP, termed ADNF-9 and NAP, respectively, have been shown to protect neuronal degeneration against the insults of oxidative stress and other

related neurodegenerative models *in vitro* and *in vivo* [Reviewed by [59, 60]]. NAP exhibits the ability to cross the blood-brain barrier and selectively interact with the tubulin and microtubule assembly, thus promoting neuronal outgrowth [61]. The neuroprotective effect of ADNF-9 and/or NAP was shown against neurotoxin β -amyloid peptide [56, 62, 63]. In addition, NAP was found to inhibit β -amyloid aggregation by binding to its 25-35 fragment at high affinity, consequently preventing its toxic effect [61].

Moreover, the neuronal calcium regulation involving NAP may contribute to neuronal survival. NAP colocalizes with microtubules, thereby regulating the mobilization of neuronal calcium [64, 65]. Another potential beneficial action of NAP has been suggested to involve the MAPK/ERK and PI3/Akt pathways [66]. It has been determined that stimulation of these two pathways leads to neuronal growth *via* the phosphorylation of cAMP response-element binding protein [66].

Furthermore, ADNP knockdown mice treated specifically with NAP demonstrated enhanced cognitive abilities [67]. It is important to note that NAP is currently under investigation in phase II clinical trial studies involving AD-related cognitive losses [68]. The most pharmaceutically relevant portion of ADNF is its active fragment, ADNF-9. ADNF-9 has been tested in a model of apolipoprotein E (ApoE)-deficient mouse; ApoE deficiency is considered as one of the risk factors of AD [56]. This study showed that daily injections of ADNF-9 to newborn ApoE-deficient mice improved the acquisition of developmental reflexes and prevented short-term memory deficits [56]. ADNF-9 has been observed to decrease A β peptide amyloids, reduce ApoE deficiencies, and prevent oxidative insults [69]. It has also been observed to increase synapse formation, promote axonal elongation *via* cAMP-dependent mechanisms, and increase chaperonin expression of heat shock protein 60 [56, 70, 71]. The increased expression of heat shock protein 60 is especially significant because it leads to additional protection against A β insult [70-72]. The use of therapeutic ADNF-9 was shown to have an effect on both memory and learning in conjunction with polyADP ribosylation catalyzed by poly(ADP-ribose)polymerase-1 [73]. Although several other ADNF-derived peptides have been tested, ADNF-9 has proven to be the most effective overall at preventing cell death [73].

New directions have been taken to synthesize the hybrid peptide, colivelin, composed of ADNF-9 and AGA-(C8R)HNG17 (PAGASRLLLTGEIDL), a potent Humanin derivative [74]. Humanin is a bioactive peptide that prevents cell death by various familial AD-causative genes and β -amyloid peptide [74]. Although the exact mechanism of action is unclear, it is believed that Humanin inhibits the cytosolic Bcl-2 family of proapoptotic proteins [75-77]. Colivelin completely suppressed neuronal cell death against β -amyloid peptide *in vitro* [65]. In contrast to ADNF-9 alone, Colivelin has the characteristic of displaying its complete neuroprotective effect at variant concentrations [65]. *In vivo* experiments demonstrated that in the model of mice treated intracerebroventricularly with β -amyloid, Colivelin has a potent neuroprotective effect on β -amyloid-induced memory dysfunction as compared to animals treated with ADNF-9 alone [74]. Two pro-survival signals have been theorized to be possible mechanisms of Colivelin's action, the first being a Humanin-mediated STAT3 pathway, which has been shown to be especially relevant to protection against memory loss related to AD [65]. The second possible pro-survival signal

is that of an ADNF-9 mediated Ca^{2+} /calmodulin-dependent protein kinase IV pathway [76]. This compound is considered a candidate for the treatment of AD, considering its great ability to cross the blood-brain barrier and prevent neurodegeneration.

4. CYTOKINE FUNCTION IN AD

4.1. Neuroprotective Abilities of Interleukin-10 in AD

The cytokine known as Interleukin-10 (IL-10) is a potential candidate for AD treatment primarily due to its anti-inflammatory properties as well as its neuroprotective abilities. IL-10 displays a broad range of potential actions on neurogenesis [78]. IL-10 produces several anti-inflammatory cytokines, including IL-1 receptor antagonists (IL-1ra), which deactivate inflammatory macrophages [79]. IL-10 has also been found to suppress inflammatory molecules TNF- α and IL-1 β , which are induced by AD's trademark A β plaques [80]. The induction of IL-1ra production is especially relevant to AD treatment because it is believed that IL-1 is a key factor in both A β plaque deposition and increased APP formation [81]. Antagonizing the effects of IL-1, IL-1ra might help reduce the severity and density of A β plaque formation and aggregation.

Furthermore, IL-10 has displayed not only anti-inflammatory effects but also neuroprotective properties, including potential nerve repairing abilities mediated through NGF [82]. It is also suggested that IL-10 has the ability to block pro-apoptotic proteins, similar to the neuroprotective action of NAP [83]. A patent indicates the use of UV-B light radiation to increase levels of IL-10 [84]. The patent details a process that involves introducing UV-B light radiation directly to inflamed target areas in the brains of AD patients. The UVB radiation triggers a cytokine cascade, which eventually produces IL-10. The UV-B radiation ultimately deactivates inflammatory cells (Th1) and activates anti-inflammatory cells (Th2), which emit IL-10 [84]. These effects may lead to immunosuppression; therefore, it is necessary to focus the UV-B radiation directly on the inflamed areas of the brain. It is suggested that systemic exposure would result in full body immunosuppression, as evidenced in animal trials [85].

4.2. Neuronal Growth and Interferon- β

The cytokine known as interferon- β (IFN- β) stimulates astrocytes to release NGF, ultimately promoting neuronal growth [86]. This property of IFN- β has already been exploited in the treatment of multiple sclerosis (MS) [87]. Recombinant human IFN- β , known under the trade name “Rebif”, has been shown to directly reduce the progression of MS as evidenced by MRI readings [88]. One patent indicates that a modified version of this compound may be used to treat AD in a similar manner [86]. The patent describes a multimeric fusion protein that consists of both IFN- β and a traditional cholinesterase inhibitor fused to an immunoglobulin. The synergistic action of the drug would potentially both increase neuron growth and stimulate neuronal function.

5. METHODS FOR TARGETING B-AMYLOID PEPTIDE

5.1. β -Secretase and -Secretase Inhibitors

The signature neuronal γ β -amyloid peptides and its associated pathway are major focuses of AD drug design. The important target to prevent amyloid formation is a β -secretase inhibitor. However, production of a safe and successful β -secretase inhibitor is a long and difficult process. β -secretase inhibitor drugs in trial are often quickly metabolized or displaced by transport systems, primarily pglycoprotein [89]. Phase I clinical trials studying a β -secretase inhibitor known as LY2811376 were terminated due to the side effect of the drug in inducing retinal damage in rat models [89]. -secretase inhibitors, which theoretically prevent the cleavage of c99 into amyloid-prone $a\beta$ 4 segments, have been developed. Recent clinical trials of the drug Semagacestat, a γ -secretase inhibitor, were terminated during phase III clinical trials due to declining cognitive abilities and an increased risk of skin cancer [90]. Additionally, a clinical trial on the drug Tarenflurbil, a γ -secretase modulator, was also terminated during phase III because of poor results [90]. It is believed that interfering with secretase activity may indirectly affect unrelated pathways, such as NOTCH, which may lead to such unforeseen reactions [90].

5.2. β -Amyloid Antibodies

How can we avoid interfering with metabolic pathways but still target amyloid aggregation? A frequently proposed method of directly inhibiting growth of β -amyloid peptide is through the use of antibodies. By targeting specific $A\beta$ β -amyloid peptides with antibodies, the goal is to inhibit the aggregation of the neurotoxic amyloid plaques in the brains of AD patients. $A\beta$ 40 and $A\beta$ 42 are both endogenous compounds, and therefore problems arise with the use of non-selective $A\beta$ 40 and $A\beta$ 42 antibodies [91]. These proteins have yet unknown functions in the human body, thus causing unforeseen adverse reactions, which may be because the immune system fails to recognize endogenous proteins as antigens or because it produces an inflammatory response to them [92]. A phase III clinical trial of an $A\beta$ 42 antibody known as AN-1792 demonstrated the dangers of such complications, as encephalitis was reported in 6% of the test subjects [91]. The phase III clinical trial for this drug was terminated due to these side effects.

Another $A\beta$ -antibody drug for which clinical trials were recently terminated is Bapineuzumab [93]. Bapineuzumab is a humanized monoclonal antibody designed to specifically target an epitope consisting of amino acids 1-10 of $A\beta$ 42 [93]. Bapineuzumab phase III clinical trials, conducted in 2012, were deemed a failure due to a lack of evident efficacy when compared to a placebo [91]. However, the monoclonal antibody drug, Solanezumab, concluded phase II clinical trial testing in 2012 [94]. Solanezumab is a humanized antibody that is designed to specifically target an epitope between amino acids 13 and 28 of the $A\beta$ peptide [95]. By targeting this specific sequence, the drug aims to sequester $A\beta$ strands, thus preventing aggregation [95]. The results of the trial proved that Solanezumab was well tolerated when given in weekly doses and had few side effects [94]. Although the trial results were not nearly as prolific as hoped, a direct correlation between increased unbound $A\beta$ levels and increased drug dose was demonstrated, indicating the

potential anti-A β aggregation properties of the drug [94]. A phase III trial has been ordered to further investigate the drug's anti-A β efficacy [94].

5.3. Targeting the A β -Arc Peptide

Rather than targeting all wild-type A β 4 peptides, one patented method indicates targeting a specific mutation that has been observed to consistently result in progressive dementia [96]. The patent indicates that protofibrils, not mature A β fibrils, are the root of neurotoxicity in AD. After nucleation, the A β precursor protofibrils have the predisposition to both elongate and laterally associate to form mature fibrils [97]. Thus, mutations during nucleation may result in abnormal aggregation [97]. The patent proposes that antibodies would be synthesized to specifically target the epitope created by the replacement of a glutamic acid with a glycine at codon 693 of APP. This single substitution of glycine with glutamic acid at position 22 in the A β arc was found to dramatically increase protofibril growth rate and capacity. The target would selectively be A β fibrils, as the A β peptide is in the protofibril conformation when used as an immunogen. Such target selectivity and specificity would greatly reduce the possibility of unforeseen adverse reactions, such as the encephalitis seen in the nonselective A β 42 vaccine [91].

The basis of another patent is the observed effects of an APP mutation in the A β sequence at codon 693 (E693G) on a family from Northern Sweden, known as the “Arctic mutation” (A β -Arc) [98]. The carriers of the Arctic mutation display progressive neurodegeneration and dementia. Interestingly, carriers also exhibit decreased A β plasma levels, which seem to contradict previous assumptions that A β plaque levels have a direct correlation to progressive dementia [99]. This observation was also supported *in vivo*, as cells transfected with the APP E693G displayed very low concentration levels of A β 42 [96]. Fibrillization studies have concluded that the A β -arc mutation produces longer and less curved A β peptides, prone to twisting, that also form at both an accelerated rate and quantity when compared to wild-type A β [96].

5.4. Modulation of NMDAR in AD

Modulation of the N-methyl-D-aspartate-type glutamate receptor (NMDAR) has been shown to prevent amyloid- β production and its subsequent aggregation [100]. As previously mentioned, improper cleavage of APP by β -secretases can lead to the A β formation characteristic of AD. NMDAR-dependent signaling pathways, however, aid in the proper regulation of APP cleavage [101]. NMDAR agonists stimulate a Ca²⁺ dependent PKC dependent pathway, leading to the activation of β -secretase [101]. Cleavage of APP by β -secretase leads to what is known as the amyloidogenic pathway, or the neurotoxic amyloid plaque forming pathway [11]. As a result, the increased A β levels can enhance the production of TNF- α and INF- which both reduce D-serine [102]. The inhibition of D-serine appears to be important in the production of neurotoxic A β . A patent indicates a synergistic NMDAR antagonist and co-agonist drug design to prevent this pathway's formation [103]. The co-administration of an NMDAR antagonist and co-agonist, the aforementioned D-serine, not only prevents the amyloidogenic pathway but also triggers the formation of the nontoxic soluble Alpha-APP fragments. Stimulation of NMDAR by D-serine increases intracellular Ca²⁺ and activates a Ca²⁺ dependent PKA and CaMKII-dependent pathway

[101]. These pathways activate α -secretase, cleaving APP into a non-amyloid forming product. The patent indicates the balance between β -secretase (amyloid forming) and α -secretase (non-amyloid forming) as crucial for preventing and treating AD.

6. TARGETING TAU-TAU INTERACTIONS

The second major pathological hallmark of AD is neurofibrillary tangles consisting of tau proteins, a condition known as “tauopathy” [104]. Under normal physiological conditions, tau proteins are necessary to stabilize the microtubules (tubulin) in the brain that facilitate movement of synaptic elements. Normal tau proteins are also rich in hydrophilic amino acid residues and are thus water soluble [104]. In AD, however, these soluble tau proteins change into an insoluble polymerized cross- β -sheet form known as paired helical filaments (PHF) [105]. PHFs are insoluble due to a protease-resistant core fragment at amino acids 93-95 [106]. These PHF forms cannot sustain the neuronal microtubules, and they inhibit axonal transport of neurologically active substances, resulting in synaptic loss and cognitive impairment [107]. The PHF form most likely features a certain conformational change that induces cross-linking between other PHF tau proteins, creating the insoluble cluster [108]. One patent indicates the development of a compound that would block the active cross-linking site on PHF, thereby inhibiting its ability to cluster into a plaque [109].

The tau proteins tangled within neurofibrillary clusters also have the distinct trait of hyperphosphorylation. Phosphorylation of tau under normal conditions allows for the formation of paired helical filaments [110]. However, in AD, the two kinases cyclin dependent kinase and glycogen synthase kinase 3B are responsible for overactive hyperphosphorylation of tau, contributing to neurofibrillary aggregation [110].

There are several key patterns, which appear in tangle-prone Tau, that can be used to distinguish it from normal Tau, such that normally functioning Tau is not also targeted by the proposed pharmaceutical compounds [108]. The patent theorizes that a monoclonal antibody could be developed to target these distinguishing traits, such as the truncation at the Glu-391 C-terminus of PHF [108]. Regular tau proteins do not feature this Glu-391 truncation, and therefore regular tau-tubulin binding would not be effected [111].

6.1. Use of Phenothiazines in Tau-Tau Targeting

The patent describes the use of Phenothiazines, which function in a similar manner as an antibody to a hypersensitive tau protein. The most effective phenothiazines highlighted in a structure-activity relationship study were toluidine blue O, thionine, azure A, azure B, and 1,9-dimethyl-methylene Blue. Phenothiazines like thionine have the capability to block neurofibrillary aggregation at high affinity tau capture sites by either aiding in the neuronal filament differentiation or by directly blocking the formation of neurofibrillary tangles [109]. According to the patent, these compounds also lack the ability to disrupt normal physiological Tau-microtubules complexes. Under normal physiological conditions, the tandem repeat region of tau serves as the normal tubulin binding site [112]. This same region is responsible for the formation of PHFs because it contains the sequence responsible for high affinity tau aggregation. Once aggregated, PHFs are difficult to eliminate because

they contain a protease-resistant core, therefore it is important to prevent aggregation altogether [112].

6.2. Use of Methylthioninium Chloride in Tau-Tau Targeting

A phenothiazine tau-tau inhibitor chemically known as methylthioninium chloride (MTC, methylene blue) completed phase II clinical trials and is starting phase III clinical trials [113]. Previous studies on mice have displayed MTC's ability to not only prevent PHF from aggregating but also dissolve existing tau aggregates [114] (Fig. 3). In rat stroke models, MTC was able to reverse neurotoxicity and reduce lesion size [115]. MTC displayed multiple mechanisms for reducing tau-tau interactions, including inhibition of heat shock protein 70 (Hsp70) ATPase [116, 117]. Hsp70 appears to be a protective factor for tau that prevents degradation (Fig. 3). Inhibiting Hsp70 ATPase removes this protection, increasing the degradation of tau proteins and subsequently preventing aggregation [116, 117]. At low doses, MTC exhibits extraordinary antioxidant abilities, yet another unique neuroprotective feature [118-120]. This is due to MTC's capability to act as an electron cyclor in the electron transport chain, which has been proven to both enhance memory and serve as a neuroprotective process [121, 122]. Data have provided evidence that in low doses, MTC also provides quick, short-term memory enhancement by oxidizing hemoglobin and thereby increasing the amount of oxygen transported to the brain [122]. MTC is extremely lipophilic and therefore capable of crossing biomembranes, including the blood-brain barrier, making it an excellent candidate as a neurologically active drug [123].

7. INSULIN'S POTENTIAL ROLE IN AD

Impairment of cerebral glucose utilization often precedes or enhances mental dysfunction; therefore compromised insulin signaling pathways may play a role in the development of cognitive diseases, including AD [124]. Improper regulation of both insulin and insulin growth factor-1 receptors has been indicated in AD patient brains [125]. Mice deficient in insulin receptor substrate-2 or the neuronal insulin receptor gene display reduced brain growth and increased tau phosphorylation, a direct link to cerebral AD conditions [126]. Much of the significant evidence reinforcing this theory comes from the study of mice treated with intracerebral doses of streptozotocin paired with oxidative stress [127]. Streptozotocin depletes insulin and insulin growth factor signaling cascades in the brains of mice, resulting in decreased brain size and activity, as well as phosphorylated tau proteins and amyloid clusters similar to AD conditions [124, 128]. Streptozotocin metabolites have an alkylating property that generates reactive oxygen species, resulting in DNA damage and a reduction of glucose metabolism in both the cortex and hippocampus [124, 129].

8. CONCLUSION AND CLOSING THOUGHTS

Although the mechanism of AD development is unknown, the pathological hallmarks are prevalent and predictable in patients. The current clinical drug treatments of AD simply slow the progression of the disease by providing neurological stimulation but do not target these characteristic developments. There is potential for the application of drugs that specifically target the neurodegenerative pathological hallmarks of β -amyloid protein plaques and tau protein aggregates. Furthermore, neurotrophic factors such as BDNF, FGF,

NGF, ADNF, and ADNP in increasing neuronal growth could potentially reverse neurodegeneration. Targeting β -amyloid protein plaques, whether directly *via* antibodies or indirectly *via* secretase inhibitors, has proven to be difficult. Several β -amyloid antibody trials were terminated before completion due to poor results, and no efficacious secretase inhibitors have been developed. An upcoming phase III clinical trial of the tau-tau aggregation inhibitor MTC, however, is very promising due to its previous success in reversing neurodegeneration and preventing tau aggregation. The current generation of clinical and experimental drugs can shed the light on potential therapies to attenuate or slow the progression of AD.

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LIST OF ABBREVIATIONS

Aβ	Amyloid β
Aβ-Arc	Arctic mutation
AD	Alzheimer's disease
ADNF	Activity-dependent neurotrophic factor
ADNP	Activity-dependent neuroprotective protein
APP	Amyloid precursor protein
BDNF	Brain derived neurotrophic factor
FGF	Fibroblast growth factor
IFN-β	Interferon- β
IL-1β	Interleukin-1 β
IL-1ra	Interleukin-1 receptor agonist
IL-6	Interleukin-6
MTC	Methylthioninium chloride
NGF	Nerve growth factor
NMDAR	N-methyl-D-aspartate-type glutamate receptor
PHF	Paired helical filament
TNF-α	Tumor necrosis factor- α

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Fig. (1).

Diagram shows three primary drug targets in AD pharmacotherapy, β -amyloid protein plaques, neuronal tau protein aggregates, and loss of neuronal growth and function. The β -amyloid protein plaques are targeted specifically by β - and γ -secretases, which lead to prevent the improper cleavage of APP and the subsequent formation of plaques. The β -amyloid protein plaques are also directly targeted by anti- β -amyloid antibodies, which bind to preexisting β -amyloid fragments and prevent their neurotoxic aggregation. NMDAR modulators prevent the activation of β -secretases, thus preventing formation of amyloid aggregates. The second AD drug target is neuronal tau aggregation, which is corrected through the use of phenothiazine drugs, primarily methylene blue. Furthermore, phenothiazines block tau proteins at their high affinity binding sites, which prevents their aggregation. The third direct AD drug target is the loss of neuronal growth and function. Several neurotrophic growth factors, including BDNF, FGF, NGF, ADNF peptide, and ADNP peptide have been shown to both reverse preexisting neurodegeneration and stimulate new neuronal growth. Cytokines have also been shown to reduce neurodegeneration *via* anti-inflammatory pathways and neuroprotective abilities.

Fig. (2).

Diagram shows the enzymatic pathway of amyloid precursor protein (APP) cleavage. Cleavage of APP is achieved first *via* one of two primary enzymes, α -secretase or β -secretase. Cleavage by α -secretase within the A β domain initiates the non-amyloid forming pathway. The product of the α -secretase reaction undergoes secondary cleavage *via* the enzyme γ -secretase, resulting in the water soluble, non amyloid forming product known as p3. If the primary cleavage of APP is performed by β -secretase, however, the amyloid forming pathway is initiated. The β -secretase cleavage site is outside the A β domain and results in the formation of two protein products, secreted amyloid precursor protein (sAPP β) and c99. The protein product c99 undergoes secondary cleavage *via* γ -secretase and produces one of several amyloid-prone A β 4 protein forms, most commonly 42 to 44 residues long. These A β 4 forms subsequently cross-link, creating the characteristic neurotoxic A β plaques. The β -amyloid end product can be prevented at three potential sites. The first is by inhibition of β -secretase enzyme ("1") by drugs such as the terminated LY2811376. The second is inhibition of the enzyme γ -secretase ("2") by drugs such as the terminated Semagacestat and Tarenfurtil. The third is prevention of β -amyloid fragment aggregation by A β -antibodies ("3") such as Bapineuzumab and Solanezumab.

Action of MTC on Tau Protein Aggregation

Fig. (3).

Diagram shows Methylthioninium chloride's (MTC) ability to inhibit tau protein aggregation. The action of MTC is two-fold, as the drug both prevents new tau aggregation *via* inhibition of heat shock protein 70 (Hsp70) and dissolves preexisting tau aggregates. Hsp70 appears to be a protective factor for tau that prevents degradation. Inhibiting Hsp70 removes this protection, increasing the degradation of tau and subsequently preventing aggregation.