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MOLECULAR DRUG TARGETS AND THERAPIES FOR ALZHEIMER'S DISEASE

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Abstract

Alzheimer's disease (AD) is a neurodegenerative disorder that is characterized by normal memory loss and cognitive impairment in humans. Many drug targets and disease-modulating therapies are available for treatment of AD, but none of these are effective enough in reducing problems associated with recognition and memory. Potential drug targets so far reported for AD are β-secretase, γ-secretase, amyloid beta (Aβ) and Aβ fibrils, glycogen synthase kinase-3 (GSK-3), acyl-coenzyme A: cholesterol acyl-transferase (ACAT) and acetylcholinesterase (AChE). Herbal remedies (antioxidants) and natural metal-chelators have shown a very significant role in reducing the risk of AD, as well as lowering the effect of Aβ in AD patients. Researchers are working in the direction of antisense and stem cell-based therapies for a cure for AD, which mainly depends on the clearance of misfolded protein deposits – including Aβ, tau, and alpha-synuclein. Computational approaches for inhibitor designing, interaction analysis, principal descriptors and an absorption, distribution, metabolism, excretion and toxicity (ADMET) study could speed up the process of drug development with higher efficacy and less chance of failure. This paper reviews the known drugs, drug targets, and existing and future therapies for the treatment of AD.

Keywords

• Alzheimer's disease • Amyloid β • Tau protein • Amyloid precursor protein • β and γ-secretases • Glycogen synthase kinase-3 • Acyl-coenzyme A: cholesterol acyl-transferase (ACAT)

Introduction

AD has become a very sensitive health issue globally because of its high social impact and economic cost. The number of persons with AD is increasing very rapidly, especially in western countries. Dementia, i.e. memory loss, is associated with old age, but AD causes neurodegeneration. Oxidative stress is considered a primary event in AD, so an antioxidant-based therapy could be a promising step against this neurodegenerative disorder [1,2]. Non-steroidal anti-inflammatory drugs (NSAIDs), estrogen, 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase inhibitors (statins), or tocopherol (vitamin E) can prevent progression of AD but have low clinical efficacy. Major progress in molecular medicine has suggested potential drug targets for AD such as β-secretase, γ-secretase, tau protein, Aβ and ACAT [3]. There is need to gain a deeper knowledge of the disease processes of AD at the molecular level for better understanding of potential targets. It has been reported that NPT002 (bacteriophage M13) may play a direct role in dissociation of misfolded proteins (aggregates) that form Aβ fibrils and tau [4]. NPT002 is an elongated, ~950 nm long particle that cannot penetrate into the brain from the blood.

AD is characterized by the formation of intracellular neurofibrillary tangles (NFT) and extracellular senile plaques in the brain cortex [5]. NFTs are composed of hyperphosphorylated microtubule-binding protein tau, while the senile plaques are aggregates of 40 to 43 residues of amyloid beta proteins (Aβ40 to Aβ43) [6] derived from proteolytic cleavage of amyloid precursor protein (APP) [7] by β and γ-secretases [8,9]. Aggregation of Aβ causes neuronal destruction and memory loss [10], which is associated with oxidative stress, mitochondrial dysfunction, disruption of membrane integrity, abnormal calcium homeostasis, and induction of apoptosis [11] Aβ42 is believed to play a crucial role in the pathogenesis of AD because it is a predominant component of the senile plaques of AD patients [12], and it is significantly neurotoxic and responsible for aggregation resulting in the formation of Aβ fibrils [13-15]; however, Aβ43 also appears to be prone to aggregate *in vitro* as Aβ42 [16]. Evolutionary tracing and protein family analysis of Aβ indicates that C-terminal residues of Aβ are highly conserved and hydrophobic in nature. Prevalence of hydrophobic residues at the C-terminal of Aβ promotes aggregation, and also provides stability to Aβ plaques due to hydrophobic–hydrophobic interactions between residues of Aβ. Cleavage sites for different secretases and formation of Aβ and Aβ fibrils from APP are represented in Figure 1.

For the development of new aggregation inhibitors, the structure and aggregation mechanism of Aβ fibrils has been studied extensively. Aβ fibrils are reported to be 7–12 nm in diameter and are composed of several proto filaments, which are 3–6 nm in diameter [17,18]. Aβ fibrils in both Aβ40 and Aβ42 have β strands lying perpendicular to the fiber axis and form intermolecular parallel β sheets [19-21]. Besides the amyloid beta pathway, the misfolding and accumulation of tau protein and alpha synuclein may also be responsible for AD,

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and the clearance of all of these has to be taken into account if a suitable cure is to be found. Patients with mild cognitive impairment (early AD) had significantly increased plasma tau levels compared with healthy persons. Plasma tau levels are negatively associated with the performance of episodic declarativememory, visual reproduction, and verbal fluency, and are also negatively associated with the total volume of cortical gray matter, particularly hippocampus and several other regions [22].

Prion-like spread of AD pathology

Once an Aβ aggregate is formed, it can escape from the cell of origin, come into contact with a connected cell, enter that cell, and induce further aggregation via conformational change [23]. This prion-like model predicts a key role for extracellular protein aggregates (Aβ) in mediating the progression of AD. This study suggests new therapeutic approaches based on blocking neuronal uptake of protein aggregates and promoting their clearance. It has been observed that filamentous tau protein seems to spread in a stereotypic manner as the disease progresses. The induced formation of tau aggregates can be propagated between mouse brains [24]. These findings suggest that once tau aggregates have formed in discrete brain areas, they become self-propagating and spread in a prion-like manner. TDP-43 is the major component protein of ubiquitin-positive inclusions in the brains of patients with frontotemporal lobar degeneration (FTLD-TDP) or amyotrophic lateral sclerosis (ALS). Prion-like properties have been reported in aggregatedTDP-43 prepared from diseased brains [25]. These results indicate that insoluble TDP-43 has prion-like properties that may play a role in the progression of TDP-43 proteinopathy. P301S tau transgenic mice express mutant human tau protein and develop progressive tau pathology. Anti-tau monoclonal antibodies were used to block seeding activity present in P301S brain lysates [26]. These antibodies markedly reduce the level of hyperphosphorylated, aggregated, and insoluble tau, and also reduce microglial activation. These data indicate a central role of extracellular tau aggregates in the development of AD pathology. The phenotype of exogenously-induced amyloidosis depends on both the host and the source of the agent, suggesting the existence of polymorphic Aβ strains with varying biological activities reminiscent of prion strains [27].

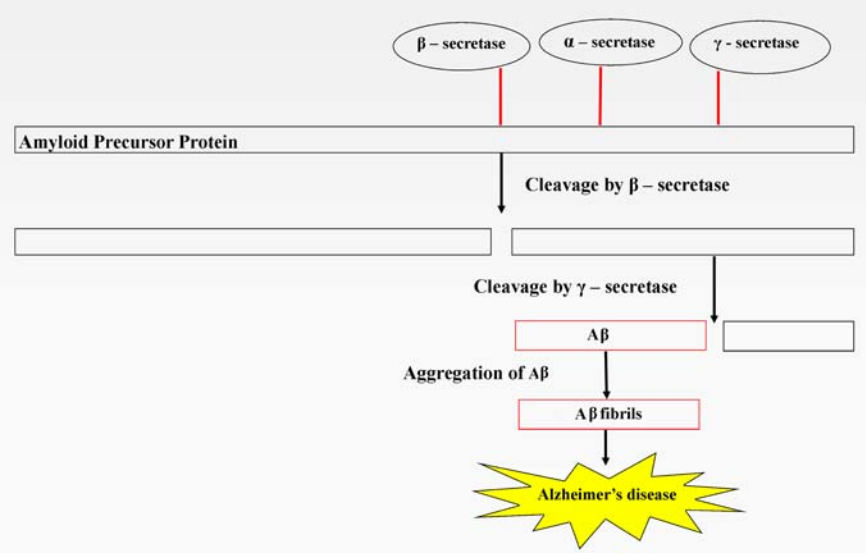

Figure 1. Formation of Aβ (Aβ40 and Aβ42) and Aβ fibrils from APP by sequential cleavage by β- and γ-secretases.

Neuroinflammation

It is well known that both Aβ fibrils and NFT are main cause of neuroinflammation in AD. Aβ fibrils are frequently associated with reactive astrocytes, activated microglia and neuroinflammatory markers such as interleukin. Mitogen-activated protein kinase (MAPK) cascades (p38 module) that mainly regulate inflammatory gene expression are activated in microglia and neurons in and around senile Aβ plaques [28]. Aβ-activated glia can produce reactive oxygen species (ROS) and reactive nitrogen species (RNS), and Aβ itself possesses interesting reduction-oxidation properties through its capacity to promote metal-catalyzed reduction-oxidation cycling reactions and ROS production [29]. Thus, an Aβ peptide itself, as well as small oligomeric forms (Aβ fibrils), are inherently neuroinflammatory. Most NSAIDs were designed to block prostaglandin synthesis through inhibition of cyclooxygenase (COX), and modern selective COX inhibitors can block inducible cyclooxygenase 2 (COX-II)-dependent prostaglandin production. Population-based studies suggest that NSAID use may diminish AD risk by 80% if used for more than two years [30]. Several clinical trials were designed to slow progression of AD through administration of NSAIDs, but they failed to produce a clear benefit. Minocycline is a tetracyclic derivative with anti-inflammatory and neuroprotective properties. Minocycline treatment corrects the up-regulation of inducible nitric oxide synthase (NOS) and COX-II levels in young transgenic placebo mice [31]. Minocycline treatment also restores the levels of BACE1 to normal, which is found to be up-regulated in transgenic placebo mice.

Small molecules as inhibitors of Aβ aggregation and misfolding

Small molecules that specifically and efficiently inhibit Aβ aggregation could be used as therapeutic agents for AD. Congo red (CR), Berberine and its analogues, and β-sheet breaker peptide are well-known inhibitors of Aβ aggregation [32,33]. CR is a histological dye that binds to Aβ fibrils because of its extensive beta-sheet structure. Binding of CR to Aβ fibrils has been proven by the change in absorbance spectrum of the dye upon binding to Aβ fibrils [34]. Spectroscopic studies reveal that CR likely binds electrostatically

to the imidazolium side chains of histidine residues that are exposed on the surface of amyloid fibrils [35]. CR binds to the positively charged surface residues of the Aβ fibril [36]. (E)-6-Methyl-4′-amino-2-styrylquinoline has been reported as a small molecule with features allowing it to potentially diagnose, deliver therapy and monitor response to therapy in protein misfolding diseases [37]. CR can bind to critical intermediate structural forms of Aβ, and therefore it has been suggested as a potential therapeutic agent against AD [38]. CR interacts with the helical form of Aβ, and the main interaction site is located at the first helical and hydrophobic core region, residues 17-25, which is assigned as a discordant helix region [39]. This study implies that any factors or molecules that can stabilize the discordant helical conformation may prevent Aβ aggregation.

Dipeptide-derived inhibitors of BACE1enzyme have shown to be useful in the treatment of AD [40]. L Carnosine (alanylhistidine) protects against the aging processes of the brain by retarding lipid peroxidation and stabilizing cell membranes. Carnosine is not able to form stable contacts with Aβ, but it has been reported to block the pathway of aggregation by disturbing the hydrogen bonding of residues involved in aggregation [41]. NMR provides new clues about how a dipeptide molecule can block the formation of toxic amyloid beta-peptide aggregates in the mouse brain.

Recent studies have shown that polyphenolic compounds from plants, specifically food products such as green tea, red wine, and curcumin (diferuloylmethane), the yellow pigment in the rhizome of the spice turmeric (*Curcuma longa*), have potent anti-aggregating activity [42-46]. Epidemiological studies report that India, where turmeric consumption is widespread, has a lower incidence of AD than the USA [47]. *In vitro* studies have shown that curcumin not only inhibits Aβ aggregation, but also disaggregates Aβ fibrils [48,49]. *In vivo* studies using AD transgenic mice have shown that dietary curcumin lowers Aβ deposition in the brain, and intravenously injected curcumin crosses the blood-brain barrier and binds to amyloid plaques [50,51]. The systemic treatment of mice with curcumin reduced new amyloid accumulation, as well as previously deposited amyloid plaques. This in all probability was due to the potency of curcumin in disaggregating Aβ fibrils and inhibiting Aβ aggregation as shown by our research group [52] Since, curcumin shows a plethora of therapeutic properties, such as anti-inflammatory, anti-oxidant, anti tumor and maintenance of protein homeostasis functions, the possibility that some of its other actions contribute to the clearance of amyloid deposits *in vivo* cannot be ignored [53].

Solid-state NMR spectroscopy studies indicate that curcumin has the capability to induce major structural changes in the Asp23-Lys28 salt bridge region and near the C terminus [54]. Electron microscopy shows that the Aβ42 fibrils are disrupted by curcumin. Curcumin (5 μM) is able to protect human neuroblastoma cells SK-N-SH against acrolein toxicity [55]. In any case, the binding of curcumin to amyloid deposits has been shown to contribute to their reduction. However, all these compounds mentioned here require further research for evaluating their safety and modifying their structures to render them suitable for human therapeutics. Many researches are moving in this direction, using different drug targets of AD for developing an effective therapy. Some potential drug targets of AD (Fig. 2) and existing drug candidates for these targets have been discussed here in this review.

BACE (β-site APP cleaving enzyme)

AD is a neurodegenerative disorder arising mainly due to accumulation of Aβ peptide. Much evidence suggests a strong correlation between Aβ and AD. Aβ results from APP via proteolytic cleavage by two enzymes: BACE and γ-secretase [56]. APP is a large type1 transmembrane protein that acts as precursor for amyloid formation. Aβ is produced in a two-step proteolytic process initiated by BACE1, followed by γ-secretase [57]. Due to the evident role of BACE1in Aβ formation, it appears to be a primary target to prevent Aβ formation in brain. BACE1 is a novel aspartic protease that initiates Aβ formation from APP. BACE2 is a transmembrane aspartic proteases homologous to BACE1. BACE1 exhibits all the functional properties of β-secretase, and has been reported as the key enzyme that initiates the formation of Aβ [58]. Studies of the human brain have revealed an increased amount of BACE protein in the cortex of AD patients [59]. BACE1 has been reported as an excellent therapeutic target for treatment of AD by lowering Aβ production in the brain

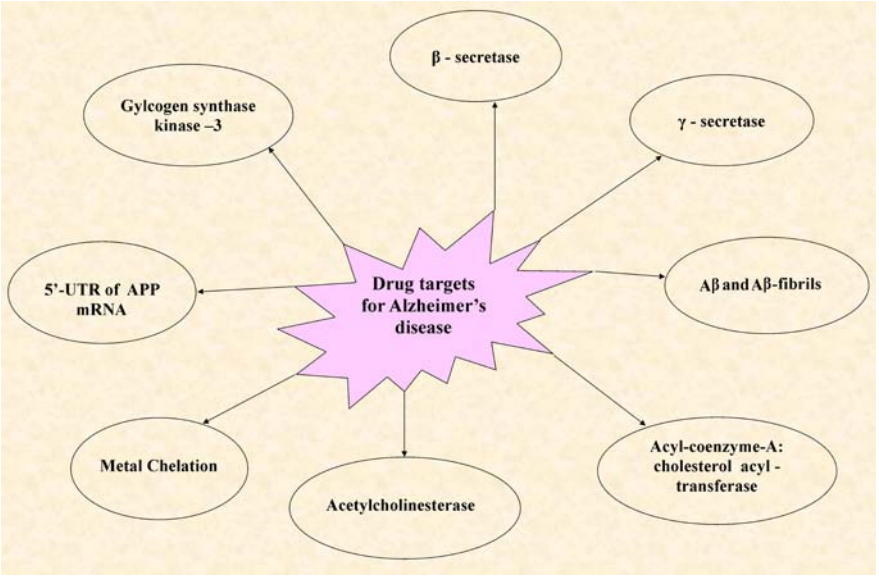

Figure 2. Drug targets for Alzheimer's disease.

of AD patients. BACE1 knockout mice do not generate Aβ and were found free from AD symptoms such as neuronal loss and certain memory deficits [60]. It was observed that complete disruption of BACE1 in knockout mice may result in some behavioral and physiological alterations [61]. Early onset of familial AD occurs due to mutations in three genes (*APP*, *PSEN1*, *PSEN2*), resulting in the formation of Aβ, which is mainly composed of 42 amino acids [62].

The mechanism of BACE mRNA recognition can be inhibited utilizing antisense action of small inhibitory nucleic acids (siNAs). This strategy can include antisense oligonucleotides, catalytic nucleic acids and small interfering RNAs (siRNAs). It has been reported that siNAs are able to inhibit *BACE* gene expression both at the mRNA and protein level, and the role of siNAs in regulating Aβ production has been observed in cortical neurons and a wide variety of cell lines [63]. Thus targeting BACE with siNAs could be useful in AD therapy and future drug design. A Statin-based peptidomimetic approach was used to inhibit the activities of β-secretase. Molecular docking and three-dimensional quantitative structure-activity relationship (3D-QSAR) and comparative molecular field analysis (CoMFA) studies of statin-based peptidomimetic analogues also demonstrated favorable interactions between the statin-based peptidomimetics and BACE1 [64]. The peptide inhibitor OM99-2 interacts with Asp32, Gly34, Pro70, Thr71, Thr72, Tyr198, Lys224, Asp228, Gly230 and Thr329 residue of BACE1 in the cavity region and has shown seven hydrogen bond interactions (Fig. 3). The selective non-peptidic BACE1 inhibitor GSK188909 was designed to potently inhibit β-cleavage of APP, resulting in a significant reduction in the level of Aβ40 and Aβ42 [65]. Oral administration of GSK188909 supports the designing of potential BACE1 inhibitors for the treatment of AD. The novel compound VIa is a small, potent, selective, and non-peptidic BACE1 inhibitor. Oral administration of VIa caused a significant decrease in $A\beta_{1-40}$ and $A\beta_{1-42}$ level in the blood of a mouse model of AD by 17.5-72.44% and 14.5-80.32%,respectively[66].Celastrolprevents NFkB activation and inhibits BACE1 expression. Celastrol was found to lower the levels of both soluble and insoluble Aβ1-38, $A\beta_{1-40}$ and $A\beta_{1-42}$ by reducing the cleavage of APP by BACE in a mouse model [67]. QSAR models could play an important role in biological assay of heterogeneous series of BACE 1inhibitors, as well as guide the synthesis of BACE1 inhibitors [68]. QSAR studies may play an important role in understanding the essential structural requirement and specificity for the binding of inhibitors with the β-secretase receptor.

Proteins involved in the production of Aβ are membrane-associated, and hence, membrane trafficking and cellular compartmentalization play important roles in the access of a drug for AD. BACE1 is mainly localized in endosomes, lysosomes, and the trans-Golgi network. BACE1 is highly expressed in neurons, and it can also be expressed in astrocytes under conditions of chronic stress [69]. BACE1 has maximal activity at an acidic pH; therefore, agents that disrupt intracellular pH inhibit BACE1 activity. The pH of endosomes (pH 4.0 – 5.0) is optimal for β-secretase activity, which explains the requirement of endocytosis for optimal BACE1 function. BACE inhibitors were designed focusing on active-site binding, neglecting the subcellular localization of the active enzyme. Rajendran and his colleagues addressed this issue by synthesizing a membrane-anchored version of a BACE transition-state inhibitor by linking it to a sterol moiety [70]. This inhibitor was found to reduce enzyme activity much more efficiently than the free inhibitor in cultured cells and *in vivo*.

γ-secretase

The γ-secretase enzyme may be a potentially attractive drug target because of its significant role in the production of Aβ fragments by creating peptides of various lengths, namely Aβ40 and Aβ42. γ-secretase comprises a molecular complex of four integral membrane proteins: presenilin (PSEN), nicastrin, APH-1 and PEN-2 [71,72]. In case of familial AD, the level of Aβ42 increases due to mutations in presenilin (*PSEN1*, *PSEN2*) or *APP* genes near the cleavage site of γ-secretase. γ-secretase inhibitors were designed by screening drug databases (ligand based) and by designing potential analogues based on the APP substrate cleavage site (structure based). Most of these inhibitors were not only specific for γ-secretase cleavage of APP, but also inhibited the processing of Notch and a subset of cell-surface receptors and proteins involved in embryonic development, hematopoiesis,

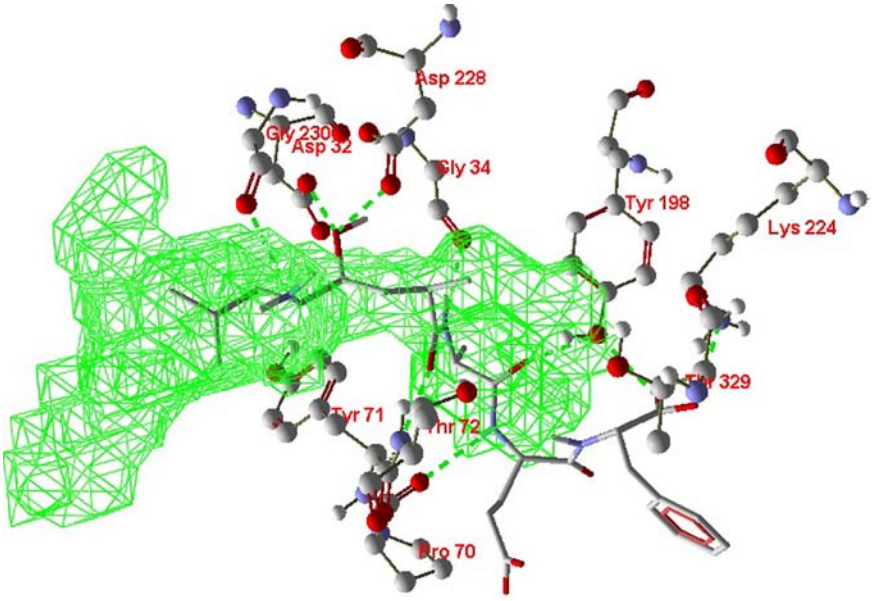

Figure 3. Interaction view of peptide inhibitor OM99-2 with Asp32, Gly34, Pro70, Thr71, Thr72, Tyr198, Lys224, Asp228, Gly230 and Thr329 residue of BACE1 in the cavity region visualized by Molegro (PDB ID: IFKN). Hydrogen bond interactions (seven) between the inhibitor and protein are shown by dotted green lines.

and cell adhesion [73]. It has been reported that NSAID analogues preferentially inhibit Aβ formation and do not affect the Notch processing and other developments; therefore, these NSAID analogues may serve as a lead for optimizing the biological activity or starting point for future drug development against AD. Thus, current research aims to design potential inhibitors that can selectively inhibit APP cleavage by γ-secretase. Inhibitors that can block the APP binding site rather than active site of γ-secretase may also be considered as an alternative approach. Recent studies point to γ-secretase as an attractive target for the therapeutic control of AD. NSAID derivatives are able to lower Aβ42 and the development of inhibitors with Notch-sparing selectivity has revived the interest in γ-secretase as an attractive target for AD [74], despite the fact that all clinical trials with NSAIDs or γ-secretase inhibitor have failed to show clinical benefit. Some studies suggest that the presenilins are in fact the active site of γ-secretase, while other data indicate that they might have a more indirect role in transporting substrates to the correct subcellular compartments for γ-secretase cleavage [75]. It has been reported that neurexins and neuroligins are proteolytically processed by presenilins at synapses. Interaction between neurexins and neuroligins promotes the formation of functional synaptic structures [76]. It is hypothesized that dysfunction of the neuroligin-neurexin pathway might be associated with AD. Semagacestat is a small-molecule γ-secretase inhibitor that was developed as a potential treatment for AD. Semagacestat does not improve cognitive status, and patients receiving higher doses had problems with functional ability, skin cancers and infections [77].

Aβ and Aβ fibrils

AD is characterized by enormous extracellular deposition of Aβ plaques composed mainly of Aβ peptide aggregates, and intracellular accumulation of hyperphosphorylated tau protein in the cerebellar region. APP is cleaved via two competing enzymes, α- and β-secretase, that are specific in their site of proteolysis and site of cleavage. In normal individuals, α-cleavage is catalysed by several enzymes followed by γ-secretase and causes cleavage of APP to generate small non-amyloidogenic fragments; in case of AD, however, the BACE1pathway involves sequential cleavages of APP by the BACE1 and γ-secretase complexes and generates Aβ. Aβ is secreted from the cell into the extracellular space where it undergoes aggregation. In the central nervous system (CNS), neurons are the major source of Aβ production. These Aβ peptides have the capability to self-aggregate in the form of Aβ fibrils. The C-terminal residues of Aβ are highly hydrophobic and clump together due to hydrophobic-hydrophobic interactions. This hydrophobic contact provides stability to the structure of Aβ fibrils [52]. Residues in Aβ42 from Asp1 - Ala42 (PDB ID: 1IYT) and the aggregation pattern of five chains A, B, C, D and E consisting of residue Leu17- Ala42 in a Aβ fibril is shown in Figure 4. Deposition of these Aβ fibrils in the brain causes inflammation, apoptosis and problems in impulse conduction

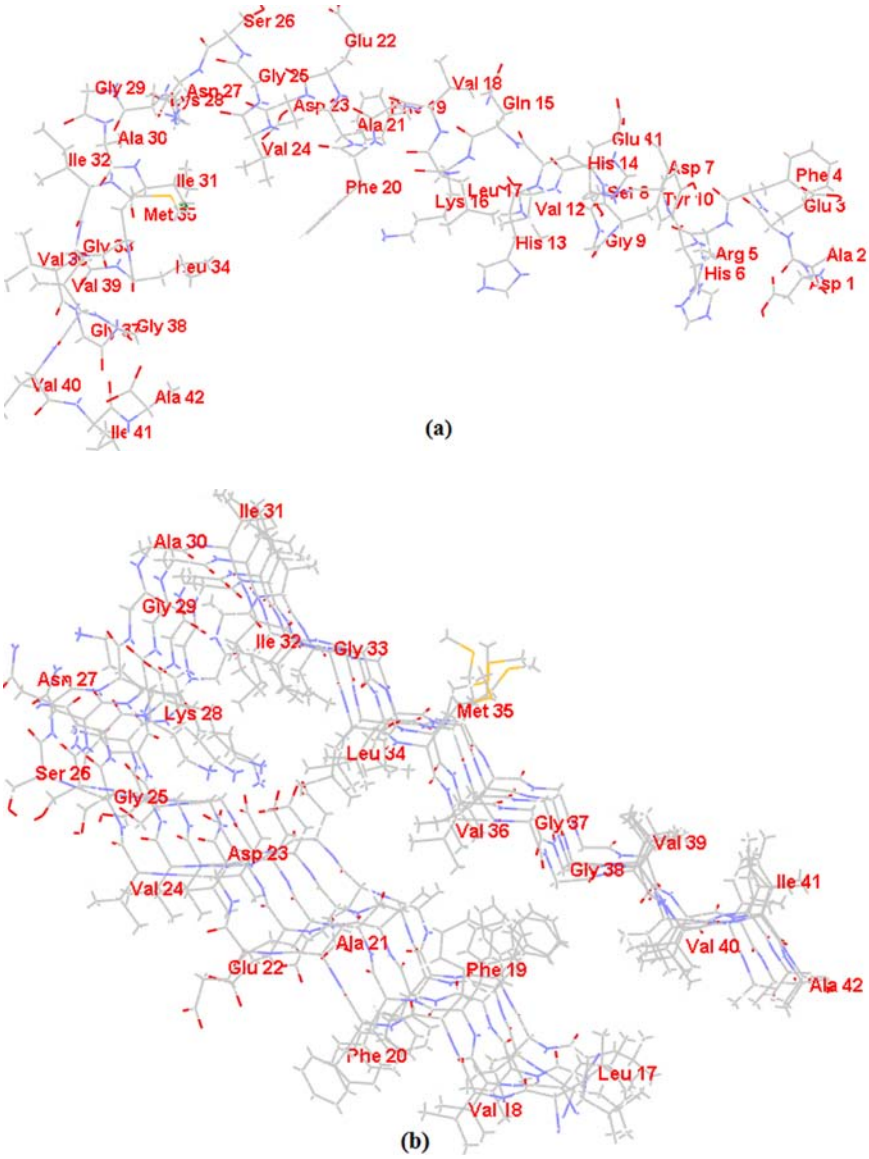

Figure 4. Presentation of (a) Aβ42 with residue Asp1- Ala42 (PDB ID: 1IYT) and (b) Aβ fibril (17-42) with five chains A, B, C, D and E consisting of residue Leu17- Ala42 (PDB ID: 2BEG).

resulting in memory loss and recognition problems. Studies have shown that oligomeric Aβ may bind with a number of surface proteins to mediate its neuronal toxicity. ATP synthase is one of the proteins present on the cell surface of neurons that binds with oligomeric Aβ. Surface ATP synthase may be involved in the neurotoxic effects of oligomeric Aβ and may be a potential target for intervention in the pathogenesis of AD [78].

Aβ is a potential disease-modifying, therapeutic target for AD, and drugs directed against Aβ were found to speed up the elimination of Aβ from the brain. Bapineuzumab is an advanced monoclonal antibody directly targeting Aβ [79]. Bapineuzumab appears capable of reducing the cerebral Aβ peptide burden in AD patients; however, its ability to slow disease progression remains uncertain after phase II trials and potentially severe adverse effects may limit its applicability. Bapineuzumab, which targets Aβ fibrils, may prove less potent than competitors like solanezumab that target soluble Aβ peptide, in addition to plaques [80]. Solanezumab (LY2062430) is a humanized monoclonal antibody that binds to Aβ. Treatment with solanezumab has reversed memory loss in transgenic mice. Phase II studies in AD patients have shown a good safety profile with encouraging results [81]. Solanezumab is currently being investigated in phase III trials. Researchers hope that solanezumab may serve as effective passive vaccine for treatment of AD in the future.

Curcumin and some of its herbal congeners were docked with Aβ and Aβ fibrils to evaluate their anti-alzheimeric property. Most of these herbal compounds (cassumunins A and B) were found to be more potent inhibitors of Aβ aggregation than known drugs. Principal descriptors, as well as ADMET properties, for these compounds were found satisfactory [52]. Indians have a low risk of AD in comparison with other countries in the world; this may be due to regular consumption of turmeric and other herbal compounds through their diet. Herbal congeners of curcumin such as cassumunin A, cassumunin B, piperine, dibenzoylmethane and cyclocurcumin have shown a higher binding affinity to Aβ than curcumin. Curcumin and other herbal congeners have shown better binding affinity (-61.25 to -92.55 kcal/mol) than known drugs of AD, such as tramiprosate (-37.36 kcal/mol). Cassumunin A was found to be involved in strong binding interaction with His13, Lys16, Leu17, Phe20 and Ala21 of Aβ [52].

Interaction site between curcumin and Aβ fibrils were investigated by a NMR study and it was reported that curcumin interacts with residues at positions 17-21 of the Aβ fibrils [82]. It is well known that the binding of curcumin to Aβ does not depend on the primary sequence, but on the fibril-related conformation. Our research group also noticed a similar result in the case of Bisdemethoxy curcumin, as reported by NMR. Bisdemethoxy curcumin has shown very favorable binding with Aβ fibrils, and has a hydrogen bonding interaction. Bisdemethoxy curcumin mostly interacts with the Leu17, Val18 and Phe19 residues of Aβ fibrils with significant contribution of Leu17 in hydrogen bonding [52]. Further structural modification of these herbal compounds could open the way for improved modality in curing AD.

Glycogen synthase kinase-3 (GSK-3)

The phosphorylated form of tau has been shown to be functionally inactive [83]. A mutation in a splice site of tau was found to be associated with the formation of four-repeat tau. Four-repeat tau that is generated by mutations in the *TAU* gene has a high capacity to aggregate rapidly. This mutation is linked to a form of dementia with NFT formation in the absence of amyloid deposition. NFT can also lead to neurodegeneration, which suggests that NFT formation may be the cause of neurodegeneration [84]. Secretion of abnormally glycosylated forms of tau into the cerebrospinal fluid (CSF) is reported to be an early event in a brain affected by AD and may be considered as an early diagnostic marker in AD. Therapeutic approaches directed against tau protein include inhibitors of GSK-3. GSK-3 is an enzyme responsible for tau phosphorylation and tau protein aggregation. GSK-3 is a monomeric, second messenger-independent protein kinase that regulates several signaling pathways, including the phosphatidylinositol 3′ kinase pathway, Wnt pathway and Hedgehog signalling. GSK-3 has the ability to activate the ATP–Mg-dependent form of type-1 protein phosphatase ("Factor A") and to phosphorylate the key rate-limiting metabolic enzyme that catalyzes the last step of glycogen synthesis, glycogen synthase [85]. GSK-3 is a dual specificity kinase differentially regulated by tyrosine and serine/threonine phosphorylation. GSK-3α is especially abundant in the hippocampus, cerebral cortex, striatum and cerebellum.

GSK-3 is a regulatory serine/threonine kinase enzyme with a plethora of molecular targets. Inhibitors of GSK-3 have shown many therapeutic uses in the treatment of AD, type II diabetes and cancer. GSK-3 dysfunction has been reported to result in a number of pathologies, including AD and other neurodegenerative diseases. NP-12 (a GSK-3 inhibitor) has been tested in a Phase II study, and methylthioninium chloride (a tau protein aggregation inhibitor) has shown good results in a 50-week study [79]. The crystal structures of unphosphorylated GSK-3 in complex with different inhibitors (indirubin-3′-monoxime, staurosporine, ANP and alsterpaullone) were determined, and the active site of the target enzyme was characterized. A comparative binding study of selective and non-selective inhibitors on the ATP binding-site of enzyme was depicted, which may play an important role in inhibitor selectivity [86].

1, 3, 4-oxadiazole derivatives were reported as potent and highly selective GSK-3 inhibitors; however, these derivatives have shown poor pharmacokinetic activities (Fig. 5). 1, 3, 4-oxadiazole derivative show hydrophobic interaction with Ile62, Ala83 and Leu188, and hydrogen bonding interaction with Lys85 of GSK-3 (PDB ID: 3GB2). These problems were solved by reducing its molecular weight and lipophilicity and newly designed compounds were found to have high selectivity and potent inhibitory activity against GSK-3, along with good pharmacokinetic and favorable blood brain barrier (BBB) penetration in the mouse brain [87]. Novel series of 2-(4-pyridyl) thienopyridinone GSK-3 inhibitors were studied and their binding mode, cellular activity and CNS penetration was also determined [88]. Novel series of GSK-3 inhibitors

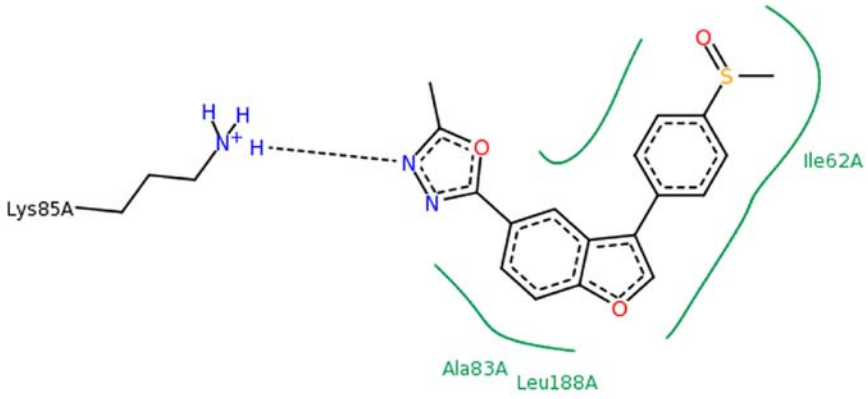

Figure 5. Interaction view of 1, 3, 4-oxadiazole derivative with GSK-3 (PDB ID: 3GB2), generated using poseView software [90] showing amino acid residues Ile62, Ala83 and Leu188 involved in hydrophobic interactions (green solid line) and Lys85 in a hydrogen bonding interaction (black dashed line).

(6-amino-4-(pyrimidin-4-yl) pyridones) were also derived from a high throughput screening [89]. These inhibitors could be a potential drug for AD after meeting the criteria of good potency, selectivity and CNS penetration.

Acyl-coenzyme A: cholesterol acyl-transferase

Acyl-coenzyme A: cholesterol acyl-transferase (ACAT) is a family of enzymes that regulates the cellular distribution of cholesterol. It converts membrane cholesterol into hydrophobic cholesteryl esters for the purpose of cholesterol storage and transport [91]. ACAT is membrane-bound protein that forms cholesteryl esters by utilizing long-chain fatty acyl-CoA and cholesterol. In mammals, ACAT1 and ACAT2 are two isoenzymes encoded by two different genes [92]. ACAT enzymes can serve as drug targets for the treatment of atherosclerosis and AD. The fraction of APP that is subject to cleavage by the secretase enzyme is regulated by the ratio of free cholesterol and cholesteryl esters in cells. ACAT inhibition results in generation of intracellular cholesteryl esters from free cholesterol and fatty acids, and finally decreases the formation of the Aβ from APP [93]. ACAT inhibition retains a small part of APP in the early secretary pathway and limits the availability of APP for proteolytic cleavage by the secretase enzyme. ACAT inhibitors were found to delay the trafficking of immature APP from the endoplasmic reticulum [94]. ACAT inhibitors have been used for the treatment and prevention of atherosclerosis; they additionally reduce cholesteryl ester (CE) levels and Aβ generation by up to 50% in cell culture models of AD [95]. It has been reported that ACAT-1 inhibition by RNAi (RNA interference) reduces the level of cellular ACAT-1 protein by 50% and cholesteryl ester levels by 22%. This results in reduced proteolytic processing of APP and a 40% decrease in Aβ secretion [96].

Various cell- and animal-based models have shown that inhibition of ACAT potentially reduces Aβ generation and prevents the progression of AD [97]. ACAT was identified as a potential therapeutic target to lower Aβ generation and accumulation. A well characterized ACAT inhibitor (CP-113,818) prevents Aβ production in cell-based experiments. Two months of treatment with CP-113,818 was found to reduce the accumulation of amyloid plaques by 88%-99% and membrane/insoluble Aβ levels by 83%-96%, while also decreasing brain cholesteryl-esters by 86% [98]. Avasimibe (CI-1011) is a well known ACAT inhibitor that is structurally different from CP-113,818. The pharmacology of avasimibe is a different from CP-113818; for example, IC_{50} values for avasimibe were reported much higher than for CP-113818 [99]. Although IC_{50} values are lower for CP-113818, ACAT selectivity is slightly better for avasimibe. CI-1011 was found appropriate for clinical study in reducing amyloid burden in both young and aged human APP transgenic mice. Treatment with CI-1011 decreases the concentration of amyloid plaque in the cortex and hippocampus, and reduces the levels of insoluble Aβ40 and Aβ42, and C-terminal fragments of APP in brain [100].

Future animal studies may evaluate the potential use of ACAT inhibitors in AD treatment. Animal studies suggest that ACAT inhibition is an effective way of reducing cerebral Aβ accumulation. The pharmacology of ACAT inhibitors may be different from that of statins, which suggests that additional therapeutic benefit might be gained by a combination of statins and ACAT inhibitors. Avasimibe is an orally bioavailable, safe and efficacious ACAT inhibitor in humans.

Acetylcholinesterase (AChE)

AChE is responsible for the breakdown of the neurotransmitter acetylcholine in the synapse region of neurons and thus terminates signal transmission. The majority of drugs currently approved for AD treatment are AChE inhibitors, which cause inhibition of the enzyme AChE [106]. Huperzine A (Hup A) is isolated from the *Huperzia serrata* (Thunb) Trev and was reported as novel, reversible, and selective AChE inhibitor [105]. Hup A has different binding sites on AChE than other AChE inhibitors and has a stronger ability to block the activity of AChE than donepezil and rivastigmine [104]. The clinical efficacy of rivastigmine (an AChE inhibitor) against AD was compared with *Ginkgo biloba*. The results indicate that there are differences in the efficacy of *Ginkgo biloba* and rivastigmine. This study suggests that acetylcholinesterase inhibitors should be used in preference to *Ginkgo biloba* for treatment of AD patients [107]. An interaction view of donepezil with AChE has been shown in Figure 6. The interaction view of donepezil with AChE (PDB ID: 4EY7) shows that amino acid residues Trp86, Trp286, Tyr337, Phe338 and Tyr341 play important roles in hydrophobic interactions, Phe295 in hydrogen bonding interaction, and Trp86 and Trp286 in pi-pi, pi-cation interaction.

Hup A has shown toxic effects due to blockage of the butyrylcholinesterase (BuChE) enzyme in the peripheral nervous system. Tacrine is another AChE inhibitor, but it also has severe side effects on the human body. The specificity and activity of Hup A for AChE can be enhanced through pharmacophore

analysis and structural modification. Several imperatorin analogs were designed and anti-cholinesterase activities of analogs were evaluated *in vitro*. All active analogs show selectivity toward BuChe over AChE [108]. A novel series of dithiocarbamates derivatives were synthesized and their ability to inhibit AChE and BuChE was evaluated. Compounds 2f and 2g were found more effective than donepezil on BuChE inhibition. Compounds 2f and 2g demonstrate an inhibitory effect on BuChE with IC_{50} values of 1.39 ± 0.041 and $3.64 \pm 0.072\,\mu M$, respectively [109]. Novel pyrazoline derivatives were evaluated for their ability to inhibit AChE and BuChE, and their cytotoxic properties were also investigated using an MTT assay. The most potent AChE inhibitor was found to be compound 7, followed by compounds 27 and 17, when compared with eserine. Compounds effective in inhibiting AChE carry the 2-dimethylaminoethyl moiety, which resembles the trimethylammonium group and the ethylene bridge of acetylcholine [110].

Metals

There is much evidence that supports interactions between Aβ and copper, iron, and zinc, which suggests that significant dyshomeostasis and mismanagement of these metals induces Aβ aggregation and neurotoxicity. Clioquinol reduces Aβ plaques, most probably by chelation of Aβ-associated zinc and copper. Accumulation of Cu^{2+} and Zn^{2+} in the brains of AD patients has been shown to be associated with aggregation of Aβ peptide. Cu^{2+} has greater binding affinity for Aβ42 in comparison to Aβ40 [111]. Environmental conditions such as metal ions, pH, and cell membranes play important roles in Aβ deposition and plaque formation. Accumulation of Aβ40 and Aβ42 is accelerated by Cu^{2+} and Zn^{2+} and results only in the formation of amorphous aggregates. In contrast, Fe^{3+} induces the formation of fibrillar Aβ plaques at neutral pH [112]. The interactions of Cu^{2+} and Zn^{2+} with monomeric Aβ40 were studied under different conditions using intrinsic Aβ fluorescence and metal-selective fluorescent dyes, and it has been reported that

Table 1. List of inhibitors and their targets for AD treatment.

No.	Compound(s)	Target/Approach	Reference
1	siRNAs	BACE mRNA	[63]
2	statin-based analogues	β-secretase	[64]
3	GSK188909	BACE1	[65]
4	Celastrol	NFkappaB	[67]
5	NSAID analogues	γ-secretase	[73]
6	Bapineuzumab	Aβ	[79]
7	Solanezumab (LY2062430)	Aβ	[81]
8	NP-12	GSK-3	[79]
9	Indirubin-3′-monoxime	GSK-3	[86]
10	Staurosporine	GSK-3	[86]
11	ANP	GSK-3	[86]
12	Alsterpaullone	GSK-3	[86]
13	1, 3, 4-oxadiazole derivatives	GSK-3	[87]
14	2-(4-pyridyl) thienopyridinone	GSK-3	[88]
15	6-amino-4-(pyrimidin-4-yl) pyridones	GSK-3	[89]
16	Methylthioninium chloride	Tau Protein	[79]
17	RNAi	*ACAT* gene	[96]
18	CP-113,818	ACAT	[98]
19	Avasimibe	ACAT	[79]
20	CI-1011	ACAT	[100]
21	Clioquinol	Chelation of Metals	[101]
22	DP-109	Chelation of Metals	[102]
23	Tacrine	Acetylcholinesterase	[103]
24	Donepezil	Acetylcholinesterase	[104]
25	Rivastigmine	Acetylcholinesterase	[104]
26	Huperzine	Acetylcholinesterase	[105]

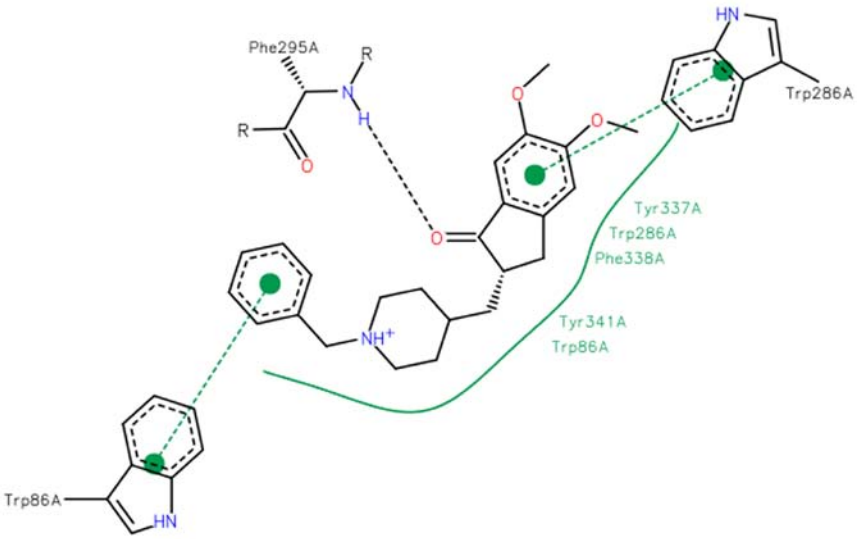

Figure 6. Interaction view of donepezil with AChE (PDB ID: 4EY7) generated using poseView software showing amino acid residues Trp86, Trp286, Tyr337, Phe338 and Tyr341 involved in hydrophobic interactions (green solid line), Phe295 in a hydrogen bonding interaction (black dashed line), and Trp86 and Trp286 in pi-pi, pi-cation interaction (green dashed line).

Cu^{2+} forms a stable and soluble complex with Aβ40 [113]. Interaction of both Zn^{2+} and Cu^{2+} ions with Aβ peptides may occur in the brain, and Zn^{2+} induces transition in the Aβ peptide that may contribute to Aβ fibril formation. High concentrations of zinc, iron, copper and aluminum within the senile plaques (SP) results in Aβ-mediated oxidative damage and also promotes Aβ aggregation. Thus, disruption of metal-Aβ peptide interactions via metal chelation therapy may prove to be a new therapeutic strategy against AD [114].

Clioquinol is a copper and zinc chelating agent that not only inhibits Aβ neurotoxicity, but also reverses the accumulation of neocortical Aβ [115,116]. Metal ions play an important role in initiating amyloidosis and oligomerization of Aβ [117]. Studies indicate that clioquinol is able to chelate metal ions from metal-Aβ complexes and assist in the disaggregation of Aβ fibrils, but it could not completely encumber the progression of Aβ aggregation [118]. DP-109 (a lipophilic metal chelator) was found to reduce the burden of Aβ plaques and facilitate the transition of Aβ from insoluble to soluble forms in the cerebrum. These results further suggest that endogenous metals such as zinc, copper and iron are involved in the deposition of aggregated Aβ in the brains of AD patients. Drugs with metal chelating properties could produce a significant reversal of Aβ plaque deposition *in vitro* and *in vivo* [119].

5′-UTR of *APP* mRNA

Studies with primary cultures of neuronal cells have shown that NGF, FGF, and IL-1 increase both *APP* mRNA and promoter levels [120,121]. In addition, some factors can also regulate protein levels by their effects at the post-transcriptional level via the 5′-untranslated region (UTR); for example, evidence for the regulation of translation of *APP* mRNA by an IL-1 responsive element has been reported [122]. Notably, a cholinesterase inhibitor, phenserine, has recently been shown to reduce the translation of APP by taking advantage of its translational regulation via the 5′-UTR of *APP* mRNA, which has been shown previously to be up-regulated in the presence of IL-1 [123]. Phenserine treatment results in decreased secretion of soluble APP and Aβ in a media of human neuroblastoma cells without toxicity [124]. Phenserine is efficient in blocking translation under conditions of intracellular iron metal chelation with desferrioxamine, which suggests that anticholinesterase drugs operate through an iron-dependent pathway at the 5′-UTR of *APP* [125]. Desferrioxamine (a high affinity Fe^{3+} chelator), clioquinol (a low affinity iron, copper and zinc chelator), piperazine-1 (an oral iron chelator) and VK-28 (an oral iron chelator), were tested for their relative modulation of 5′ UTR of *APP*, and they have been reported to slow the progression of AD [126]. (-)-Epigallocatechin-3-gallate (EGCG) is the main polyphenol constituent of green tea and it possesses metal-chelating and radical-scavenging properties. The role of EGCG in the regulation of the iron metabolism-related proteins of APP and the transferrin receptor (TfR) were studied and it was found that EGCG exhibits potent iron-chelating activity comparable to that of the iron chelator desferrioxamine [127]. Thus, the natural non-toxic brain-permeable EGCG may provide a potential therapeutic approach for AD and other iron-associated disorders. The 5′-UTR of APP thus represents a novel target for AD drug development; however, our current understanding of the cellular and molecular mechanisms that connect inflammatory cytokines to *APP* gene regulation is limited. Similarly, whether or not the large 3′-UTR APP mRNA can control protein levels and to what extent the 3′-UTR can be used as a potential drug target remains to be fully explored.

Aβ immunotherapy

AN1792 (an Aβ vaccine) consists of synthetic aggregated $Aβ_{1-42}$ peptide and the surface-active saponin adjuvant, QS-21 [128]. It was found that Aβ antibodies reduce the Aβ load, and the extent of Aβ removal is strongly correlated with mean anti-Aβ antibody titers [129]. Aβ vaccines activate T helper cells (T_H1) that promote humoral immunity and suppress autoimmune encephalitis. A clinical study of AN1792 suggests that that the T_H1 response results from the presence of both T-cell and B-cell epitopes within $Aβ_{1-42}$ peptide [130]. B-cell epitopes are characterized in the N-terminal of the peptide (11-15 amino acids). T-cell epitopes have been reported on the C-terminal of the peptide (15-42 amino acids). It is believed that the C-terminus of the peptide is responsible for triggering meningoencephalitis in the AN1792 trial. Active vaccination of AN-1792 was developed but it has immunological side-effects. Recently, several active and passive immunization therapies are in progress for AD [131]. Studies have shown that both active and passive Aβ immunization also reduces cognitive dysfunction in transgenic mouse models of AD; however, the first clinical trial of active immunization with a pre-aggregated synthetic Aβ42 preparation (AN-1792 vaccine) was not successful, as several patients experienced meningoencephalitis [132]. Immunization against Aβ in humans results in the clearance of amyloid plaques from the cerebral cortex.

Discussion

The most frequent therapies for AD aim to reduce the production and deposition of Aβ peptide. A good number of targets are available for the cure of AD, and many efforts have been made in this direction; however, no effective drug therapy has yet been developed. There is need to validate the therapeutic relevance of such drug targets, which possibly remains one of the central challenges in drug discovery. Simultaneous exploitation of several drug targets for the treatment of AD may also be problematic due to the complexity of cellular signaling and drug–protein interactions in the human body. Inhibitors of BACE1 can regulate amyloidogenic metabolism of APP, but these inhibitors were not found very successful, and only one compound, CTS-21166, has reached clinical trials. Similarly, NSAIDs and statins are useful in the treatment of AD, but they are not successful in symptomatic treatment. Several compounds that inhibit γ-secretase have been studied, and their effect was validated, the most advanced being LY-450139, which is in Phase III clinical studies [79]. β- and γ-secretase inhibitors with low molecular weight, high specificity to target, favorable ADMET and high penetrability through the BBB could provide a new hope for AD patients.

Overactivation of glutamate receptors, particularly of *N*-methyl-*D*-aspartate (NMDA) receptors, leads to an immediate rise in calcium ions, ultimately leading to nerve cell death. The NMDA receptor itself is, therefore, a target for the suppression of acute and chronic excitatory nerve cell death. Memantine is a non-competitive NMDA antagonist, which blocks NMDA receptor-mediated excitotoxicity [133]. Memantine only functions in pathophysiological conditions (i.e. NMDA receptor overactivation) and leaves physiological neurotransmission unchanged. Memantine, an NMDA channel blocker, is an approved pharmacological drug available for the treatment of AD [134,135]. Pharmacological properties of acetylcholinesterase inhibitors (donepezil, rivastigmine, and galantamine) were also studied and tested, but there is still need for more effective therapy for AD. It is important to design analogues of these inhibitors with high specificity to their target, and their QSAR and ADMET properties must be predicted before synthesis and clinical trial [136]. Cu^{2+} and Zn^{2+} ion chelators are a promising approach for the inhibition of aggregation of the Aβ peptide.

Studies reveal that antioxidant-based therapies may become the most potent approach against AD. AD is caused due to a high degree of oxidative damage to nerve cells, and natural therapies could be a promising step to aid in the prevention and cure of AD. Significant amounts of metal have been reported in the brain of AD patients, and natural antioxidants can decrease the level of metals in an AD brain by various mechanisms, such as metal chelation. The role of vitamins in the prevention of AD is widely studied and it has been reported that vitamin E, C, B_{12}, and thiamine significantly reduce the incidence of AD. A mixture of tocotrienols and tocopherols offer synergistic protective action against AD. Tocotrienols are known to cross the BBB, and are potent protectors of neuron cells that may be destroyed through stroke and neurodegenerative diseases [137]. Treatment with high-doses of B vitamins (folic acid 0.8 mg, vitamin B_6 20 mg, and vitamin B_{12} 0.5 mg) slows the shrinkage of brain volume. B vitamins decrease the level of homocysteine, which directly leads to a decrease in gray matter atrophy, thereby slowing cognitive decline [138].

It is important to consider the timing of drugs administered in the treatment of AD, since different targets exist for AD therapy that contribute to the pathology at distinct times. Natural polyphenols and antioxidants, which possess antioxidant, metal-chelating and radical-scavenging properties, should be consumed to prevent the future risk of AD. Drugs targeting BACE and γ-secretase can be beneficial at the initial stage of AD to prevent the production of Aβ from APP, as well after the onset of AD, to prevent the future burden of Aβ. Bapineuzumab and solanezumab, which target Aβ fibrils, may prove more effective in late stages of AD. GSK-3 inhibitors can be useful during the middle stages of AD, as they prevent tau phosphorylation and tau protein aggregation. Drugs inhibiting ACAT limit the availability of APP for proteolytic cleavage by secretases enzyme and also delay the trafficking of immature APP from the endoplasmic reticulum. Therefore, ACAT inhibitors may be recommended in the early stage of AD to limit the cleavage of APP by secretases. Aβ fibrils cause activation of microglia and neuroinflammatory markers such as interleukin, which results in neuroinflammation. NSAID drugs can be taken during the late stage of AD to block prostaglandin synthesis through inhibition of COX.

Recent advancement

Immunotherapy for AD has shown that targeting Aβ with antibodies can reduce AD pathology in both mouse models and the human brain. These findings encourage the efforts to use antibody therapy against other CNS targets, such as tau and BACE; however, it is also evident that antibody penetration across the BBB is limited, with an estimated 0.1-0.2 % of circulating antibodies found in the brain [139]. Some small, lipid-soluble drugs do cross the BBB simply by diffusion through the cell membrane, and others, like caffeine, enter successfully via specialized transporter proteins. The BBB is a major obstacle preventing drug delivery to the brain, particularly for large protein therapies. Watts and Dennis designed bispecific antibodies to fulfill at least two functionalities: one that facilitates transport, and the other to provide therapeutic benefit [140]. One arm of the antibody was designed to sneak the drug into the brain by binding to the transferrin receptor (TfR) on BBB endothelial cells, and the second arm binds to and inhibits BACE1 inside the brain. However, the first antibody designed to bind to TfR was not successful in crossing the BBB because the antibodies were trapped within the capillaries of the BBB. The high affinity of antibodies for TfR might be the problem [141]; therefore, low affinity antibodies were designed, and it was observed that this strategy was successful in crossing the entering the brain. Furthermore, studies have shown that bispecific antibodies against TfR and BACE1 traverse the BBB and effectively reduce Aβ levels [142].

Villeda *et al.* reported that exposure of an aged animal to young blood can counteract and reverse pre-existing effects of brain aging at the molecular, structural, functional and cognitive level. Structural and cognitive enhancements elicited by exposure to young blood are mediated by activation of the cyclic AMP response element binding protein (CREB) in the aged hippocampus [143]. It has been observed that impaired regeneration in aged mice is reversible by exposure to young blood products, suggesting that young blood contains a growth differentiation factor (GDF11) that can restore regenerative function [144]. GDF11 is a rejuvenating factor for skeletal muscle. This indicates that GDF11 systemically regulates muscle aging and may be therapeutically useful for reversing age-related skeletal muscle and stem cell dysfunction.

Future perspective

Development of effective therapy for the cure of AD has now become a challenging task, as most of the drugs fail in Phase III clinical trials. Researchers are trying to optimize the problem of AD by looking at all approaches that can be effective treatment strategies for AD. AD can be prevented by lowering the formation of Aβ from APP, clearance of Aβ already formed,

or by preventing of aggregation of Aβ into Aβ fibrils. These potentially disease modifying treatments include Aβ-peptide vaccination, β- and γ-secretase inhibitors, cholesterol-lowering drugs (ACAT inhibitors), metal chelators, anti-oxidants and anti-inflammatory agents. These approaches for disease-modifying therapy with future advancement may become potential and successful forms of AD therapy in the future. Active immunization against Aβ in humans has shown the ability to clear the burden of Aβ deposits in the brain, but it cannot be used due to its adverse effects. Vaccine designing against Aβ could be a potential therapy for AD.

Studies in a mouse model of AD have confirmed that either intravenous or intra-cerebral injections of human adipose-derived stem cells lead to significant improvements in memory and learning, and reduce the levels of Aβ fibrils and Aβ in the brain; therefore, future improvement in stem cell therapy may open a new and exciting way for the treatment of AD. Computational approaches of drug designing such as modeling, docking, molecular dynamics simulation, QSAR analysis and ADMET prediction can speed up the drug development process and reduce the cost and risk of drug failure in clinical trials.

Neurodegenerative diseases such as AD, Parkinson disease and Huntington disease are associated with aggregates of toxic proteins/peptides. Antisense therapy for AD could be used to reduce the levels of Aβ and Aβ fibrils by down-regulating the expression of *APP* gene and its protein product. Antisense therapy could also be considered for the treatment of other CNS diseases, but the delivery of antisense molecule to the CNS is challenging due to the limited permeability of the blood-brain barrier. This difficulty can be overcome by directly injecting a viral vector encoding a transcription-based siRNA into the human brain.

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