1. INTRODUCTION

Dr. Alois Alzheimer was the first to describe Alzheimer's disease over 100 years ago as an unknown brain disease. Presence of two abnormal structures mainly senile plaques and neurofibrillary tangles was revealed in neuropathlogical analysis of the post-mortem of brain of a dementia patient.[1] Alzheimer's disease (AD) is an irreversible neurodegenerative brain disorder which is one of the most common forms of dementia. Dementia is not a specific disease but a collection of symptoms that can be produced by a number of disorders which affect the brain.[2] Memory loss and impairment in at least one or the other area of cognition involving language, problem solving, judgment, calculation, attention, perception abnormal behaviour and personality changes, are the two main abnormalities found in dementia patients, leading ultimately to full dementia and death. [3, 4]

In the current era of innovative medication, Alzheimer's disease (AD) has emerged as a disease of prime concern amongst all other neurodegenerative diseases. As on today almost 46 million people live with dementia worldwide.[3] The total population affected by AD has been expected to rise three fold by 2050, especially in the developed countries like U.S. and Europe.[5, 6] Although the exact cause of the AD is not yet fully known, reports suggest multiple factors responsible for the development of the disease, including low levels of acetyl choline (ACh) in the hippocampus and cortex area of brain. The deposition of amyloid β (Aβ) peptide, neurofibrillary tangles (*p*-Tau) and oxidative stress have also been known to play vital roles in the pathogenesis of AD.[7, 8] As per the cholinergic hypothesis, AD can be attributed to the reduced levels of Ach, an important neurotransmitter present in the brain involved in memory and learning.[9] Acetyl cholinesterase (AChE), a hydrolase enzyme, hydrolyzes ACh and helps in terminating cholinergic synaptic transmission more quickly.[10] Studies have also suggested involvement of butyryl cholinesterase (BuChE), another major cholinesterase present in the brain capable of hydrolyzing ACh, and therefore inhibition of BuChE may further enhance cholinergic transmission in AD.[11-13] Thus, the use of acetyl cholinesterase inhibitors (AChEIs) and butyryl cholinesterase inhibitors (BuChIs) have laid the foundation for the current therapy of AD.

1.1 Etiology of AD

A number of risk factors like age, genetic/family history, smoking, alcohol consumption and oxidative damage have been suggested as the possible causes for the development of AD. [14]

Age: The older you grow the greater your risk of developing AD. There are two types of ADs depending on whether the disease starts before or after the age of 65.

- Early onset: Less than 5% of patients usually develop AD before 65 years. It occurs due to gene mutations on chromosomes 1, 14, or 21.
- Late onset: Majorities of AD cases are late onsets that usually develop after the age of 65 years. Only one risk factor gene identified so far is apolipoprotein E gene which is found on chromosome 19.

Genetic/Family history: For early onset form of AD, three genes have been identified so far, Amyloid precursor protein (APP) gene on chromosome 21, presenilin-1 (ps1) gene on chromosome 14 and presenilin-2 (ps2) gene on chromosome 1.[15]

Presenilin-1 (ps1), an integral membrane protein located in endoplasmic reticulum (ER) disturbs ER calcium homeostasis resulting in increased release of calcium through ryanodine receptors and inositol-1,4,5-triphosphate receptors after undergoing mutation in ps1. Enhanced calcium release triggers further calcium influx through calcium releasing channel in plasma membrane leading to calcium homeostasis which makes neurons vulnerable to apoptosis and excitotoxicty. [15] Increased neuronal degradation may be promoted by presenilin mutations owing to enhanced γ-secretase cleavage of APP thereby increasing production of neurotoxic $A\beta_{1-42}$. Due to mutation of presenilin-1 protein, neurons become susceptible to death induced by trophic-factor withdrawal, exposure to $A\beta_{1-42}$ or glutamate, and energy deprivation. Mutant presenilin-1 acts at an early step before Par-4 production, mitochondrial dysfunction and caspase activation. Presenilin-2 may also facilitate apoptosis, although the mechanism by which it causes so has not been established till date.

For late onset (sporadic) AD, apolipoprotein E (ApoE4) gene has been identified as the risk factor. ApoE gene found on chromosome-19 codes for a protein that helps carrying cholesterol in the blood stream. ApoE is found in several forms or alleles. The ε 4 allele occurs in about 40 % of people with AD but it is neither a pre-requisite nor does its presence confirm development of AD. Additional risk factor genes may lay on regions of chromosomes 9, 10 and 12.

Oxidative damage: Excess free radicals may cause substantial neuronal damage, contributing to AD development.[16]

Smoking and alcohol use

Other risk factors include diseases like atherosclerosis, diabetes, down syndrome, long lasting high blood pressure etc.

Plasma homocysteine: Individuals with low dietary folate intake are at increased risk of AD as an apparent consequence of increased levels of homocysteine.

1.2 Pathophysiology of AD

AD is associated with shrinkage of brain tissue accompanied by localised loss of neurons mainly in hippocampus and basal forebrain which play an important role in learning and long-term memory. Damage to this part produces inability to retain new information. Pathologists have considered the defining characteristic of amyloid β (Aβ) deposits in senile plaques and neurofibrillary tangles (NFT) as the hallmarks of the disease. Amyloid β (Aβ) mainly consists of paired helical filaments of abnormally phosphorylated τ protein. Neuronal death appears with the progress of the disease (**Figure 1**). Particularly, cholinergic neurons and synapses of the basal forebrain are selectively lost, which account for the development of cognitive impairments. This finding forms the basis of "cholinergic hypothesis", which proposed cholinergic enhancement as an approach for improving cognitive function in AD. [17] This approach has produced majority of the drugs approved so far for treating AD. [18]

1.2.1 β-Amyloid plaques

β-Amyloid (Aβ) plaques accumulate outside of the neurons mainly as deposits of β-amyloid protein formed due to breaking down of Amyloid Precursor Protein (APP). In AD patients, a large amount of Aβ accumulates which can clump and leads to formation of fibrils (insoluble particles) which make up the amyloid plaques, the hallmark of AD.[20] APP can be proteolytically processed as mentioned in **Figure 2.**[21] Aβ protein containing polypeptides of 40/42 amino acids derived from a larger integral membrane protein called APP. Among various amyloid proteins, $A\beta_{40}$ and $A\beta_{42}$ are more toxic. APP, a membrane glycoprotein is encoded by a gene on chromosome-21, and composed of an approximately 41-kDa peptide. The normal functions of APP are regulating neuronal survival, neurite outgrowth, synaptic plasticity and cell adhesion.

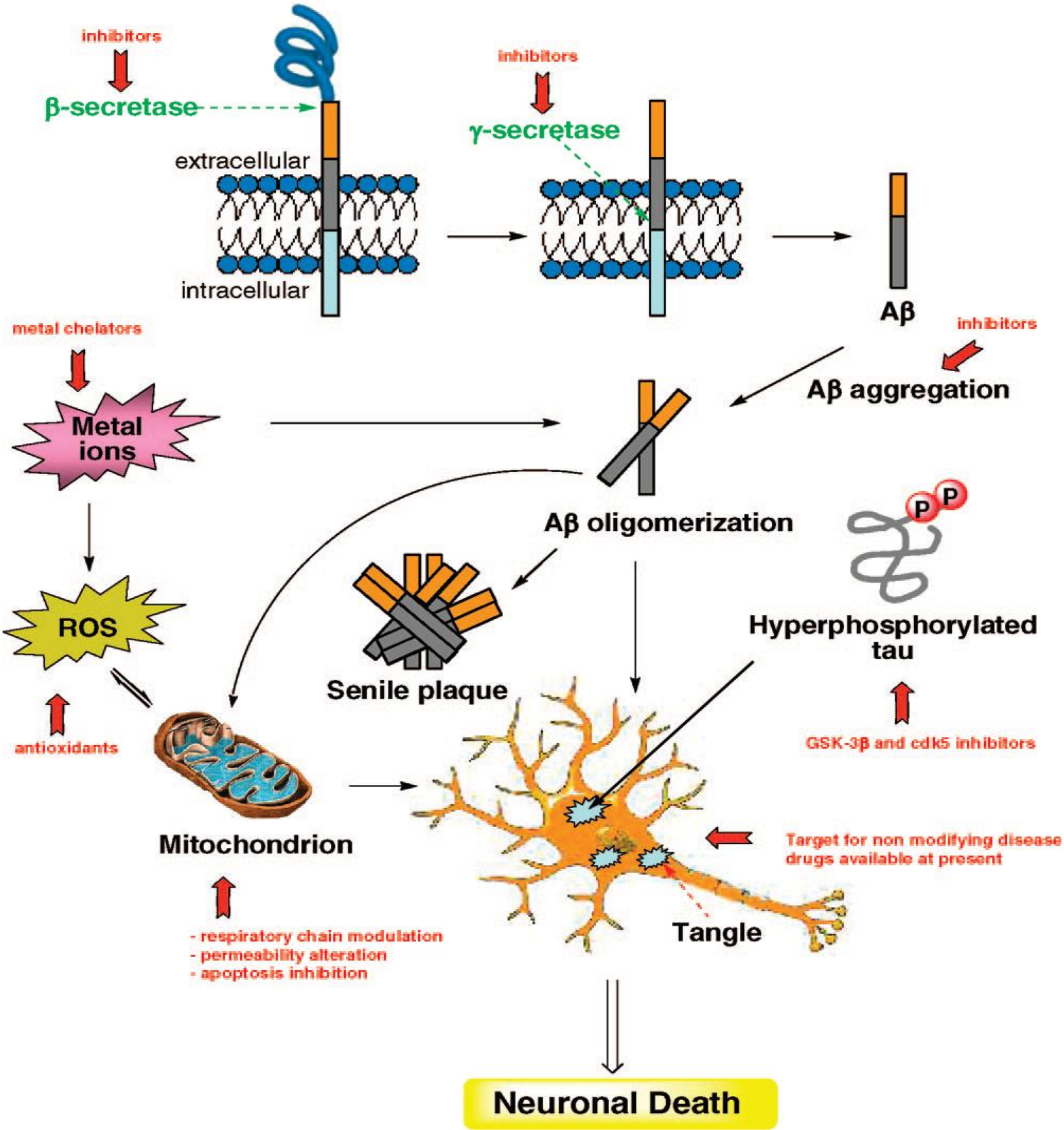


Figure 1. Possible molecular causes of neuronal death and protective mechanisms in AD. [19] The central event in AD pathogenesis is an imbalance between Aβ production and clearance. The enhanced activity of β and *γ*-secretases leads to increased release of amyloidogenic Aβ42, which forms oligomers and then extracellular deposits (senile plaques). One way to confront AD pathogenesis may be to combat the oligomerization by means of small molecules. A role for metal ions and ROS in the A oligomerization has also been advanced. Therefore, metal chelation and antioxidant activity are two general mechanisms to be considered in the search for disease-modifying anti-AD drug candidates. Also, β and *γ*-secretase inhibitors may be promising lead compounds because they tackle an early event in AD pathogenesis. Mitochondrial dysfunction plays a fundamental role in the neuronal death associated with AD, as it is likely that intracellular Aβ could compromise the function of this organelle. *τ* hyperphosphorylation leading to tangle formation is regarded as a downstream event but could contribute to reinforcing neuronal dysfunction and cognitive impairment.

α-Secretase (a metalloprotease) initially degrades a small subset of the APP molecules to produce c83 and soluble version of APP (sAPP), followed by γ-secretase enzyme (resembling aspartyl protease) to give rise to a harmless, non toxic peptide p3. However, when APP is cleaved initially by β-secretase enzyme to produce c99 and β-amyloid, followed by γ-secretase cleavage, either a 40 amino acid Aβ peptide (Aβ40) or a 42 amino acid Aβ peptide ($A\beta_{42}$) is formed which are more toxic as these are hydrophobic and form aggregates rapidly in the extracellular space.

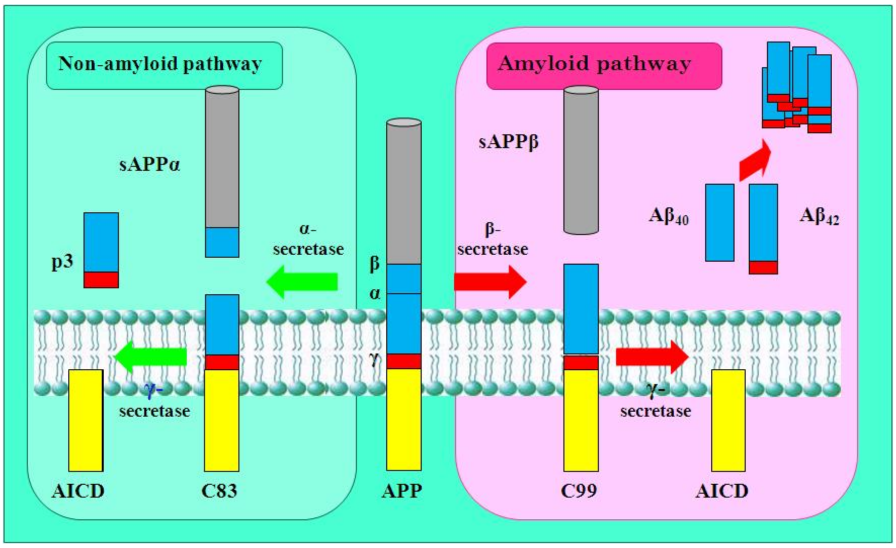


Figure 2. Amyloid processing in AD

1.2.2 Neurofibrillary tangles [22]

Healthy neurons of brain have support structures called microtubules, which guide nutrients and molecules from the cell body down to the ends and back. Microtubules are stabilised by a special kind of protein TAU which is changed chemically in people with AD and begins to pair with other threads of Tau to become tangled up together. Due to tangling, microtubules disintegrate, collapsing the neuron's transport system. This may result first in communication malfunctions between neurons and later cell death (**Figure 3**).

Small number of senile plaques and neurofibrillary tangles can be observed in intellectually normal individuals, but they are much more abundant in AD patients. Paired helical filaments (PHF) constitute NFTs that contain an abnormal hyperphosphorylated form of the microtubule-associated protein τ. Reduction in ability of τ protein to stabilize microtubules assembly results due to phosphorylation within the microtubule binding domain which leads to the disruption of neuronal transport followed by accelerated synaptic loss and cell death.[23] Abundance of tangles is roughly proportional to the severity of cognitive impairment. They are most abundant in hippocampus and associate region of the cortex whereas in areas such as the visual and motor cortices they are relatively spared.

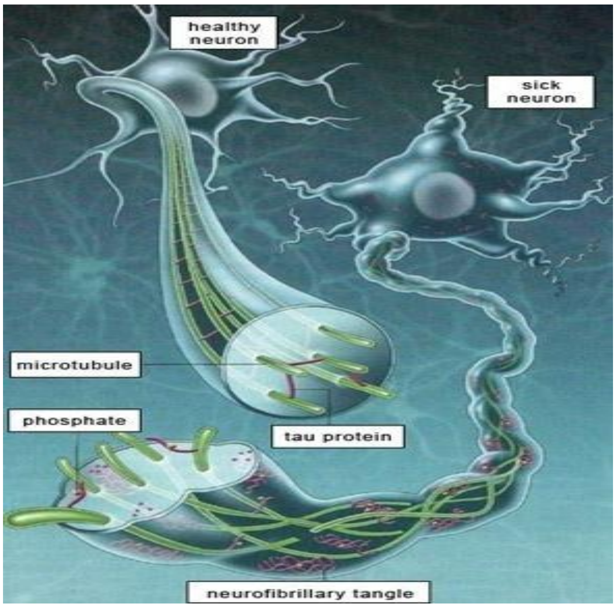


Figure 3. Neurofibrillary tangle processing

1.2.3 Cholinergic hypothesis [22]

Neurochemical basis of AD is a "cholinergic deficiency hypothesis" which proposes that "the memory disturbances in AD are caused by loss of cholinergic neurons functioning". Perhaps this is because Aβ is deposited in cholinergic neurons that destroys them.[24] Reduction in the levels of ACh synthesis can be observed in cortex and hippocampus of AD patients. Muscarinic antagonists (such as atropine) can produce memory disturbances in normal human volunteers that have some similarity to the memory disturbances in AD. Procholinergic agents (i.e. agents that enhance cholinergic functioning) not only can reverse atropine induced memory impairments in normal human volunteers but can also enhance memory functioning in AD patients. In addition, central nicotinic receptor density also gets decreased in the brain of AD patients, but muscarinic receptors activity is not affected. [25]

1.2.4 Oxidative stress

A pathogenic role of oxidative damage in the process of neurodegeneration has been well reported.[26-28] Oxidative stress leads to conditions like deposition of senile plaques, neurofibrillary tangles [27, 29] and the deposition of Aβ.[30] Some studies have suggested that the neurotoxicity of aggregated Aβ is mediated through its ability to induce oxidative stress via spontaneous generation of free radicals and reactive oxygen species (ROS).[31-33] Oxidative and nitrative stress results in the formation of ROS and reactive nitrogen species (RNS) [34] inducing oxidative damage in the form of protein oxidation, which ultimately leads to the cellular dysfunction and cell death.[35, 36] Thus, a successful protection of neuronal cells from oxidative damage could potentially prevent AD. [37]

1.2.5 Possible role of *D*-serine in the pathophysiology of AD

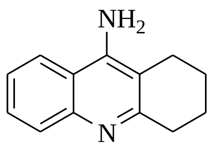
Several lines of evidence suggest that *D*-serine may function as an endogenous agonist of the glycine site on the NMDA receptor that has been implicated in the pathophysiology of AD. [38]

1.2.6 Endosomes

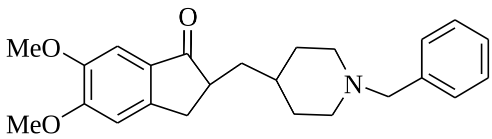
Membrane-bound endosomal vesicles have an integral role to play in multiple cellular events like protein processing and turnover along with critical regulation of the cell-surface availability of receptors and other plasma membrane proteins in various cell types. Neurons too are dependent on endosomal function for housekeeping and synaptic events. Various events suggest a link between neuronal endosomal function and AD pathophysiology. Endosomal abnormalities invariably occur within neurons in AD brains, and endocytic compartments are the most likely sites for the production of the pathogenic Aβ, that accumulates in the brain during the disease and generated by proteolytic processing of APP. Therapeutic agents target the enzymes and events involved in APP processing aimed at slowing or reversing the pathogenesis of AD. The neuronal endosome may well prove to be the intracellular site of action for inhibitors of β-amyloidogenic APP processing. [39]

1.3 Treatment strategies for AD

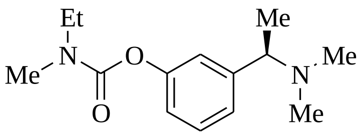
Various approaches have been used for the treatment of AD over the past several years.[40] Currently, no single drug is available which can completely cure AD.[41] Primary therapeutic option approved by US FDA for the treatment of AD is the use of cholinesterase inhibitors **(Figure. 4)** like tacrine **(I)**, donepezil **(II)**, rivastigmine **(III)** and galantamine **(IV)**. Tacrine **(I)** exhibited hepatotoxicity via elevation of serum alanine aminotransferase levels, resulting in its limited clinical use and consequent withdrawl from the market shortly after its approval.[42] Donepezil **(II)** is the most effective pharmacological agent used to treat AD efficiently amongst the list of approved drugs.[43]



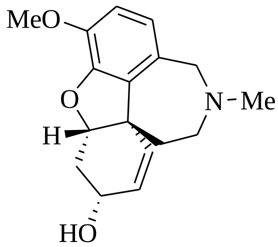
Tacrine **(I)**



Donepezil **(II)**



Rivastigmine **(III)**



Galantamine **(IV)**

Figure 4. AChE inhibitors **(I-IV)** used for the treatment of AD

2. REVIEW OF LITERATURE

2.1 Role of cholinesterases in AD

Literature review reveals that neuritic amyloid plaques and neurofibrillary tangles comprising of hyper phosphorylated tau proteins represent the basic neuropathologic features of AD [44, 45] that is characterized by a low concentration of acetylcholine (ACh) in hippocampus and cortex [24] owing to dysfunctions in the system of cholinergic neurotransmission of the central nervous system (CNS).[46] In the CNS, the cholinergic system regulates memory and learning processes, in which two enzymes, acetylcholinesterase (AChE) and butyrylcholinesterase (BuChE) degrade Ach.[47] Thus, these two enzymes, acetylcholinesterase (AChE) and butyrylcholinesterase (BuChE) have been identified as the most potential targets for the treatment of AD, myasthenia gravis, glaucoma, and in the recovery of victims of nerve gas exposure.[48]

AChE is a key component of cholinergic brain synapses and neuromuscular junctions that plays the main role in the termination of impulse transmission by rapid hydrolysis of the cationic neurotransmitter acetylcholine.[49] BuChE is produced in the liver and enriched in the circulation. The exact physiological role of BuChE is still elusive, but it is generally viewed as a backup for the homologous AChE.[50, 51] The activity of AChE decreases progressively in certain regions of brain along with progression of AD from mild to severe stages to reach 10–15% of the normal values, while BuChE activity remains unchanged or even increases by 20%. Hence, a large pool of BuChE is available in neurons and neuritic plaques.[10] Selective cholinesterase inhibitor (ChEI) for AChE may not be advantageous; on the contrary, a good balance between AChE and BChE may prove beneficial owing to higher efficacy.[52]

It has been reported that the genesis of amyloid protein plaques associated with AD is connected to both AChE and BuChE as a significant decrease in plaques has been observed in AD patients using ChE inhibitors (ChEIs).[53-56] It is important to note that ChEIs have demonstrated more promising results in the treatment of AD as compared to any other therapeutic strategy explored.[57-60]

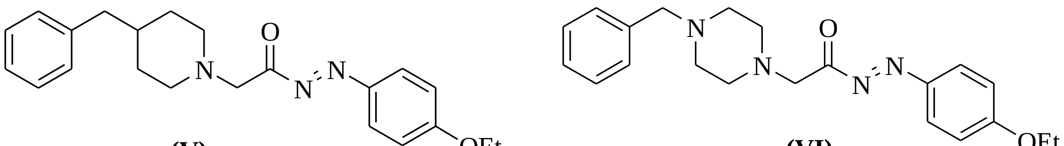
The catalytic active site (CAS) of AChE has been found to be located at the bottom of a narrow gorge which consists of two subsites, a negatively charged or 'anionic' site, and an esteratic site, containing the actual catalytic residues, also called the catalytic triad.[61] BuChE possesses a larger void at the active site gorge. AChE contains a second 'anionic' site, which became known as the 'peripheral anionic' site (PAS) and lies around 14Å away from the active site.[62]

Recent studies have identified that AChE could also play a key role in accelerating senile amyloid β-peptide (Aβ) plaques' deposition.[63] It is likely that AChE interacted with (Aβ) and promoted amyloid fibril formation through a pool of amino acids located in the proximity of peripheral anionic site (PAS).[64]

2.2 Multi-target directed ligand design (MTDL) as cholinesterase inhibitors.

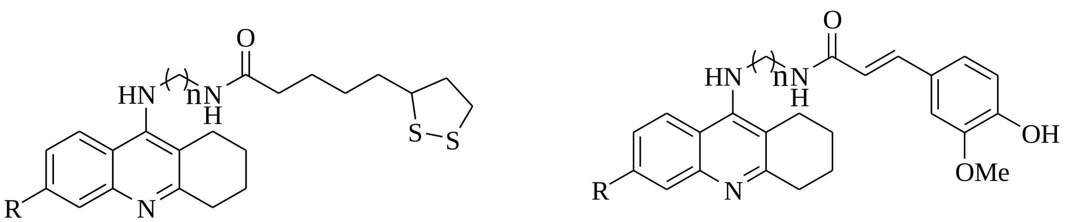
Due to complex pathophysiology of AD and involvement of a number of new potential targets, an approach called multi-target directed ligand design (MTDL) has been applied in search of better drugs to treat AD.[65, 66] Recently various bispharmacophore/ bivalent ligands have been reported in the literature in which two of the same or different pharmacophoric moieties are linked together via a spacer for the treatment of AD, which include donepezil-propargylamine, donepezil-pyridyl, donepezil-ebselen, tacrine-ferulic acid, huprine-tacrine etc.[67-70] Additionally, a bivalency approach (formerly applied mainly to GPCR ligands, like opioid agonists and dopamine antagonists)[71, 72] has been applied to AChE inhibitors (i.e., to connect two inhibitor molecules covalently by a long hydrocarbon spacer).

Özer *et al.*[73] designed a new series of compounds on the basis of donepezil structure, which were expected to inhibit acetylcholinesterase, butyrylcholinesterase and Aβ aggregation. This class of dual-acting compounds was a combination of 4-benzylpiperidine/piperazine and a differently substituted benzene ring connected by an *N*-acylhydrazone moiety. The benzene fragment contained one or two methoxy/ethoxy groups. The results of Ellman's test showed that these compounds were moderate and non-selective inhibitors of both AChE and BuChE, having IC_{50} values in the micromolar range (53.1-88.5 µM for *h*AChE and 48.8-98.8 µM for *Eq*BuChE, respectively). Compound **(V)**, bearing a 4-ethoxybenzene fragment and 4-benzylpiperidine, was found to be the most potent *h*AChE inhibitor. Compound **(VI)**, bearing a 4-ethoxybenzene fragment and 4-benzylpiperazine, was found to be the most potent *h*AChE and *Eq*BuChE inhibitor. All the compounds were able to inhibit 69% to 90% aggregation of $A\beta_{1-40}$ and $A\beta_{1-42}$, taking rifampicin (69-90% at 100 µM) as a standard.



(V) **(VI)**

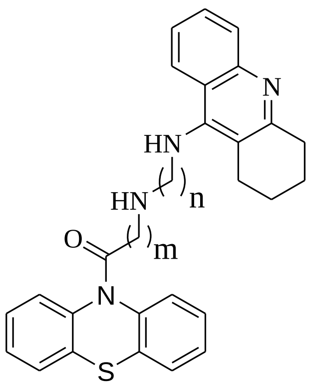
Some hybrids have been reported as active compounds containing tacrine moiety and PAS binding moiety linked via suitable linkers. Leonetti *et al.*[74] reported tacrine and lipoic acid hybrids (**VII**) whose biological profiles were markedly improved relative to those of the



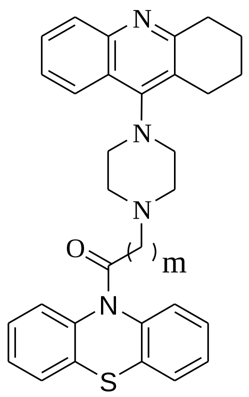
(VII) n = 3-8 **(VIII)**

prototypes tacrine and lipoic acid and were also found to inhibit AChE induced β amyloid aggregation indicating interaction of the hybrids **(VII)** with the peripheral anionic site (PAS), which accelerated amyloid fibril formation.[74, 75] Optimum inhibition was obtained in case of n=3. Decker *et al*,[76] reported a series of tacrine-ferulic acid hybrids (**VIII**), among which a compound having n=6 was found to be the most potent one. It was able to inhibit β-amyloid fibril formation, which was proved by kinetic measurements that showed mixed type of inhibition by interacting with the active site and the PAS both.

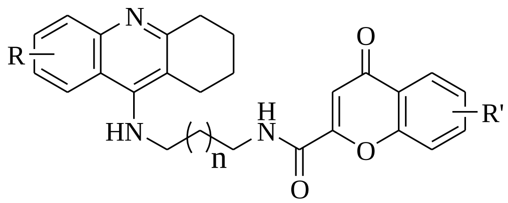
Hui *et al.*[77] reported a series of tacrine-phenothiazine hybrids (**IX, X**) as multitarget drugs for AD which inhibited not only the degradation of the neurotransmitter ACh but also the aggregation of the beta-amyloid protein and phosphorylation of the Tau protein. Tacrine–4-oxo-4*H*-chromene hybrids[78] (**XI**) have been designed as multifunctional agents capable of inhibiting ChE and β-secretase. Li *et al.* reported tacrine–rhein hybrids (**XII**) as multifunctional agents to inhibit CAS as well as PAS.[79]



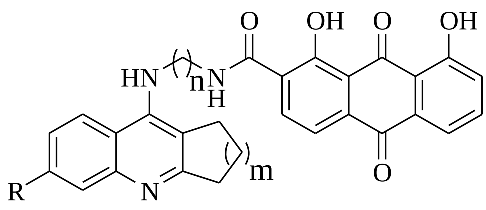
(IX)
n = 2-9,
m = 1-3



(X)
m = 1-3

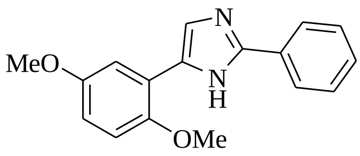


(XI)
n = 5-8, 10

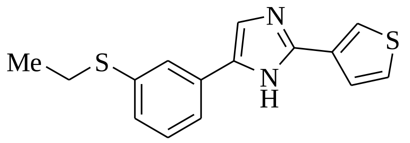


(XII)
n = 4-10,
m = 1-3

Promising dual-acting diarylimidazole hybrids have been reported recently.[80] The main objective of the project was to obtain a lead compound, as a selective and potent *Eq*BuChE and Aβ-aggregation inhibitor. A chemical library of nearly seven hundred natural and synthetic compounds containing flavonoids, alkaloids, coumarins, chalcones, imidazoles, benzimidazoles and thiophenes was screened for inhibition of *Eq*BuChE. The screening assay led to the selection of imidazole derivatives as hits which displayed *Eq*BuChE inhibitory activities with IC_{50} values in the range of 0.2 to 4 µM. The most potent and selective BuChE inhibitor was found to be compound (**XIII)**. A series of imidazole derivatives was developed on the basis of this lead and the molecular modelling studies. Compound (**XIV)** was an analog of compound **(XIII)** with a thienyl group and a thioethyl substituent instead of phenyl and methoxy group. It was found to be the most active *Eq*BuChE inhibitor (IC_{50} = 0.10 µM) in the series. Extended biological studies revealed that compound (**XIV)** displayed a high potency for inhibition of $A\beta_{1-40}$ fibril formation with an IC_{50} value of 5.8 µM. It was proposed that due to the presence of a thiophene moiety, this compound participated in the binding to prefibrils and therefore could prevent or delay the formation of Aβ assembly.

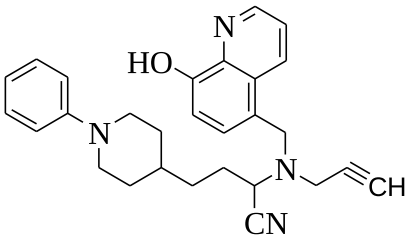


(XIII)

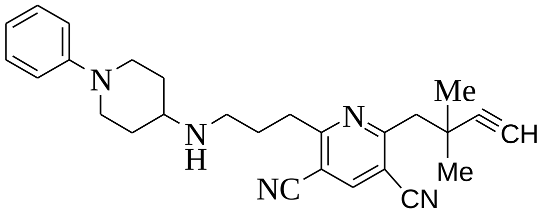


(XIV)

Wang *et al.*[81] studied donepezil-propargylamine-8-hydroxyquinoline hybrids as new multifunctional metal-chelators, and ChE and MAO inhibitors for the potential treatment of AD, and found that compound **(XV)** exhibited the best profile [EeAChE (IC_{50}=1.8mM); eqBuChE (IC_{50}=1.6 mM)].



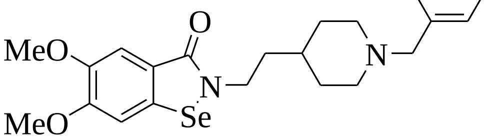
(XV)



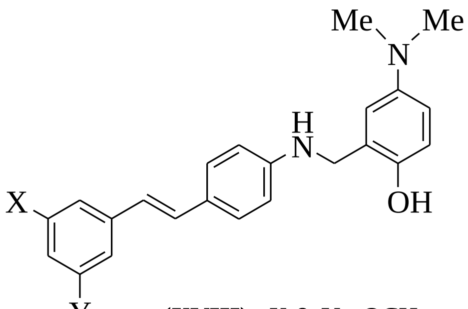
(XVI)

Oscar *et al*.[82] worked on novel donepezil-pyridyl hybrids and concluded that these hybrids were very active and selective EeAChE versus (vs) EqBuChE inhibitors. Compound **(XVI)** exhibited one of the best ChE inhibitory potencies (AChE: IC_{50}=1.1±0.3 nM; BuChE: IC_{50}=600±80 nM) ever determined. By comparison with donepezil, compound **(XVI)** was 11.8- and 11.5-fold more potent at inhibiting AChE and BuChE respectively.

Zonghua Luo *et al.*[68] worked on compounds by fusing donepezil and the antioxidant ebselen. An *in vitro* assay showed that among the molecules, compound **(XVII)** one of the most potent AChE inhibitors (IC_{50}values of 0.042 µM for EeAChE and 0.097 µM for human AChE), a strong BuChE inhibitor (IC_{50}= 1.586 µM) and also possessed rapid H_2O_2 and peroxynitrite scavenging activity and glutathione peroxidase-like activity (ν_0 = 123.5 μM min^{-1}). Toxicity studies in mice demonstrated absence of acute toxicity upto a dose of 2000 mg/kg for compound **(XVII)**. *In vitro* blood-brain barrier model showed that compound **(XVII)** successfully penetrated the central nervous system.

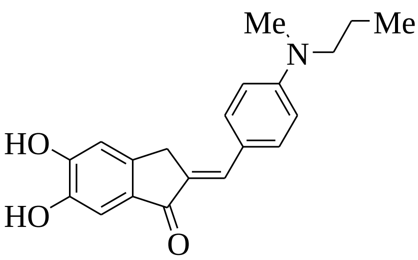


(XVII)



(XVIII) X & Y= OCH_3
(XIX) X & Y= OH

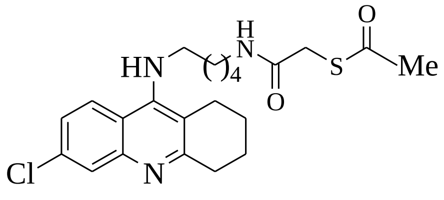
Chuanjun Lu *et al.*[67] worked on a series of multitarget-directed resveratrol derivatives for the treatment of AD. *In vitro* studies indicated that compounds **XVIII** and **XIX** are potential lead molecules for AD therapy (**XVIII**, IC_{50} = 7.56 μM and **XIX**, IC_{50} = 6.51 μM for self-induced Aβ aggregation; the oxygen radical absorbance capacity assay using fluorescein (ORAC-FL) values are 4.72 and 4.70, respectively). These compounds have been shown to be capable of disassembling the highly structured Aβ fibrils generated by self- and Cu(II)-induced Aβ aggregation. Furthermore, compound **(XVIII)** crossed the blood−brain barrier (BBB) *in vitro* without demonstrating any significant acute toxicity in mice at doses of up to 2000 mg/kg. Taken together, the data indicate that compound **(XVIII)** is a very promising lead compound for AD.



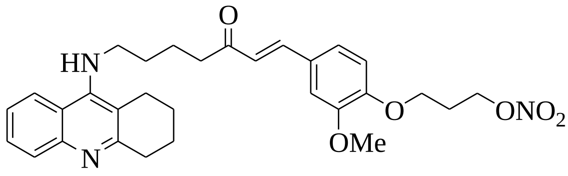
(XX)

Ling Huang *et al.*[69] reported benzylideneindanone derivatives as multitarget-directed ligands against AD. *In vitro* studies demonstrated that compound **(XX)** had the greatest ability to inhibit $A\beta_{1-42}$ aggregation (80.1%) and MAO-B (IC_{50} = 7.5 μM), and behaved as an excellent antioxidant and metal chelator. Moreover, it was capable of inhibiting Cu(II)-induced $A\beta_{1-42}$ aggregation and disassembling the well-structured Aβ fibrils. These results indicated that compound **(XX)** was an excellent multifunctional agent for the treatment of AD.

Yue Wang *et al.*[83] reported multifunctional mercapto-tacrine derivatives for treatment of age-related neurodegenerative diseases. Among all of the tested compounds, compound **(XXI)** displayed the most potent inhibitory activity against AchE. However, compared to tacrine compound **(XXI)** showed higher selectivity for AchE. The reduced potency of **(XXI)** against BuChE may be due to steric hindrance caused by the chloro group to some specific residues of the BuChE binding site.



(XXI)



(XXII)

Yao Chen *et al.*[84] synthesized tacrine−ferulic acid−NO donor trihybrids as cholinesterase inhibitors. Release of nitric oxide, vasodilator properties, cognition improving potency, and hepatotoxic potential were also tested. The trihybrid compound **(XXII)** exhibited better performance in improving the scopolamine-induced cognition impairment (mice) and less hepatotoxicity than tacrine.