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REVIEW ARTICLE

Changing Paradigm from one Target one Ligand Towards Multi-target Directed Ligand Design for Key Drug Targets of Alzheimer Disease: An Important Role of *In Silico* Methods in Multi-target Directed Ligands Design

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Abstract: Alzheimer disease (AD) is now considered as a multifactorial neurodegenerative disorder and rapidly increasing to an alarming situation and causing higher death rate. One target one ligand hypothesis does not provide complete solution of AD due to multifactorial nature of the disease and one target one drug fails to provide better treatment against AD. Moreover, currently available treatments are limited and most of the upcoming treatments under clinical trials are based on modulating single target. So, the current AD drug discovery research is shifting towards a new approach for a better solution that simultaneously modulates more than one targets in the neurodegenerative cascade. This can be achieved by network pharmacology, multi-modal therapies, multifaceted, and/or the more recently proposed term "multi-targeted designed drugs". Drug discovery project is a tedious, costly and long-term project. Moreover, multi-target AD drug discovery added extra challenges such as the good binding affinity of ligands for multiple targets, optimal ADME/T properties, no/less off-target side effect and crossing of the blood-brain barrier. These hurdles may be addressed by insilico methods for an efficient solution in less time and cost as computational methods successfully applied to single target drug discovery project. Here, we are summarizing some of the most prominent and computationally explored single targets against AD and further, we discussed a successful example of dual or multiple inhibitors for same targets. Moreover, we focused on ligand and structure-based computational approach to design MTDL against AD. However, it is not an easy task to balance dual activity in a single molecule but computational approach such as virtual screening docking, QSAR, simulation and free energy is useful in future MTDLs drug discovery alone or in combination with a fragment-based method. However, rational and logical implementations of computational drug designing methods are capable of assisting AD drug discovery and play an important role in optimizing multi-target drug discovery.

Keywords: Alzheimer's disease, AChE inhibitor, anti-amyloid inhibitor, BACE1 inhibitor, multi-target-directed-ligands (MTDLs), classical and non classical drug targets, computational drug discovery.

1. INTRODUCTION

Alzheimer's disease (AD) is a progressive terminal neurodegenerative disorder for which clinical and neuropathological characteristics were first presented by Alois Alzheimer on 3 November 1906 [1]. AD symptoms include memory loss, a defect in problem-solving ability and other cognitive and behavioural skills that affect a person to perform its daily activities [2]. Alzheimer's disease death rate increased to an alarming 71% and it was estimated that in 700,000 Americans, aged ≥65 years will die with Alzheimer's disease in 2016 [3]. It was also estimated that health care services for people aged ≥65 years will be $236 billion for the year 2016 [3]. Such an alarming data indicates the need to prevent and cure AD and other related dementias in near future. The AD research till now has revealed much about disease pathologies, pathways, and therapeutic drug targets. However, many issues such as biological changes that trigger AD, why it progresses more quickly in some cases than in others and how the disease can be prevented, slowed or stopped completely are still unclear [3].

Recent research in neurodegenerative diseases suggests that AD occurs due to multiple factors such as genetic, environmental and endogenous factors. In this disease, one can observe aggregation of small peptide, protein misfolding, oxidative stress, metal dyshomeostasis, mitochondrial dysfunction, and tau hyperphosphorylation occurring at the same time [4]. The two major hallmark pathologies of AD

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are the progressive accumulation and deposition of the abnormal protein fragments that include extracellular senile neuritic plaques (SNP) and intracellular neurofibrillary tangles (NFT) [5]. SNP are insoluble aggregates of β-amyloid protein (Aβ) deposited outside the neurons and NFT are paired helical filaments of hyperphosphorylated tau protein inside neurons [5]. These changes eventually lead to the damage and death of neurons [5]. Amyloid [6], cholinergic [7, 8], glutamatergic [9], oxidative stress [10], metal dyshomeostasis [11] and neuroinflammation [12] are the various hypotheses that explain the underlying cause of disease progression but none of them alone are sufficient to explain the root cause of AD. It was also observed that disease conditions involve multiple pathways that are indirectly or directly linked to each other. Thus, now AD is considered as multifactorial diseases as more than one pathway are responsible for the disease progression. The major AD etiologies are cholinergic systems, Aβ peptides, tau proteins, clearance of misfolded, aggregated peptides and aberrant signalling pathways. The Cholinergic hypothesis which is based on the presynaptic deficits found in the AD patients is the oldest hypothesis [7]. Acetylcholine is a neurotransmitter involved in learning and memory. However, in AD, the concentration of acetylcholine in the brain is very low, resulting in substantial loss of memory and behavioural decline. The main function of AD drugs is to support communication between nerve cells. Currently, available AD drugs are based on hypothesis, of acetylcholinesterase (AChE) inhibition. There are several evidences where the inhibition of AChE not only restores the cholinergic system but also interferes with the progression of the disease [13].

2. IMPORTANT DRUG TARGETS FOR ALZHEIMER'S DISEASE

AD drug targets can be divided into classical and non-classical or disease-modifying drug targets. Classical drug targets include acetylcholinesterase (AChE), butyrylcholinesterase (BChE) and N-methyl-D-aspartate (NMDA), whereas nonclassical drug targets include secreatse, sirtuins-2, caspases, glycogen synthase kinase-3, autophagy enhancers and synaptogenesis enhancer, muscarinic acetylcholine receptors *etc*. Other AD drugs strategies include clearance of misfolded and aggregated peptides, defective proteins like amyloid beta and tau protein, autophagy, calcium and metal dyshomeostasis, ApoE4, oxidative and nitrosative stress, mitochondrial damage and neuroinfmammation [14]. A list of important therapeutic drug targets based on their mechanism are given in Table **1**.

2.1. *In Silico* Identification of Lead Molecules Against Important AD Drug Targets

In silico methods such as virtual screening, docking pharmacophore modeling, QSAR and molecular dynamics are successfully used to identify and design better inhibitors for AD targets. Here, we discuss some of the important targets that have been explored for single target inhibitor with the help of various computational approaches and validated with experiment. Later, we describe the few privilege chemical moieties that discovered as MTDLs agent against various AD drug targets. Further, we have described ligand based and structure-based computational methods used to design MTDLs.

2.1.1. AChE Inhibitors

A large number of molecules have been tested against this target as this belong to an oldest hypothesis which explains the occurrence of AD. Moreover, four FDA approved drugs for AD are AChE inhibitors (Fig. **1**). Various natural as well as synthetic molecules have been reported as AChE inhibitors [30, 31]. The target has been extensively studied through experiment as well as computational methods.

Table 1. List of AD therapeutic strategies and their targets.

Therapeutic strategies	Mechanism	Targets
Amyloid based therapy	Aβ aggregation inhibitors	Amyloid peptide [6] Amyloid fibril [15]
	Reduction of Aβ production	β-Secretase inhibitors [16]
		γ-Secretase inhibitors [17, 18]
		α-Secretase modulators [19]
	Targeting Aβ-induced neurotoxic effects	Anti-inflammatory agents [12, 20]
	Oxidative stress reduction	Antioxidant agents [10, 21]
Modulation of neurotransmission	Modulation of Aβ-induced neurotransmitter effects	Cholinesterase enzyme inhibitors [7, 8]
		N-methyl-d-aspartate antagonists [22]
Tau based strategies	Targeting tau-induced neurotoxicity	Tau anti-aggregants [23, 24]
		Preventing tau oligomerization [25]
	Tau phosphorylation inhibitor	Glycogen synthase kinase-3 enzyme inhibitors [26, 27]
Oxidative stress	Reduction in reactive oxygen species (ROS)	Monoamine oxidases inhibitor [28, 29]
Other	Metal regulation	Metal chelators

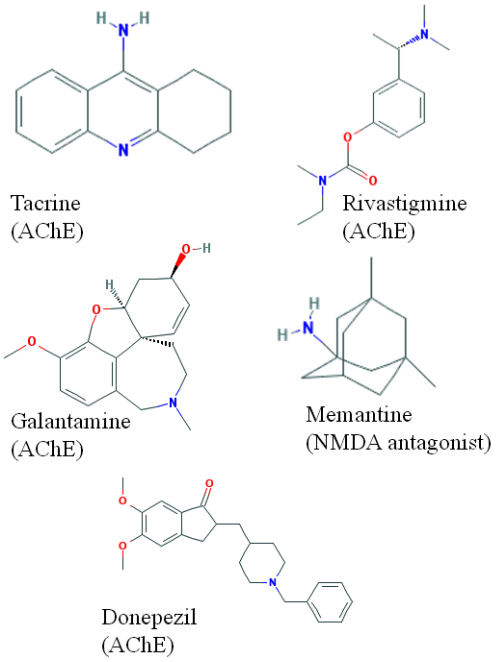


Fig. (1). FDA approved drugs for AD.

In one of the vitro and docking studies of natural flavonoid molecule quercetin, rutin, kaempferol 3-O-beta-D-galactoside and macluraxanthone showed good inhibition of AChE and BChE, and study identifies the important amino acid residues [32]. Tacrine moiety was well studied against AChE and many derivatives were synthesized and tested. In one of 3D-QSAR, molecular docking and MD simulations of 60 tacrine-based derivatives revealed that Tyr70, Trp84, Tyr121, Trp279, and Phe330 are the key residues for the tacrine binding into the active site [33]. 4-Aryl-4-oxo-2-aminylbutanamides were evaluated for anticholinesterase activity through docking and molecular dynamics studies. They suggested that AChE selectivity is due to cycloalkylamino moiety, and a hydrogen bond between ligand –NH– group and AChE Tyr 124 –OH has a very important interaction for activity [34]. Many carbamates derivativesdesigned and synthesized chemically have shown better AChE inhibitory activity than the already present rivastigmine. Docking studies revealed important direct/indirect interactions contributing to the stabilization of the AChE–carbamate complexes [35]. Molecular docking studies of new pyridopyrimidine derivatives were performed with the 3D structure of Torpedo californica AChE (TcAChE) and human butyrylcholinesterase (hBChE) enzymes to understand the binding interaction and orientation of these molecules into the active site of receptors [36]. Pyridonepezil [37] and 4-hydroxycoumarins [38] derivatives also displayed significant AChE inhibitory activity and docking studies revealed that the Phe 330, Trp279 and π- π interaction stabilize the complex [39]. 6-chloro-pyridonepezils and piperzine derivatives showed dual inhibitory activity that binds at the catalytic site and PAS of AChE [37, 40]. Virtual screening of the diverse natural products identified nordihydroguaiaretic acid, a phenolic lignin showing anti-aggregation as well as AChE inhibitor properties similar to the marketed drugs [41]. One group has demonstrated that certain pyridopyrimidine derivatives have higher AChE inhibitory activity than the drug galantamine.

AChE active site offers hydrophobic and anionic interaction sites for ligand binding. The active site contains a highly conserved catalytic triad (S200, E327 and H440) and a PAS site. Another remarkable feature near catalytic triad is active-site gorge made up of mostly aromatic residues side chain and few acidic residues, which include D285 and E273 at the top, D72, hydrogen-bonded to Y334, in middle and E199, near the base. Virtual screening and docking will be performed in these regions to identify the new lead and binding mode of the inhibitor with the apo structure of human AChE 1B41. Receptor flexibility is very important in inhibitor design. The AChE also showed conformation flexibility. Interaction study of the anti-Alzheimer drug, rivastigmine and huperzine with AChE reported the movement of acyl pocket [42] and rearrangement of active site residues [43]. The residue W279 at PAS site reported to adopt several alternative conformations. These ligand-induced conformation change studied through molecular dynamics simulation may help in explored binding and inhibitory mechanism.

3. ANTI-AMYLOID INHIBITORS

Amyloid hypothesis is the most explored hypothesis after acetylcholinesterase (AChE) inhibition in which Aβ aggregation and deposition are considered as the main cause of AD [44, 45]. Aβ leads to different pathways like oxidative stress, inflammation, neural injury and ultimately neural cell death [45]. Thus, targeting Aβ generation, deposition and focusing on anti-aggregating small molecule for the treatment of AD seem rational approach. In this regard, computational approaches are successfully applied to identify small molecule inhibitors against Aβ. In one of the pharmacophore modeling, NCI database screening and docking studied, identified two anti AD lead which were able to reverse amyloid aggregation and also reduced neurotoxocity [46, 47]. Vitamin K3 analogues were also reported to inhibit Aβ aggregation and reduced the free radical *in vitro*. Binding affinity and binding mode between vitamin K3 analogue and Aβ were studied by docking and MD simulation [48]. The binding affinity, binding mode of ligand with Aβ and ligand anti-aggregation Aβ mechanism of various small molecules were studied using docking and MD simulations [49-51] and suggested that flat small polyphenol molecule like morin, myricetin and flavonoid derivative binds at the amphiphilic core and disrupts the salt bridge between Asp32 and Lys28 which is responsible for Aβ stability. Similarly binding of EGCG, Ibuprofen (non steroidal anti inflammatory drug), ThCT and ThNT (β-sheet breaker), DMF (fullerene derivatives), ThT (fluorescent dye), Wgx-50 were studied through molecular dynamics simulations [52-56]. These studies concluded that various molecules bind at the various site on protofibril such as dye ThT, Wgx-50, and DMF, EGCG binds at the protofibril surface whereas, ibuprofen bind on the edge and other polyphenol and flavnoid bind inside the D23, K28, I32 and L34 [57].

Similarly, computational docking and MD simulations were used to design novel peptide inhibitors against Aβ aggregation. In this study, a novel methodology has been adopted by mutating RGTFEGKF peptide inhibitor and perform docking, MD and binding energy calculation of each

mutated peptide to identify potential inhibitor [58]. The docking and MD simulation techniques provide useful information about the binding mode and molecular insight into the destabilizing mechanism of small molecule against Aβ. Another computational method QSAR was applied on benzyloxybenzene derivatives and synthesized and evaluated as ligand towards Aβ plaques. Further suggested that binding affinities declined significantly from para-substituted ligands to ortho-substituted ones. Docking predicted the binding at the hydrophobic Val18_Phe20 channel on the flat surface of Aβ fiber and act as an efficient tracer [59]. Docking and simulation studies have been successfully applied to identify both acetylcholinesterase activity and amyloid-β aggregation inhibitors from marine metabolites [60]. Computationally designed peptide inhibitors and mutational analysis suggested that GxMxG motif is the major factor creating the compatibility between two amyloid surfaces [58]. MD simulation also played an important role in assessing the stability, fibril formation and the development of inhibitors against amyloid β-peptide [61, 62].

As Amyloid beta is a short and very flexible fragment and does not have a definite binding site, thus inhibitor design for Aβ is a difficult task. As reported that Aβ adopts a different conformation in solution one may use ensemble docking approach in the case of Aβ docking to design anti-aggregating inhibitors to predict the correct pose one should perform docking with least two reliable docking algorithm. The results from molecular docking, thus indicate that many binding poses may be possible on any given structure and that binding affinity calculations should be interpreted with care. Similar amyloid fibril pentapeptide does not have a distinct binding site, however, it has been reported that various marked binding at the upper surface through hydrophobic interaction, near amphiphilic pore or at the end of the peptide. As the clear binding site is not available for Aβ and fibril one should perform robust docking with a long run and cluster the generated conformation on the basis of RMSD and then perform molecular dynamics simulation for each representative of the cluster. Molecular dynamics simulation must perform longer time scale with replicates. A good Aβ must have hydrophobic aromatics ring that may be helpful in binding the hydrophobic surface of the Aβ protofibril. Inhibitor must be a flat and small molecule, which enters into the amphiphilic core and disrupts the salt bridge to destablize preformed Aβ protofibril. A polar group is necessary to destabilise the salt bridge between chain A and chain B. Larger hydrophobic molecules bind to the hydrophobic channel formed by the amino acid side chain at the surface of the protofibril. Binding at this site may compete with the upcoming peptide and inhibit the growth of plaque formation.

4. BACE1 INHIBITORS

BACE-1 is important disease-modifying therapeutic drug target for AD [16, 63]. In recent years, a large number of BACE-1 crystal structures and inhibitors are reported due to their importance as AD drug targets [64]. Various computational studies were carried out against BACE-1 in recent years [65-67]. In one of the states of art study, important computational methods to design BACE-1 inhibitor have been discussed [67]. Various virtual screening methods have been reported to identify the BACE-1 inhibitors from natural as well as synthetic databases [66, 68, 69]. Ligand and structure-based hybrid techniques were used for the identification of small molecule inhibitors against BACE-1 [70]. Virtual screening and docking studies have been extensively used for identifying BACE-1 inhibitors [71-76]. Various studies suggested the important role of Asp dyad protonation state, the flexibility of BACE-1, consensus scoring and principal component analysis in virtual screening against BACE-1 [77-79]. Virtual screening, in combination with pharmacophore modeling and docking study, has been used to identify BACE-1 hits [69, 80]. Similar studies have utilized the molecular docking and pharmacophore filtering, for the identification of potent inhibitors against BACE-1 with MD simulation [81]. 3D QSAR model based on topomer CoMFA was building on ninety-nine known inhibitors of BACE-1. This model suggested the key interacting residue important for binding which was further used to screen lead like molecules from ZINC database [82]. Apart from virtual screening and docking study, molecular dynamics have been widely applied to understand protein folding, perturbation and conformational changes in BACE-1 [69, 83-85]. The flexibility of BACE-1 flap and various inserts in apo form as well as an inhibitor bound state has been explored by MD simulation [69, 83]. In a similar study, CHARMM force field has been used to explore the conformational changes upon substrate/inhibitor binding [86]. Normal mode analysis has been performed for a large conformation change in transition with substrate binding domain of BACE-1 which is further implemented for inhibitor recognition [87]. In another conformational study, a Gaussian model was used to understand the effect of amino acid mutations on active binding sites of BACE-1 and further virtual screening was performed to identify novel flap up BACE-1 inhibitor [88, 89]. MD simulation and docking study of BACE-1 with complex suggested the importance of protonation state of Asp 32 and Asp 228 [17, 90, 91]. A reasonable correlation exists between the calculated ligand-binding and the experimentally determined binding affinities are obtained by using an adequate MD simulation time scale in BACE-1 inhibitors [92]. Molecular docking studies, CoMFA and CoMSIA QSAR model were used to investigate statine-based peptidomimetics inhibitory activities against BACE-1. Another study based on molecular modeling and *invitro* studies reported benzodiazepine as BACE-1 inhibitor [93]. Recently, an oral efficacious hydroxyethylamine derivative was designed against BACE-1 [94]. Both hydroxyethylamine derivatives and TAK-070 were tested in animal model [94, 95]. Both AZD3293 and MK-8931 are currently in clinical phase I and III trial, respectively [39].

5. GSK-3β INHIBITORS

GSK-3β is a serine/threonine kinase enzyme involved in type-2 diabetes mellitus, neurodegenerative diseases, cancer and chronic inflammation and considered as an important target for drug discovery. Virtual screening of NCI, Maybridge and Leadquest databases was performed to identify selective inhibitors against GSK-3β that were based on

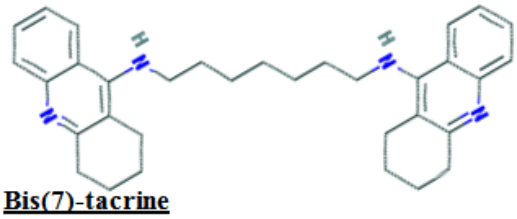
pharmacophore model build from known inhibitors using DISCO methods [96]. Two small molecules KRM-189 and KRM-191 were reported as ATP competitive inhibitors for GSK-3β with the help of virtual screening [27]. Three nanomolar compounds cimetidine, gemifloxacin and hydroxychloroquine were found to be potent inhibitors against GSK-3β in one of the QSAR and pharmacophore modeling study [97]. Docking and pharmacophore models using HypoGen algorithm was applied on twenty three structurally diverse flavonoid inhibitors to identify hits against GSK-3β from Zinc and NCI database [98]. A sequential virtual screening method combined with common feature pharmacophore model was used to discover potent micromolar GSK-3β inhibitor [99]. Structure and ligand based hybrid virtual screening identified two potent inhibitors 2-anilino-5-phenyl-1,3,4-oxadiazole and phenylmethylene hydantoin [100]. These two molecules showed good blood-brain permeability and activity in both *in vitro* and *in vivo*. In one of the study, different programmes FlexX, FlexX-Pharm and FlexE were used to screen out known GSK-3β inhibitors and inactive compounds. This study compared the screening protocol by comparative experimental and virtual high-throughput screens. Virtual screening protocol was reported as an effective tool in GSK-3β-based library focusing [101]. Fragment based method was used to develop virtual library and knowledge-based approach and the further comparative model was used to predict the activities to identify the GSK-β inhibitor [102]. This study also matched the important H-bond interaction with VAL135 and ARG141.

Recently, computational guided virtual screening, docking, molecule dynamics and binding affinity calculations were used to identify the potent inhibitors against GSK inhibitor [103]. The study identified (Z)-2-(3-chlorobenzylidene)-3,4-dihydro-N-(2-methoxyethyl)-3-oxo-2H-benzo[b][1,4]oxazine-6-carboxamide with IC_{50} value of 1.6 μM. Further dynamics simulations were performed to understand the interactions of the inhibitor with GSK-3β. In another study, energy-based pharmacophore induces fit docking, quantum polarized ligand docking and 50 ns molecular dynamics simulation was performed to identify a potent antagonist for GSK-3β [104]. Virtual screening of Zinc database and in-house database by GOLD software identified a micromolar inhibitory activity lead molecule in enzyme and cell-based assays [105]. *In silico* structure-based virtual screening and docking studies lead to the identification of 8 hit compound that showed pIC50 values ranging from 4.9 to 5.5 and also reported a novel structural class 1H-Indazole-3-carboxamides as a GSK-3β inhibitor [106]. In another ligand and structure-based virtual screening, ensemble docking and pharmacophore modeling studies identified sub-micromolar inhibitors of GSK-3β [107]. In one of the QSAR and pharmacophore study, support vector machines and random forests algorithm were used to build a QSAR model for 728 GSK-3β inhibitors with diverse structural scaffolds predicted the hit compound [108]. Various structure-based methods have been used to design GSK-3β [109] and most of the inhibitors were design for the ATP binding site of GSK-3β. However, one of the virtual screening experiment identified a novel scaffold benzothiazepinones as a non-competitive inhibitor of GSK-3β [110].

6. PARADIGM SHIFT IN AD THERAPEUTICS FROM SINGLE TARGET TO MULTI-TARGET

Currently, available treatments for AD are mainly based on the inhibition of AChE. Tacrine, donepezil (Aricept; Eisai/Pfizer), galantamine (Razadyne; Johnson & Johnson) and rivastigmine (Exelon; Novartis) are being used for the treatment of moderate to severe AD. The effects of these drugs are limited as they improve only the symptoms not the main cause of the disease. Now, it 's clear that cholinergic dysfunction may not cause cognitive impairment directly but it is involved indirectly in disease progression [7]. Another FDA approved drug is memantine which is a NMDA antagonist. As earlier evidences suggested that symptoms based drugs does not provide effective treatment thus, researchers are now in search for other disease modifying strategies for AD. For many years, AD drug research followed the one target one ligand approach for symptoms as well as disease modifying targets to treat AD. However, due to multifactorial nature of the disease, this strategy did not show promising results. Whereas, accumulating evidences suggested that one molecule hitting multiple targets provides better treatment and effective strategy to treat complex disease [111]. This approach has already been proven successful in the treatment of similar complex diseases such as cancer, HIV and hypertension, where it achieves maximum efficacy by attacking several drug targets [112]. One of the studies on neurodegenerative diseases suggested that multi target drug ligand (MTDL) is better for neurodegenerative disorder [112]. This study also suggested that MTDL design strategy represents a natural evolution and may emerge as valuable tools for hitting the multiple targets of AD. Some interesting compounds that are under investigation for the treatment of neurodegenerative diseases are curcumin and other polyphenols having anti-inflammatory and antioxidant properties. These natural molecules supported the concept of MTDLs [112-114]. Moreover, many examples such as salicylate, non-steroidal anti-inflammatory drugs (NSAIDs), metformin, antidepressants, anti-neurodegenerative agents and multi-target kinase inhibitors (such as Gleevec™) affect many targets simultaneously support that MTDLs are likely to be better than single target inhibitor. Many MTDLs agents against AD have been reported so far however, most of them were discovered by combining two chemical moieties into single molecules *via* a linker or some of the agents were discovered by chance and/or yielded by approaches relying on high-throughput screening (HTS) against a panel of selected molecular targets. Whereas, rational design of ligands with a predefined multitarget profile is very difficult and a new challenges for medicinal chemists. It is not easy to adopt a structure-based drug design approach, in which ligands are designed to display balanced activities towards the targets of interest, while simultaneously achieving a wider selectivity and a suitable pharmacokinetic profile. Thus, computational tools might be helpful in designing better MTDLs in combination with already existing knowledge of medicinal chemistry.

The multifactorial nature of AD gives the opportunity to target many possible therapeutic targets. Current single target treatments focus mainly on acetylcholinesterase (AChE) inhibition due to the early cholinergic hypothesis. In case of MTDLs design, acetylcholinesterase is well explored in



Bis(7)-tacrine

AChE IC_{50}=0.40 nM,

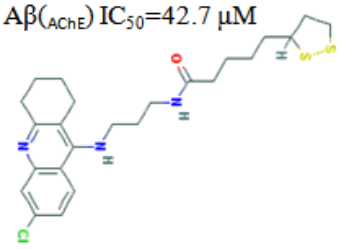
BuChE IC_{50}= 390 nM

NMDA blockade IC_{50}= 0.76μM

GABAA blockade IC_{50}=5.6 μM

BACE-1 IC_{50}=7.5μM

$A\beta_{(AChE)}$ IC_{50}=42.7 μM



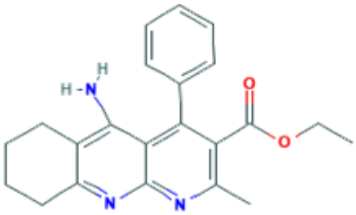
Lipocrine

AChE IC_{50}=0.25 nM,

BuChE IC_{50}= 10.8 nM

$A\beta_{(AChE\ agg)}$ IC_{50} =45 μM

% inh ROS 51% at 10 μM



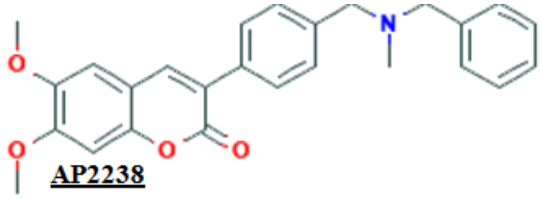
ITH4012

AChE IC_{50}=0.82 μM,

BuChE IC_{50}= 5.0 μM

VDCC blockade 20.4% at 3 μM

Ca2+ promoting act at 10 nM

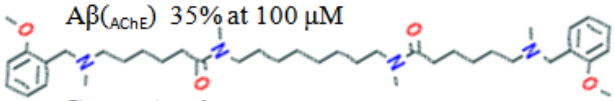


AP2238

AChE IC_{50}=8.8 nM,

BuChE IC_{50}= 81.5 nM

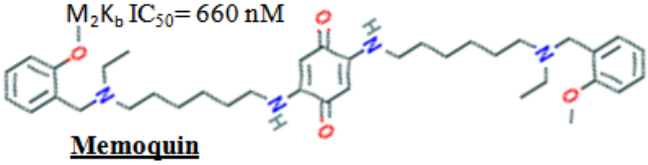
$A\beta_{(AChE)}$ 35% at 100 μM



Caproctamine

AChE IC_{50}=0.17 μM,

M_2K_b IC_{50}= 660 nM



Memoquin

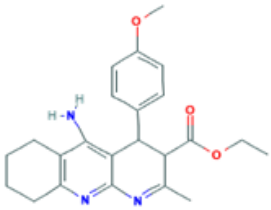
AChE IC_{50}=1.55 nM,

BACE-1 IC_{50}= 108 nM

NQO1 Km= 12.7μM

$A\beta_{(AChE)}$ IC_{50}=28.3 μM

$A\beta_{(self\ agg.)}$ IC_{50}=5.93 μM



Tacripyrine

AChE IC_{50}=45 nM,

BuChE IC_{50} >100 μM

VDCC blockade 32.7 % at 0.3 μM

$A\beta_{(AChE)}$ 30% at 100 μM

$A\beta_{(self\ agg.)}$ 35% at 50 μM

Fig. (2). Multi-target-directed ligands for key target of AD.

combination with other drug targets. First dual inhibitor for AChE based on the hypothesis that interaction of Aβ at the PAS site of AChE catalyze some conformational changes in Aβ which promote the β-sheet formation and accelerate aggregation [115]. So donepezil derived inhibitor design to interact with the catalytic site and PAS site of AChE. This idea leads to a new area of research in which know inhibitors for targets are combined with different chemical moiety heterodimer or combine with same moiety homodimer. Bis(7)tacrine is first reported homodimer which inhibits AChE and BuChE. Later years, scientist discovers that Bis(7) tacrine also interact with PAS site and Aβ moreover, also reported to inhibit BACE-1 [116]. Molecular modelling and docking studies were used to design inhibitor AP2238 that binds at both the catalytic and the peripheral sites of the human enzyme AChE and also have Aβ antiaggregating properties [117]. These inhibitors AP2238 contain a benzylamino and coumarin moiety.

Apart from AChE- Aβ inhibitors, many researcher design molecules like lipocrine which also deal with oxidative stress that is an important aspect of AD [118]. Lipocrine was the first compound that also inhibits the AChE and AChE-induced amyloid-β aggregation and protects against reactive oxygen species (ROS) [118]. To deal with ROS, two moiety tacrine and melatonin were combined. This heterodimer was reported to have antioxidant properties with anti AChE and BuChE activity [119]. Their derivatives were further evaluated against toxic Aβ. In one of the MTDL study, a new hybrid compound was introduced based on lipocrine and memoquin against AD by combining benzoquinone fragment and a lipoyl function which have potent selective and inhibi-

tory activity towards AChE and reduce Aβ self-aggregation and also decrease in reactive oxygen species (ROS) production [120]. To deal with oxidative stress, researchers try to inhibit monoamino oxidase (MAO) which release ROS during catalytic deamination of neurotransmission. Inhibition of MAO reduces ROS and decreases oxidative stress in AD patient. Thus, few chemical classes such as ladostigil [121], propargylamine (ASS234) moieties [122] have been reported to inhibit both MAO and AChE. Ladostigil is currently in phaseII clinical trials. Few compounds are also reported which inhibit calcium channel and AChE as earlier research have shown that Ca^{2+} dysfunction is involved in the pathogenesis of AD. ITH4012 was potent AChE inhibitor and block the Ca^{2+} influx. Tacripyrine is another compound which inhibits Ca^{2+} channel, AChE, BuChE Aβ aggregation and it also crosses the blood brain barrier (BBB).

BACE-1 is another important target in AD drug discovery but very few candidates are under clinical trials. BACE-1 drug discovery is very challenging because of its large active site and flexible nature. Larger molecules always cross BBB that made it further difficult to design lead. However, few multi target BACE-1 inhibitor are reported in combination with AChE, Aβ,α-secretase and GSK-3β. Bis(7) –tacrine was found to a moderate activator of α-secretase and selective inhibitor of BACE-1. Both the enzyme BACE-1 and AChE are involved in Aβ generation and aggregation process so designing dual inhibitor for these enzyme seems a good approach. However, only few structures are reported as dual inhibitor of AChE and BACE-1. First dual inhibitor designed by combining the AChE dual inhibitor AP2238 with BACE-1 inhibitory dihalophenyl acid moiety and docking study suggested that ligand bind into S1 and S1' pocket of BACE-1 [123]. Similarly, fragment based strategies were used to design dual inhibitor for BACE-1 and AChE by merging isophthalamide from BACE-1 inhibitor GRL-8234 and donepezil into a single molecule and used different docking strategies by splitting the molecule into two and each part docked into the respective target to know the binding mode of dual inhibitor [124]. Quinoxaline-based hybrid compounds [125] and coumarin derivatives [123] also reported to inhibit AChE and BACE-1. Further, new derivatives of memoquin were design to inhibit AChE, BACE-, Aβ self aggregation and also deal with oxidative stress [126]. BACE-1 combinations with metal chelator have been also reported. In this study, a database consisted of 1,3-diphenylurea derivatives was built by combine LR-90 with BACE-1 inhibitor compounds and screened by the pharmacophore model of BACE-1 [127].

Another hallmark pathology is the formation of NFTs, caused by hyperphosphorylation of the tau protein, for which several protein kinases are discussed to play major roles, including GSK-3β, PKA, CDK5, and Dyrk1A. GSK -3β is most widely explored kinase drug target in AD as well as in cancer research. Recently, first dual inhibitor of BACE-1 and GSK-3β were reported [128]. They have synthesized and perform SAR of trizinone (by combining two motif a guanidino and cyclic amide) derivatives and evaluated 34 compounds against BACE-1 and GSK-3β also showed neuroprotection. Further, curcumin scaffold also reported to dual inhibitory activity against BACE-1 and GSK-3β [113]. Roscovitine has finished IIb clinical trial against nonsmall-cell lung cancer completed which also show the interaction with Dyrk1A, CK1, pyridoxal kinase. Recently bis(hydroxyphenyl)-substituted thiophenes as a novel class of selective, dual inhibitors of the tau kinase Dyrk1A and of the Aβ aggregation has been reported. Non ATP competitive inhibitor of GSK-3β has been also reported as PPARγ agonist and showed anti-inflammatory and neuroprotective properties. This thiadiazolidindinones has finished a safety study with NP031112 for AD treatment. One of the tri substituted purine reported to inhibit CK1, CDK5, CDK1 and GSK-3β.

7. LIGAND BASED DESIGN OF MTDLS AGAINST AD

MTDLs are now proven as better strategies than single target in AD. There are two different methods viz., serendipitous screening in which ligand known for one target are screened against other targets and a rational approach also referred as "framework approach" [129]. In the framework approach, two frameworks are combined into a single chemical entity as discussed earlier. The major drawback is that most of these molecule are bulky with very poor ADME/T properties [130]. This problem may overcome if a chemical skeleton alone is known to bind various targets. One such scaffold is polyamine and considered as a universal template. Polyamine skeleton has flexibility, charge and adopt various conformation, thus, able to interact with various biological macromolecule [131]. Several derivatives have been developed as a neurotransmitter receptor, neuroprotactive and anti proliferative agents. Memoquin [132] is a suitable example of MTDLs in AD and is designed by incorporating benzoquinone fragment into the backbone of caprocatamine [15, 133, 134]. As a lead, identification memoquin was able to target AChE, Aβ and has radical scavenging properties. Another chemical class of dual target-directed drug was alkylxanthines which inhibit the monoamine oxidase and adenosine receptors. These caffeine derivative methylxanthine block both the receptors. Various ligand based side chain modification in development of caffeine-derived MTDLs suggested that 1,3-diethyl substitution of the xanthinyl core leads to enhanced A2A antagonism [131, 135]. While 1-, 3-, and 7-trimethyl substitution is probably optimal for the design of xanthine-based reversible MAO-B inhibitors, ethyl or propyl functional groups at C-1 and C-3 are optimal for A2A antagonism [131]. Ladostigil is another multi-target drug candidate for AD as well as PD. Ladostigil was designed by combining the rasagiline MAO-B inhibitor and rivastigmine a cholinesterase inhibitor. Ladostigil also have neuroprotective propargyl moiety. Ladostigil has been shown to be involved in AChE inhibitory activity, brain selective irreversible MAO-A and B inhibition, regulation of APP processing, neurotrophic factors and protective against oxidative stress [131, 136]. Virtual ligand, screening QSAR analysis and molecular modelling were used to identify donepezil-indolyl hybrids as multipotent cholinesterase/monoamine oxidase inhibitors [137]. The 3D-QSAR analysis was carried out both to explain the binding of these compounds to the active sites of the enzymes and to predict substitutions that would increase binding. Out of 19, seven molecules showed activity against all the four enzymes [138]. Thus, *in silico* screening can be used to modify a lead compound and generate effective multi-potent inhibitors

[137, 138]. Lead optimization of two donepezil hybrid compounds shown previously to inhibit multiple target MAO-A, MAO-B, AChE, and BuChE [139]. Similarly, one research group recently reported ASS234 as a new MTDL agent [122]. This hybrid compound was able to bind to all the AChE/BuChE and MAO A/B enzymes as well as prevented β-amyloid-induced aggregation and also showed good permeability, neuroprotection and antioxidant properties.

8. STRUCTURE-BASED MTDL DESIGN FOR ALZHEIMER'S DISEASE

Structure-based drug design is based on the structural requirement for the active site of the target protein. In one of the states of art studies on multi target amyloid, cascade pharmacophore requirement for BACE-1 and AChE and amyloid fibril has been discussed [92]. Author screened and identified carabzole and indole-based MTDL candidate. Dimebon was identified as a lead molecule in computer-assisted pharmacophore search in this study. They further identified the dimebon and carbazole derivatives for MTDL. The study suggested that the hydroxyethylamine moiety provides an anchor for BACE-1 binding *via* interacting with Asp dyad, while the aromatic moieties on both ends (carbazole and substituted phenyl groups) could be a source of affinity of these compounds for AChE and Aβ peptide oligomers which are required pharmacophore for the BACE-1, AChE and Aβ [92]. In this MTDL study, authors used pharmacophore based hit identification after they performed the docking experiment with the BACE-1, AChE and docking and dynamics studies for Aβ. Similar strategies were applied to screen indole based derivatives. BACE-1, AChE and Aβ target have different binding sites, designing of MTDL against three of them are challenging task and therefore, Pharamcophore, docking and MD have been successfully applied to identify MTDLs.

9. ROLE COMPUTATIONAL METHODS IN MTDLS DESIGN AGAINST AD AND FUTURE PROSPECTS

Virtual screening, quantitative structure-activity relationships, molecular modeling approaches, machine learning, data mining, and molecular simulations are useful in drug discovery and optimization of new leads with enhanced affinity to a drug target. Recently, Triazin moiety was identified as a first dual inhibitor against BACE-1 and GSK-3β, after that curcumin was also reported as a dual inhibitor for both the targets [113, 128]. Both these target are disease modifying drug target and directly link with the two major pathologies of AD, amyloid and tau.

Various chemical motifs have privileged to interact with more than one target. In the AD MTDL drug discovery tacrine, donepezil, xantostigmine, benzofuran, polycyclic, dibenzothiadiazepine, dihydropyridine *etc*. were reported to have multiple activities. However, a large number of class and dual or multiple inhibitors are designed to target AChE, symptoms based target compare to disease modifying targets. Fewer chemical moieties are reported to disease modifying targets. We need more chemical moieties which hit multiple disease modifying targets with anti-oxidant, antiinflammotry and/or metal chelating properties to add extra advantage. However, the rational drug design is a tedious job because for multiple target active sites requirement is different and lead optimization will require great physical or mental effort. Moreover, most of the lead molecules are failing into clinical trials and success rate is even lower for CNS drug discovery. MTDL is still in its initial stage and most of the molecules are designed by combine two chemical moieties into a single molecule with the linker molecule. Thus, most of the dual or multi target inhibitors are designed for already existing and well explored AD target such as AChE and Aβ.

Single target drug discovery is very costly and time consuming so one can imagine the time and cost that will be required to design dual or multiple inhibitors with optimal activity and desired ADME/T. As insilico methods are widely used and well established in single target drug discovery, results are promising in MTDL lead discovery. Computational methods increase the probability of finding dual or multiple inhibitors. Such as virtual screening important tool in single target drug discovery may be applied to dual inhibitor design in which screening of multiple targets was performed to identify the top lead from both the target and further rank them on the basis of docking score. However, virtual screenings of the large database are computationally expensive for the screening of multiple targets. Then, if the large number and chemical diverse molecule are known for both the target then one should calculate the chemical space from physicochemical properties for both the inhibitor then try to screen database that is relevant for both the target eg. In the case of BACE-1 and GSK-3β, we have calculated the chemical space from known inhibitor and we found that for most of the properties ranges were overlap from BACE-1 and GSK-3β. In this way one can filter the molecule and may generate focused library from large database and screen desired chemical space only which reduce time and computational cost.

Another important strategy in drug discovery is drug repurposing, re-profiling, re-tasking or therapeutic switching to deal with time and cost. In drug repurposing, we screen agents that are already approved and their detailed information is available like pharmacology, formulation and potential toxicity. This lead can directly go into clinical trials that speed up the drug discovery as BBB is a major hurdle in CNS drug discovery. In drug repurposing, all the compound were known to cross BBB screening for AD drug target. For identifying initial dual or multiple lead, one may screen known inhibitors of one target to another target and vice a versa. The same principle may be used for designing dual or multiple inhibitors. For example, one has to design dual inhibitor for one classical drug target Aβ in combination with disease modifying target BACE-1. We can screen all the anti aggregating compound against BACE-1 active site using virtual screening and select on the basis of docking score. Further, we may use robust docking and manual inspect of top lead which interacts with flap and Asp dyad of BACE-1. The selected molecule can further be studied with molecular dynamics and binding free energy analysis and atlast, for *in vitro* validation. Virtual screening for dual inhibitors from the large database may also be performed by SVM. In one of the states of art study, they build individual SVM model for each kinase target and train each model with known single-

target inhibitors and further tested with the known dual inhibitor for respective target. For a different pair of kinase, retrieval rate is different and marginal satisfactory. So we think of a new kind of target-specific descriptor that can deal and provide better results for multi or dual inhibitors prediction.

Docking and pharmacophore searches are efficient tools for designing dual inhibitor design. For dual inhibitor screening, Pharmacophore approach can be applied *via* superimposition/ alignment of two pharmacophores into one or screen of the database with the first pharmacophore, than filter compound should further screen for another target pharmacophore. Molecule screen from both the pharmacophore can be filtered out as a potential dual inhibitor and further checked with docking and molecular dynamics simulation. Docking of the top lead may be performed with great care and run and a number of conformation generation must be large enough to cover sample space and after conformation, generation clustering must perform to get the idea about binding mode. After that each cluster representative must be taken and MD simulation and binding free energy performed to calculate the biding affinity with the target.

These computational methods and knowledge of multi-target privileged structure might be useful for AD drug discovery. Recently, various natural products and recent ligands development of potential multifunctional agents are reported to act on central nervous system [140, 141]. Various chemical moiety and hybrid compound reported to have multiple properties. The earlier reports combine with *in silico* methods may be applied to identify various targets for a multifunctional agent, atomistic detail of binding mechanism of MTDLs, the discovery of new lead for an unexplored combination of drug targets, activity prediction and lead optimization. However, focused may be given to more disease-modifying target compare to symptoms based target. One screen already approveds failure drug for potential dual or multi target inhibitor for AD.

CONCLUSION

AD is related to Aβ, hyperphosphorylated tau protein, oxidative stress, metal ion deregulation, inflammation and other disease conditions. Most of the validated drug target come under symptoms and disease-modifying based categories. Dual or multi-target inhibitors may provide better treatment for a multifactorial complex disease like AD. MTDL approach may provide a better solution for AD-like multifactorial complex disorders.

Computational methods are used for screening and identification of new chemical class that is potent inhibitor and also can act as a lead molecule for AD drug discovery process. These methods can also predict the binding pose of the ligand into the active site and also provide structural insights of the binding site to guide the development of new potent molecules. QSAR models are used for the prediction of new chemical entities, while MD simulation provides atomistic details and binding mechanism with ligand-induced conformational changes in drug target. Prediction models are also used to optimize the ADME/T properties of important ligands in CNS drug discovery. All these *in silico* methods identify lead molecules that can be validated through experiments. Thus, *in silico* methods have been applied to identify effective drugs for the classical as well as disease-modifying drug targets of AD. Dual or multiple inhibitors that can inhibit two or more targets of AD may also be investigated through virtual screening, docking, and MD simulation methods. *In silico* methods have a clear application in filtering compound databases, predicting the physicochemical profiles, structure-activity relationship, designing of synthetic and natural analogues, and optimizing lead for better ADME/T profiling in AD drug discovery. Currently, available drugs for AD are only symptoms based as they do not address the root cause of disease. Therefore, current AD drug discovery shifted towards disease-modifying drug targets.

AD is a multifactorial disease as various pathways are involved in the disease progression. Therefore, current AD drug discovery is focused on drugs that can act on the root cause of disease like Aβ generation and aggregation, Aβ clearance, tau hyperphosphoryaltion and tangle formation *etc*. Various MTDLs have been designed but most of them are based on a framework or known inhibitor fragments. Owing to various limitations, the numbers of lead MTDLs in clinical trials are less in number. Computational methods may speed up AD drug discovery process due to recent advancement in *in silico* techniques that include molecular docking, pharmacophore, QSAR, MD simulations, binding free energy and linear interaction energy. Docking and MD simulation studies can identify the binding conformation and binding free energy. All these techniques are essential in MTDLs discovery because of less time consumption and cost-effective drug discovery. Pharmacophore model for more than one drug targets depicts the structural requirement for the dual inhibitors. While multi QSAR model predicts MTDLs activity for multiple targets.

The major challenge in AD till now is to understand the disease mechanism and pathways involved. Understanding disease condition can guide us to design better therapeutic treatments. In this context, MTDLs drug discovery that target symptoms, as well as other novel targets, constitute a promising strategy against AD. Computational methods and tools have the potential to fulfill MTDLs drug designing challenges with selected target activity and less off-target side effect. Conclusively, various novel virtual screening, docking, pharmacophore methods and polypharmacology algorithms need to be developed for reducing the time for multiple inhibitor screening.

CONSENT FOR PUBLICATION

Not applicable.

CONFLICT OF INTEREST

Financial contributions and any potential conflict of interest must be clearly acknowledged under the heading 'Conflict of Interest'. Authors must list the source(s) of funding for the study. This should be done by each author.

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