RESEARCH ARTICLE

BENTHAM
SCIENCE ***In silico* Structure-based Identification of Novel Acetylcholinesterase Inhibitors Against Alzheimer's Disease**

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Abstract: Objective and Background: Inhibition of acetylcholinesterase (AChE) has gained much importance since the discovery of the involvement of peripheral anionic site as an allosteric regulator of AChE. Characterized by the formation of β -amyloid plaques, Alzheimer's disease (AD) is currently one of the leading causes of death across the world. Progression in this neurodegenerative disorder causes deficit in the cholinergic activity that leads towards cognitive decline. Therapeutic interventions in AD are largely focused upon AChE inhibitors designed essentially to prevent the loss of cholinergic function. The multifactorial AD pathology calls for Multitarget-directed ligands (MTDLs) to follow up on various components of the disease. Considering this approach, other related AD targets were also selected. Structure-based virtual screening was relied upon for the identification of lead compounds with anti-AD effect.

Method: Several chemoinformatics approaches were used in this study, reporting four multi-target inhibitors: MCULE-7149246649-0-1, MCULE-6730554226-0-4, MCULE-1176268617-0-6 and MCULE-8592892575-0-1 with high binding energies that indicate better AChE inhibitory activity. Additional *in-silico* analysis hypothesized the abundant presence of aromatic interactions to be pivotal for interaction of selected compounds to the acetyl-cholinesterase. Additionally, we presented an alternative approach to determine protein-ligand stability by calculating the Gibbs-free energy change over time. Furthermore, this allows to rank potential hits for further *in-vitro* testing.

Results and Conclusion: With no predicted indication of adverse effects on humans, this study unravels four active multi-target inhibitors against AChE with promising affinities and good ADMET profile for the potential use in AD treatment.

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1. INTRODUCTION

Alzheimer's disease (AD) is the 5th leading cause of death in Americans of age 65 years or older. According to a survey, more than 5.2 million people in US are suffering from AD. In the coming era, the baby boom generation is

projected to add approximately 10 million to the total number of people in the US with AD. It is accurate to say that someone somewhere in the US develops AD every 68 seconds. By 2015, it is expected that this will happen every 33 seconds [1]. The major signs and symptoms of AD include: dementia, confusion, memory lapses, misinterpreting spatial relationships, and decline in the ability to speak, write, think, reason, making decisions and planning. Personality and behavioral changes have also been observed including depression, anxiety, agitation, social isolation, mood swings, diurnal rhythm disturbances and delusions [2, 3]. AD is charac-

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terized by various markers in the brain including large number of amyloid plaques surrounded by neurofibrillary tangles, vascular damage from plaque deposition and neuronal cell degradation. The main component of plaques is amyloid β protein, and also the major causative factor of AD. The deposition of this notorious protein leads to the other symptoms [4-6]. Head injuries, progression of age, sequelae of delivery, ataxic fever, paralysis, mania, apoplexy, mercury abuse, wine abuse, political upheavals, unhappy love, dietary excess, masturbation, unfulfilled love, domestic problems, poverty and fears are among the causes of AD that emerged in the last century [7, 8].

Acetylcholine (ACh) is the major player as far as AD is concerned. It is synthesized by choline acetyl transferase (CAT), with the major source of CAT being the nucleus basalis of Meynert in the basal forebrain. This region of the brain is affected early in the pathophysiological phenomenon of AD. This causes, ultimately, a marked discrepancy in CAT and ACh synthesis. CAT levels are reduced 58-90% in AD, which increases the severity of dementia [9-11]. Acetylcholinesterase is the enzyme responsible for the degradation of the Acetylcholine, which is markedly reduced in AD.

Muscarinic and nicotinic cholinergic receptors are present in the brain. Five kinds of muscarinic receptors, M1-M5, have been found [12, 13]. M1 is identified in hippocampus and in the upper and lower levels of cerebral cortex. M2 is found in brainstem and nucleus basalis. M1 receptors are not markedly disturbed in AD but M2 and nicotinic receptors are profoundly decreased. Additionally, the M1 receptors can be less functional in the patients with AD. Decoupling from G-protein second messenger can happen to M1 cholinergic receptors [14, 15]. Other potential targets include: *glutamate receptors* responsible for excitotoxicity causing neurodegeneration [16], *secretase* as a target in order to inhibit APP cleavage [17], *amyloid-beta* ($A\beta$) aggregation inhibition [18], *tau* phosphorylation inhibition [18, 19], inhibition of *butyrylcholinesterase* (BChE), which catalyzes the hydrolysis of choline esters [20]; nitric oxide (NO) produced by nitric oxide synthase (NOS), the induction of which causes neurodegeneration [21], tumor necrosis factor- α (TNF- α), a proinflammatory cytokine, responsible for neuroinflammation leading to neurodegeneration [22, 23], glycogen synthase kinase 3 (GSK-3 β) involved in tangle formation through paired helical filament- τ (PHF- τ) production [24], JNK involved in hyperphosphorylation of τ [25], human carboxylesterase (hCE -1) being similar in structure to cholinesterases (ChE) [26], Cyclooxygenase (COX 1 and 2) for being involved in $A\beta$ deposition [27], inhibition of phosphodiesterase-5 (PD-5) for enhancing neuroprotection and cognitive function [28] and inhibition of angiotensin converting enzyme (ACE) for slowing down the progression of disease [29].

Complexities of AD also include reduction in the non-cholinergic neurotransmitters and neuromodulators. Serotonin is decreased by 50-70 %, gamma-aminobutyric acid (GABA) by 50%, somatostatin by 40-60 and norepinephrine by 30-70 % [30-32]. Receptors for serotonin, glutamate and somatostatin are also lessened in number in AD [33-35].

Familial AD involves mutations in the chromosome number 21, 14 and 1 [36, 37]. These mutations are inherited

in an autosomal dominant mode of transmission. When such mutations are there, it can cause familial early onset of AD. In families with such mutations, there is variability in the age of onset and in phenotypic heterogeneity. Such differences in family members having identical mutations explain that environmental factors change and modify both the symptom and timing profile of disease representation [38, 39]. Reduction in the AD frequency can occur by encouraging awareness and environmental enrichment strategies in the childhood and by reducing the likelihood of head injury through safe use of seat belts, protective helmets and better head protection in contact sports.

Nowadays, two types of therapeutical approaches are used in AD. One is to increase the cholinergic function, and the second is to provide neuroprotection using anti-oxidants. Moderate enrichment of cognitive functions have been observed by the administration of Acetylcholinesterase inhibitors (donepezil, galantamine, rivastigmine and tacrine), with varying duration of action, provide physicians with a wide range of cholinergic therapy options [40-44]. Apart from it, acetyl cholinesterase inhibitors and cholinergic receptor agonists lessen the behavioral abnormalities accompanied by AD [45, 46]. The mechanism by which cholinesterase inhibitors improve cognitive function is through an increased residual cholinergic activity. Furthermore, cholinergic disturbance may contribute to abnormal APP processing and cholinergic therapies may modulate this effect [47]. Neuroprotective agents also show efficacious results in AD. Alpha-tocopherol or selegiline, a MAO inhibitor, slows the progression of AD. Both of these agents do not improve the existing symptoms [48]. Inflammatory pathways are activated in the neuritic plaques of AD with NSAIDs and steroids as available treatments [49]. Cox-II inhibitors are under investigation. Other neuroprotective agents such as MAO inhibitors and antioxidants have protective and modulating roles in improving the disease state. It is well settled that calcium influx is also a major factor in causing AD, hence, Calcium Channel blockers (CCBs) and Calcium Channel modulators are also beneficial but yet to be illustrated as far as their efficacy is concerned [50, 51].

Studies have also shown that the role of N-methyl-D-aspartate (NMDA) receptors blockade can be helpful in AD [52-56]. Memantine is currently administered in this respect with its believed principal mechanism of action the blockade of ion current flow through NMDA channels [57]. These NMDA channels are a subfamily of glutamate receptors involved in brain function. Other drugs, that block the NMDA receptors like ketamine, exhibit potential deleterious actions. Such differences in these drugs include effects on gating of blockade channels and binding of memantine to two sites on NMDA receptors [57]. Moreover, psychotropic agents, including antipsychotics, antidepressants, anxiolytics, sedative hypnotics and anti-agitation drugs can be employed for the behavioral disturbances that occur in many AD patients [58]. Currently FDA has approved drugs targeting acetylcholinesterase inhibitors and NMDA receptor antagonists. This study demonstrates structure-based virtual screening (SBVS) to reveal novel inhibitors against AD.

Over the recent years, virtual screening proved to be a successful strategy as part of the drug discovery process. *In*

silico approaches have paved the way to solve many biological problems [59-64], which have led to the identification of novel inhibitors against neurological disorders [65]. The process has made it possible to look for compounds with specific key features in order to obtain potential leads. By screening through the Lipinski's rule of five [66], assessment of a drugs' pharmacological activity is possible. Through the process of molecular docking, the best possible orientations forming stable ligand protein complexes are achieved. Based on this principle, the goal of the current study was to virtually screen and unravel potential inhibitors of acetylcholinesterase. The selected compounds screened from ZINC databank, were filtered by Lipinski's rule of five followed by further filtration based on pharmacokinetic parameters. The compounds were additionally assessed for their ability to penetrate blood-brain barrier (BBB), cell permeability of Caco2, human intestinal absorption (HIA) and plasma protein binding. Potentially pharmacologically active leads were docked and hit compounds were assessed for absorption, distribution, metabolism, excretion and toxicity (ADMET) analysis. The results allow the interpretation of the efficacy of four screened compounds as inhibitors of AChE, thus suggesting their usefulness in the treatment of AD, after which the compounds with best drug-like properties can be synthesized.

2. MATERIALS AND METHOD

2.1. Structure-based Virtual Screening

SBVS was utilized to screen substantial databases in quest of finding novel and potential lead compounds. They represented essential data with respect to structural features similar to the search query. In this work, SBVS was applied to Mcule drug discovery platform [67] that consists of approximately 35 million compounds, employing the 'Structure-based virtual screening' module. The top compounds were chosen based on favorable binding energies and were subjected to visual inspection *via* UCSF Chimera for ligand bound conformation with a reference co-crystallized structure of each complex.

2.2. Preparation of Dataset

The coordinates for AChE were retrieved from the protein data bank (PDB ID: 4BDT, resolution [Å]: 3.1, *R*-Value: 0.159) [68]. To find out the binding potential of screened hits on other related AD targets, resolved crystal structures *i.e.* BChE, TACE, hACE, PDE-5, hCE, GSK-3, BACE-1, hPreP and MAO with potential inhibitors with the respective codes 4BDS [69], 2DDF [70], 3BKL [71], 1TBF [72], 1MX1 [73], 1Q5K [74], 2VIZ [75], 4RPU [76], and 4CRT [77] were analyzed for their relevant role in binding. Binding residues lining the binding pocket of AChE and other targets were further analyzed using Q-site finder [78] which uses the interaction energy to locate between protein under study and a van der Waals probe, to help predict the favorable binding sites energetically. Another tool for binding site analysis, Pocket Finder [79] was used which employs Ligsite algorithm which is intended to search probable target-ligand interactions, heuristically and creates the profiles of best binding sites.

2.2.1. Drug Likeness Filtration

Drugs representing poor pharmacokinetic properties fail to progress during the process of drug development. In order to obtain a best lead compound, the hits obtained from ZINC database were further filtered through Lipinski's Rule of Five (Ro5) [66] for their oral bioavailability and pharmacokinetics parameters to check whether the compounds are able to cross the blood-brain barrier (BBB), have low toxicity with good solubility and human intestinal absorption (HIA). The compounds that fulfilled the aforementioned properties were selected for molecular docking studies.

2.2.2. Pre-docking Procedure

Hydrogens and Gasteiger charges were added with a specific end goal to set up the chosen compounds for docking. Energy optimization methods were adopted and solvent molecules were eliminated from protein structure. This step was followed by energy minimization using UCSF Chimera 1.9 [80] to exclude bad steric clashes. Using AutoGrid program, a grid map with dimensions 30 Å × 30 Å × 30 Å was generated which covered the binding pocket of AChE. AutoDockVina [81] was utilized to perform molecular docking using a standard autodock protocol. AutoDockVina was installed on a DELL Inspiron Core i7 workstation which had Ubuntu 12 as an operating system. In depth analysis of the protein complexes of all the ligands was performed using UCSF Chimera 1.9 [80], Discovery Studio 4.0 [82] and Py-MOL [83].

2.3. Molecular Docking

Molecular docking is used to generate a score for every compound considering the interaction between a target protein and the ligand. AutoDockVina [81] was employed to study the molecular interactions on the basis of binding affinity and number of hydrogen and hydrophobic interactions. AutoDockVina uses Monte-Carlo algorithm and using a sophisticated gradient optimization method, it permits flexibility in docking, is speedier and significantly enhances the average accuracy of binding mode predictions compared to other docking programs. The hits from virtual screening and through the aforementioned filters, were docked in the binding site of AChE to locate the best binding affinity and conformation. Amid the docking process, the docking score was calculated and the docked poses were analyzed by UCSF Chimera 1.9 [80].

2.4. In Silico ADMET Assessment

Top scoring compounds were assessed *in silico* for their ADMET (Absorption, Distribution, Metabolism, Excretion and Toxicity) properties. Compounds violating Lipinski Ro5 were eliminated using Mcule [67] and their physiochemical properties were calculated using the server, Molinspiration [84]. In order to find the possible adverse effects of these compounds in humans, the ADMET properties of the filtered compounds were found using the OSIRIS property explorer [85] which is designed to highlight the undesired effects if posed by the compound being analyzed, AdmetSAR [86] for QSAR-based ADMET properties prediction, ADMET prediction suite by ACD/Labs [87] for rapid profiling and screening of compounds utilizing ADME and toxicity pa-

rameters and Molsoft [88] for calculation of drug-likeness properties.

2.5. Post-docking Analysis Methods and Materials

MD simulations of 20 ns were performed to check the protein backbone and ligand conformational stability of the top ranked compounds in complex with AChE using AMBER 16.0 software package [89]. All calculations were performed on a Linux-based operating system. Counter ions and solvent were added using the Amber ff14SB force field [90]. The build-in antechamber method was used with the AMBER force field (GAFF) to generate force field parameters for all selected compounds. The tleap module was used to produce all essential parameters by considering residues at their default protonation states at a neutral pH value. Prior to energy minimization, all complexes were neutralized by adding Na⁺ ions around the complex at different locations and centered in a dodecahedral simulation box with a 12 Å distance from the edge of the box, and solvated using TIP3P water molecules. Particle mesh Ewald method with a non-bounded cut-off of 10 Å was used to set the periodic boundary conditions. Integration steps of 2 fs were set and the SHAKE algorithm was used to constrain X-H bond lengths involving hydrogen bonds. The solvated and neutralized system was subjected to energetic minimization using 5000 steps of steepest descent method followed by conjugate gradient method. Hereafter, the system was gradually heated from 0 to 300 K with 50 ps and maintained at 300 K with restraints on the complex. Solvent density was reached after 500 ps after which the system was equilibrated for another 500 ps. Finally, a 20 ns relaxed MD calculation was performed under isobaric and isothermal conditions (p = 1.0 atm, T = 300 K). Coordinate trajectories were collected after every 2 ps for the whole production run and CPPTRAJ modules of AMBER 16 were implied for the analysis of trajectories [91]. The root-mean-square-deviation (rmsd) was computed using the CPPTRAJ modules in AmberTools, indicating protein conformational stability. The same module was used to create respective PDB files after and before the extensive simulation, providing valuable insights into ligand movement and ligand-binding induced fit in the target. Intermolecular contacts (hydrogen bonds and non-bonded contacts) were analyzed with the Discovery Studio software. The default settings were used (3.9 Å heavy atoms distance cut-off for non-bonded contacts; 2.7 and 3.5 Å proton-acceptor and donor-acceptor distance cut-offs, respectively, with minimum 90° angles (D-H-A, H-A-AA, D-A-AA) for hydrogen bonds). The MMGBSA module [89] in AmberTools was used to calculate the Gibbs free energy before and after 20 ns simulation indicating protein-ligand affinity together with the (de)stabilizing effect of ligand binding. Van der Waals and electrostatic energy, as calculated by the MM force field, were analyzed separately to comprehend the ΔG_{bind} energy-shift. These combined insights can then be compared to future biochemical validate and rank the selected compounds.

$$\Delta G_{\text{bind}} = \Delta E_{\text{EEL}} + \Delta E_{\text{VDW}} + \Delta G_{\text{GB}} + \Delta G_{\text{SURF}} \quad (1)$$

$$\Delta G_{\text{bind}} = \Delta G_{\text{bind},20\text{ns}} + \Delta G_{\text{bind},1\text{ns}} \quad (2)$$

3. RESULTS AND DISCUSSION

The objective of the present study was to discover multi-target inhibitors with high binding energies. To evaluate the drug likeness, *in silico* virtual screening was performed on the premise of Lipinski's Ro5 and physicochemical properties. The compounds demonstrating high binding energies were chosen for further examinations. Later, these filtered compounds were subjected to pharmacokinetic filters. The ADMET profile was investigated to obtain the appropriate lead compounds. The criteria determining top compounds were high binding energies and great pharmacological properties. In the present study, the results of best four compounds have been examined through MD simulations. Further, we also predicted the free binding affinities of these compounds that have the potential to be lead inhibitors for AD treatment.

3.1. Binding Site Analysis

The binding site of AChE was carefully analyzed from co-crystallized structures of Huperzine A, Galantamine, Donepezil, dihydrotanshinone I with AChE. The analysis of co-crystallized X-ray structures of AChE with the ligands mentioned provided an understanding of the integral structural elements involved in the processing of acetylcholine (ACh). The presence of ~20 Å deep hydrophobic gorge lined by aromatic aminoacids, is the most prominent feature in the structure of AChE [92]. It was further investigated that the substantial portion of the gorge which widens at the end and penetrates halfway into the enzyme, was covered by twelve highly conserved residues that were interacting with inhibitors included: Trp86, Gly120, Gly121, Gly122, Tyr124, Tyr133, Glu202, Ser203, Ala204, Trp286, Phe295, Tyr337, Phe338 and Tyr341. These binding site residues were further validated by Q-site finder and Pocket finder. The active site comprises residues Ser203, His447, and Glu334 forming a catalytic triad inside the deep gorge. The inhibitory compounds form a covalent bond to this active site's serine (Ser203) in order to inhibit AChE's function [92]. The peripheral anionic site (PAS) containing residues Tyr72, Tyr124, Trp286, Tyr341 and Asp74, is situated at the lip of the gorge, providing a binding site to allosteric modulators and inhibitors. The indole group of tryptophan is flanked by the aromatic rings of these tyrosine residues, which provide a site of interaction with the charged group of ligands [93]. PAS involved in the allosteric regulation of AChE, assists in ACh catalysis [94]. The interaction between quaternary ligands and AChE is characterized as the cation- π interaction. The π electrons of aromatic residues, particularly Trp86 and Phe337, and the quaternary nitrogen of the ligands interact and, are considered to be involved in recognizing substrate through dipole-dipole interactions [92]. Furthermore, the π - π stacking between the aromatic residues of AChE and those of the inhibitors, along with interactions between anionic Asp72 and protonated nitrogen of the ligands, together, assists in ligand binding [95]. A unique feature about the structure of the inhibitors analyzed, is their inability to cause hindrance or blockage of the entrance of active site gorge, which thus provides efficient binding to not only the active site but peripheral anionic site (PAS) as well [96]. However, ligands larger in size like donepezil are seen to reach out to the mouth of the hydrophobic gorge, causing it to open [95].

Among the residues that line the active site in AChE and BChE, a few differences have been observed. In BChE, Gln119 is positioned at the same site as that of Tyr123 in AChE. At the position where Ser125 in AChE exists, in BChE, Thr120 is present and Met437 in BChE exists at approximately the same position where Pro446 in AChE is positioned. Important binding site residues for other targets include: TACE (Thr347), ACE (Gln281, Thr282, Glu376, His383, Asp415, Tyr520, Tyr523, Gln281), hCE-1 (Gly1143, Ser1221), GSK-3 β (Asp200, Pro136), BACE-1 (Thr133), HPP (Ser347, Ser453), and MAO-B (Ser590).

3.2. Structure-based Virtual Screening

Virtual screening of small compound databases may assist the robust screening for novel and the most potent lead compounds with good pharmacological properties. Virtual screening based methods can easily identify the preferred compounds from commercially available resources for biological testing as compared with other *de novo* drug design methods. Structure-based drug design (SBDD) provides information about ligand-target interaction and therefore allows the formulation of highly specific ligand structures. SBVS is used efficiently to find the most potent and novel lead compounds having good pharmacological properties for further development from small compound databases. In the current systematic study, Mcule [67] database containing millions of purchasable compounds was selected for SBVS against AChE. Considering high binding affinity and the best possible orientation with respect to the target molecule under

study, a number of candidate compounds can be virtually screened through molecular docking. The compounds chosen represent strong binding interaction with the active site of target protein. As a result, 10,997 compounds were obtained that can fit into the AChE binding site efficiently which were subjected to drug-likeness filters. The redundancy in the small molecules database was reduced by removing similar structures for the analysis of variable structures with slight differences. The compounds with similar structures were rejected for acquiring new scaffolds.

3.3. Drug-likeness, BBB and HIA Screening

According to Lipinski's rule of five (Ro5); a molecular mass under 500 KDa, the log P-value under five, five or less hydrogen bond donor sites and ten or less hydrogen bond acceptor sites represent orally administered drugs, anticipating their oral bioavailability [66]. 10,997 compounds were further subjected to Lipinski's Ro5 and drug safety profile which resulted in 3,472 compounds. Drug-likeness is the most important criteria to characterize novel hits by screening large chemical libraries. Compounds violating more than one of these parameters may have issues with bioavailability; therefore, such compounds were eliminated from the study. The filtered hits were further submitted to *in silico* application of pharmacokinetic filters: ability to penetrate blood-brain barrier (BBB) and human intestine absorption (HIA) hence, resulting in ~800 compounds. The schematic representation of chronological virtual screening process is depicted in Fig. (1).

Fig. (1). Schematic workflow representing the screening of top four proposed virtual hits.

A total of 789 compounds were subjected to visual inspection to check for those highly superimposable with the reference co-crystallized structure (PDB ID: 4BDT). This led to the process of filtering out the best candidates that bind AChE and inhibit its activity. Total of 123 compounds, highly superimposable with the lowest RMSD (Root-Mean-Square-Deviation), were subjected to PAINS filters. These filters removed the toxicophores resulted in 77 compounds. The ADMET (absorption distribution metabolism elimination toxicity) prediction of these compounds was carried out and the reported activities of these compounds were checked. On the basis of promising ADMET profile criteria, 4 best compounds were obtained. These compounds were also docked with each the rest nine targets to check the multitargeted potential against AD.

3.4. Molecular Docking Studies

It is through molecular docking that a number of candidate compounds can be screened virtually, considering high binding affinity and the best possible orientation with respect to target molecule under study. It likewise permits one to choose compounds with strong binding interaction with the active site of target protein. Molecular docking simulation was performed by using AutoDock Vina.

All top four compounds (cmp): cmp 1 (MCULE-7149246649-0-1), cmp 2 (MCULE-6730554226-0-4), cmp 3 (MCULE-1176268617-0-6) and cmp 4 (MCULE-8592892575-0-1) possessed strong binding energies against AChE (-11.8, -11.3, -10.9 and -10.9 kcal/mol respectively) and docked perfectly inside the deep gorge of enzyme as analyzed from the binding mode of inhibitors in co-crystallized structures of AChE. The efficiency of ligand-protein complex can be quantified by calculating binding energy of the ligand and its half-life. With an increase in the value of binding energy, the rate of dissociation slows down. For weaker interactions, the rate of dissociation is rather fast [97]. This proposes, that the drugs with strong binding energies take longer time to dissociate and thus, have greater

half-life. Interestingly, all four compounds showed strong binding energy than tacrine with promising ADMET properties as well. Further post docking analysis was performed by investigating the particular pose of compound inside the gorge, number of hydrogen bonds and hydrophobic interactions. The representation of molecular structures of four lead compounds is shown in Fig. (2). All four hits against AChE observed through molecular docking interactions inferred hydrophobic and hydrophilic interactions (Table 1).

To further analyze the binding potential of these specific hits, related AD targets were allowed to dock with these four compounds (Table 1). Butyrylcholinesterase (BuChE) has a role in amyloid-beta aggregation [98]. Three of these four compounds indicated binding energies greater than -10 kcal/mol with BChE: compound 1-4 (-10.2, -11.3, -9.0 and -9.6 kcal/mol respectively). A proinflammatory cytokine, tumor necrosis factor (TNF)-alpha, is upregulated in AD. The TNF α -converting enzyme (TACE) inhibition is thus, of significant importance in the AD brain [99]. Binding energies observed for compounds 1-4 were -8.4, -9.0, -6.6 and -8.2 kcal/mol respectively. Glycogen synthase kinase (GSK-3) is a tau kinase that induces hyperphosphorylation event modifying tau protein and leading to the formation of neurofibrillary tangles. Furthermore, GSK-3 has a role in the formation of amyloid-beta by enhancing β -secretase activity. Thus, it has been proposed that glycogen synthase kinase (GSK)-3 are linked with the formation of plaques and tangles in AD brain. The overexpression of GSK-3 has also been associated with neuronal death, tau hyperphosphorylation, and cognitive decline [100]. Thus inhibition of GSK-3 has been proposed as an effective strategy for treating AD. Compounds 1-4 indicated binding energies -9.9, -9.3, -9.2 and -8.7 kcal/mol respectively. The phosphodiesterase inhibitors enhance cAMP and cGMP signaling by reducing their degradation. Since these signaling moieties are essential for neuroprotection, these inhibitors have received much attention to treat Alzheimer's disease. PD-5 inhibitors have shown to improve memory performance in both aged animals

Fig. (2). Chemical structures of the top four compounds in 2D format.

Table 1. Important interacting residues of top four compounds with ten potential targets of AD.

Sr. No.	PDB Code	Target	Docking Results	1	2	3	4
				MCULE-7149246649-0-1	MCULE-6730554226-0-4	MCULE-1176268617-0-6	MCULE-8592892575-0-1
1	4BDT	AChE	Binding Energy	-11.8	-11.3	-10.9	-10.9
			Number of H-bonds	-	-	-	2
			Residues involved in H-bonding	-	-	-	Ser125, Ser203
			Bond length (Å)	-	-	-	3.33, 3.21
			Hydrophobic interactions	Asp74, Trp86, Gly120, Gly121, Tyr124, Ser125, Gly126, Leu130, Tyr133, Tyr337, Trp439, His447, Tyr449	Tyr72, Val73, Asp74, Trp86, Asn87, Gly121, Ser125, Gly126, Ser203, Phe297, Tyr337, Phe338, Trp439, His447, Tyr449	Trp86, Gly120, Gly121, Tyr133, Glu202, Tyr337, Trp439, Pro446, His447, Gly448, Tyr449	Trp86, Gly120, Gly121, Tyr124, Glu202, Phe297, Tyr337, Tyr341, Trp439, His447, Tyr449
2	4BDS	BuChE	Binding Energy	-10.2	-11.3	-9	-9.6
			Number of H-bonds	1	-	-	-
			Residues involved in H-bonding	Ser198	-	-	-
			Bond length (Å)	3.1	-	-	-
			Hydrophobic interactions	Asp70, Trp82, Gly116, Thr120, Glu197, Trp231, Pro285, Leu286, Pro329, His438	Ile69, Asp70, Trp82, Gly115, Gly116, Trp128, Glu197, Trp231, Pro285, Leu286, Phe329, Trp332	Asp70, Trp82, Ser198, Trp231, Pro285, Leu286, Phe329, Phe398, His438	Asp70, Trp82, Gly115, Thr120, Gly121, Leu125, Ser198, Trp231, Leu286, Phe329, Phe398, His438
3	2DDF	TACE	Binding Energy	-8.4	-9	-6.6	-8.2
			Number of H-bonds	-	1	-	-
			Residues involved in H-bonding	-	Thr347	-	-
			Bond length (Å)	-	3.14	-	-
			Hydrophobic interactions	Gly346, Thr347, Leu348, Gly349, Leu350, Leu401, Val402, His405, Glu406, Tyr436, Pro437, Ala439	Met345, Gly346, Leu348, Leu401, Val402, His405, His415, Tyr436, Pro437, Ile438, Ala439	Gly346, Leu348, Gly349, Leu350, His405, Glu406, His409, Pro437, Ala439	Val314, Gly346, Thr347, Gly349, Leu348, Leu350, Leu401, Val402, His405, Val434, Tyr436, Pro437, Ala439, Val440
4	3BKL	ACE	Binding Energy	-8.5	-8.9	-8.1	-8
			Number of H-bonds	1	2	-	3
			Residues involved in H-bonding	Glu376	Gln281, Thr282	-	His383, Asp415, Tyr520
			Bond length (Å)	2.99	3.34, 2.95	-	3.01, 2.97, 2.95
			Hydrophobic interactions	Asn277, Trp279, Thr282, His383, Asp415, Phe457, Tyr523, Phe527	Glu162, Thr166, Asn277, Trp279, Ala354, Val379, His383, Glu384, Asp453, Ly454, Tyr523, Phe527	Glu162, His353, Ala354, Val380, Phe457, Tyr523, Phe527	Thr282, His353, Ala354, Val379, Val380, Glu384, Lys454, Tyr523, Phe527
5	1TBF	PDE-5A	Binding Energy	-10.2	-9.4	-9	-9.1
			Number of H-bonds	-	-	-	-
			Residues involved in H-bonding	-	-	-	-
			Bond length (Å)	-	-	-	-

(Table 1) Contd....

Sr. No.	PDB Code	Target	Docking Results	1	2	3	4
				MCULE-7149246649-0-1	MCULE-6730554226-0-4	MCULE-1176268617-0-6	MCULE-8592892575-0-1
			Hydrophobic interactions	Tyr612, Leu725, Asp764, Leu765, Asp767, Val782, Phe786, Leu804, Met816, Glu817, Phe820	Tyr612, His613, Asp654, His657, Glu682, Leu725, Leu765, Ala767, Val782, Phe786, Phe820, Ile824	Tyr612, Leu725, Leu765, Ala767, Gln775, Val782, Phe786, Leu804, Gln817, Phe820	Tyr612, His613, Glu682, Thr723, Leu725, Asp754, Asp764, Val782, Phe786, Gln817, Phe820
6	1MX1	hCE1	Binding Energy	-11.3	-8.4	-8.7	-9.9
			Number of H-bonds	1	-	1	2
			Residues involved in H-bonding	Ser1221	-	Ser1221	Gly1143, Ser1221
			Bond length (Å)	3.26	-	2.92	3.26, 3.24
			Hydrophobic interactions	Ala1093, Leu1097, Phe1101, Gly1142, Gly1143, Val1146, Val1254, Leu1255, Leu1304, Leu1318, Leu1363, His1468	Ala1093, Leu1097, Phe1101, Gly1141, Gly1142, Tyr1152, Glu1220, Ser1221, Val1254, Leu1255, Leu1304, Ile1359, Leu1363, Met1364, Phe1426, His1468	Ala1093, Leu1097, Gly1142, Val1146, Thr1252, Val1254, Leu1304, Ile1359, Leu1363, Met1364, Phe1426	Ala1093, Leu1097, Gly1142, Val1146, Thr1252, Val1254, Leu1255, Leu1304, Leu1318, Leu1363, Met1364, Leu1388, Met1425, Phe1426
7	1Q5K	GSK-3β	Binding Energy	-9.9	-9.3	-9.2	-8.7
			Number of H-bonds	1	-	-	-
			Residues involved in H-bonding	Asp200	-	-	-
			Bond length (Å)	3.15	-	-	-
			Hydrophobic interactions	Ile62, Val70, Ala83, Lys85, Asn186, Leu132, Asp133, Tyr134, Val135, Leu188, Cys199	Ile62, Gly65, Val70, Val110, Leu132, Thr138, Thr140, Arg141, Val170, Lys183, Gln185, Leu188, Asn196, Cys199, Asp200	Ile62, Gly65, Val70, Ala83, Lys85, Leu132, Asp133, Tyr134, Val135, Leu188, Cys199, Asp200	Ile62, Gly65, Phe67, Gly68, Val70, Ala83, Lys85, Asp133, Tyr134, Val135, Lys183, Asn186, Leu188, Cys199, Asp200,
8	2VIZ	BACE-1	Binding Energy	-8.8	-7.2	-8.4	-9.1
			Number of H-bonds	-	1	-	-
			Residues involved in H-bonding	-	Thr133	-	-
			Bond length (Å)	-	2.76	-	-
			Hydrophobic interactions	Tyr132, Thr133, Gln134, Phe169, Trp176, Gly291, Thr292, Thr293, Asn294, Arg296	Asp93, Gly95, Ser96, Ser97, Asn98, Val130, Phe131, Tyr132, Thr187, Ala188, Tyr259, Asp289, Gly291, Thr292	Leu91, Tyr132, Thr133, Gln134, Phe169, Trp176, Ile179, Gly291, Thr292, Thr293	Leu91, Asp93, Gly95, Tyr132, Thr133, Gln134, Phe169, Trp176, Ile179, Tyr259, Ile267, Asp289, Gly291, Val393
9	4RPU	HPP	Binding Energy	-8.7	-8.8	-7.7	-8.5
			Number of H-bonds	1	-	-	1
			Residues involved in H-bonding	Ser347	-	-	Ser453
			Bond length (Å)	3.16	-	-	3.21
			Hydrophobic interactions	Thr340, Phe344, Gly382, Tyr383, Lys450, Ser453, Glu463, Leu464, Leu467, Gln470	Ile337, Phe344, Ser347, Tyr380, Ile432, Met436, Tyr450, Ser453, Cys454, Leu464, Leu467	Ile337, Ala343, Phe344, Ser347, Tyr450, Ser453, Cys454, Leu464	Ile337, Thr340, Phe344, Ser347, Tyr380, Gly382, Tyr383, Tyr450, Cys454, Glu463, Leu464, Leu467, Gln470

(Table 1) Contd....

Sr. No.	PDB Code	Target	Docking Results	1	2	3	4
				MCULE-7149246649-0-1	MCULE-6730554226-0-4	MCULE-1176268617-0-6	MCULE-8592892575-0-1
10	4CRT	MAO-B	Binding Energy	-11.6	-8.4	-9.2	-10.3
			Number of H-bonds	-	1	-	-
			Residues involved in H-bonding	-	Ser59	-	-
			Bond length (Å)	-	2.98	-	-
			Hydrophobic interactions	Tyr60, Leu171, Cys172, Ile198, Gln206, Tyr326, Leu328, Met341, Phe343, Tyr398, Tyr425	Arg42, Gly57, Gly58, Tyr60, Val61, Gln65, Leu171, Cys172, Gln206, Phe343, Cys397, Tyr398, Tyr435, Met436, Glu437	Leu171, Cys172, Ile198, Ile199, Gln206, Tyr326, Phe343, Tyr398, Tyr435	Gly58, Tyr60, Leu171, Cys172, Gln206, Tyr326, Leu328, Phe343, Tyr398, Tyr435

Fig. (3). Molecular surface representation of target proteins with the respective ligands. (A) HCE-1 (1MX1); (B) GSK-3B (1Q5K); (C) PDE-5 (1TBF); (D) TACE (2DDF); (E) BACE-1 (2VIZ); (F) ACE (3BKL); (G) AChE (4BDT) (H) MAO-B (4CRT); (I) HPP (4RPV); (J) BChE (4BDS), each docked with compounds MCULE-7149246649-0-1, MCULE-6730554226-0-4, MCULE-1176268617-0-6 and MCULE-8592892575-0-1.

and models of AD [101]. Two of the four compounds indicated binding energies greater than -10 kcal/mol. For compounds 1-4, the binding energies observed were -10.2, -9.4, -9.0 and -9.1 kcal/mol, respectively. Among the amyloid-beta degrading enzymes, angiotensin-converting enzyme (ACE) is the most commonly targeted enzyme by inhibitors for it plays a significant role in the regulation of blood pressure and hypertension. A study suggests that ACE inhibitors are associated with an increased risk of AD [102], while two other studies argued that ACE inhibitors reduce dementia risk [103, 104]. Compounds 1-4 indicated binding energies -8.5, -8.9, -8.1 and -8.0 kcal/mol, respectively. Given that hCE1 and the ChEs show significant structural homology, the inhibitors that inhibit the latter (rivastigmine) have activity towards the former. The binding energies observed for compounds 1-4 were -11.3, -8.4, -8.7 and -9.9 kcal/mol respectively. The β -secretase, generally known as β -site amyloid precursor protein cleaving enzyme 1 (BACE1), initiates the generation of the toxic amyloid β ($A\beta$) that has a critical influence on AD pathogenesis [105]. Binding energies observed for compounds 1-4 were -8.8, -7.2, -8.4 and -9.1 kcal/mol, respectively. In human neuronal cells, human

presequence protease (hPreP) plays a role in degrading mitochondrial amyloid- β peptide and is subsequently an alluring target to enhance $A\beta$ proteolysis [106]. Compounds 1-4 indicated binding energies: -8.7, -8.8, -7.7 and -8.5 kcal/mol respectively. The enzyme, monoamine oxidase, catalyzes the oxidative deamination of different biogenic amines and thus, MAO inhibitors may enhance amine neurotransmission and may be beneficial for the treatment of AD. As per one study [107] an MAO-B inhibitor, for example, rasagiline, causes neuroprotection, antiapoptosis and inhibition of $A\beta$ accumulation. All four compounds indicated binding energies greater than -10 kcal/mol. The binding energies observed for compounds 1-4 were -11.6, -8.4, -9.2 and -10.3 kcal/mol respectively. Molecular surface representation of target proteins with these ligands have been represented in Fig. (3).

3.5. Molecular Dynamics Simulations and Free Binding Energy Calculations

The current study mainly focuses on potential hits against AChE. Therefore the protein-ligand stability in time was assessed by carrying out 20ns MD simulations for all four complexes of AChE followed by free binding energy calcu-

Table 2. Predicted MMGBSA calculations.

	MMGBSA										
	Initial (1ns)					MD (20ns)					Energy Shift
	VDW	EEL	EGB	ESURF	ΔG	VDW	EEL	EGB	ESURF	ΔG	ΔΔG
Cmp1	-40.4009	-1.5255	29.995	-4.8604	-16.7918	-46.8131	-3.3233	30.2759	-4.9502	-24.8107	-8.0189
Cmp2	-39.6206	-0.8336	38.9227	-7.0512	-8.5827	-56.295	-11.9545	38.7286	-6.7549	-36.2759	-27.6932
Cmp3	-40.994	2.9919	31.448	-4.2312	-10.7853	-41.2957	-6.2299	35.2727	-4.2082	-16.4611	-5.6758
Cmp4	-51.0811	-4.4388	39.466	-6.2928	-22.3467	-47.1256	-11.9269	35.9049	-5.8254	-28.9729	-6.6262

lations. The MD simulated conformations of all four compounds were further examined in 3-D visualization console of Discovery studio. Interestingly, all compounds were found to remain deep inside the gorge. Interaction analysis using Discovery studio software is shown in Fig. (4A). Quantification of ligand interaction using MMGBSA calculations is shown in Table 2. All selected compounds are rich in aromatic systems. Therefore aromatic pi-pi, pi-anion and pi-alkyl interactions with the surrounding amino-acids have an important contribution to this biological system, providing a significant amount of binding enthalpy. Non-covalent interactions involving π systems are pivotal to protein-ligand recognition. In this analysis, ΔG_{bind} estimates binding affinity whereas ΔE_{eel} and ΔE_{vdw} are electrostatic and Van-der-Waals contributions. Cmp2 was found to have the highest affinity after 20 ns simulation with a total energy of -36.2759 kcal/mol. With three pi-pi stacking interactions (TRP82, TYR445 and TYR333), four additional pi-alkyl interactions (TRP435, TYR337, TYR68 and TYR120), and 16 van-der-Waals interactions, the difference in binding energy with the other three compounds can be explained. Cmp4 ($\Delta G_{\text{bind}} = -28.9729$ kcal/mol) shows three pi-pi stacking interactions, of which, two aromatic amino acids (TRP82 and TYR333) are commonly found interacting with cmp2. TRP435 is responsible for the third stacking interaction. No pi-alkyl interactions were found and only 14 residues interacted through Van-der-Waals forces. However, one hydrogen bond was found with HIE443, significantly increasing the binding affinity towards a comparable value as seen in cmp1 ($\Delta G_{\text{bind}} = -24.8107$ kcal/mol). With no significant difference in van-der-Waals energy (Table 2), the two nitro-groups and central ester group provide more energetically rich regions to cmp4 in comparison to cmp1 and can therefore explain the difference in electrostatic energy (EEL, Table 2). Cmp1 also shows three pi-pi stacking interactions, of which two aromatic amino acids (TRP82 and TYR333) are commonly found interacting with cmp2 and cmp4. PHE334 is responsible for the third stabilizing stacking interaction. The lower electrostatic contribution of cmp1 to its total free interaction energy in comparison to the contribution in cmp2 can be explained by the more electronically rich central pyrimidine-(2,4,6)-trione scaffold. However, the presence of pi-anion stacking residue ASP70 and hydrogen-bond forming residue THR79, contribute to the comparable binding free energy. With 15 van-der-Waals interacting residues in cmp3, only

two residues were shown to interact *via* pi-pi interaction (TRP82 and HIE443). HIE443 was also found to be a hydrogen donor, while TRP82 interacts similarly in all compounds. This analysis shows aromatic interactions to be pivotal in explaining the binding affinity of selected compounds with the acetylcholinesterase. Fig. (4B) displays the RMSD convergence of all complexes between 1.2-1.5 Å, indicating the overall protein stability for all interacting compounds. To assess the binding stability for 20ns, $\Delta\Delta G_{\text{bind}}$ was calculated, which additionally allowed ranking of the selected compounds. Corresponding with the converging RMSD values (Fig. 4B), all compounds showed stabilizing effect with, more specifically, cmp2 as the most stabilizing (-27.6932 kcal/mol) and the less flexible thienopyrimido-quinazolinone moiety of cmp132 as the least stabilizing. Next to the comprehensive findings of aromatic interactions to be pivotal in the interaction of the selected compounds with the acetylcholinesterase, a certain degree of conformational flexibility is required which is reduced by the presence of bi- tri- or tetracyclic scaffolds.

The strong connections of interacting residues for all four compounds were evident for the possibility that their binding with inhibitory compounds stabilizes them to lead to the inhibitory response. Furthermore, the binding poses inside the gorge with interacting residues and molecular interactions of all four hits were evident from the investigation as determined through co-crystallized structures of specific inhibitors (Huperzine A, Galantamine, Donepezil and dihydrotan-shinone I) with AChE. Greater binding efficiencies indicate a decreased rate of dissociation [97]. Therefore, the strong binding energies of these compounds depict their ability to form stable complexes because of great binding efficiency and decreased rate of dissociation.

3.6. In Silico ADMET Assessment

The top 4 screened compounds had the most promising ADMET profile as tabulated in Table 3. The ADMET criteria included; blood-brain barrier penetration, hepatotoxicity, CYP2D6 binding, aqueous solubility, plasma-protein binding and intestinal absorption, which was considered as the initial step towards examining the novel chemical compounds by checking the failure of lead candidates alongside. The failed lead candidates can either be metabolized by the body or might result in toxicity and an inability to cross membranes.

Fig. (4). **A**) 2D interaction plot of top four compounds (MCULE-7149246649-0-1, MCULE-6730554226-0-4, MCULE-1176268617-0-6 and MCULE-8592892575-0-1) inside the AChE binding pocket. **B**) Root-mean-square-deviation (RMSD) trajectories with representative color codes calculated during 20 ns MD simulations.

None of the compounds were *in silico* predicted to have adverse effects. The uptake and delivery of drugs are inhibited by the highly resistant tight junctions that exist between the endothelial cells of brain capillaries [108]. The ability of a compound to penetrate is dependent upon molecular size, lipophilicity, pK_a -to-charge ratio, desolvation potential and hydrogen bond [109, 110]. In the current study, similar results were obtained for Caco-2 permeability and HIA. All four hits indicated positive results for BBB and HIA. In the case of renal organic cation transporter (ROCT), all four hits were observed to exhibit non-inhibition. Moreover, all four

hits were labeled non-substrate in the P-gp substrate analysis and non-inhibitors for the P-gp inhibitor except compound 4. The analysis highlighted low CYP inhibitory activity for all hits. CYP450, a group of isozymes, demonstrated a significant role in metabolizing drugs, xenobiotics, steroids, fatty acids, bile acids and carcinogens [111]. Therefore during ADMET analysis, various CYP450 substrate (2C9, 2D6, and 3A4) and CYP450 inhibitor (1A2, 2C9, 2D6, 2C19 and 3A4) models were calculated. As per the results, compound 2 was non-substrate for all CYP450 substrates (2C9, 2D6, 3A4)

Table 3. Predicted pharmacokinetic and ADMET properties of top four virtual hits.

Investigated Compounds	Compound 1	Compound 2	Compound 3	Compound 4
Formula	C19 H15 N O	C25 H22 N2 O3	C15 H15 N O3	C17 H10 N2 O6
Pharmacokinetic Data				
Mass	273.12	398.16	257.11	338.05
Log P	4.11	4.77	1.92	4.05
H bond acceptors	1	3	3	6
H bond donors	1	0	1	0
PSA	22.26	43.83	43.86	87.11
RO5 violation	0	0	0	0
Drug likeness	-0.5	-0.21	0.53	-0.86
Bioavailability	yes	yes	yes	yes
AD Vina (Docking energy) kcal/mol	-11.8	-11.3	-10.9	-10.9
Number of H-bonds	-	-	-	2
Residues involved in H-bonding	-	-	-	Ser125, Ser203
ADMET data				
BBB	+	+	+	+
HIA	+	+	+	+
Caco-2 permeable	+	+	+	+
Aqueous Solubility	-2.8621	-3.4407	-2.582	-4.1843
P-gp				
Substrate	-	-	-	-
Inhibitor	-	-	-	+
CYP450 substrate				
CYP450 2C9	-	-	-	-
CYP450 2D6	-	-	-	-
CYP450 3A4	+	-	-	+
CYP450 inhibitor				
CYP450 1A2 Inhibitor	+	-	-	-
CYP450 2C9 Inhibitor	-	-	-	-
CYP450 2D6 Inhibitor	-	-	-	-
CYP450 2C19 Inhibitor	-	-	-	-
CYP450 3A4 Inhibitor	-	-	-	-
CYP IP	low	low	low	low
ROCT	-	-	-	-

while all others were found to be substrates for CYP450 3A4 enzyme. The inhibition of CYP450 isoforms affects the metabolism of drugs resulting in toxicity [112]. Since all four compounds were found capable of inhibiting most of the CYP450 inhibitors, the analysis indicated low CYP inhibitory promiscuity [113]. One of the major findings was the detection of these compounds as non-inhibitors of almost all CYP450 enzymes, including isoform CYP450 1A2 (except compound 1). CYP450 1A2 is particularly responsible for the metabolism of various therapeutic drugs including tacrine

[114, 115]. According to the results, compounds 2 and 3 represent a promising ADME profile with good HIA, BBB properties additional to being non-inhibitors of most of the CYP450 isoforms, assisting in the metabolism of the drug and its clearance from the body [114].

CONCLUSION

The progressively increasing disease, Alzheimer's, is one of the leading causes of deaths across the globe. Acetylcho-

line plays a major role in AD, thus, AChE inhibition is the most widely studied mechanism for the design and synthesis of drugs against AD. Based on their high binding affinities, good pharmacological properties and ability to interact with multiple targets, four compounds have been reported for treating Alzheimer's. Obtained after careful analysis, these compounds fulfill ADMET criteria and thus could subsequently be further experimentally screened using *in vitro* or *in vivo* strategies for drug formulation. The stability of these compounds and free binding affinity change over time anticipated in our findings, provide evidence for potential multi-targeted inhibition against AD. Furthermore, these compounds not only have shown good binding affinities with the rest of the chosen targets but also represented promising ADMET properties along with no adverse effects on humans, demonstrating them good enough for human consumption. The compounds identified in the present study have a high potential as an alternative for existing AD treatments.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

Not applicable.

HUMAN AND ANIMAL RIGHTS

No Animals/Humans were used for studies that are base of this research.

CONSENT FOR PUBLICATION

Not applicable.

CONFLICT OF INTEREST

The authors declare no conflict of interest, financial or otherwise.

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Declared none.

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