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Discovery of dual binding site acetylcholinesterase inhibitors identified by pharmacophore modeling and sequential virtual screening techniques

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ABSTRACT

Dual binding site acetylcholinesterase (AChE) inhibitors are promising for the treatment of Alzheimer's disease (AD). They alleviate the cognitive deficits and AD-modifying agents, by inhibiting the β-amyloid (Aβ) peptide aggregation, through binding to both the catalytic and peripheral anionic sites, the so called dual binding site of the AChE enzyme. In this Letter, chemical features based 3D-pharmacophore models were developed based on the eight potent and structurally diverse AChE inhibitors (**I–VIII**) obtained from high-throughput in vitro screening technique. The best 3D-pharmacophore model, Hypo1, consists of two hydrogen-bond acceptor lipid, one hydrophobe, and two hydrophobic aliphatic features obtained by Catalyst/HIPHOP algorithm adopted in Discovery studio program. Hypo1 was used as a 3D query in sequential virtual screening study to filter three small compound databases. Further, a total of nine compounds were selected and followed on in vitro analysis. Finally, we identified two leads—**Specs1** (IC_{50} = 3.279 μM) and **Spec2** (IC_{50} = 5.986 μM) dual binding site compounds from Specs database, having good AChE enzyme inhibitory activity.

Alzheimer's disease (AD) is a chronic, irreversible and progressive neurodegenerative disorder with both genetic and non-genetic causes. Two distinct histological changes in the nerve cells of Alzheimer brain are the formation of extracellular amyloid ('senile') plaques and intracellular neurofibrillary tangles, which lead to neurotoxicity. One of the major therapeutic strategies adopted for primarily symptomatic AD is based on the cholinergic hypothesis targeting acetylcholinesterase (AChE) enzyme. AChE is a substrate-specific enzyme that degrades the neuro-transmitter acetylcholine in the nerve synapses. An optimum level of acetylcholine should be maintained in the brain for its proper function.[1]

Cholinesterase inhibitors such as rivastigmine, tacrine, donepezil and galantamine, which block the breakdown of acetylcholine, have been used for the treatment of mild to moderate AD.[2–5] All of the above mentioned drugs are reversible inhibitors of AChE, and can restore the level of acetylcholine in the brain of AD patients. The most common adverse effects of these drugs include nausea and vomiting, both of which are linked to the presence of excess cholinergic neurons. Less common, secondary adverse effects include bradycardia, muscle cramps, decreased appetite and weight loss as well as increased gastric acid production.[6–8] Therefore, new and potentially more effective classes of AChE inhibitors are needed to minimize these side effects.

Recently, interference on amyloid beta (Aβ) aggregation by AChE inhibitors directed for the development of a novel class of dual binding site inhibitors. Crystal structure analysis showed that the active site gorge of AChE enzyme span about 20 Å long which is lined by 14 aromatics residues, and is conserved across different species. The active site gorge of Torpedo californica AChE (TcAChE) enzyme consists mainly of two sub-sites: catalytic site (CS) at the bottom of the gorge that contains the catalytic triad (H440, E327 and S200) with W84 and F330 called the anionic sub-site (AS), and a peripheral anionic site (PAS) composed of Y70, Y121 and W279 residues. The distance between AS and PAS is 14 Å apart.[9–11] The function of the AChE enzyme is mainly controlled by tryptophan residues at AS and PAS, that is, W84 will participate in the orientation of the charged part of the substrate that enters the active center; AS has another interesting feature which is involved in the 'cross-talk' mechanism with the PAS in which mainly W84 and W279 residues will participate. Thus, dual binding site inhibitors should target both these residues simultaneously at the AS and PAS contributing to its tighter binding and thereby increasing its efficacy as a drug

Abbreviations: AD, Alzheimer's disease; AChE, acetylcholinesterase enzyme; CS, catalytic site; PAS, peripheral anionic site; VS, virtual screening; DTNB, 5,5′-dithiobis-2-nitrobenzoic acid; ATCI, acetylthiocholine iodide; TcAChE, *Torpedo californica* AChE; PDB, protein data bank; GFS, GOLD fitness score; BBB, blood–brain barrier; CNS, central nervous system.

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candidate for the treatment of AD.[11] It was also experimentally observed that the effect of PAS ligands on AChE moderates the rate of amyloid deposition.[9,10] The bifunctional role, anti-aggregating Aβ (characteristic pathological role in AD) and anti-cholinesterase activity are mainly contributed by these dual binding site interactions on this enzyme, as discussed above. Therefore, identifying selective dual binding site AChE inhibitors is highly demanding for CNS penetration and good pharmacokinetic properties for the treatment of AD.

Donepezil is a well known FDA approved dual binding site AChE inhibitor.[12] The crystal structure of donepezil-AChE complex showed that donepezil interact with these dual sites simultaneously by making aromatic stacking interactions with that of the W84 and Y279 residues of the AChE enzyme. Another highly potent, selective, and low cost bifunctional (dual binding site) inhibitor is bis-7 tacrine developed by utilizing computer modeling of ligand docking with AChE enzyme.[12] This exercise was performed by identifying low affinity sites and design bifunctional analogs capable of simultaneously binding at the CS and PAS of the enzyme. Applying this strategy to 9-amino-1,2,3,4-tetrahydroacridine (THA) or tacrine (an FDA approved drug for AD), the alkylene linked bis-THA analogs were synthesized and tested. These analogs were up to 10,000-fold more selective and 1000-fold more potent than THA in inhibiting AChE enzyme. Thus, dual binding site inhibitors provide a structural basis for the design of improved anti-cholinesterase activity for treating AD.[12] Other research groups also successfully employed computational and synthetic strategy to identify and design dual binding site AChE inhibitors.[13–15]

The main objective of the present work is to identify dual binding site AChE inhibitors with good pharmacokinetic parameters by 3D-pharmacophore modeling and sequential virtual screening (VS) techniques. The dataset used for pharmacophore generation consists of potent and selective AChE inhibitors obtained from high-throughput in vitro screening, previously reported by our research group.[16] This pharmacophore model will then be used as 3D query for screening three different small compounds databases, including national cancer institute (NCI), Specs and Inter Bio (IB) Screen, to identify novel dual binding site AChE inhibitors using sequential VS techniques. During VS, we used several filters such as $(CH_3)_3N$, Lipinski's rule of five, molecular docking, and absorption, distribution, metabolism, excretion and toxicity (ADMET), to refine the retrieved hits. Finally, some of the refined hit compounds were purchased from the vendor and conducted an in vitro inhibitory assay against AChE was performed, as well as in depth molecular docking analysis, in order to understand the mode of interactions at the dual binding sites of this enzyme. We thus adopted a strategy, by systematically integrating the in vitro and in silico techniques throughout the present work, to discover new lead AChE inhibitors having good ADMET parameters for the treatment of AD.

Dataset for 3D-pharmacophore modeling consists of eight compounds (**I–VIII**), which were structurally diverse and potent AChE inhibitors, with an IC_{50} <0.35 μM, from previous published article by our research group.[16] The compounds were selected as positive hits from an initial high-throughput in vitro screening study involving a collection of about 56,000 compounds. Details of the enzyme assay and inhibition studies are provided in Supplementary data. The chemical structures as well as the experimental

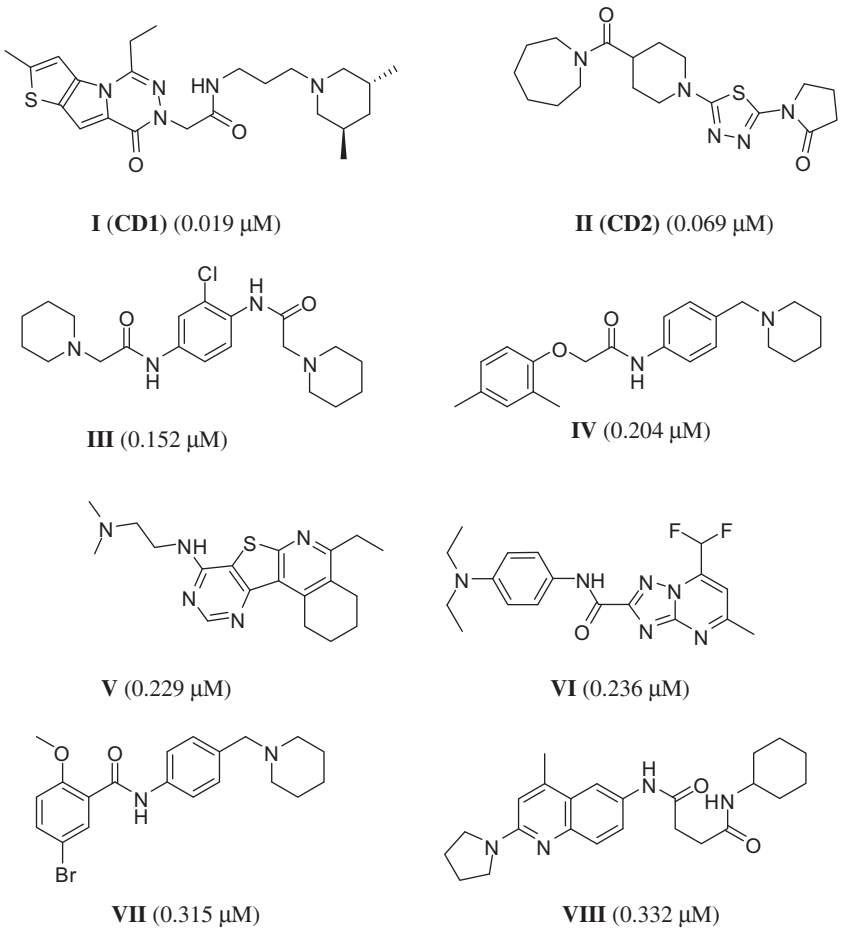


Figure 1. Structurally diverse eight most potent AChE inhibitors (**I–VIII**) obtained by high throughput in vitro screening method.

IC_{50} values of the eight training set compounds (**I–VIII**) are presented in Figure 1.

We have selected these eight compounds (**I–VIII**) on the basis of structure-based molecular docking analysis, which revealed the possible molecular determinants responsible for its binding affinities and dual binding site (CS and PAS) abilities towards Torpedo californica AChE (TcAChE) enzyme. Among the available crystal structures of the AChE enzyme, docking studies were performed on the donepezil bound crystal structure of TcAChE (PDB code: 1EVE).[17] We have selected this crystal structure by considering that the size and shape complementarity as well as the dual binding site nature of the donepezil compound was similar to our training set lead compound **CD1**. TcAChE has almost identical amino acid residues with the human AChE (hAChE) (PDB code: 1B41)[18] at both the CS and PAS, apart from the substitution of F330 (Tc) with Y337 (human). GOLD (genetic optimization for ligand docking) program[19] was used for docking study and the GOLD score option was selected as the fitness function, denoted in short form as GOLD fitness score (GFS). The X-ray coordinates of donepezil bound to the active site of the AChE enzyme were used to define active site region with an active site radius of 9.5 Å, by keeping all the water molecules within the active site. The annealing parameters of van der Waals and hydrogen-bonding interactions were considered within 4.0 and 2.5 Å, respectively, and other parameters were kept at the default setting. Superimposition of the docked donepezil onto the crystallographic geometry yielded RMSD of 0.55 Å, which revealed that GOLD program performed well in reproducing experimentally observed binding conformation of donepezil with a GFS of 66.79 kJ/mol. This study confirmed its suitability for the present docking analysis. Finally, using the above docking protocol all the eight molecules (**I–VIII**) were successfully docked at the dual binding site of the AChE enzyme.

Computational analysis was performed on Linux and SGI workstations. The compounds under study were built using the SYBYL7.1[20] molecular modeling package installed on a SGI work station running IRIX 6.5. Gasteiger-Hückel partial atomic charges[21] were assigned to the compounds and their conformational energy was minimized using the Powell[22] method and the Tripos force field,[23] with a convergence criterion for the energy gradient of 0.001 kcal/mol/Å. The methodology adopted for compound generation, HipHop pharmacophore model development and sequential virtual screening (VS) is explained below.

The eight compounds (**I–VIII**) used in this study was also tested for its inhibitory activity in BChE enzyme to understand its selectivity with respect to that of the AChE enzyme. Earlier, we reported AChE enzyme inhibition for the compounds **I** (0.019 μM) and **II** (0.069 μM), and BChE enzyme inhibition for the compounds **I** (13.27 μM) and **II** (23.58 μM), respectively.[16] The corresponding selectivity index (BChE/AChE) was 698 for compound **I** and 342 for compound **II**, respectively. Thus, these compounds are selective towards AChE enzyme and thereby increasing its chances of having the dual binding site ability, as mentioned earlier. Also, our previous published work on structure-based molecular docking studies on these compounds (**I** and **II**) on TcAChE enzyme also showed binding at the AS and PAS, and thereby confirming its dual binding site abilities.[16] These experimental results further supports our theoretical observation that the compounds are indeed making interactions at the dual binding sites of the AChE enzyme, which thus forms the main basis of the present investigation.

We have developed a common feature 3D-pharmacophore model from structurally diverse and most active eight potent AChE inhibitors (**I–VIII**), obtained from high-throughput in vitro screening, using HipHop module of DS2.5.[24] Compounds which are to be used in HipHop pharmacophore generation and which are to be ignored can be specified by using 'Principal number' and MaxOmitFeat option given in the program. The resultant pharmacophores are ranked as they are built. The ranking is a measure of how well the active training set compounds map onto the proposed pharmacophores, as well as the rarity of the pharmacophore model. HipHop was instructed to explore up to five featured pharmacophoric space for the following possible features: hydrogen bond acceptor lipid (HBAL), hydrogen bond acceptor (HBA), hydrophobic (HY), hydrophobic aliphatic (HY_Al), and ring aromatic (RA). Furthermore, the number of features of any particular type was allowed to vary from 0 to 5. The most potent compound of the training set **I** (**CD1**) having AChE inhibitory value of 0.019 μM was considered as 'reference compound'. HipHop returns by default a maximum of 10 high-ranking pharmacophores from each automatic run. The details of top 10 hypothesis generated by HipHop-Refine run are presented in Table 1s (Supplementary data). The best hypothesis referred as Hypo1, which has the highest ranking score 97.7, contains two hydrogen-bond acceptor lipid, one hydrophobic (HY), and two hydrophobic aliphatic (HY_Al) features, and is presented in Figure 2.

As described earlier, the active site regions of AChE enzyme is filled with hydrophobic residues and the dual binding sites (AS and PAS) are separated by ~14 Å, respectively.[11] The present generated 3D-pharmacophore model also clearly supports the above observation, as depicted by the pharmacophoric distance between different chemical features. Notably, the distance between the two hydrophobic aliphatic (HY_Al) features are ($\geqslant$15 Å) apart (Fig. 2), which is in good agreement with that of the dual binding site distances in the AChE enzyme crystal structure.[17] Also the charge complementarity principle at these regions, that is, hydrophobic character of the active site residues clearly follows the corresponding chemical features obtained in our pharmacophore model, favoring stacking interactions between the ligand and hydrophobic residues of AChE enzyme, which is explained below in structure-based molecular docking section.

The success of pharmacophore-based virtual screening (VS) largely depends on the accuracy and specificity of the pharmacophore query employed. Keeping in mind the caveats regarding false positives during VS, a statistical approach was used to estimate the enrichment ability of the 'dual binding site pharmacophore' Hypo1. The Hypo1 model was used to query the 'Decoy dataset' by HipHop Pharmacophore Mapping protocol using the fast flexible search option.

Decoy dataset consist of 56 AChE active compounds obtained from the present in vitro screening, and 500 decoys from AChE benchmarking dataset (consisting of 3732 decoys) developed by Shoichet and Irwin.[25] The decoys have similar physical properties, such as molecular weight, hydrogen bond donors and acceptors, log *P*, etc. but with chemical and topological differences, so that they might not bind to AChE active site. These later class of

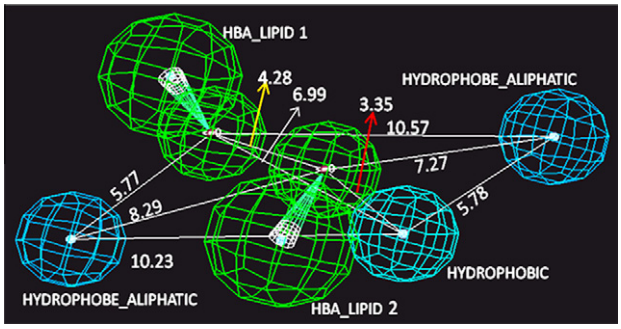


Figure 2. The best HipHop pharmacophore model for AChE inhibitors. The features are color coded with green, hydrogen-bond acceptor lipid (HBAL); light blue, hydrophobic aliphatic feature (HYAl), and hydrophobic aromatic feature (HY). The pharmacophoric distances are given in Angstrom units.

compounds (decoys) were chosen in order to test the robustness of our developed pharmacophore model in VS.

The efficiency of the pharmacophore Hypo1 for VS was determined by computing different statistical metrics namely sensitivity, goodness of fit (G) and enrichment (E)[26] using the above mentioned 556 compounds. The VS was done by using the HipHop pharmacophore mapping module in DS2.5. The parameters such as total number of compounds present in the hit list (Ht), percentage sensitivity (%S), percentage ratio of actives maps in the hit list (%A), enrichment (E), false negative (Fn), false positive (Fp) and goodness of fit score (G) is calculated using the formula

$$G = [(\mathrm{Ha}/4\mathrm{HtA})(3\mathrm{A} + \mathrm{Ht}) \times (1 - ((\mathrm{Ht} - \mathrm{Ha})/(\mathrm{N} - \mathrm{A})))] \quad (1)$$

$$E = (\mathrm{Ha}/\mathrm{Ht})/(\mathrm{A}/\mathrm{N}) \quad (2)$$

Different statistical values are computed after VS which mainly includes; Percentage ratio of actives (%A) = 89.3; %Sensitivity (%S) = 73.5; E = 7.3 and G = 0.75, respectively. The details are presented in Table 2s (Supplementary data). These statistical values are significant showing high efficiency of Hypo1, and which could then be confidently used for VS of different small compound databases.

VS techniques are widely employed in computational drug discovery programs to evaluate large chemical databases in order to discover new lead compounds for a drug target of interest.[27–29] We adopted sequential VS strategies to reduce the search space for potential drug candidates by filtering out compounds unlikely to interact with the AChE enzyme. Three different small compound databases containing 888,762 compounds were used for sequential VS, among which Specs database had 199,501 compounds, National Cancer Institute (NCI) database had 260,071 compounds, and Inter BioScreen (IB Screen) database had 429,190 compounds, respectively.[30–32] Therefore, a small subset of these databases which should include promising leads that likely bind with the AChE enzyme should be preferred for experimental in vitro screening. All three database compounds in 2D SD form were transferred into multi-conformer with DS2.5. During the process of database generation, the FAST method was selected, and the maximum number of conformers generated was set to 100. Different steps adopted by us for sequential VS strategy is explained below and its flowchart is depicted in Figure 3.

In the first stage of VS there is a need for computationally inexpensive filters, because databases may contain millions of compounds, most of which have little chance of being hits for the specific drug target. The simple physico-chemical properties of the compound were included in the initial phase of VS, that is, $(CH_3)_3N$ and Lipinski's rule of five. We have included $(CH_3)_3N$ functional group in the first phase of VS, because ionizable nitrogen is required for better AChE inhibitory activity of the compound, in order to cross the blood–brain barrier (BBB). The basic reason of this might be that the nitrogen atom of the inhibitor should be protonated for interaction with the anionic site of the AChE enzyme. Piazzi et al. suggested that the presence of basic (protonable) nitrogen in the AChE inhibitor is crucial for the compound to cross the BBB.[33,34] Thus, our first stage of filtering was computationally demanding, and the number of compounds was drastically decreased from 888,762 compounds to 162,372 compounds on the basis of this VS, and is depicted in Figure 3, respectively.

In the second step, developed AChE enzyme specific 3D-HipHop pharmacophore model (Hypo1) was used as a query for prioritization of database compounds, on the hits (162,372 compounds) obtained from first step of VS. The computed compound similarities on the basis of the best fit values ($\geqslant$4.0) between the structures of the database compounds, filtered using the Hypo1 pharmacophore model are then used to prioritize compounds in the second stage of VS. Hypo1 aligned with the most active training set compound **I** (**CD1**) is presented in Figure 4a. Quantitative estimation of the goodness of match between a compound and the pharmacophore was estimated using the (Fit) option and the values are reported as fit values in Table 3s (Supplementary data). This stage of VS technique was very effective and the 162,372 compounds from previous VS step was finally filtered to 117 compounds which include: 26 compounds from Specs database, 2 compounds from NCI database and 89 compounds from IB screen database, respectively, as shown in Figure 3.

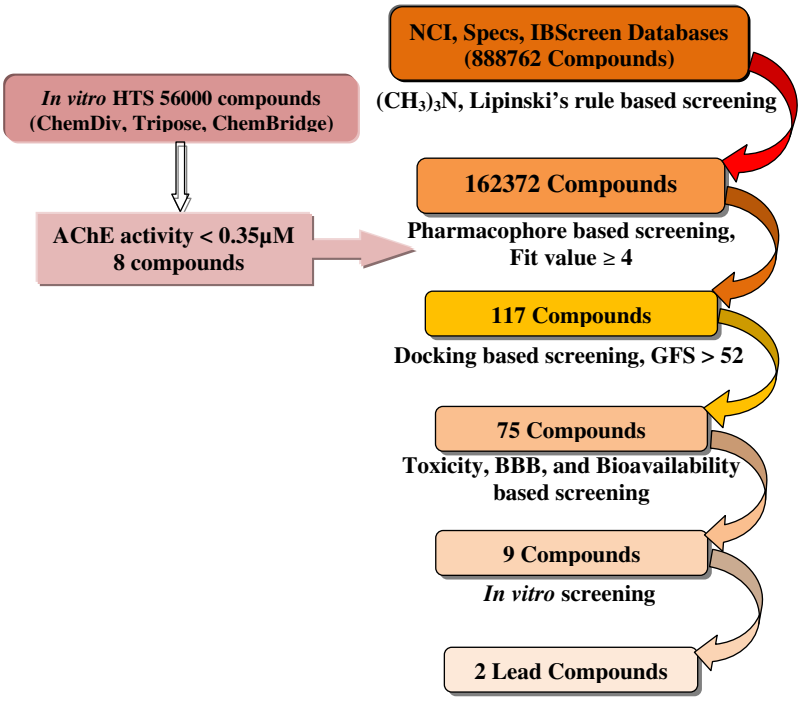


Figure 3. Flow chart showing sequential virtual screening techniques used in the present study.

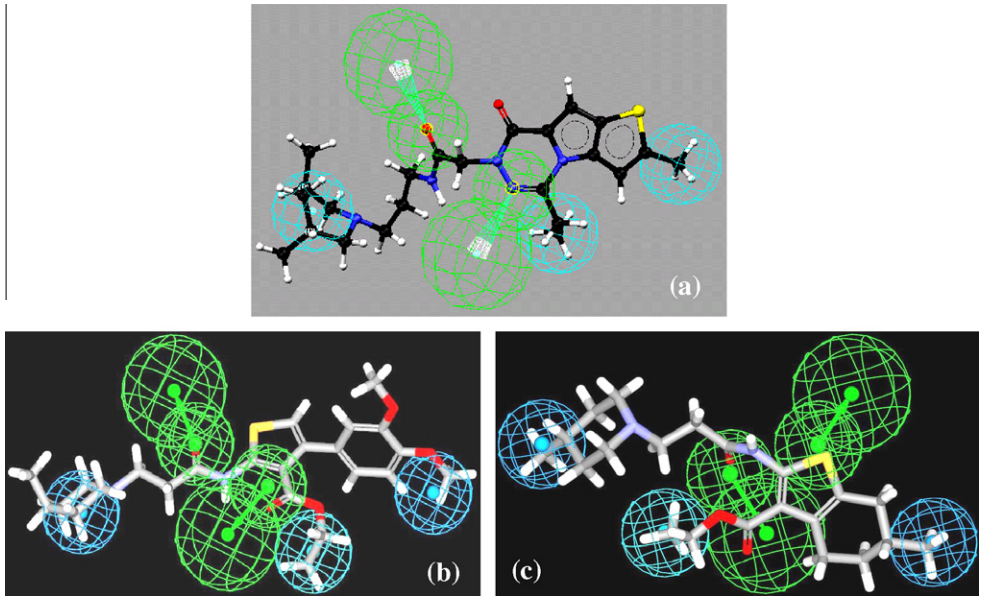


Figure 4. The best HipHop model mapped with the most active compound **CD1** from ChemDiv database (a). HipHop pharmacophore map fitting for the two lead compounds **Specs1** (b) and **Specs2** (c) obtained from Specs database using sequential VS followed by in vitro analysis. The features are color coded with green, hydrogen-bond acceptor lipid (HBAL); light blue, hydrophobic aliphatic feature (HYAl), and hydrophobic aromatic feature (HY).

The third step of VS includes high-throughput molecular docking, which is a computationally intensive structure-based VS technique that generates and scores possible protein–ligand interactions according to their computed binding affinities, and is illustrated as flow chart in Figure 3. The docking procedure adopted by us for sequential VS study is described earlier. GOLD docking results were reported as the highest scoring pose for each compound, and also on the basis of their ability to form favorable interactions within the dual binding site of the AChE enzyme. 117 compounds from our previous stage of VS were then further prioritized, and 75 compounds were selected on the basis of GFS higher or equal to 52 kJ/mol (GFS $\geqslant$ 52 kJ/mol) obtained by docking at the dual binding (AS and PAS) site of the AChE enzyme (Fig. 3). In this context, it is worth mentioning that the TcAChE crystal structure is very similar to the AChE structure of both mouse and human. Thus, the conclusions drawn from the present docking analysis should be valid for the mammalian enzyme. Recently, we have reported the docking analysis of coumarin 106 to the TcAChE enzyme, and promising results in consonance with the experimental results were obtained.[35]

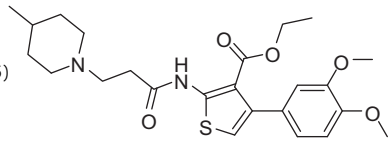
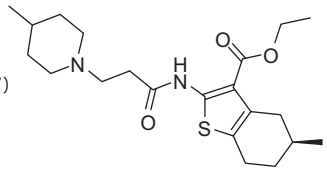
Computational approaches are being used nowadays in drug discovery program to assess the ADMET properties of the compounds at the early stages of the discovery/development. Computed physico-chemical properties associated with the compounds that have good oral bioavailability, less or no toxicity and the capacity to penetrate the BBB are key decision filter for CNS drug discovery.[36–38] The last stage of our sequential VS strategy was filtering the compounds on the basis of ADMET properties. The computational results are an aid to decision making, but solubility, oral bioavailability, brain uptake and efficacy are experimentally determined features of a compound.[39] However, properties such as bioavailability, BBB penetration and aqueous solubility often correlate with the profiles seen in computed physico-chemical property analysis, thereby allowing computational predictions to be used as a cautious aid to prioritize experimental efforts.[36] Specifically, some key computed parameters that we examined as a part of multiple property filter of lead compounds were octanol/water partition coefficient (Log *P*), distribution coefficient (Log *D*), computed aqueous solubility (Log *S*), polar surface area (PSA), percent human oral absorption, BBB penetration, CNS activity and toxicity. In this context, we have computed the above pharmacokinetic properties of the compounds using different well known software, which includes: ADME module of DS2.5 and QikProp module of Schrödinger software. These software have inbuilt screening filters for this type of analysis. Toxicity profiles of the compounds were assessed using DEREK software.[40]

After strictly applying all the above mentioned ADMET profile, we eventually filtered 9 compounds from 75 compounds in our last stage of the VS strategy, and this is illustrated in Figure 3. All calculated ADMET parameters of these nine compounds are presented in Table 4s (Supplementary data). We expect these nine compounds to posses promising drug-like property, have good CNS activity and no organ toxicity (Supplementary data Table 4s). The structural diversity of these nine compounds demonstrated that the 3D-HipHop pharmacophore model was able to retrieve hits with similar features to existing AChE inhibitors. We purchased these nine compounds from the respective Specs and InterBio Screen vendors for bioassay (in vitro) analysis.

In vitro AChE inhibitory assays were performed on nine compounds obtained from sequential VS, as described above, which led us to identify two Specs compounds, denoted as **Specs1** and **Specs2** showing good AChE inhibitory activity of 3.279 μM (**Specs1**) and 5.986 μM (**Specs2**) respectively (Table 1). In vitro analysis on **Specs1** and **Specs2** compounds were also performed for BChE activity and no inhibition (<10%) was found in the Ellman's based kinetic assay, at least at a concentration (10 μM) higher than the IC_{50} required to inhibit AChE. These results clearly support that these compounds are selective towards AChE enzyme.

The 3D-HipHop pharmacophore map fitting for the most potent compound **CD1** (a), and two lead compounds **Specs1** (b) and **Specs2** (c) are represented in Figure 4, respectively. The map clearly depicts that the pharmacophoric distance between two HYAl features of these compounds are in good agreement with the distance between the AS and PAS of AChE enzyme, which is separated by ~14 Å.[11] The activity of VS compounds, are 100-fold less than the activity of the training set compound **CD1**. But, still the VS compounds showed promising pharmacokinetic properties predicted by different ADMET software, and no organ toxicity,

Table 1
Structure of the two lead compounds along with the AChE enzyme inhibition, and its GOLD fitness score (GFS)

Compound Structure[a] (vendor ID)	IC_{50}[b] (μM) for AChE inhibition	GFS in AChE enzyme
(Specs (**Specs1**), (ID = AF-399/15128515) 	3.279	55.93
(Specs (**Specs2**), (ID = AF-399/40654557) 	5.986	53.29

[a] IUPAC names: **Specs1**: ethyl 4-(3,4-dimethoxyphenyl)-2-(3-(4-methylpiperidin-1-yl) propanamido) thiophene-3-carboxylate; **Specs2**: (*S*)-ethyl 5-methyl-2-(3-(4-methylpiperidin-1-yl) propanamido) 4,5,6,7-tetrahydrobenzo[*b*]thiophene-3-carboxylate. All the compounds have >95% purity.
[b] AChE potency obtained using in vitro assay.

compared to the two most active training set compounds **CD1** and **CD2**, and is tabulated in Table 2, respectively.

Structure-based molecular docking analysis using GOLD software on two lead compounds **Specs1** and **Specs2** revealed the possible molecular determinants responsible for its binding affinities and dual binding site (CS and PAS) abilities towards TcAChE enzyme. Structures of these two lead compounds and its in vitro AChE enzyme inhibition, along with the GFS are presented in Table 1. We have used crystal structure of TcAChE for docking analysis and Electric eel AChE for the in vitro analysis. The primary sequence analysis showed that the active site residues of both these enzymes are conserved, and there is only one residue difference, F330 in TcAChE was replaced with Y330 in Electric eel of AChE.

Molecular docking derived binding pose of the two VS compounds **Specs1** and **Specs2** in the dual binding site of the TcAChE enzyme are shown in Figure 4a and b, respectively. The best compound **Specs1** identified from VS showed GFS of 55.9 kJ/mol and formed two π–π stacking interactions (i) between the thiophene ring of **Specs1** and aromatic ring of F330 (AS) in AChE enzyme, and (ii) between dimethoxy benzene ring of **Specs1** stacked against the indole ring of W84 at the anionic subsite (AS) of the AChE enzyme (Fig. 5a). In the same manner, piperidine nitrogen atom and benzene ring of donepezil also formed cation–π and π–π interaction with the W84 of AChE enzyme, respectively. In addition, three water mediated hydrogen-bonding interactions were observed between (i) one of the methoxy group oxygen of **Specs1** and carbonyl oxygen (C═O) of W84 through water (WAT1157), (ii) second water (WAT1350) mediated hydrogen-bonding interaction was observed between the carbonyl oxygen (C═O) of **Specs1** and carboxylate group of D72, in the middle of the gorge site (CS) of the AChE enzyme. Third water mediated (WAT1254) hydrogen-bonding interaction was found between the piperidine nitrogen atom of **Specs1** and amide (NH) group of F288 (PAS). Donepezil also showed same water mediated (WAT1254) hydrogen-bonding interaction with the F288 of the AChE enzyme (Fig. 5a).

Similarly, docking simulations revealed that the top-scored docking pose (GFS = 53.13 kJ/mol) of the second most active virtual screened **Specs2** compound, showed a binding pattern similar to that of **Specs1** compound (Fig. 5b). However, the hydrogen-bonding pattern was slightly different from that of the **Specs1** compound. **Specs2** compound showed four hydrogen-bonding interactions. In which, one water molecule (WAT1234) act as a bridge to form two hydrogen bonds between the carbonyl oxygen (C═O) atom of acetate group of **Specs2** compound and carbonyl oxygen atom of S81 (CS), and carboxylate oxygen atom of D72 (CS). The other two hydrogen-bonding interaction were observed between the carbonyl oxygen (C═O) and piperidine nitrogen atom of **Specs2** compound and hydroxyl group of Y121 (Fig. 5b) of AChE enzyme (PAS). In addition, the trend of variation of the GFS with that of the AChE inhibitory activity of both these compounds showed good correlation (Table 1).

The dynamical motion of F330 and W279 residues are well known in the crystal structure of AChE bound with different ligands. An open conformation of F330 on binding of the AChE enzyme with the compound donepezil drug molecule was established in the crystal structure 1EVE.[17] In the present study also F330 adopted an open conformation on binding with the **Specs1** and **Specs2** compounds (Fig. 5), in consonance with the above crystal structure analysis. Also W279 residue adopted same conformational trend in donepezil (1EVE), which is in agreement with the **Specs1** and **Specs2** docked compounds, as shown in Figure 5.

Table 2
ADMET parameters of training set and VS lead compounds obtained from ChemDiv and Specs database

Compound	Toxicity[a]	CNS[b]	log *S*[b]	% H_O_Abs[b]	*A* log *P*[c]	log *D*[c]	log BB[b]	PSA[b]
CD1	Skin sensitization	1	−5.64	97	3.46	1.89	−0.23	80.5
CD2	None	−1	−3.53	89.6	1.23	1.23	−0.39	78.5
Specs1	None	1	−3.62	100	3.85	2.36	0.18	74.3
Specs2	None	1	−4.61	100	3.99	2.51	−0.11	66.1

[a] Toxicity prediction using DEREK software.
[b] Prediction using Schrödinger software.
[c] Prediction using Discovery studio ADME module software; CNS = Predicted central nervous system activity on a −2 (inactive) to +2 (active) scale; Log *S* = Predicted aqueous solubility, log *S* on a −6.5% to 0.5; % H_O_Abs = Percent Human Oral Absorption; *A* log *P* = Log of the octanol–water partition coefficient using Ghose and Crippen's method; Log *D* = The octanol–water partition coefficient calculated taking into account the ionization states of the compound; Log BB = Predicted brain/blood partition coefficient on a −3 to 1.2 scale; PSA = Van der Waals surface area of polar nitrogen and oxygen atoms.

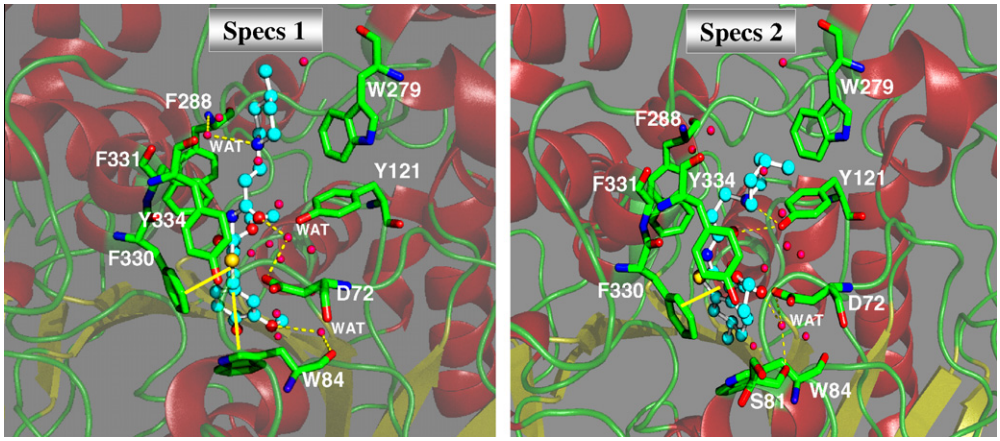


Figure 5. Molecular docking derived binding pose of the VS lead compounds **Specs1** (a) and **Specs2** (b) in the dual binding site (CS and PAS) of AChE enzyme. The inhibitor is shown as ball and stick model in the surface representation of the enzyme. Water molecules are shown as red dotted spheres. Hydrogen bonding is shown as dotted lines (yellow color) and cation–π (or π–π) interaction by straight line (yellow color). GOLD software was used to derive the binding mode and the picture was generated from PYMOL software.

In summary, we report here an integrated in vitro–in silico approach to identify new lead compounds, that might be further used for designing dual binding site AChE inhibitors. The 3D-pharmacophore model was developed based on the eight potent and structurally diverse AChE inhibitors, obtained from high-throughput in vitro screening, with the aid of Catalyst/HIPHOP algorithm in DS2.5.

VS strategy was applied on three different small compound databases including Specs, NCI, and IBScreen containing 888,762 compounds, using multi-filtering approaches, (i) simple physicochemical properties screening such as $(CH_3)_3N$ and Lipinski's rule of five, (ii) 3D-HipHop pharmacophore model based screening, (iii) molecular docking based screening at the dual binding site of the AChE enzyme, (iv) pharmacokinetic and toxicity parameters based screening such as central nervous system activity, aqueous solubility, bioavailability, and BBB partition level and compound toxicity. These sequential VS finally identified nine putative dual binding site AChE leads which were further validated by in vitro AChE inhibitory assay. In vitro analysis on these nine leads enabled us to identify two potent and selective dual binding site AChE inhibitors from Specs database, **Specs1** and **Specs2**. Both VS compounds showed potent inhibitory activity against AChE enzyme, with promising BBB penetration and other pharmacokinetic parameters. Moreover, both VS lead compounds (**Specs1** and **Specs2**) showed better pharmacokinetic profiles than the most potent compounds (**CD1** and **CD2**) obtained by in vitro analysis. Molecular docking studies on **Specs1** and **Specs2** compounds allowed an in depth atomic level analysis, and interpretation of the dual binding site (AS and PAS) interactions of the inhibitors with the AChE enzyme.

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Supplementary data

Supplementary data ((A) Details of enzyme assay and inhibition studies of high-throughput in vitro screening of AChE inhibitors; Table 1s: Details of top ten hypotheses generated by HipHop-Refine; Table 2s: Statistical parameters from screening decoy external test set compounds; Table 3s: Eight compounds along with the activity value and Principal, MaxOmitFeat and Fit value obtained by best hypothesis Hypo-1using HipHop-Refine module; Table 4s: In silico ADMET parameters of top 9 virtually screened lead compounds predicted using different software; (B) Purity of the **Specs1** and **Specs2** compounds) associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2010.12.131.

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