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VIRTUAL SCREENING OF ACETYLCHOLINESTERASE-CENTERED INHIBITORS AS POTENTIAL THERAPIES FOR ALZHEIMER'S DISEASE

Benjamin Liu [1]

[1] United States of America

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Abstract

Acetylcholinesterase inhibitors(AChE-Is) are currently one of the most popular treatments for Alzheimer's disease(AD). Despite their proven effectiveness in easing symptoms of cognitive decline, their limited efficacy and strong adverse side effects demand the urgent need to develop better treatment for AD patients. This study explores a new series of ligands targeted towards inhibiting AChE as an anti-AD drug. Findings from various binding site detection methods, such as geometric, machine learning, and energetic-based methods, showed that AChE is a suitable binding target for ligands. The research utilized ZINCPharmer to identify compounds with good binding interactions with different pharmacophore maps of AChE. Molecular docking using SwissDock revealed multiple ligands with an impressive SwissParam score range of –7.2 to –8.9 kcal/mol, confirming their strong binding interaction with AChE binding sites. The top compounds were tested for their absorption, distribution, metabolism, and excretion(ADME) using SwissADME. Three promising compounds L_6(ZINC12232928), L_7(ZINC92176885), and L_9(ZINC92189850) were able to cross the Blood Brain Barrier while adhering to Lipinski's rule. The toxicity of the compounds was also examined using a computational prediction tool ProTox 3.0. Most compounds have acceptable toxicity, with compound L_18(ZINC03302264) having the best predicted LD50 of 5240 mg/kg and predicted toxicity class 6. Finally, ligands L_7 and L_9 are the most promising candidates as potential lead compounds for future AChE-I studies as they maintained excellent results in all experiments.

Keywords: *Alzheimer's Disease, Acetylcholinesterase, Virtual Screening, Drug Discovery, Therapeutics*

Introduction

Alzheimer's disease (AD) is a progressive neurodegenerative disease characterized by memory loss, cognitive deficit, and difficulties conducting personal daily activities. It is the main cause of dementia, accounting for

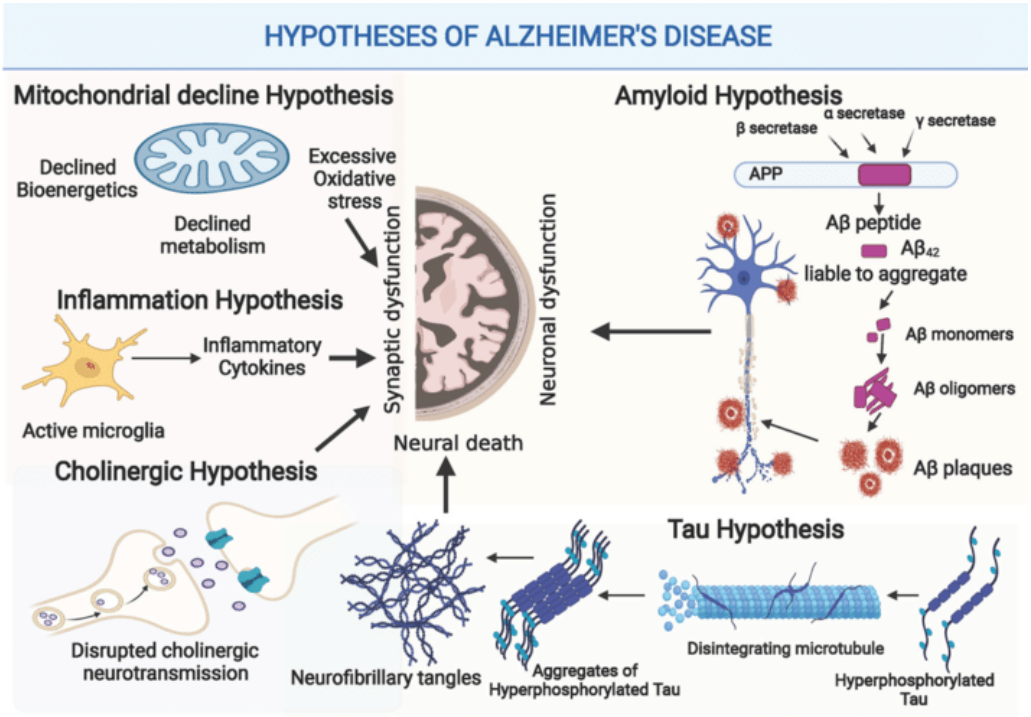
60–70% of the 50 million cases worldwide. It is estimated that there are around 50 million AD patients worldwide, and the numbers are projected to increase to 152 million by 2050 (Breijyeh et al., 2020; Chen et al, 2022). A meta-analysis of treatment costs for Alzheimer's disease estimates that the total costs per patient per year determined by the meta-analysis is $20,461(Marešová et al, 2019). These studies emphasize the severity of the physical and mental toll and the financial burden on the patients and their families, demonstrating the urgent need for improved treatment for the disease (Tahami et al, 2022).

Although the trigger and driving force behind the progression of AD remain unclear, two main hypotheses have been proposed: the cholinergic hypothesis and the amyloid hypothesis. The cholinergic hypothesis attributes the cause of AD to the degeneration of cholinergic neurons, neurons that use and synthesize acetylcholine (ACh) (Stanciu et al, 2020).

ACh, first discovered in 1913 (Tansey, 2006), is an excitatory neurotransmitter involved in several physiological processes such as memory, learning, attention, arousal, and involuntary muscle movement (Sam et al, 2024). Due to its important role in cognition, the deficiency of ACh and the cholinergic neurons is considered to play a significant role in the pathogenesis of AD (Ferreira-Vieira et al, 2016). ACh is synthesized by an enzyme called choline acetyltransferase (ChAT), which causes a reaction between choline and the acetyl group to create acetylcholine. To transmit chemical messages across nerve cells, ACh binds to two types of receptors: nicotinic receptors and muscarinic receptors. To repeat the process, ACh is broken down by the enzyme acetylcholinesterase (AChE) in the synapse into choline and acetate, which can then be reused. These compounds are reabsorbed and recycled to be reused in transmitting additional chemical messages (Cleveland Clinic Medical, n.d.).

The amyloid hypothesis attributes the cause of AD to the buildup of amyloid-β (Aβ) plaque in the brain parenchyma and the cerebral vasculature (Ricciarelli et al, 2017). Although amyloid plaques are found in healthy brains, the human body's ability to properly break down Aβ decreases with age or pathological conditions, thus leading to abnormal accumulation of Aβ peptides. As a result, the buildup creates neurotoxicity and causes neuronal cell death and neurodegeneration (Murphy et al, 2010).

Figure 1. *Illustration of the Cholinergic, Amyloid, Mitochondrial, Inflammation, and Tau Hypothesis of AD (Abuelezz et al, 2021)*



Currently, there are only two types of drugs approved to treat Alzheimer's disease (AD). The most common type includes cholinesterase inhibitors, and the other type consists of N-methyl D-aspartate (NMDA) receptor antagonists such as memantine (Breijyeh, 2020). Recent clinical data has shown that the brains of patients with AD show severe damage to cholinergic neurons, a decrease in ACh levels, and reduced ChAT activity (Chen, 2022). Acetylcholinesterase inhibitors (AChE-Is) help restore cholinergic functions by blocking the breakdown of ACh by AChE and butyrylcholinesterase (BChE), which increase the ACh levels in the synaptic cleft. Currently, the three most used Food-and-Drug-Administration(FDA)-approved AChE-Is are donepezil, galantamine, and rivastigmine. All three types showed their ability to improve AD symptoms. However, rivastigmine has stood out as the most promising drug, with the most desirable safety, tolerability, and efficacy profile, likely due to its ability to inhibit both AChE and BChE (Grossberg, 2003). In July 2023, the FDA approved lecanemab, a new monoclonal antibody. A clinical trial demonstrated that lecanemab effectively removed Aβ plaques in early Alzheimer's disease and showed moderate success in reducing cognitive decline (Van Dyck et al, 2023). Despite showing favorable results, these drugs are associated with adverse side effects such as loss of appetite, diarrhea or vomiting, headaches, feeling tired or dizzy, and difficulty sleeping well ("Dementia Medication", n.d.). Therefore, further research is needed to develop new compounds that minimize these side effects.

Literature review

To tackle the complex multifactorial pathogenesis of AD, recent AChE-Is development focused on the multi-target-directed ligands (MTDLs) strategy which emerges as an advantageous approach compared to combination therapy, which involves using multiple medications to treat a single disease. The MTDL strategy demonstrated its potential by effectively addressing multiple pathological pathways using a single medication, offering additional benefits. These include avoiding potential risks from drug-drug interactions, reducing the likelihood of worsening side effects, and providing a more convenient dosing regimen (Zou et al, 2023). Although the resulting compounds in this study are not experimented to be multi-targeted, they are strong starting points for the future development of this advantageous anti-AD therapy.

Dual AChE and MAO-B inhibitors

Despite AChE being the primary target in AD drug development, the discovery of overexpression of monoamine oxidase B (MAO-B) in the brains of AD patients has identified it as another promising enzyme target for AD therapy. To discover, design, and screen for AChE and MAO-B dual inhibitors, the research utilized nanotechnology and computer-aided drug design (CADD) and incorporated the pharmacophores of anti-AD molecules or drugs. Inhibitors such as chalcone, coumarin, chromone, benzo five-membered ring, imine, and hydrazone scaffolds were systematically classified based on their structure and analyzed by their design strategies, docking studies, and structure-activity relationships (SARs). While dual AChE and MAO-B inhibitors have strong potential to provide significant treatment to AD, there are limiting factors to overcome while developing such inhibitors. One limitation is the structural difference between the catalytic anionic site (CAS) of AChE and the active sites of MAO-B. This structural difference makes it extremely difficult to discover effective dual inhibitors for AChE and MAO-B, as both enzymes often cannot share the same pharmacophores (Zou et al, 2023).

Dual AChE and BACE-1 inhibitors

A new series of multi-targeted donepezil analogues as dual AChE and β-secretase 1 (BACE-1) inhibitors have been designed, synthesized, and evaluated. This new design targets both cholinergic dysfunction and amyloid-β plaque formation and is achieved by introducing backbone amide linkers to enhance BACE-1 inhibition and reduce extracellular cleavage of the amyloid precursor protein (APP). Molecular docking studies confirm the analogues' capability to inhibit both AChE and BACE-1. Additionally, in vitro cytotoxicity testing on SH-SY5Y neuroblastoma cells showed that the new analogues exhibited tolerable toxicity levels and did not negatively

impact cell viability compared to the controls. Furthermore, the analogues demonstrated passive permeability of the blood-brain barrier (BBB) comparable to donepezil, as measured by the parallel artificial membrane permeability assay for BBB (PAMPA-BBB). These encouraging results, particularly with compound 4, highlight the strong potential of these compounds as strong candidates for further therapeutic development (Moustafa et al, 2018).

Dual Targeting of AChE and Tau Aggregation

In search for inhibitors of AChE, BACE1, Aβ1-42 fibrillation, tau aggregation, and α-syn aggregation, deoxyvasicinone analogues are designed, synthesized, and evaluated as potential multi-targeted therapy for AD. The research utilized a pharmacophore combination strategy to design new MTDLs. The compounds were then screened using biological assays to confirm their performance in inhibiting the five targets. Molecular docking studies were also conducted using the Autodock software to predict the binding interaction of the compounds. Finally, physicochemical properties and BBB permeability were analyzed to test their suitability as drug candidates. The result concluded that several MTDLs demonstrated their ability to effectively inhibit both AChE and cellular tau oligomerization. Interestingly, compound 11f has demonstrated greater neuroprotective efficacy, showing the effectiveness of multi-targeted drugs as therapeutic agents for AD and urging further investigation (Shoaib et al, 2021).

Lecanemab clinical trial

Targeted for soluble aggregated Aβ plaques, Lecanemab, a humanized IgG1 monoclonal antibody, was approved by the FDA on July 6, 2023, and is currently undergoing clinical trials ("FDA Converts", 2023). The phase 2 clinical trial for lecanemab, specifically study 201 blinded period(core), was a multinational, multicenter, double-blind, placebo-controlled study of 856 patients randomized to one of five dose regimens or placebo. Subsequently, during the open-label extension (OLE) of the study, the patients were allowed to receive open-label lecanemab 10 mg/kg biweekly for up to 24 months, with an off-treatment period (gap period) ranging from 9 to 59 months (mean 24 months). The study found a significant difference between the drug and placebo group over time and observed key changes to the pathophysiology of the AD patients and a continued drug effect during the gap period. These results indicated lecanemab's strong potential for reducing amyloid plaques and improving symptoms in AD patients. The study also proved the effectiveness of plasma biomarkers as indicators for lecanemab treatment responses. The ongoing phase three clinical trials will further explore these results and the therapeutic potential for lecanemab (Mc Dade et al, 2022).

Research method

Binding Sites Identification

The availability of compatible binding sites for small molecules is crucial in developing drug candidates for enzymes. Multiple factors such as the size, composition, and energy interaction could indicate if an enzyme has suitable binding sites for ligands (Agu et al, 2023). Geometric, machine learning, and energetic-based methods were used to confirm the potential for AChE to provide binding sites for future drug developments. For the purpose of studying binding interactions with small molecules, we used Protein Data Bank(PDB) ID 3LII for AChE for all binding site prediction experiments (Berman et al, 2000).

Geometric Method

DoGSiteScorer is an automatic algorithm from Protein Plus that identifies potential binding pockets in a protein only using the 3D structure of the protein. It also assigns each pocket with a druggability score ranging from 0 to 1 using a support vector machine(SVM) algorithm trained and tested by a druggability dataset consisting of 1069 targets (Volkamer et al, 2010; Volkamer et al, 2012).

Machine Learning Method

Prankweb is an online tool that uses a combination of the 3D structure of a protein accessed through the PDB, evolutionary conservation analysis, and a previously developed machine learning algorithm P2Rank to predict potential ligand-binding sites in proteins (Dávid et al, 2022; Lukáš et al, 2019; Radoslav et al, 2018).

Energetic Based Method

FTSite is an energy-based binding sites detection method with a 94% success rate in

the LIGSITE test set (Kozakov et al, 2015; Ngan et al, 2012; Brenke et al, 2009).

Virtual Screening

Pharmacophore Identification with PocketQuery

To identify lead compounds that can modulate the function of AChE, we first used pocketquery with PDB code 4QWW to search for pharmacophore models that have a higher likelihood of supporting ligand binding. 4 pharmacophore maps were selected based on their high scores and their location in chains C and E which are the antibody chains.

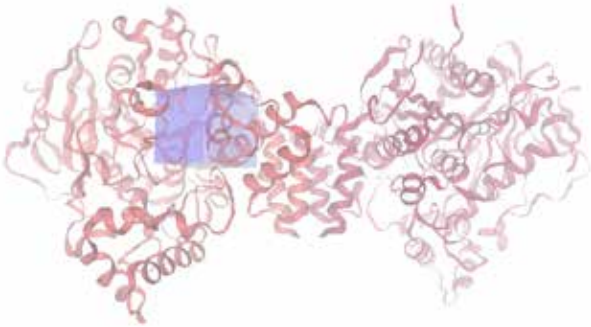
Large-scale Screening with ZINCPharmer

The 4 selected pharmacophore maps are then exported to the online tool ZINCPharmer. A large library of around 18 million compounds was virtually screened to identify ones that have desired fitting with the features of the imported pharmacophores. A few pharmacophore features are deselected before the query to prevent having 0 hits. Afterward, 5 compounds with the lowest Root-mean-square deviation(RMSD) from each of the 4 maps were selected and recorded. Compounds with lower RMSD values have better matching with the pharmacophore models and are thus selected for further experimentation. The 20 compounds are displayed in Tables 4, 6, 8, and 10.

Molecular Docking with SwissDock

The binding affinity of the compounds to AChE was further examined using the Attracting Cavities (AC) 2.0 docking engine from the web service SwissDock (Bugnon et al, 2024; Röhrig et al, 2023). To start the docking session, follow these steps: (i) For the ligand preparations, submit the SMILES for each ligand. These can be accessed through the ZINC database. (ii) For the protein target preparation, submit the PDB ID 4QWW for AChE with only chains A and B being selected. In addition, select "None" for the heteroatoms parameter. (iii) The dimensions of the search box are 15Å by 15Å by 15Å with the center coordinates at (18, -75, -4)Å (Fig. 2.). This search region is considered as it is in proximity to the binding sites with high druggability score found from the results in the binding site identification experiments (4.1). (iv) The number of Random Initial Conditions (RIC) was kept at 1 and other parameters were kept as default. (v) Lastly, click on the "Starting Docking" button to initialize the session. The results from this experiment are displayed in Table 11 with the compound analyzed based on their SwissParam score.

Figure 2. *Position and Size of the Purple Search Box Used for the SwissDock Experiment*



Drug Effectiveness with SwissADME

In order to determine the drug effectiveness of our top compound from the SwissDock experiment, we assessed our compounds based on their absorption, distribution, metabolism, and excretion (ADME) properties. We utilized SwissADME, a free web tool, to determine the physicochemical properties, lipophilicity, water solubility, druglikeness, and pharmacokinetics of our compounds. For lipophilicity specifically, we used the partition coefficient between n-octanol and water (logP) from SwissADME's in-house physics-based iLogP method (Daina et al, 2017). Using these pieces of information, we were able to first determine if each compound passed Lipinski's rule of 5 for good absorption and permeation. Specifically, we ex-

amined if the ligand had a molecular mass of less than 500 daltons, no more than 5 hydrogen bond donors, no more than 10 hydrogen bond acceptors, and no more than 5 for log P. Lastly, we also considered the compound's ability to permeate the BBB and its GI absorption. To run SwissADME, we entered a list of SMILES files for our compounds and clicked "run" to start the calculations. Our results for the compounds with the top ADME result and compounds with the top SwissParam scores are recorded in Table 13.

Toxicity test with ProTox 3.0

Determining the toxicity of a ligand is crucial in developing lead drug compounds. Our study utilized ProTox 3.0, a virtual lab that runs the prediction of toxicities of small molecules (Banerjee et al, 2024). To run Tox-Prediction, we entered the SMILES files for our compounds and clicked "smiles" to upload the compound information. We then selected "all" for our prediction models and clicked "start Tox-Prediction" to run the prediction.

Results and discussion

Binding Sites Identification

AChE is well-suited as a target for drug development as we observed the availability of multiple binding sites for ligands and small molecules in all three of our used binding site detection methods.

Geometric Method Results

Figure 2. *Molecular Model of AChE Binding Sites Predictions Using the DoGSiteScorer Method*

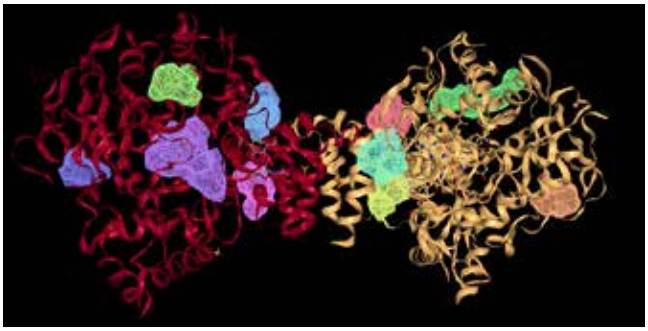


Table 1. *Potential AChE Binding Subpockets And Their Drug Scores Predicted By DoGSiteScorer Ranked By Volume Using PDB ID: 3LII*

	Binding Pockets	Volume (cubic Å)	Surface (square Å)	Drug Score
	P_0	796.24	650.39	0.82
	P_1	771.85	643.47	0.83
	P_10	235.02	378.65	0.35
	P_11	228.41	169.54	0.36
	P_12	223.39	318.79	0.49
	P_13	221.57	341.31	0.34
	P_14	193.3	134.38	0.55
	P_15	188.06	315.58	0.38
	P_16	186.01	147.47	0.57
	P_17	176.89	286.69	0.45

The table above displays the top 10 binding sites predicted by DoGSiteScorer, ranked based on their volume. Binding sites P_0 and P_1 are the largest, with volumes of 796.24 and 771.85 cubic Å, surface areas of 650.39 and 643.47 square Å, and the best drug scores of 0.82 and 0.83. Smaller binding sites like P_15 and P_17 showed significantly worse drug scores, indicating that larger binding sites potentially have better drug scores and stronger binding affinity for small molecules. One notable binding site is P_1

from Protein Plus. It showed up in similar locations as Rank 2 from Prankweb and the green highlighted site from FTSites. Results from DoGSiteScorer display that this binding site consists of 37 residues with the most abundant residues being tryptophan (TRP), tyrosine(TYR), and glycine (GLY), each appearing 7, 6, and 4 times respectively. Another promising binding site is P_0 from Protein Plus and Rank 1 from Prankweb. This binding site consists of 44 residues with the most abundant residues being glycine (GLY), tyrosine (TYR), and serine (SER), each appearing 8, 7, and 4 times respectively.

Machine Learning Method Results

Figure 3. *Molecular Model of Predicted Binding Sites in AChE (PDB ID: 3LII) by PrankWeb*

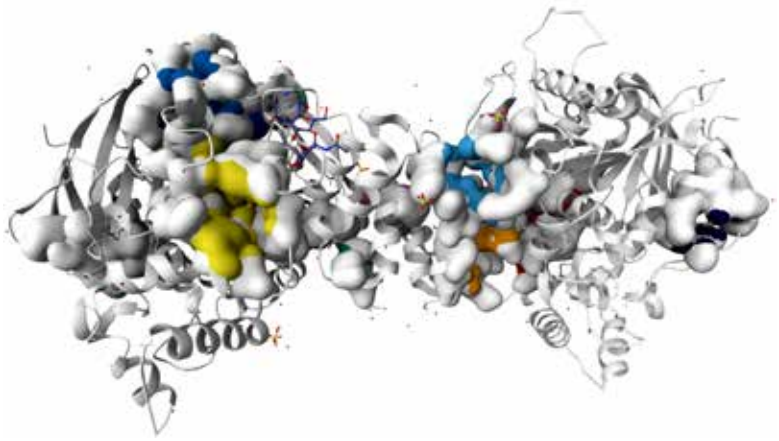


Figure 4. *Close Up of Rank 1 AChE Binding Site*

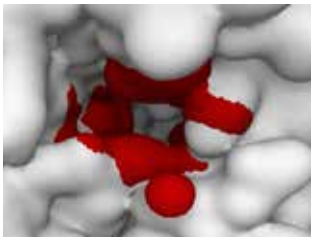


Figure 5. *Close Up of Rank 2 AChE Binding Sites*

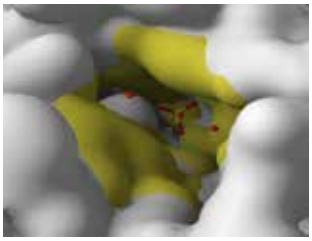


Table 2. *Potential AChE Binding Pockets Predictions by PrankWeb3 Ordered by Score Using PDB Code 3LII*

Rank	Score	Probability	# of residues	Avg conservation
1	29.65	0.915	24	0.765
2	24.12	0.877	25	0.808
3	1.8	0.033	10	0.082
4	1.78	0.032	14	0
5	1.77	0.032	7	0.146
6	1.5	0.021	12	0.09
7	1.4	0.018	6	0
8	1.21	0.012	12	1.606
9	1.18	0.011	12	1.916
10	1.12	0.009	8	0

The table above displays the top 10 binding sites predicted by PrankWeb3, ranked based on their score. Binding sites Rank 1 and Rank 2 are the largest, with 24 and 25 residues and significantly higher drug scores of 29.65 and 24.12.

Energetic-Based Method Results

Figure 6. *Molecular Model of Predicted Binding Sites by FTSite Server in AChE Using PDB code 3LII. Binding Sites are Highlighted with the Colors Pink, Green, and Purple*

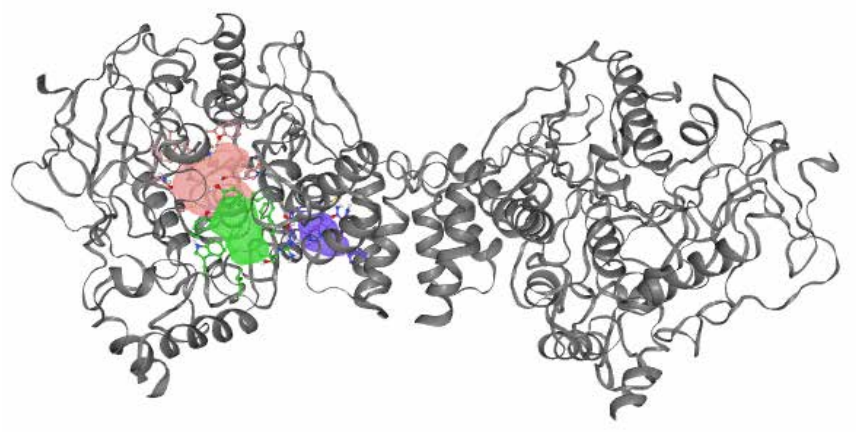


Figure 7. *Molecular Model of Predicted Binding Sites In AChE By PyMol Session Using PDB Code 3LII. Binding Sites are the Blob Shapes Highlighted with the Colors Pink, Green, And Purple. FTsite Identified Three Binding Sites for AChE*

ZINCPharmer Ligand Binding Result

Table 3. *Pharmacophore Map 1 Information Containing Cluster Model, Chain Letter, Residues, Score and Features Considered*

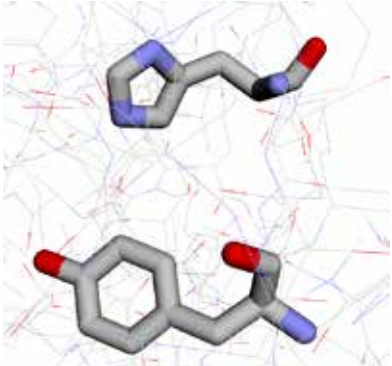
Figure 8. *Pharm Map 1 Model*	**Chain**	**Residue**	**#**	**Pocket Query Score**	**Features Considered**
	C	TYR	90	0.986921	Hydrogen Donor Hydrogen Donor Hydrogen Acceptor Hydrophobic
		HIS	93		

Table 4. *ZINCPharmer Results Based on Pharmacophore Map 1 for the Interaction of 5 Top Compounds with AChE (PDB ID: 4QWW)*

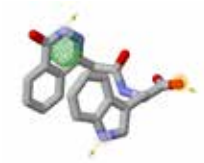
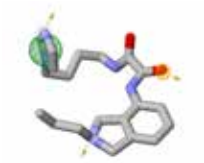
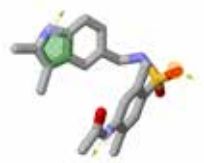
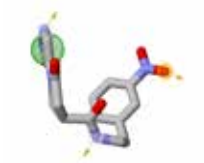
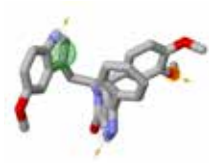
Name	ZINC64380746	ZINC67533629	ZINC19567459	ZINC89306446	ZINC95101290
Structure					
RMSD	0.137	0.137	0.177	0.180	0.184
Mass	390	371	400	343	509

Table 3 provides information about pharmacophore map 1. PocketQuery identifies that pharmacophore map 1 belongs in the C chain with two crucial residues Tyrosine at position 90 and Histidine at position 93. It has a high PocketQuery score of 0.986921. The key features we used for the ZINCPharmer query are 2 hydrogen donors, 1 hydrogen acceptor, and 1 hydrophobic interaction. Table 4 presents the top 5 ligands for pharmacophore map 1. The RMSD values for these ligands range from 0.137 to 0.184, with the median value at 0.177. This indicates that these ligands are a good fit for the Pharm Map 1.

Table 5. *Pharmacophore Map 2 Information Containing Cluster Model, Chain Letter, Residues, Score and Features Considered*

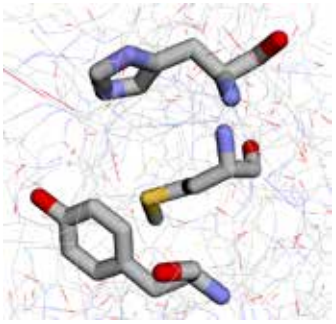
Figure 9. *Pharm Map 2 Model*	**Chain**	**Residue**	**#**	**PocketQuery Score**	**Features Considered**
	C	TYR	90	0.984095	Hydrogen Donor Hydrogen Acceptor Hydrophobic
		HIS	93		
		MET	95		

Table 6. *ZINCPharmer Results Based on Pharmacophore Map 2 for the Interaction of 5 Top Compounds with AChE (PDB ID: 4QWW)*

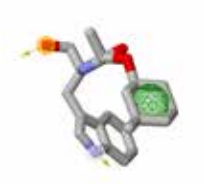
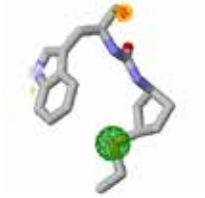
Name	ZINC12232928	ZINC92176885	ZINC69328766	ZINC92189850	ZINC74888813
Structure					
RMSD	0.005	0.007	0.007	0.008	0.010
Mass	388	371	396	358	362

Table 5 provides information about pharmacophore map 2. PocketQuery identifies that pharmacophore map 2 belongs in the C chain with three crucial residues Tyrosine at position 90, Histidine at position 93, and Methionine at position 95. It has a high PocketQuery score of 0.984095. The key features we used for the ZINCPharmer

query are 1 hydrogen donor, 1 hydrogen acceptor, and 1 hydrophobic interaction. Table 6 presents the top 5 ligands for pharmacophore map 2. The RMSD values for these ligands range from 0.005 to 0.010, with the median value at 0.007. This indicates that these ligands are an exceptional fit for the PharmMap 2.

Table 7. *Pharmacophore Map 3 Information Containing Cluster Model, Chain Letter, Residues, Score and Features Considered*

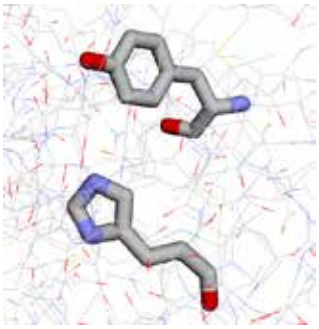
Figure 10. *Pharm Map 3 Model*	**Chain**	**Residue**	**#**	**Pocket Query Score**	**Features Considered**
	E	TYR HIS	90 93	0.981743	Hydrogen Donor Hydrogen Acceptor Hydrogen Acceptor Hydrophobic

Table 8. *ZINCPharmer Results Based on Pharmacophore Map 3 for the Interaction of 5 Top Compounds with AChE (PDB ID: 4QWW)*

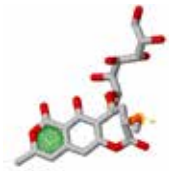
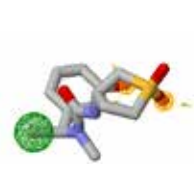
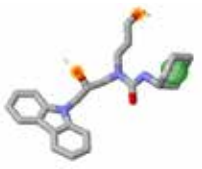
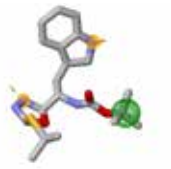
Name	ZINC64684329	ZINC31156228	ZINC67580654	ZINC12249875	ZINC63426782
Structure					
RMSD	0.018	0.021	0.022	0.024	0.025
Mass	426	486	312	424	406

Table 7 provides information about pharmacophore map 3. PocketQuery identifies that pharmacophore map 3 belongs in the E chain with 2 crucial residues Tyrosine at position 90 and Histidine at position 93. It has a high PocketQuery score of 0.981743. The key features we used for the ZINCPharmer query are 1 hydrogen donor, 2 hydrogen acceptors, and 1 hydrophobic interaction. Table 8 presents the top 5 ligands for pharmacophore map 3. The RMSD values for these ligands range from 0.018 to 0.025, with the median value at 0.022. This indicates that these ligands are a great fit for the PharmMap 3.

Table 9. *Pharmacophore Map 4 Information Containing Cluster Model, Chain Letter, Residues, Score and Features Considered*

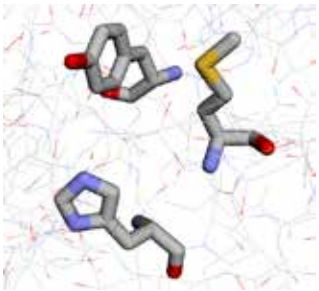
Figure 11. *PharmMap 4 Model*	**Chain**	**Residue**	**#**	**Pocket Query Score**	**Features Considered**
	E	TYR HIS MET	90 93 95	0.981391	Hydrogen Donor Hydrogen Acceptor Hydrophobic

Table 10. *ZINCPharmer Results Based on Pharmacophore Map 4 for the Interaction of 5 Top Compounds with AChE (PDB ID: 4QWW)*

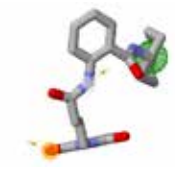
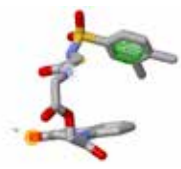
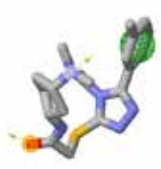
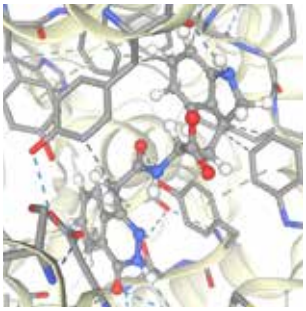
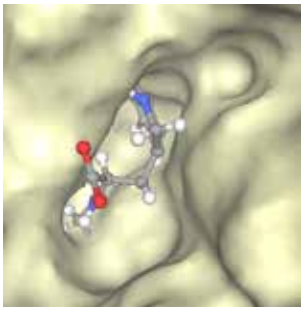
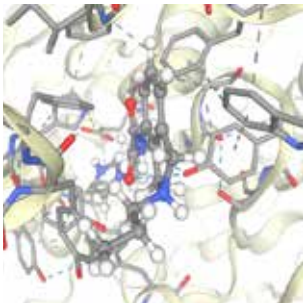
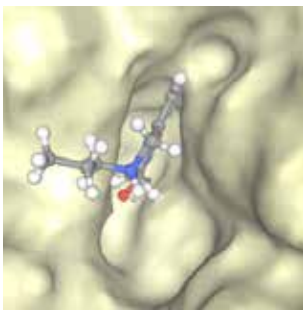
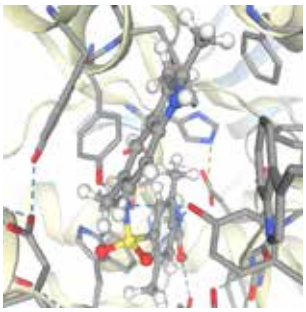
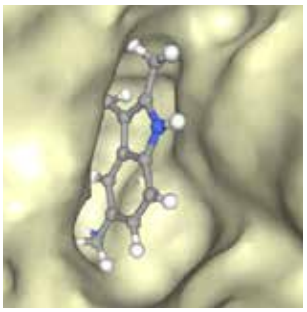
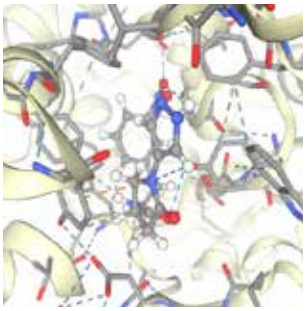
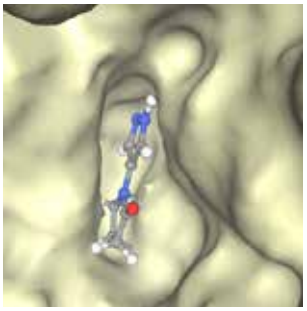
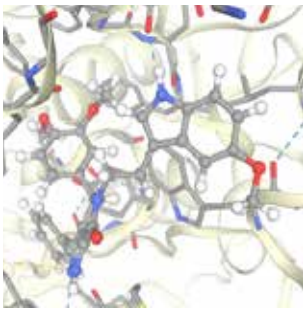
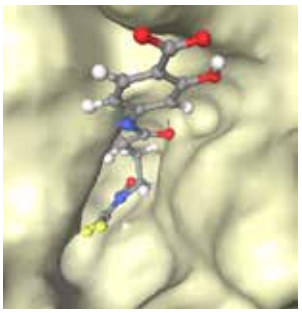
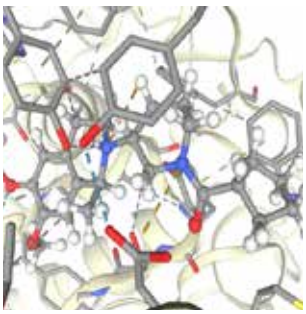
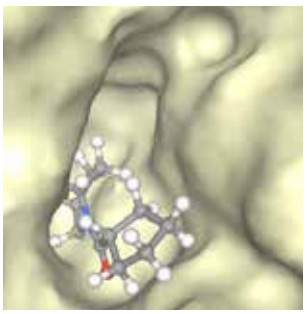
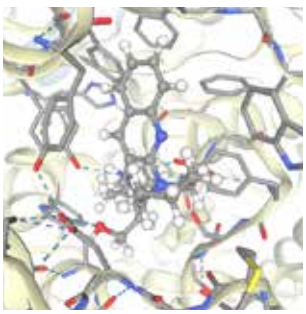
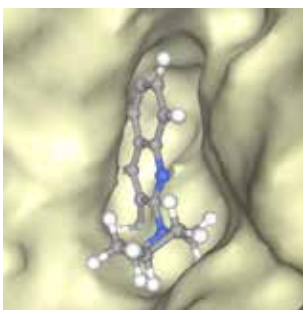
Name	ZINC01211703	ZINC33417589	ZINC03302264	ZINC35638852	ZINC93796225
Structure					
RMSD	0.016	0.018	0.020	0.021	0.021
Mass (dalton)	448	469	489	502	389

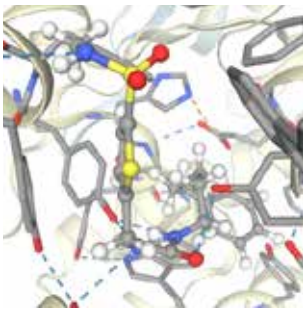
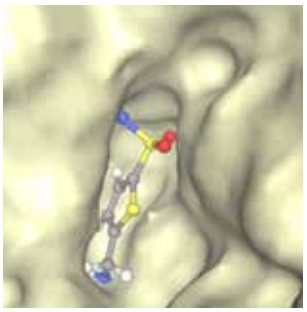
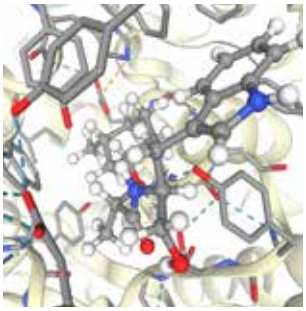
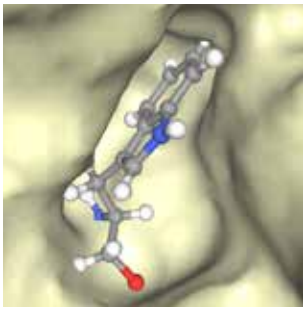
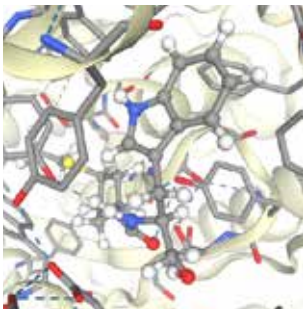
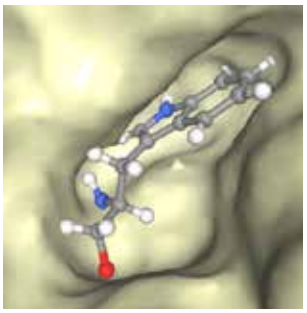
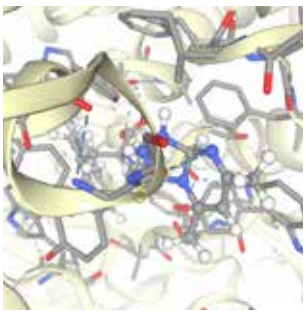
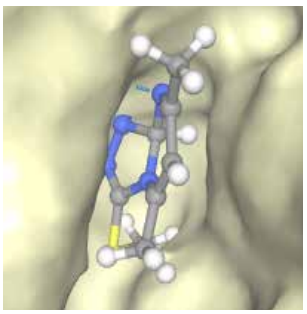
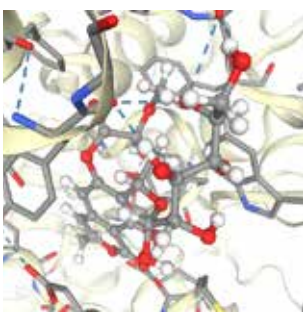
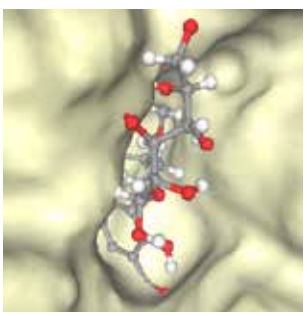
Table 9 provides information about pharmacophore map 4. PocketQuery identifies that pharmacophore map 2 belongs in the C chain with three crucial residues Tyrosine at position 90, Histidine at position 93, and Methionine at position 95. It has a high PocketQuery score of 0.981391. The key features we used for the ZINCPharmer query are 1 hydrogen donor, 1 hydrogen acceptor, and 1 hydrophobic interaction. Table 10 presents the top 5 ligands for pharmacophore map 4. The RMSD values for these ligands range from 0.016 to 0.021, with the median value at 0.020. This indicates that these ligands are a great fit for the PharmMap 4.

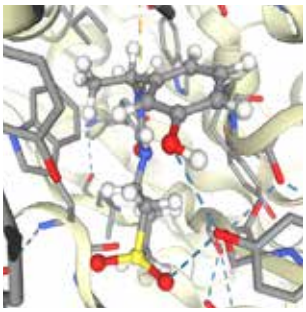
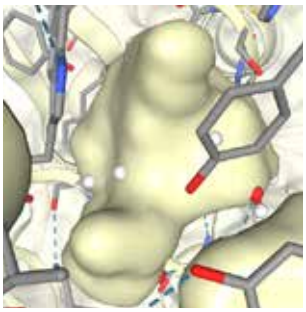
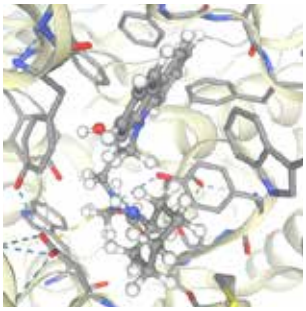
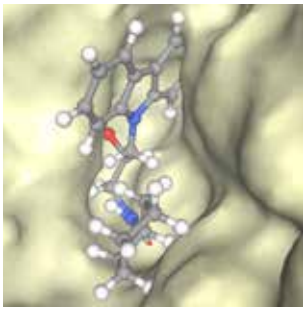
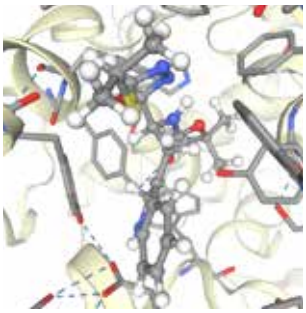
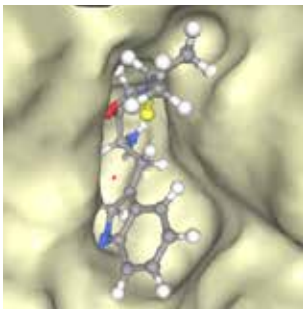
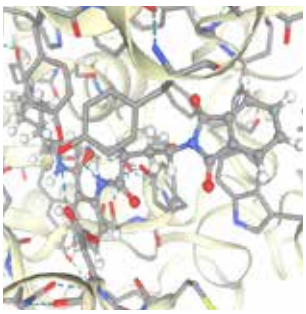
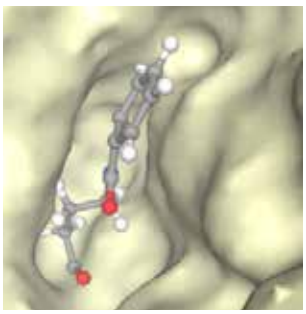
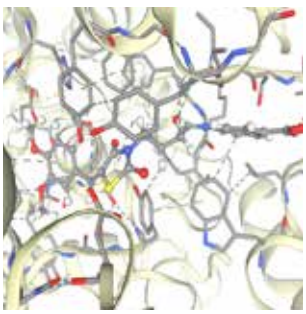
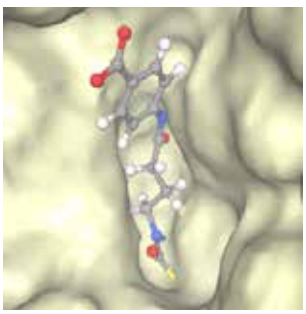
SwissDock Molecular Docking Results

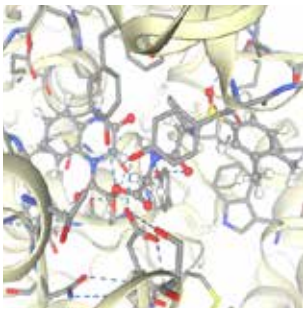
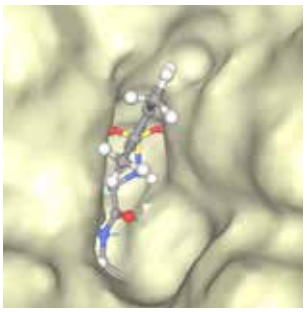
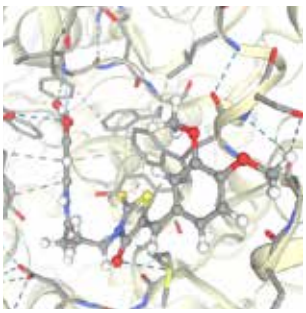
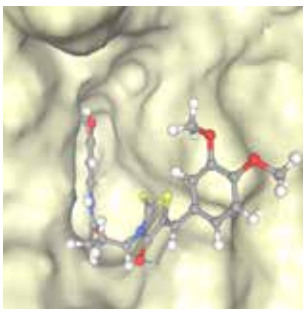
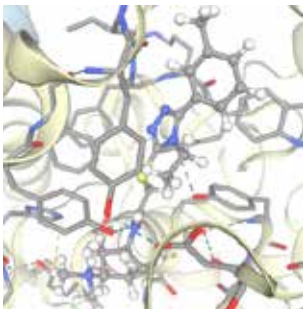
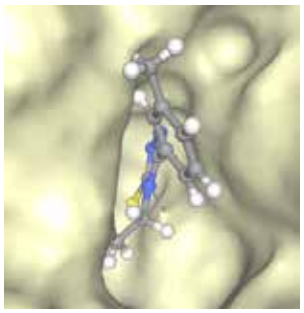
Table 11. *Swissdock Results Showing the Docking of the 20 Compounds with AChE. Two Models (Interactions and Protein Surface) are Presented for each Compound, Along with the Top SwissParam Score*

Name	Pharm Map #	ZINC ID	Cluster#	Model (with interactions)	Model (with protein surface)	Swiss Param Score (kcal/mol)
L_1	1	64380746	13			–7.1876
L_2	1	67533629	0			–8.1535

Name	Pharm Map #	ZINC ID	Cluster#	Model (with interactions)	Model (with protein surface)	Swiss Param Score (kcal/mol)
L_3	1	19567459	0			–8.2314
L_4	1	89306446	0			–7.9297
L_5	1	95101290	4			–8.1756
L_6	2	12232928	2			–7.0061
L_7	2	92176885	2			–7.1795

Name	Pharm Map #	ZINC ID	Cluster#	Model (with interactions)	Model (with protein surface)	Swiss Param Score (kcal/mol)
L_8	2	69328766	6			–8.3006
L_9	2	92189850	1			–7.5941
L_10	2	74888813	2			–7.6825
L_11	3	64684329	1			–7.9125
L_12	3	31156228	0			–7.7121

Name	Pharm Map #	ZINC ID	Cluster#	Model (with interactions)	Model (with protein surface)	Swiss Param Score (kcal/mol)
L_13	3	67580654	1			–7.4535
L_14	3	12249875	7			–7.3507
L_15	3	63426782	0			–7.5266
L_16	4	01211703	0			–8.6741
L_17	4	33417589	1			–7.5992

Name	Pharm Map #	ZINC ID	Cluster#	Model (with interactions)	Model (with protein surface)	Swiss Param Score (kcal/mol)
L_18	4	03302264	1			–8.9425
L_19	4	35638852	8			–7.2905
L_20	4	93796225	2			–7.9142

The data table above shows 20 selected ligands categorized by 4 pharmacophore map groups. The SwissParam score indicates the binding affinity between the ligand and the specific binding site, with the larger negative value indicating a stronger interaction. The SwissParam score for this dataset ranges from -8.9425 to -7.0061, with the median score being at -7.6973. These promising results indicate that many ligands interact well with AChE and are suitable as potential lead compounds for further investigation. For each ligand, the top cluster number and the member with the best SwissParam score are recorded. The cluster number represents a specific binding site on the target protein and the cluster member represents one of many ways a ligand can bind to a cluster. Only the cluster number is displayed. The most common cluster numbers are 0, 1, and 2, with 6, 5, and 4 occurrences respectively. For each ligand, the left side figure shows the interaction between the ligand and its environment, including hydrogen bonds, ionic interactions, cation-π interactions, hydrophobic contacts, and π-stacking interactions. The right side figure shows the ligand docking with the protein surface displayed. Visual analysis of these figures suggests that most ligands used in this experiment bind favorably to one specific site on AChE.

Table 12. *SwissParam Scores of Top Ligands Compared with Popular AChE-Is*

Ligand	Cluster	SwissParam Score (kcal/mol)
Donepezil	1	–7.3369
Rivastigmine	1	–7.3763
Galantamine	1	–6.8899
L_18	1	–8.9425
L_16	0	–8.6741
L_8	6	–8.3006
L_3	4	–8.2314
L_5	0	–8.1756

Comparing the SwissParam score of our compounds with popular AChE-I like donepezil, rivastigmine, and galantamine, we found that our top compounds have stronger binding interaction scores than these FDA-approved compounds.

Ligand Absorption, Distribution, Metabolism and Excretion(ADME) Results

Table 13. *SwissADME Result of Top Ligands with Information on the Physicochemical Properties, Water Solubility, Lipophilicity, and Pharmacokinetics of each Compound*

Name	Lipinski	LogP	#H-bond acceptor	#H-bond donor	Molecular Weight (g/mol)	BBB permeant	Water Solubility	GI Absorption
L_6	Yes	3.66	3	1	387.54	Yes	Moderately Soluble	High
L_7	Yes	3.31	3	2	370.51	Yes	Moderately Soluble	High
L_9	Yes	3.01	3	3	358.47	Yes	Moderately Soluble	High
L_16	Yes	3.38	4	2	447.53	No	Moderately Soluble	High
L_3	Yes	2.57	4	3	399.51	No	Moderately Soluble	High
L_8	Yes	2.76	4	3	395.54	No	Soluble	High
L_5	No	2.84	5	2	508.57	No	Poorly Soluble	High
L_18	No	1.59	8	3	488.51	No	Soluble	Low

Table 13 shows that most compounds are able to pass Lipinski's rule with the last two compounds each having 1 violation. Overall, L_6(ZINC12232928), L_7(ZINC92176885), and L_9(ZINC92189850) are our top 3 drug candidates as they showed promising ADME results while having BBB permeability and high GI absorption. The 5 compounds with the highest SwissParam score had poor ADME results with none of them being able to have BBB permeability and three of them being less soluble than other compounds.

Ligand Toxicity Prediction Results

Figure 12. *Toxicity Radar Chart for L_16*

Compound **L_16:**
Predicted LD50: **2450 mg/kg**
Predicted Toxicity Class: **5**

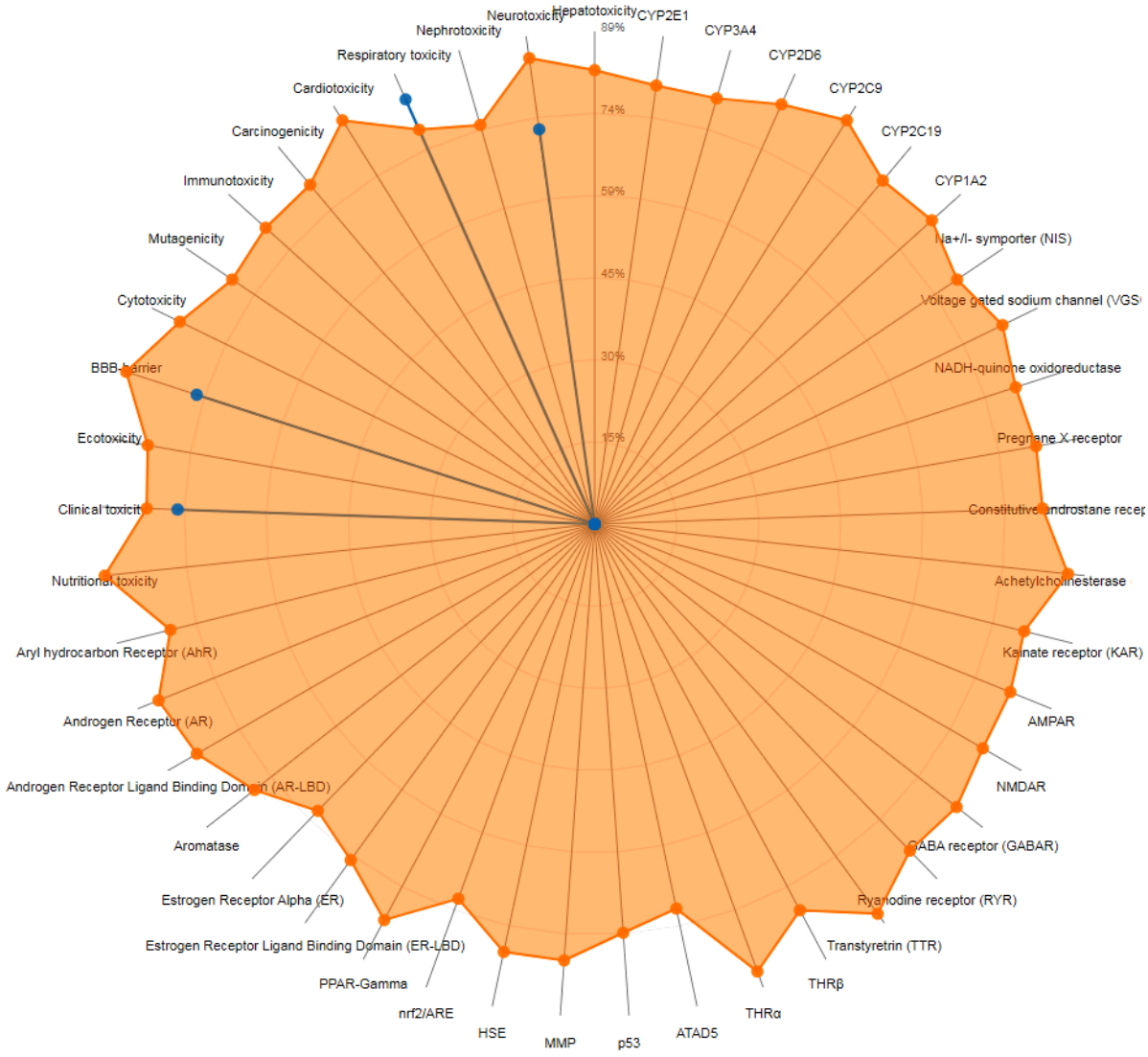


Figure 13. *Network Chart for L_16*

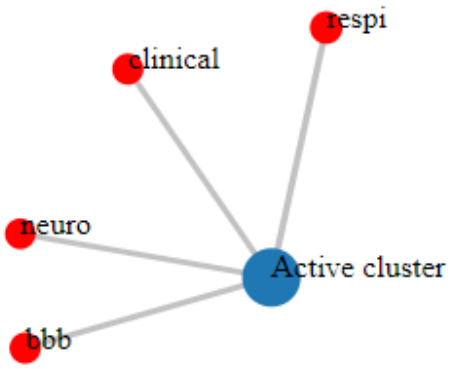


Figure 14. *Toxicity Radar Chart for L_3*

Compound **L_3:**
Predicted LD50: **2800 mg/kg**
Predicted Toxicity Class: **5**

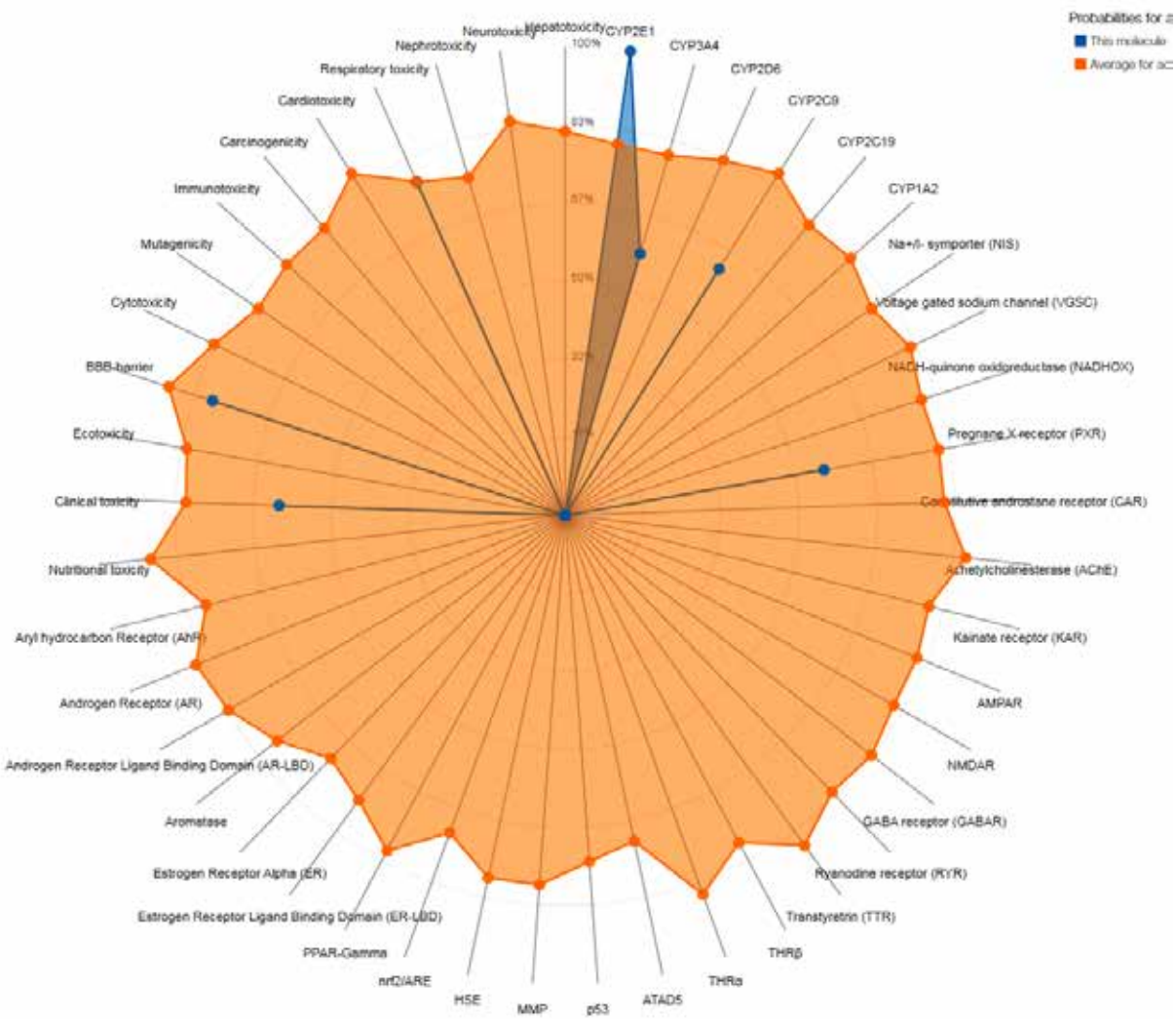


Figure 15. *Network Chart for L_3*

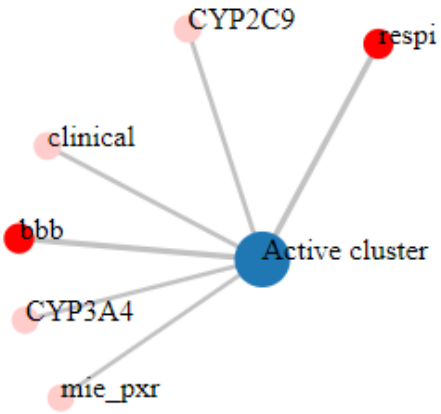


Figure 16. *Toxicity Radar Chart for L_8*

Compound **L_8:**
Predicted LD50: **1000 mg/kg**
Predicted Toxicity Class: **4**

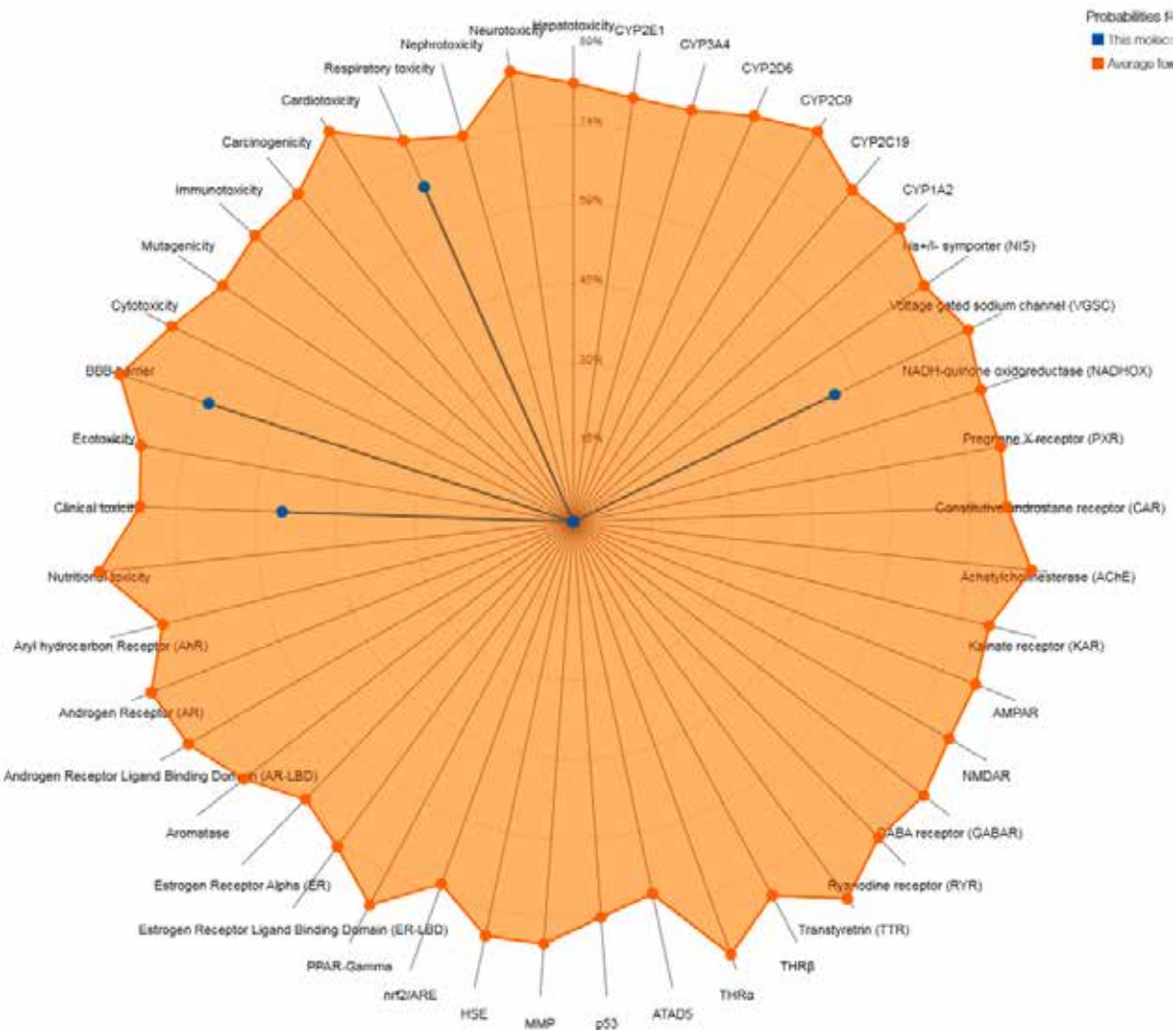


Figure 17. *Network Chart for L_8*

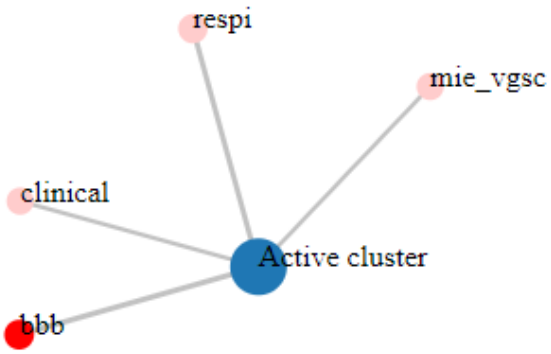


Figure 18. *Toxicity Radar Chart for L_5*

Compound **L_5:**
Predicted LD50: **2800 mg/kg**
Predicted Toxicity Class: **5**

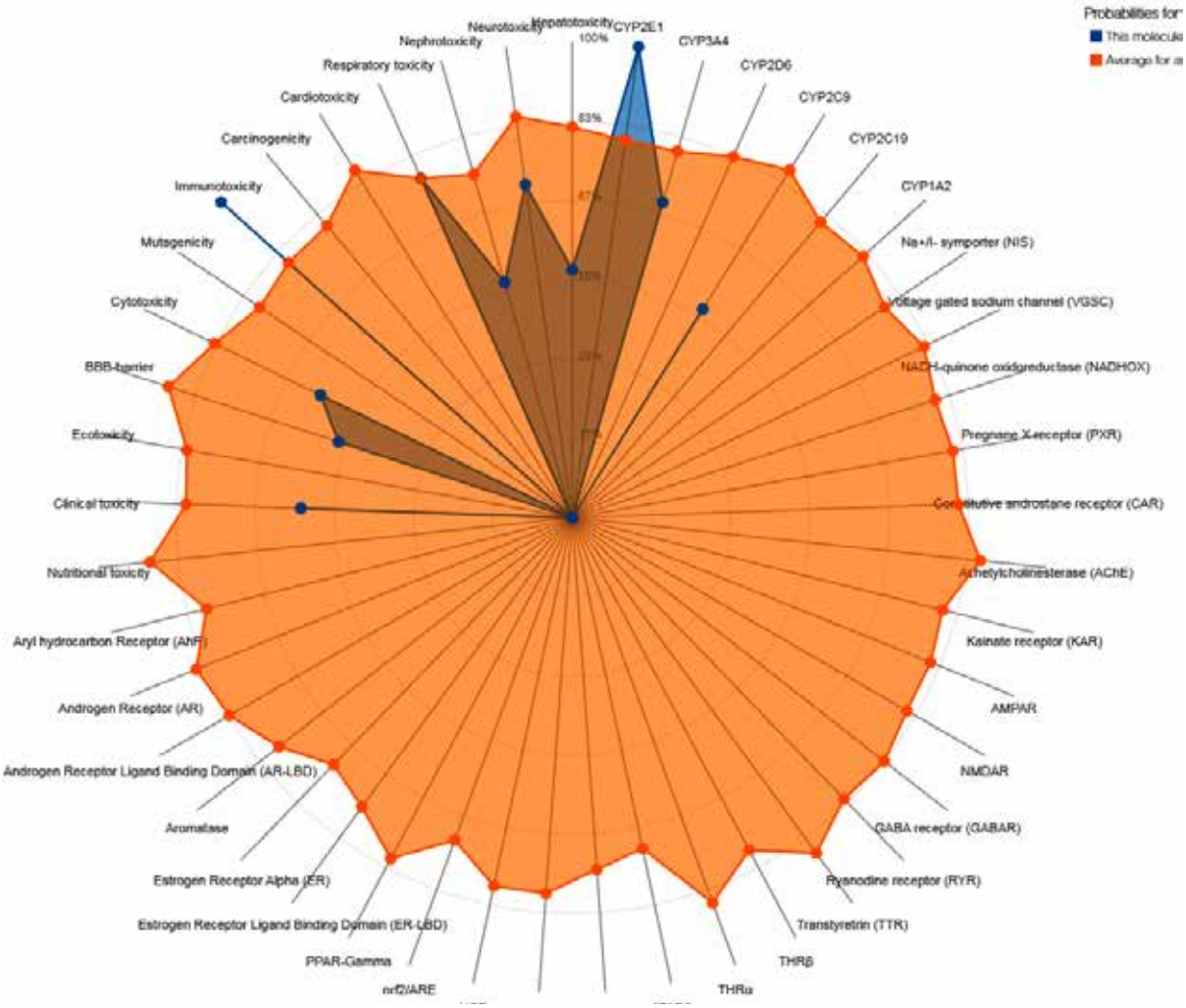


Figure 19. *Network Chart for L_5*

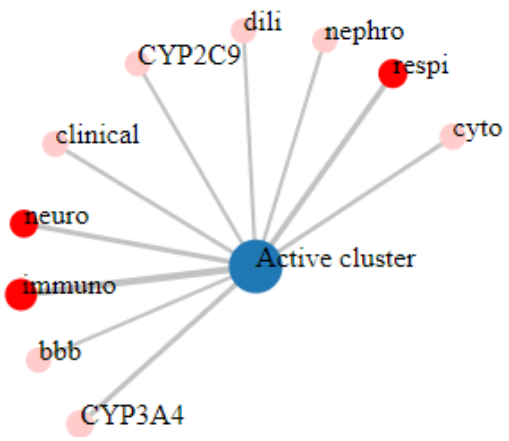


Figure 20. *Toxicity Radar Chart for L_18*

Compound **L_18:**
Predicted LD50: **5240 mg/kg**
Predicted Toxicity Class: **6**

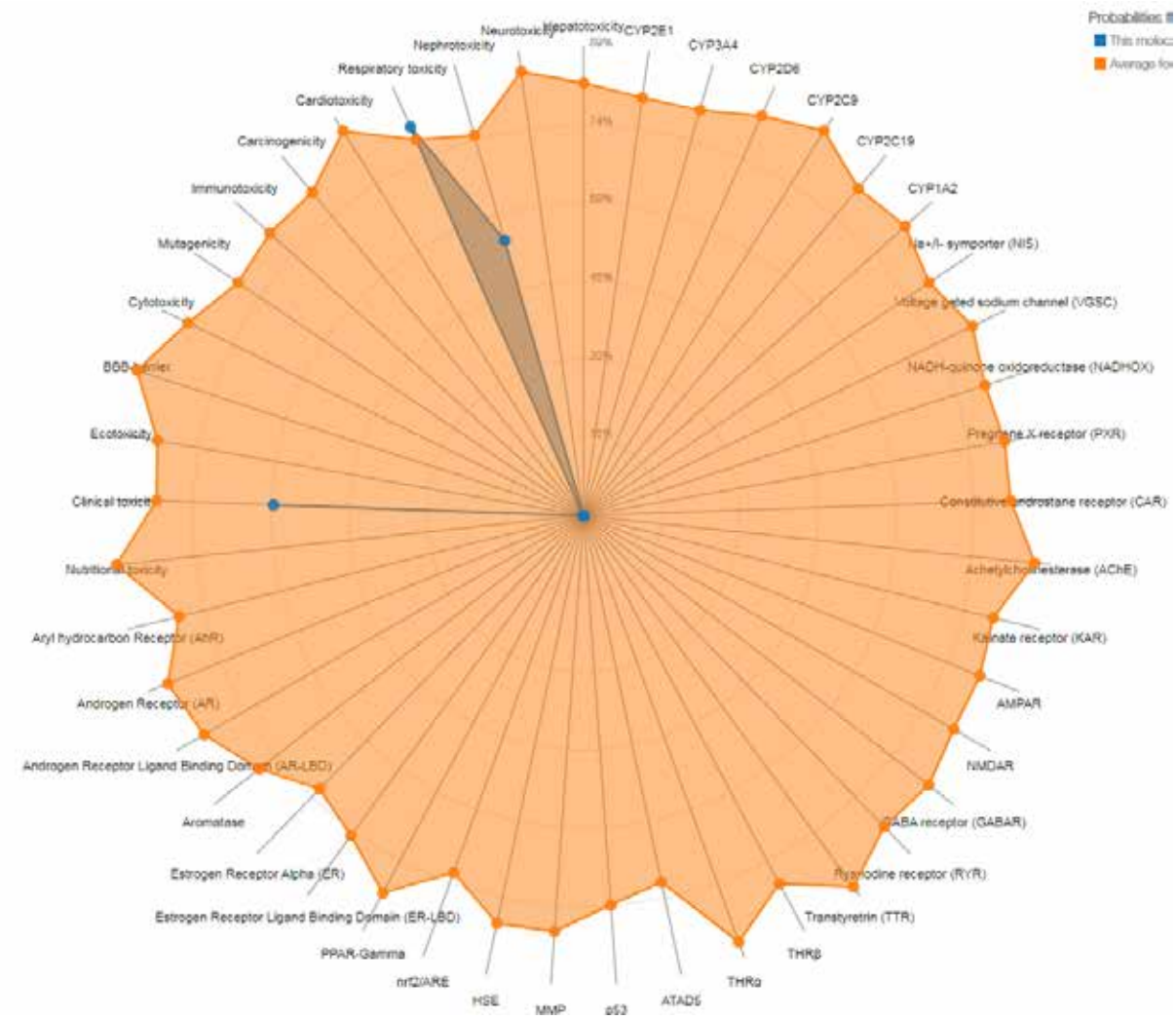


Figure 21. *Network Chart for for L_18*

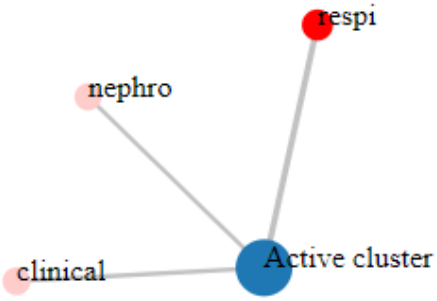


Figure 22. *Toxicity Radar Chart for L_6*

Compound **L_6:**
Predicted LD50: **75mg/kg**
Predicted Toxicity Class: **3**

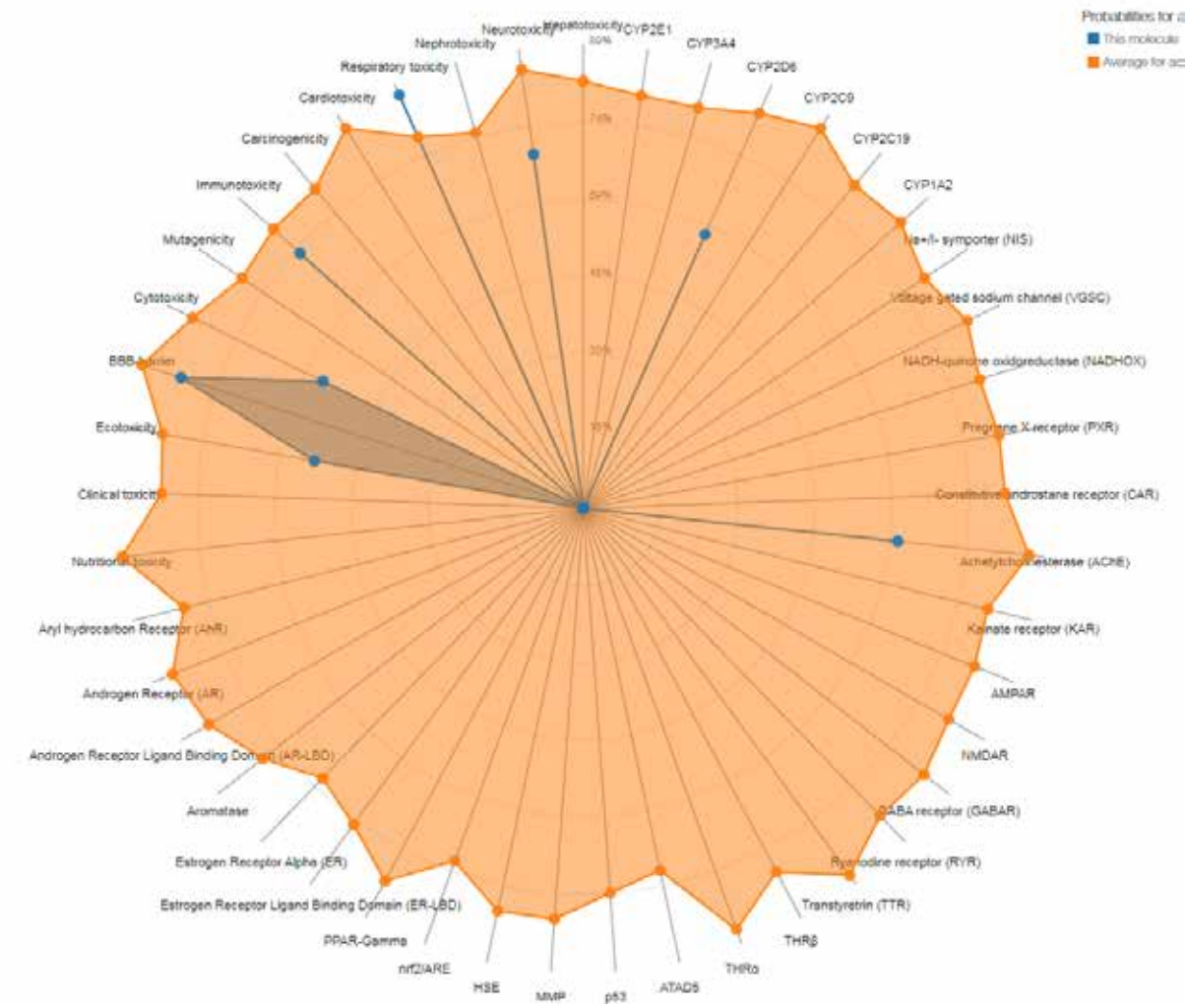


Figure 23. *Network Chart for L_6*

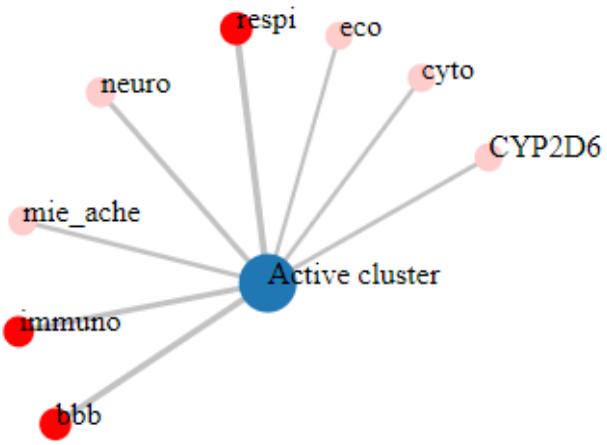


Figure 24. *Toxicity Radar Chart for L_7*

Compound **L_7:**
Predicted LD50: **1190 mg/kg**
Predicted Toxicity Class: **4**

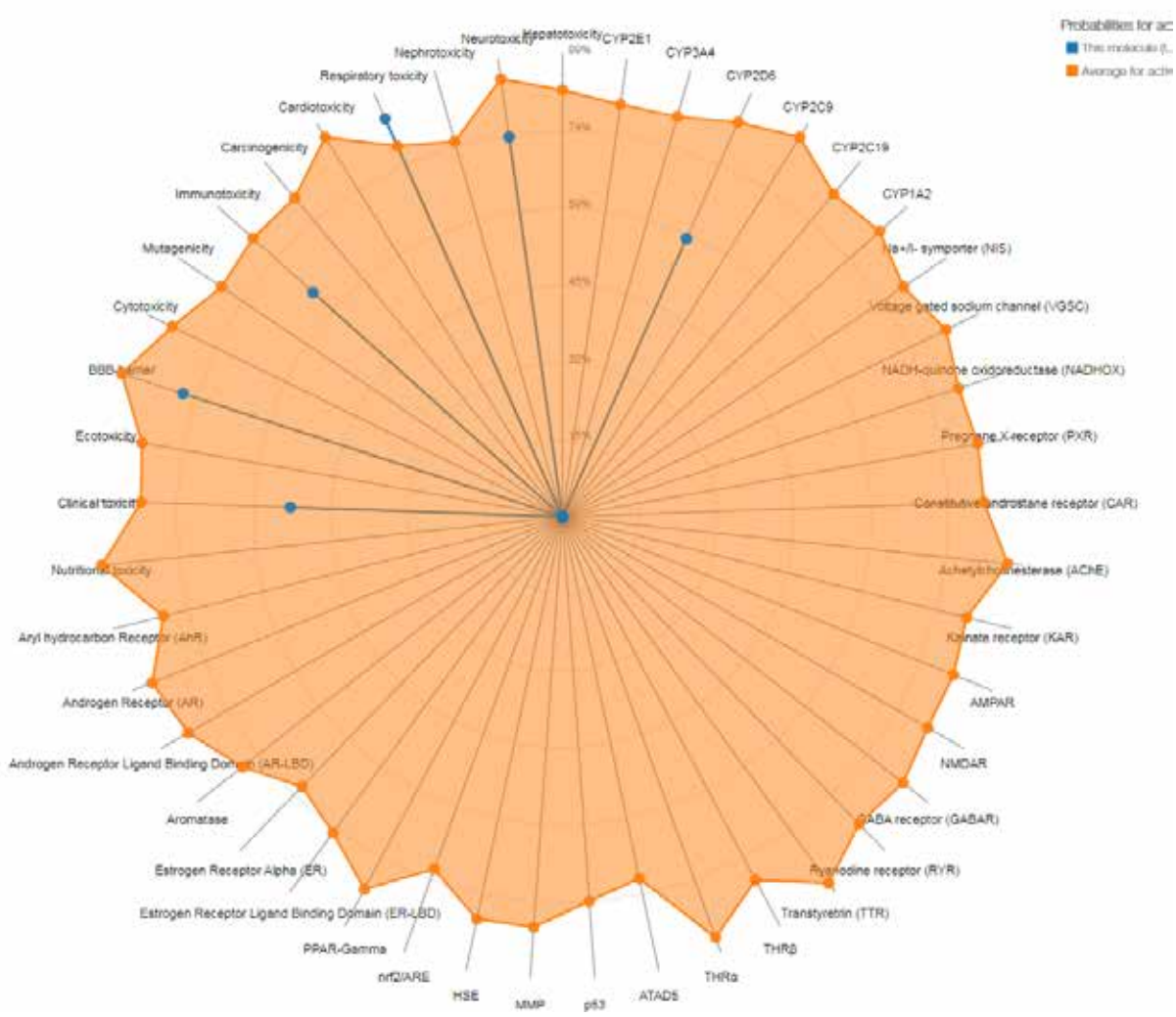


Figure 25. *Network Chart for L_7*

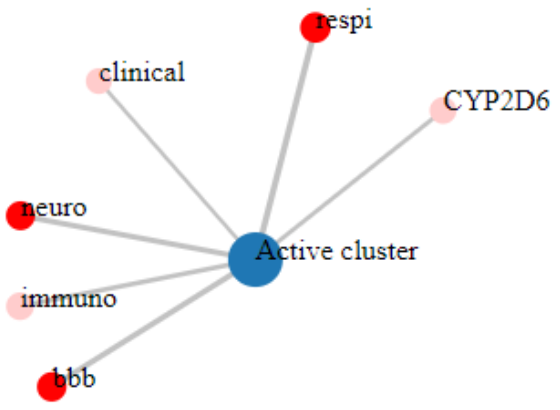


Figure 26. *Toxicity Radar Chart for L_9*

Compound **L_9:**
Predicted LD50: **800 mg/kg**
Predicted Toxicity Class: **4**

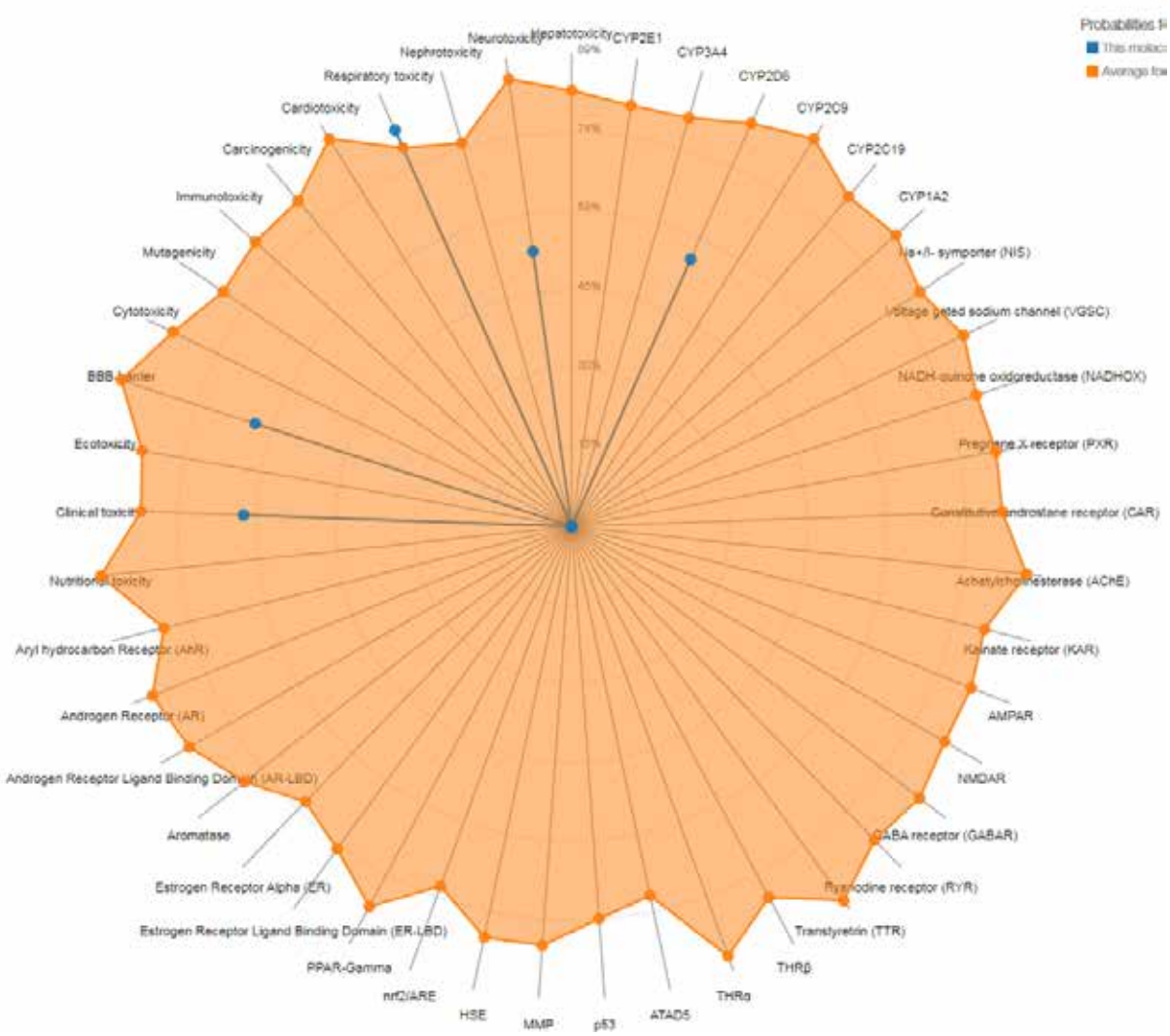


Figure 27. *Network Chart for L_9*

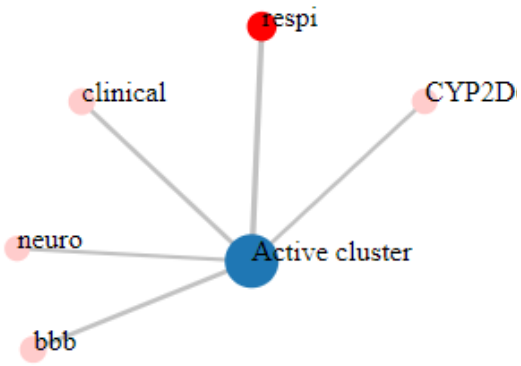


Table 14. *ProTox Toxicity Class Description*

Class 1	fatal if swallowed	(LD50 ≤ 5)
Class 2	fatal if swallowed	(5 < LD50 ≤ 50)
Class 3	toxic if swallowed	(50 < LD50 ≤ 300)
Class 4	harmful if swallowed	(300 < LD50 ≤ 2000)
Class 5	may be harmful if swallowed	(2000 < LD50 ≤ 5000)
Class 6	non-toxic	(LD50 > 5000)

LD50: the median lethal dose meaning the dose at which 50% of test subjects die upon exposure to a compound (SwissADME. Retrieved from URL: http://www.swissadme.ch/. Accessed July 16 2024)

Our results from Pro Tox 3.0 show that most compounds have a toxicity class of 4 or 5 and are harmful or may be harmful if swallowed. The network chart showed us that the clusters most heavily affected by the drugs are respiratory, neurological, clinical, and BBB. Overall, our compound has a median LD50 of 1820 mg/kg with outliers L_18 being surprisingly non-toxic(LD50 of 5240 mg/kg) and L_6 being toxic if swallowed(LD50 of 75 mg/kg). The data for L_6 specifically suggest that although these compounds have strong binding interaction and favorable ADME results, they could be harmful and might require modifications to improve toxicity levels. Regardless, most compounds tested in the Pro-Tox experiment showed appropriate toxicity classes of between 4 and 5.

Statement of limitation

This research has potential limitations. Due to the time constraint of the study, only the top compounds are reported as the results, leading to a limited sample size. Only 4 pharmacophore maps with the top scores were selected from Pocketquery to be used for the ZINCPharmer experiment. In Pocketquery, there are hundreds of other pharmacophore maps for AChE with scores above 0.9 that could potentially lead to new promising ligands. In addition, only 5 compounds with the highest RMSD scores for each of the 4 pharmacophore maps are recorded and analyzed from ZINCPharmer. This limits our sample size for the following SwissDock, SwissADME, and ProTox experiments to only 20 compounds. Additionally, our results from the SwissDock experiment showed that most of our top compounds only dock to one specific binding site on AChE. This is in contrast to the results from our binding site detection experiment, which showed multiple promising binding sites for small compounds. This discrepancy is likely due to the time limit constraint for the search box on SwissDock, leading to only a portion of the whole AChE enzyme being examined. These limitations could lead to the exclusion of many compounds with potentially promising results. To overcome these limitations, one direction for future studies in the short term is to expand the sample size of the study and examine a larger series of compounds. In addition, the study could explore other regions of AChE and examine the molecular docking of compounds with other binding clusters.

In this study, the 20 compounds selected for further experimentation had the highest RMSD scores from the ZINCPharmer experiment. The series of compounds explored in this study could be biased towards their structural affinity to their respective pharmacophore maps. This bias potentially excluded other important factors in drug development such as binding energy interactions, ADME, and toxicity. This leads to tradeoffs in different characteristics of the compound. For example, L_16(ZINC01211703) has the highest SwissParam score of –8.9425 kcal/mol and the best toxicity result, however, it has unacceptable ADME results. Additionally, L_6(ZINC12232928) has a SwissParam score of – 7.0061 kcal/mol and has favorable ADME results, however it is highly toxic, with a predicted LD50 of 75 mg/kg. One potential way to avoid this limitation in future virtual screening studies is to select the top compounds based on a multifactorial analysis of their characteristics and test results instead of only focusing on their RMSD scores. Re-

searchers can give a score for the compound for each factor considered for drug development, and create a composite score with the scores for each factor weighted based on their respective importance. This method might lead to more consistent results as opposed to considering only one factor to narrow compounds for further experimentation.

All experiments in this study are computational and done on free online web tools. These online models and algorithms might produce inaccurate results and fail to precisely simulate real-world interactions. Not all pharmacophore features were selected in the ZINCPharmer experiment which might lead to slightly inaccurate results. Although virtual screening offers a speedier and relatively accurate way to narrow down a large library of compounds, physical screening is needed to validate these results.

Conclusion

Alzheimer's disease (AD), a neurodegenerative disease characterized by memory loss and cognitive deficit, is currently accounting for 60–70% of the 50 million dementia cases worldwide. The costliness and adverse side effects of current AD treatments call for new improved therapy of AD that offers strong efficacy while having tolerable toxicity and favorable ADME. Acetylcholinesterase inhibitors (AChE-I), compounds that are about to reduce the breakdown of acetylcholinesterase (AChE), have shown promising efficacy in recent years in reducing cognitive decline symptoms in AD patients. Our research identifies and examines potential lead compounds for developing better AChE-I using a variety of online virtual screening tools. We first utilized geometric, machine learning, and energetic-based methods to confirm the availability of binding sites for small compounds on AChE. Using Pocketquery and then ZINCPharmer, we were able to narrow down a large library of compounds based on their structural affinity to the features of the top 4 pharmacophore maps we selected from AChE. Using SwissDock, we further narrow down ligands L_18(ZINC03302264), L_16(ZINC01211703), L_ 8(ZINC69328766), L_3(ZINC19567459), and L_ 5(ZINC95101290) as the compounds with the strongest binding interaction with AChE. This series of ligands has a median SwissParam score of -8.3006 kcal/mol, which is nearly 1 kcal/mol higher compared to currently FDA-approved AChE-I. However, in ADME screening, ligands L_6(ZINC12232928), L_7(ZINC92176885), and L_9(ZINC92189850) with slightly worse binding interaction score were the only compounds able to cross the BBB and pass Lipinski's rule. After testing these compounds for their toxicity using ProTox 3.0, ligands L_7 and L_9 maintained excellent testing results as they are our most promising compounds with high SwissParam scores of – 7.1795 and -7.5941 kcal/mol, favorable ADME results, and acceptable toxicity levels. In future studies, enzymatic assays and biological screening for these compounds can further investigate and confirm the drug properties of these compounds. The efficacy and toxicity of the compounds can be further verified through in vivo and in vitro studies. Additionally, other laboratory techniques such as microscale thermophoresis (MST), surface plasmon resonance (SPR), isothermal titration calorimetry (ITC), and Kd calculations can be conducted to validate the molecular binding interactions of these ligands.

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Contact: benjaminliu0701@gmail.com