Novel dual binding site inhibitors of acetylcholinesterase designed with a carbazole ring using molecular modeling

Dr. Faten Alchab[*]
Mahmood Hilal[**]

(Received 24 / 6 / 2023. Accepted 9 / 8 / 2023)

□ ABSTRACT □

Acetylcholinesterase (AChE) inhibitors play a significant role in Alzheimer's disease (AD). These inhibitors are the FDA-approved first-line AD therapy as they increase the levels of acetylcholine (ACh). In addition, they can also interfere with AD pathophysiology by preventing the accumulation of amyloid beta (Aβ) plaques. This study aimed to find effective and dual binding site inhibitors of AChE using molecular modeling. In this work, a library of chemical compounds derived from carbazole was designed to meet the necessary structural criteria of AChE inhibitors. Molecular docking experiments were conducted for the compounds, and interaction modes with enzyme active sites were evaluated. Docking studies indicated a robust interaction between the active site of AChE and C16, C22 from the carbazole core. Additionally, authors examined the pharmacokinetic properties and blood-brain barrier permeability of these compounds.

Keywords: Acetylcholinesterase; Molecular Modeling; Docking; AChE; Alzheimer's disease.

[*]**Assistant Profeesor, Department of Pharmaceutical Chemistry and Drug Control, Faculty of Pharmacy, Tishreen University, Lattakia, Syria.**
****Postgraduate Student, Department of Pharmaceutical Chemistry and Drug Control, Faculty of Pharmacy, Tishreen University, Lattakia, Syria. mahmoudhilal@tishreen.edu.sy**

مثبطات جديدة ترتبط بموقعين فعالين لأنزيم أستيل كولين استيراز مصممة بوساطة حلقة الكاربازول باستخدام النمذجة الجزيئية

د. فاتن الشب*

محمود هلال**

(تاريخ الإيداع 24 / 6 / 2023. قبل للنشر في 9 / 8 / 2023)

□ ملخّص □

تؤدي مثبطات الأستيل الكولين استيراز (AChE) دورًا مهمًا في مرض الزهايمر (AD). هذه المثبطات هي الخط الأول للعلاج المعتمد من قبل إدارة الغذاء والدواء لداء الزهايمر، حيث تزيد من مستويات الأستيل كولين (ACh). بالإضافة إلى ذلك، يمكن أن تتداخل أيضًا مع إمراضية داء الزهايمر عن طريق منع تراكم لويحات بيتا أميلويد (Aβ). هدفت هذه الدراسة إلى إيجاد مثبطات فعالة وذات ارتباط مزدوج لـ AChE باستخدام النمذجة الجزيئية. في هذا العمل، تم تصميم مكتبة من المركبات الكيميائية المشتقة من الكاربازول لتلبية المتطلبات الهيكلية لمثبطات AChE. تم إجراء دراسات الإرساء الجزيئي للمركبات، وتم تحديد أوضاع التداخل مع الموقع الفعال في الإنزيم. أشارت دراسات الإرساء الجزيئي إلى تفاعل قوي بين الموقع الفعال لـ AChE و المركبين C16 و C22 من نواة الكاربازول. تمت دراسة الخصائص الحركية الدوائية وقابلية عبور الحاجز الدماغي الدموي للمركبات.

الكلمات المفتاحية: أستيل كولين استيراز، النمذجة الجزيئية، الإرساء، AChE، داء الزهايمر.

* مدرس- قسم الكيمياء الصيدلية والمراقبة الدوائية، كلية الصيدلة، جامعة تشرين، اللاذقية، سورية.

** طالب ماجستير -قسم الكيمياء الصيدلية والمراقبة الدوائية، كلية الصيدلة، جامعة تشرين، اللاذقية، سورية.

mahmoudhilal@tishreen.edu.sy

Introduction

Acetylcholinesterase (AChE, EC 3.1.1.7) plays a crucial role in cholinergic brain synapses and neuromuscular junctions. The primary biological role of the enzyme is to rapidly break down the neurotransmitter acetylcholine (ACh) into acetate and choline, thereby terminating impulse transmission AChE inhibitors are commonly used as pesticides, therapeutic agents for various diseases, and unfortunately, as chemical weapons [1]. Butyrylcholinesterase (BChE, EC 3.1.1.8), also known as pseudocholinesterase or plasma cholinesterase, is a nonspecific cholinesterase (ChE) enzyme that hydrolyses many different choline-based esters and primarily found in the liver [2]. Different from AChE, BChE exhibits a faster hydrolysis rate for butyrylcholine compared to Ach [2].

AChE is an enzyme that primarily exists in neuromuscular junctions and synapses associated with ACh transmission in the brain. The molecule displays an ellipsoidal form with the dimensions ~ 45 A° by 60 A° by 65 A°. The monomer of the enzyme is composed of an alpha/beta protein structure that consists of a central mixed beta sheet consisting of twelve strands and is enclosed by fourteen alpha helices [3]. One notable characteristic of the structure is a narrow and deep gorge, which extends for about 20 A° into the enzyme and widens near its base [3].

The catalytic active site (CAS) of AChE is located 4 A° from the bottom of the molecule [4]. Studies on AChE kinetics carried out in the early stages revealed that CAS comprises two distinct subsites, namely the esteratic and anionic subsites. In addition to the two subsites of the catalytic center, AChE has been found to have one or more extra binding sites for ACh and other quaternary ligands, these additional binding sites, known as "peripheral" anionic site(s), are clearly different from the choline-binding pocket in the active site [5-7].

The conversion of ACh occurs within the esteratic region of the AChE active site. This site comprises three crucial amino acid residues that play a role in the breakdown of ACh, namely Ser200, His440, and Glu327 [8]. The right position of ACh in the active site is monitored by the anionic site through the interaction with the ACh quaternary ammonium ion. This site is composed of residues Trp84, Tyr121، and Phe330 [8, 9].

When ACh enter the active site, it passes through the peripheral anionic site (PAS) and the aromatic gorge, wich contains high aromatic amino acid residues content, that is responsible for cation-π interactions between the aromatic amino acids and ACh[10]. Fourteen aromatic amino acid residues including Phe330, Trp279, Phe120, Trp233, Trp432, Tyr70, Tyr121, Phe288, Phe290، and Phe331 are located in the gorge of AChE [10]. PAS forms a barrier around the entrance to the aromatic gorge and is situated on the surface of the enzyme molecule [10]. The non-cholinergic functions of AChE, such as cell adhesion and differentiation, are attributed to PAS [10]. There are some sources that suggest a potential involvement of PAS in the formation of amyloid plaques in Alzheimer's disease [11, 12]. The characteristics of PAS are defined by five specific amino acid residues, namely Tyr70, Asp72, Tyr121, Trp279, and Tyr334, which are numbered according to AChE from *Torpedo californica* [13].

Inhibitors of AChE are known to obstruct the breakdown of ACh, thereby increasing the concentration and duration of the neurotransmitter’s activity. AChE inhibitors are classified as either reversible or irreversible based on their mode of action[14]. Reversible inhibitors, such as competitive or non-competitive ones, are commonly employed therapeutically. However, irreversible AChE modulators are often associated with harmful side effects [14]. Reversible inhibitors of AChE have significant implications in the

pharmacological manipulation of enzyme activity. These inhibitors include various compounds with different functional groups, such as carbamates and quaternary or tertiary ammonium groups. They have been used in the diagnosis and/or treatment of several diseases, including myasthenia gravis, Alzheimer's disease (AD), post-operative ileus, bladder distention, and glaucoma, as well as acting as an antidote to anticholinergic overdose [14].

AD is an irreversible, progressive neurodegenerative disorder associated with memory impairment and cognitive deficit. While the exact neurotoxic mechanism is still a topic of debate, there is compelling pathological and genetic evidence to suggest that Alzheimer's disease is primarily caused by two processes: depletion of ACh [15] and aggregation of amyloid β-proteins (βA) [16, 17]. To alleviate the symptoms of AD, the US Food and Drug Administration (FDA) has approved four drugs that inhibit AChE: which are tacrine, donepezil, galantamine, and rivastigmine. These drugs are used as palliative treatment to manage the symptoms of this debilitating condition [14, 18]. Compounds with tricyclic and heterocyclic structures, such as tacrine, quinolizidinyl, and coumarin derivatives, can interact with both PAS and CAS of AChE by engaging in hydrophobic interactions and T-stacking with the aromatic residues in the enzyme gorge [19-22]. The temporary improvement of cognitive and memory impairment resulting from AChE inhibitors is attributed to the enhancement of cholinergic neurotransmission [23]. The interaction between AChE and the Aβ protein can form stable complexes of AChE-Aβ, which can increase the neurotoxicity of Aβ fibrils [24]. It is known by now that CAS is related to acetylcholine hydrolysis, and PAS to Aβ interactions. Thus, the search for a new dual binding site AChE inhibitor is urgently required for a potential disease-modifying strategy. This leads to studying new molecules through molecular modeling and synthesizing them.

Drug discovery and development can be a lengthy and costly process that demands a lot of effort. Therefore, computer-assisted drug design (CADD) strategies can significantly reduce the time and cost involved in attaining at optimal drug candidates. CADD studies utilize specialized systems to simulate drug-receptor interactions to detect whether the chemical compound can bind to the target of interest or not, as well as to quantify the affinity of the binding domains. Indeed, one key advantage of CADD is the predictive power that enables it to generate *in silico* models to evaluate the pharmacokinetic and toxicological aspects of candidate compounds. This prediction process, specifically with absorption, distribution, metabolism, excretion, and toxicity (ADMET) properties, can help with narrowing down the pool of potential compounds, thereby saving time and resources.

METHODOLOGY

Selection of the crystal structure

When preparing a set of data, all the available X-ray crystalline forms of AChE were collected from the protein data bank (PDB) which is an extensive repository comprising numerous crystalline proteins and macromolecules, some with compounds bound to them. The crystal form (PDB 4w63) was chosen for this study. The authors ensured their study's credibility by conducting a validation process where they used the original ligand extracted from the active site as a point of reference. Following the consistent protocol used, the authors re-docked the ligand into the same site and confirmed the validity of the method by comparing Root Mean Square Deviation (RMSD) values. The results revealed that the RMSD values between the original ligand and the resulting poses were minimal, measuring no more than 2 A° (Table 1) [25].

Table 1. The RMSD report for re-docking ligand4 within the active site of AChE.

Name	Reference	RMSD(A°)
4w63 1	4w63 11	0.4952
4w63 2	4w63 11	0.7443
4w63 3	4w63 11	0.7151
4w63 4	4w63 11	0.6949
4w63 5	4w63 11	0.6832
4w63 6	4w63 11	0.6937
4w63 7	4w63 11	0.6779
4w63 8	4w63 11	0.6060
4w63 9	4w63 11	0.6316
4w63 10	4w63 11	0.6275

Preparing the crystal structure

In order to prepare the protein structure in the 4w63 form for docking, several steps were taken. Firstly, the Chemistry at Harvard Macromolecular Mechanics (CHARMM) force field was applied to the structure and hydrogen atoms were added. This was followed by global system minimization to decrease the energy of the structure without affecting its remaining heavy atoms [26]. Finally, a 9.5 A° sphere was determined around the binding site to identify the region where docking would take place (figure 1). These steps were crucial to ensure that the protein structure was appropriately prepared for docking experiments aimed at designing novel AChE inhibitors.

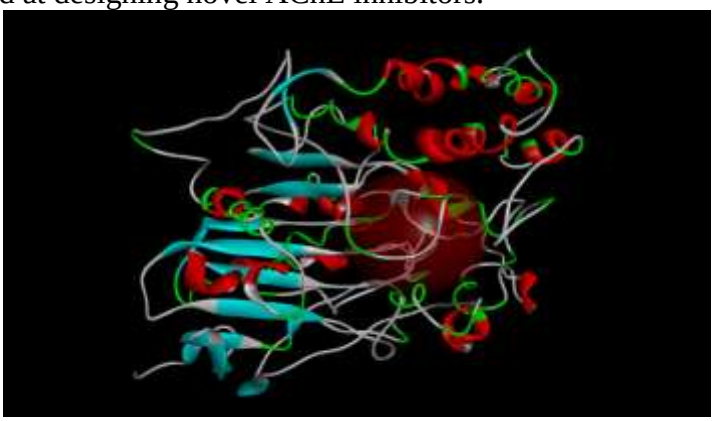


Figure 1. The crystalline form of AChE including 9.5A° sphere

Drawing and filtering compounds

ChemDraw Pro 12.0 program was used to draw the compounds in this study. The authors used Lipinski's Rule of Five to filter all the compounds in their study. This rule outlines the following criteria:

1. The number of hydrogen bond donor groups should not exceed five.
2. The number of hydrogen bond acceptor groups should not exceed ten.
3. The molecular weight should be greater than or equal to 500 Daltons.
4. The Log P value (octanol/water) should be less than or equal to 5.
5. The number of rotatable bonds should be less than 10.

Based on these criteria, the authors filtered out any compounds that did not meet Lipinski's Rule of Five [27].

Molecular docking calculations

The docking study was done using the CDocker model in Discovery Studio 2016 (DS) program, where the CDocker model is an algorithm based on molecular kinetics simulation using CHARMM force field [28]. This study requires supply preparation of all the designed ligands and the previously prepared protein. Each ligand is docked at the active site specified previously, and 10 steric positions were obtained for each ligand. Upon completion of the docking process, the binding mode was studied, and the binding affinity was measured through the -CDocker energy score function.

ADMET methods

The ADMET descriptors protocol in the DS program was employed to examine the properties of absorption, distribution, metabolism, elimination, and toxicity of the compounds. This protocol calculates various parameters such as aqueous solubility, blood-brain barrier (BBB) permeability, CYP2D6 binding, hepatotoxicity, intestinal absorption, and plasma protein binding. Additionally, it computes AlogP98 and PSA_2D, which are utilized in generating confidence ellipses.

It is crucial to compute ADMET properties at an early stage of drug development to prevent the need to discard compounds with unfavorable ADMET properties later in the process, ideally before synthesis. The models utilized to forecast the ADMET characteristics in this protocol are based on diverse experimental data sources.

RESULTS AND DISCUSSION

The significance of inhibiting AChE, coupled with the critical role that molecular modeling plays in the development of new inhibitors for diverse enzymes, prompted the use of molecular modeling in this study to devise novel AChE inhibitors. DS programming and PDB were used to facilitate the design of novel inhibitors.

Data set

To compile a data set, a comprehensive collection of all available crystalline structures of AChE was obtained from PDB. For this particular study, a specific crystalline form of the enzyme in complex with an inhibitor was selected (PDB code: 4w63). The accuracy of the shape was notably precise with an R value of 2.8 A°. The AChE protein crystalline form 4w63 was bound to an inhibitor derived from the Tacrine-Benzofuran hybrid core. A range of amino acid residues located in the active site were deemed significant for binding, including catalytic triad residues Ser200, His440, and Glu327, as well as peripheral anionic site residues Tyr70, Asp72, Tyr121, Trp279, and Tyr334, along with amino acids found in the aromatic gorge Phe330, Trp279, Phe120, Trp233, Trp432, Tyr70, Tyr121, Phe288, Phe290, and Phe331 [29].

Set a library of new chemical compounds

Through a comprehensive review of previous studies and compounds currently used to inhibit AChE, which targets one or more sites within the enzyme structure, and the important amino acids involved in binding, it was found that the presence of aromatic rings in the compound structure is significant to position the compound within the active site. It also forms important bonds with amino acids within the PAS. To achieve inhibition of CAS, a long carbon chain and the presence of a nitrogen atom, which assists in forming a hydrogen bond with SER200 or HIS440, were necessary. The carbazole core was chosen to design new inhibitors for AChE (Figure 2). Functional groups were added to the proposed structure through virtual exchanges, aiding in the binding process with the active site (Table 2).

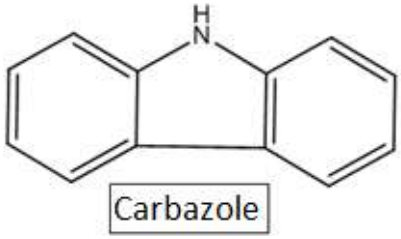
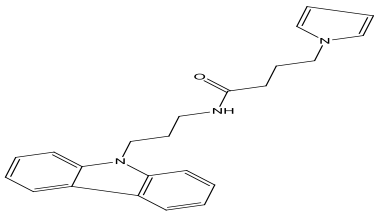
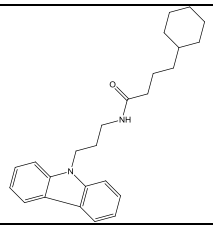
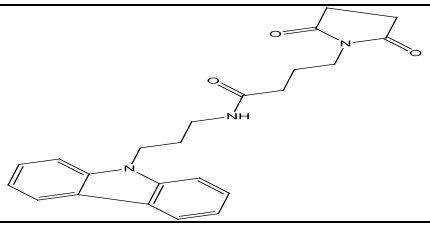
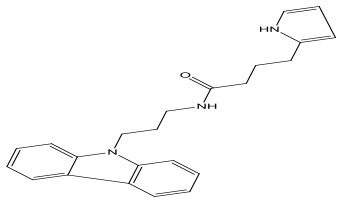
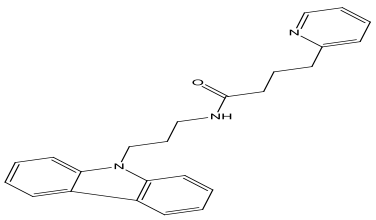
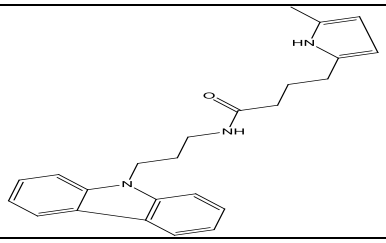
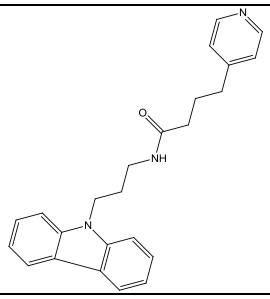
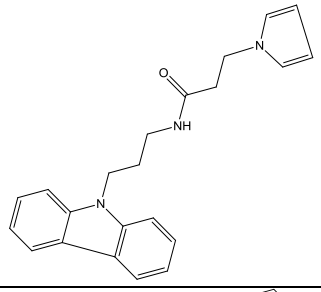
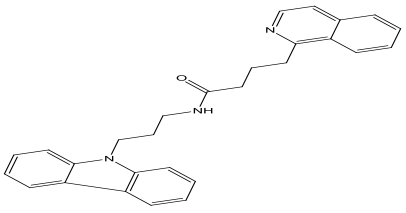
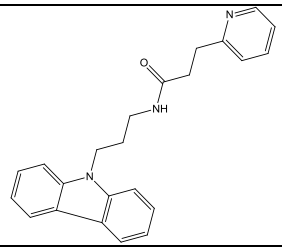
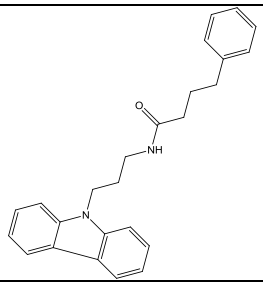
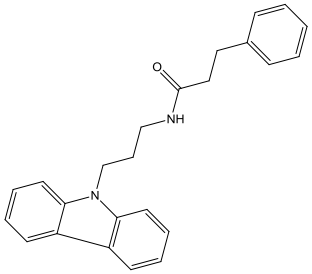
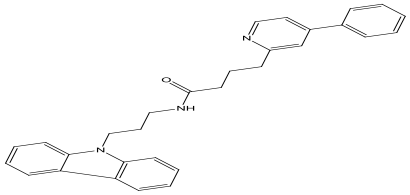
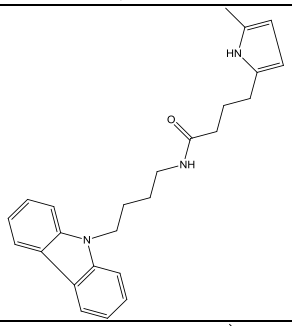
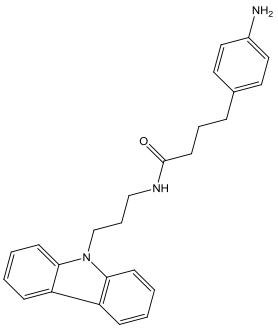
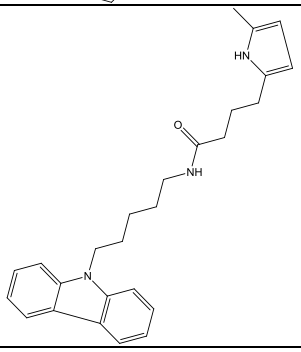
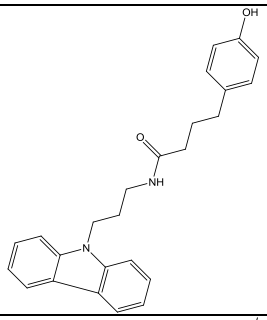
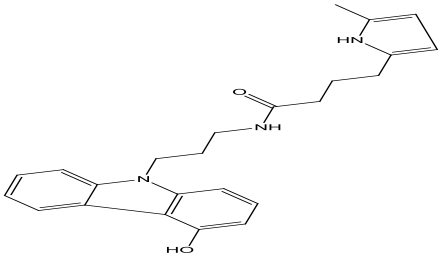
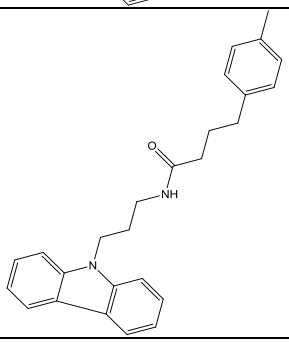
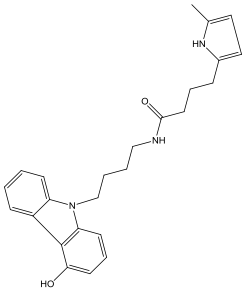
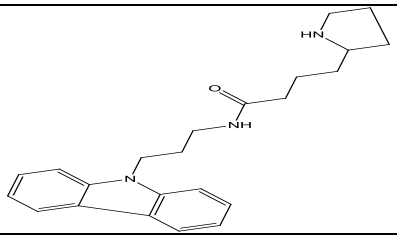
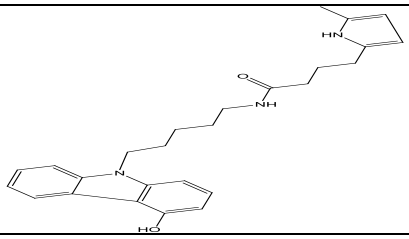


Figure 2. The core used in the study

Table 2. The designed compounds derived from carbazole core.

Compounds	Structures	Compounds	Structures
C1		C12	
C2		C13	
C3		C14	
C4		C15	
C5		C16	

C6		C17	
C7		C18	
C8		C19	
C9		C20	
C10		C21	

C11		C22	

Analysis of docking studies

Table 2 displays the ligands designed based on the carbazole core. The optimal compound was selected based on two key parameters. The first parameter was the score function (-CDocker energy), and the second parameter was its ability to effectively bind with vital amino acid residues within the active sites of AChE (Table 3). The analysis of the ligands also involved a study of the individual contribution of different substituents towards the positioning of the ligand within the active site. The addition of various substituents to the carbazole core allowed a comprehensive study of potential inhibitors within the active pocket, as *per* the principles mentioned previously. The results were extensively analyzed and discussed. Table 4 shows (-CDocker energy) values for the designed compounds.

Table 3. Important amino acid residues according to the active site.

Active site	Important amino acid residues
Catalytic site	Esteratic part: Ser200, His440, and Glu327 Anionic part: Trp84, Tyr121, and Phe330
Peripheral anionic site	Tyr70, Asp72, Tyr121, Trp279, and Tyr334
Aromatic gorge	Phe330, Trp279, Phe120, Trp233, Trp432, Tyr70, Tyr121, Phe288, Phe290, and Phe331

Table 4. -CDocker energy and -CDocker interaction energy values of the compounds derived from carbazole core.

Compounds	-CDocker energy	Compounds	-CDocker energy
C1	47.6414	C12	45.9115
C2	52.4438	C13	51.8913
C3	50.2515	C14	53.3912
C4	50.7992	C15	44.2836
C5	36.3494	C16	44.0698
C6	51.4997	C17	43.7988
C7	42.8107	C18	51.2196
C8	51.2051	C19	54.7889
C9	50.253	C20	54.3604
C10	47.3792	C21	53.5507
C11	35.5943	C22	54.062

The binding of compounds derived from the carbazole core was discussed as mentioned previously, based on the compound's affinity represented by the negative value of CDocker Energy, as well as the compound's important bindings with the amino acid residues in the active sites of AChE. As shown in Table 4, most of the compounds have a very good binding affinity. However, only C16 and C22 managed to make the necessary bonds to inhabit PAS and CAS.

By studying the binding of C16, the authors noticed that the compound has good affinity and was able to achieve bindings by the interactions of the aromatic rings, where the carbazole ring interacted with the amino acids TRP84 - PHE330, and the pyridine ring

interacted with PHE330. Additionally, C16 was found to form important bindings within the active site, as it was able to bind with TYR121 through a hydrogen bond, which is a basic amino acid in PAS. As for CAS, C16 was able to form a bond with SER200 through a hydrogen bond with the nitrogen of the pyridine ring. Consequently, it is expected that C16 can inhibit both CAS and PAS (Figure 3).

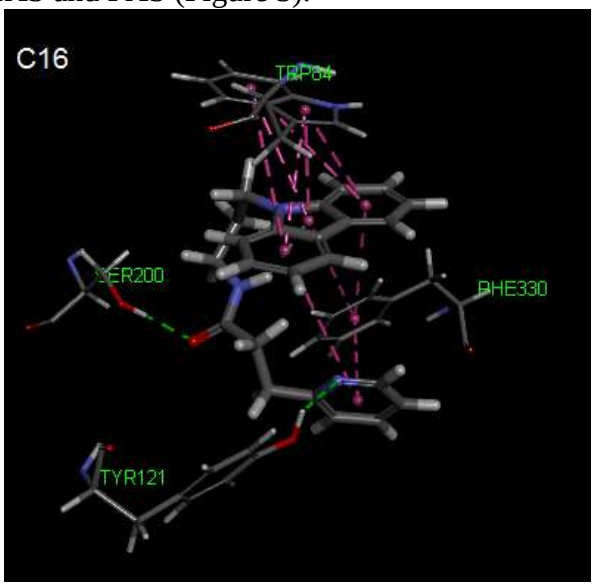


Figure 3. The binding mode between C16 and the active site of AchE.

Studying the binding of the proposed C22 compound, the authors noticed that adding an OH group on position 4 of the carbazole ring and extending the connecting chain between the carbazole ring and the amide by 5 carbon atoms led to a drastic change in the formed bindings. The compound was able to bind with TYR121 through a hydrogen bond, in addition to binding with TYR334 through aromatic ring stacking, which is also a basic amino acid in PAS, ensuring that this compound reaches and inhibits PAS. C22 was able aslo to bind with the amino acid HIS440 through a hydrogen bond with the nitrogen of the pyrrole ring; therefore, CAS was inhibited. In addition there was an aromatic interaction with the amino acid PHE330 between the carbazole and pyrrole rings, providing additional stability for the compound within the active site (Figure 4).

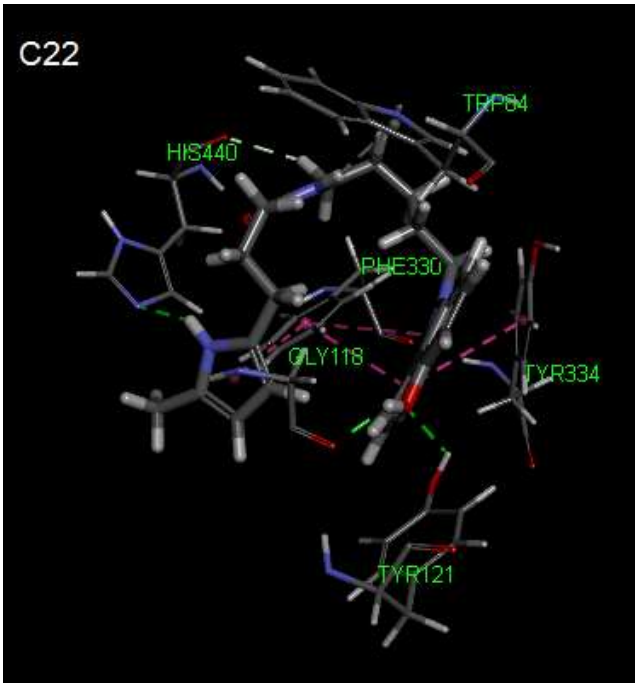


Figure 4. The binding mode between C22 and the active site of AChE.

BBB and ADMET investigation

Discovery Studio 2016 has been employed to investigate the ADMET properties using the protocol-ADMET Descriptors.

the properties of the compounds were analyzed concerning kinetic behavior. Seven crucial properties were analyzed, including intestinal absorption, the capacity to cross the blood brain barrier (BBB), solubility, cytochrome enzyme inhibition likelihood (CYP2D6), hepatotoxicity, binding to plasma proteins. Evaluating these properties is vital to determine the safety and effectiveness of the compounds [30]. (Table 5) and (Figure 5) summarize the results.

Table 5. Pharmacokinetic and toxicological properties of the proposed compounds.

Compound	Absorption	BBB penetration	Solubility	CYP26D inhibition	Hepatotoxicity	PPB	AlogP98	PSA-2D
C16	good	high permeability	low	Non-inhibitor.	true	Binding is <90%.	3.795	46.72
C22	good	high permeability	low	Non-inhibitor.	true	Binding is <90%.	5.298	71.329

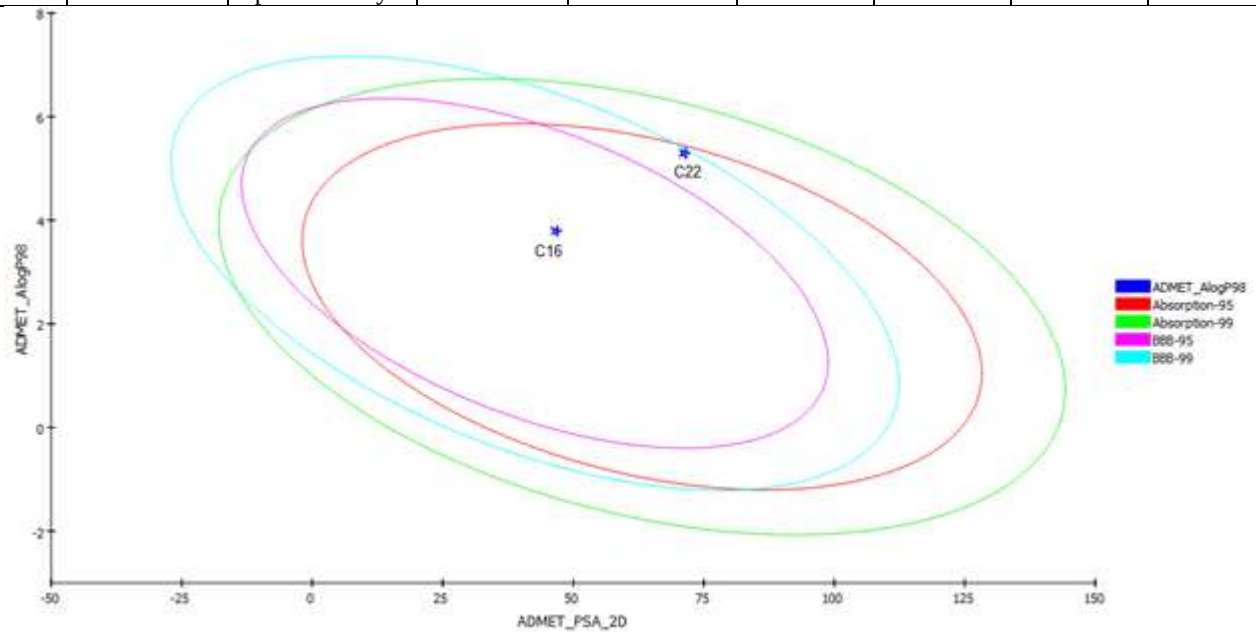


Figure 5. Representative 2D plot of the pharmacokinetic properties of the proposed compound

As *per* the pharmacokinetic model, a molecule should fulfill the below requirements to ensure ideal cellular permeability; the PSA-2D must be less than 140 $A^{\circ 2}$, and the AlogP98 should be less than 5. According to the results, C16 and C22 are expected to have good intestinal absorption. As for the BBB permeability, the compounds were predicted to have high permeability. It was noticed that the coumpounds molecules demonstrates poor water solubility. According to the findings, it is anticipated that C16 and C22 will not interfere with the function of the CYP2D6 enzyme. This outcome suggests that these compounds can be effectively metabolized during the initial phase of metabolism. The forecast implies that the compounds can cause hepatic toxicity. Upon analyzing the predicted value for binding to plasma proteins, it was determined that C16 and C22 hold good bioavailability and they are improbable that they will strongly bind to transport proteins in the bloodstream. In fact, the predictive value for binding to plasma proteins demonstrated a percentage of less than 90%.

CONCLUSION

In summary, it can be noticed that the carbazole ring holds significant importance in positioning within the active site of AChE due to its richness in aromatic amino acids. This leads to an increase in the binding affinity of the compounds based on their -CDocker energy value. Most of the compounds could establish bonds with the basic amino acids of one of the targeted sites, *i.e.*, either CAS or PAS. By modifying the proposed structures, C16 and C22 were able to achieve a high binding affinity and establish important bonds with vital amino acid residues in the active sites PAS and CAS. Therefore, the active sites

get occupied, leading to no interactions with Aβ, and disrupting the hydrolysis of acetylcholine, as well as predicting its high permeability to BBB and a good ADMET profile. These compounds were selected as a promising dual binding site inhibitor for AChE. The Authors' recommendation is to choose these compounds for further examination by conducting synthesis followed by biological tests *in vitro* and *in vivo*.

ACKNOWLEDGEMENTS

I extend my gratitude to the faculty of pharmacy at Tishreen University for their support during this practical examination. Their assistance in providing the necessary protocols and practical techniques for the process was deeply appreciated.

Reference

[1] Tougu, V. (2001). Acetylcholinesterase: mechanism of catalysis and inhibition. Current Medicinal Chemistry-Central Nervous System Agents, 1(2), 155–170. https://doi.org/10.2174/1568015013358536

[2] Huang, Y.-J., Huang, Y., Baldassarre, H., Wang, B., Lazaris, A., Leduc, M., Bilodeau, A. S., Bellemare, A., Côté, M., Herskovits, P., Touati, M., Turcotte, C., Valeanu, L., Lemée, N., Wilgus, H., Bégin, I., Bhatia, B., Rao, K., Neveu, N., … Langermann, S. (2007). Recombinant human butyrylcholinesterase from milk of transgenic animals to protect against organophosphate poisoning. Proceedings of the National Academy of Sciences, 104(34), 13603–13608. https://doi.org/10.1073/pnas.0702756104

[3] Manavalan, P., Taylor, P., & Curtis Johnson, W. (1985). Circular dichroism studies of acetylcholine sterase conformation. Comparison of the 11 S and 5.6 S species and the differences induced by inhibitory ligands. Biochimica et Biophysica Acta (BBA) - Protein Structure and Molecular Enzymology, 829(3), 365–370. https://doi.org/10.1016/0167-4838(85)90246-8

[4] Nachmansohn, D., & Wilson, I. B. (2006). The enzymic hydrolysis and synthesis of acetylcholine. advances in enzymology and related areas of molecular biology, 259–339. https://doi.org/10.1002/9780470122570.ch5

[5] Mooser, G., & Sigman, D. S. (1974). Ligand binding properties of acetylcholinesterase determined with fluorescent probes. Biochemistry, 13(11), 2299–2307. https://doi.org/10.1021/bi00708a010

[6] Cohen, J. B., & Changeux, J. P. (1973). Interaction of a fluorescent ligand with membrane-bound cholinergic receptor from *Torpedo marmorata*. Biochemistry, 12(24), 4855–4864. https://doi.org/10.1021/bi00748a008

[7] Bergmann, F., Wilson, I. B., & Nachmansohn, D. (1950). The inhibitory effect of stilbamidine, curare and related compounds and its relationship to the active groups of acetylcholine esterase. Action of stilbamidine upon nerve impulse conduction. Biochimica et Biophysica Acta, 6, 217–224. https://doi.org/10.1016/0006-3002(50)9009

[8] Silman, I., & Sussman, J. L. (2008). Acetylcholinesterase: How is structure related to function? Chemico-Biological Interactions, 175(1–3), 3–10. https://doi.org/10.1016/j.cbi.2008.05.035

[9] Kreienkamp, H. J., Weise, C., Raba, R., Aaviksaar, A., & Hucho, F. (1991). Anionic subsites of the catalytic center of acetylcholinesterase from *Torpedo* and from *cobra* venom. Proceedings of the National Academy of Sciences, 88(14), 6117–6121. https://doi.org/10.1073/pnas.88.14.6117

[10] Xu, Y., Colletier, J.-P., Weik, M., Jiang, H., Moult, J., Silman, I., & Sussman, J. L. (2008). Flexibility of aromatic residues in the active-site gorge of acetylcholinesterase: X-ray *versus* molecular dynamics. Biophysical Journal, 95(5), 2500–2511. https://doi.org/10.1529/biophysj.108.129601
[11] Holzgrabe, U., Kapková, P., Alptüzün, V., Scheiber, J., & Kugelmann, E. (2007). Targeting acetylcholinesterase to treat neurodegeneration. Expert opinion on therapeutic targets, 11(2), 161–179. https://doi.org/10.1517/14728222.11.2.161
[12] Pohanka, M. (2011). Cholinesterases, a target of pharmacology and toxicology. Biomedical Papers, 155(3), 219–223. https://doi.org/10.5507/bp.2011.036
[13] Johnson, G., & Moore, S. W. (1999). The adhesion function on acetylcholinesterase is located at the peripheral anionic site. Biochemical and Biophysical Research Communications, 258(3), 758–762. https://doi.org/10.1006/bbrc.1999.0705
[14] McGleenon, Dynan, & Passmore. (1999). Acetylcholinesterase inhibitors in Alzheimer's disease. British Journal of Clinical Pharmacology, 48(4), 471–480. Portico. https://doi.org/10.1046/j.1365-2125.1999.00026.x
[15] Ibach, B., & Haen, E. (2004). Acetylcholinesterase Inhibition in Alzheimers Disease. Current Pharmaceutical Design, 10(3), 231–251. https://doi.org/10.2174/1381612043386509
[16] Schenk, D., Barbour, R., Dunn, W., Gordon, G., Grajeda, H., Guido, T., Hu, K., Huang, J., Johnson-Wood, K., Khan, K., Kholodenko, D., Lee, M., Liao, Z., Lieberburg, I., Motter, R., Mutter, L., Soriano, F., Shopp, G., Vasquez, N., Seubert, P. (1999). Immunization with amyloid-β attenuates Alzheimer-disease-like pathology in the PDAPP mouse. Nature, 400(6740), 173–177. https://doi.org/10.1038/22124
[17] Haass, C., & Selkoe, D. J. (1998). A technical KO of amyloid-β peptide. Nature, 391(6665), 339–340. https://doi.org/10.1038/34800
[18] Kumar, A., A. Singh, and Ekavali, A review on Alzheimer's disease pathophysiology and its management: an update. Pharmacol Rep, 2015. 67(2): p. 195-203. https://doi.org/10.1016/j.pharep.2014.09.004
[19] Mao, F., Li, J., Wei, H., Huang, L., & Li, X. (2015). Tacrine–propargylamine derivatives with improved acetylcholinesterase inhibitory activity and lower hepatotoxicity as a potential lead compound for the treatment of Alzheimer's disease. Journal of Enzyme Inhibition and Medicinal Chemistry, 30(6), 995–1001. https://doi.org/10.3109/14756366.2014.1003212
[20] Bolognesi, M. L., Cavalli, A., Valgimigli, L., Bartolini, M., Rosini, M., Andrisano, V., Recanatini, M., & Melchiorre, C. (2007). Multi-Target-Directed Drug Design Strategy: From a Dual Binding Site Acetylcholinesterase Inhibitor to a Trifunctional Compound against Alzheimer's Disease. Journal of Medicinal Chemistry, 50(26), 6446–6449. https://doi.org/10.1021/jm701225u
[21] Tasso, B., Catto, M., Nicolotti, O., Novelli, F., Tonelli, M., Giangreco, I., Pisani, L., Sparatore, A., Boido, V., Carotti, A., & Sparatore, F. (2011). Quinolizidinyl derivatives of bi- and tricyclic systems as potent inhibitors of acetyl- and butyrylcholinesterase with potential in Alzheimer's disease. European Journal of Medicinal Chemistry, 46(6), 2170–2184. https://doi.org/10.1016/j.ejmech.2011.02.071
[22] Nam, S. O., Park, D. H., Lee, Y. H., Ryu, J. H., Lee, Y. S. (2014). Synthesis of aminoalkyl-substituted coumarin derivatives as acetylcholinesterase inhibitors. Bioorganic & Medicinal Chemistry, 22(4), 1262–1267. https://doi.org/10.1016/j.bmc.2014.01.010

[23] Hogan, D. B. (2014). Long-Term efficacy and toxicity of cholinesterase inhibitors in the treatment of Alzheimer disease. The Canadian Journal of Psychiatry, 59(12), 618–623. https://doi.org/10.1177/070674371405901202

[24] Castro, A., & Martinez, A. (2001). Peripheral and dual binding site acetylcholinesterase inhibitors: implications in treatment of Alzheimers disease. Mini-Reviews in Medicinal Chemistry, 1(3), 267–272. https://doi.org/10.2174/1389557013406864

[25] Wang, S., Jiang, J.-H., Li, R.-Y., & Deng, P. (2020). Docking-based virtual screening of TβR1 inhibitors: evaluation of pose prediction and scoring functions. BMC Chemistry, 14(1).https://doi.org/10.1186/s13065-020-00704-3

[26] Brooks, B. R., Bruccoleri, R. E., Olafson, B. D., States, D. J., Swaminathan, S., & Karplus, M. (1983). CHARMM: A program for macromolecular energy, minimization, and dynamics calculations. Journal of Computational Chemistry, 4(2), 187–217.https://doi.org/10.1002/jcc.540040211

[27] Lipinski, C. A., Lombardo, F., Dominy, B. W., & Feeney, P. J. (2012). Experimental and computational approaches to estimate solubility and permeability in drug discovery and development settings. Advanced Drug Delivery Reviews, 64, 4–17.https://doi.org/10.1016/j.addr.2012.09.019

[28] Wang, Q., He, J., Wu, D., Wang, J., Yan, J., & Li, H. (2015). Interaction of α-cyperone with human serum albumin: Determination of the binding site by using Discovery Studio and *via* spectroscopic methods. Journal of Luminescence, 164, 81–85. https://doi.org/10.1016/j.jlumin.2015.03.025

[29] Fancellu, G., Chand, K., Tomás, D., Orlandini, E., Piemontese, L., Silva, D. F., Cardoso, S. M., Chaves, S., & Santos, M. A. (2019). Novel tacrine–benzofuran hybrids as potential multi-target drug candidates for the treatment of Alzheimer's Disease. Journal of Enzyme Inhibition and Medicinal Chemistry, 35(1), 211–226. https://doi.org/10.1080/14756366.2019.1689237

[30] Ghanem Kattoub, R., Sliman, F. and Kousara, M., Development of novel benzimidazole derivates as potent and selective Akt kinase inhibitors using *in-silico* approaches. International Journal of Organic Chemistry, (2021) 11: p. 106-127. https://doi.org/10.4236/ijoc.2021.113009