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ARAŞTIRMA MAKALESİ / RESEARCH ARTICLE

Molecular Docking Study for Phenazine Derivative Compounds: Cholinesterases and Alzheimer's disease

Fenazin Türevi Bileşikler İçin Moleküler Docking Çalışması: Kolinesterazlar ve Alzheimer Hastalığı

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ABSTRACT

Introduction: Alzheimer's disease (AD) is a progressive neurodegenerative disorder with no definitive treatment to date. According to the cholinergic hypothesis, decreased acetylcholine (ACh) levels contribute to the cognitive symptoms of the disease. Therefore, inhibition of AChE and BChE enzymes is an important strategy in next-generation drug design. In this study, the inhibitory potentials of four phenazine-derived dyes (Safranin-O, Phenosafranine, Pyocyanine and Janus Green B) on AChE and BChE enzymes were evaluated by molecular docking method.

Material and Methods: Ligand structures were obtained from PubChem database and energy minimization was performed using MMFF94 force field with Avogadro software. The structures of target proteins, human AChE (PDB: 4M0E) and BChE (PDB: 6QAA), were obtained from RCSB Protein Data Bank and preprocessed to make them ready for docking analyses. Molecular docking analyses were performed with AutoDock Vina (v1.2.5). The complexes obtained were analyzed with Discovery Studio to evaluate binding energies and interaction types.

Results: Janus Green B showed the highest binding score for AChE (–9.2 kcal/mol). The strongest binding for BChE was obtained with Safranin-O (–9.1 kcal/mol). The interaction of Phenosafranine with BChE (–8.9 kcal/mol) is also remarkable. Molecular interaction analyses showed that aromatic residues such as TRP286, TYR341 and TYR124 for AChE and residues such as TRP82, PHE329 and HIS438 for BChE play a central role in ligand binding. All ligands exhibited hydrogen bonds, π-alkyl and electrostatic interactions in the active sites of the enzymes. The findings of the study show that phenazine-derived dyes, especially Janus Green B and Phenosafranine, exhibit strong binding to AChE and BChE enzymes. These molecules can be evaluated among the new inhibitor candidates to be developed against Alzheimer's disease. Although the findings are based on *in silico* data, they should be supported by further *in vitro* and *in vivo* studies for biological validity.

Keywords: Alzheimer's disease, phenazine, cholinesterase inhibition, acetylcholinesterase, butyrylcholinesterase

ÖZ

Giriş: Alzheimer hastalığı (AH), günümüzde kesin tedavisi bulunmayan, ilerleyici nörodejeneratif bir bozukluktur. Kolinerjik hipoteze göre, asetilkolin (ACh) düzeylerinin azalması hastalığın bilişsel belirtilerine katkıda bulunur. Bu nedenle AChE ve BChE enzimlerinin inhibisyonu, yeni nesil ilaç tasarımlarında önemli bir stratejidir. Bu çalışmada, fenazin türevi dört boyanın (Safranin-O, Phenosafranine, Pyocyanine ve Janus Green B) AChE ve BChE enzimleri üzerindeki inhibitör potansiyelleri moleküler yerleştirme (docking) yöntemiyle değerlendirilmiştir.

Materyal ve Metodlar: Ligand yapıları PubChem veri tabanından temin edilerek Avogadro yazılımı ile MMFF94 kuvvet alanı kullanılarak enerji minimizasyonu yapılmıştır. Hedef proteinler olan insan AChE (PDB: 4M0E) ve BChE (PDB: 6QAA) yapıları RCSB Protein Data Bank'tan elde edilmiş, ön işlemden geçirilerek docking analizlerine hazır hale getirilmiştir. Moleküler yerleştirme analizleri AutoDock Vina (v1.2.5) ile gerçekleştirilmiştir. Elde edilen kompleksler Discovery Studio ile analiz edilerek bağlanma enerjileri ve etkileşim tipleri değerlendirilmiştir.

Bulgular: Janus Green B, AChE için en yüksek bağlanma skorunu (–9.2 kcal/mol) göstermiştir. BChE için ise en güçlü bağlanma Safranin-O (–9.1 kcal/mol) ile elde edilmiştir. Phenosafranine'nin BChE ile etkileşimi (–8.9 kcal/mol) de dikkat çekicidir. Moleküler etkileşim analizleri; AChE için TRP286, TYR341 ve TYR124 gibi aromatik kalıntıların; BChE için TRP82, PHE329 ve HIS438 gibi kalıntıların ligand bağlanmasında merkezi rol oynadığını göstermiştir. Tüm ligandlar enzimlerin aktif bölgelerinde hidrojen bağları, π-alkil ve elektrostatik etkileşimler sergilemiştir. Çalışmanın bulguları, fenazin türevli boyaların, özellikle Janus Green B ve Phenosafranine'in, AChE ve BChE enzimlerine karşı güçlü bağlanma gösterdiğini ortaya koymaktadır. Bu moleküller, Alzheimer hastalığına karşı geliştirilecek yeni inhibitör adayları arasında değerlendirilebilir. Bulgular *in silico* verilere dayanmakla birlikte, biyolojik geçerlilik için ileri *in vitro* ve *in vivo* çalışmalarla desteklenmelidir.

Anahtar Sözcükler: Alzheimer hastalığı, fenazin, kolinesteraz inhibisyonu, asetilkolinesteraz, bütirilkolinesteraz

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Introduction

Alzheimer's disease (AD) is a progressive and ultimately terminal condition characterized by a decline in cognitive function and memory loss (1). Cerebral amyloid angiopathy may coexist with parenchymal abnormalities in the disease pathogenesis. Molecular investigations have revealed that the primary constituent of amyloid plaques is amyloid beta (Aβ) (2), whereas neurofibrillary tangles consist of tau protein (3). The loss of neurons and synapses constitutes essential pathological features of AD. Although the accurate mechanism of the disease has not been understood for 118 years since its discovery (4), the enzymes and cholinergic system that regulate the biochemical pathways related to the progression of the disease have been well documented by studies conducted to date (5). Acetylcholine (ACh), which is used by cholinergic neurons that are crucial both in the peripheral and central nervous systems and contributes to several physiological processes, including memory, emotional processing, and learning (6), is the first neurotransmitter to be identified (7). ACh is hydrolyzed by acetylcholinesterase (AChE) and butyrylcholinesterase (BChE) enzymes (8). AChE performs hydrolysis more rapidly than BChE. These ChE enzymes are structurally very similar enzymes. The differences between them are (i) their tissues, (ii) substrates, and (iii) inhibitor sensitivities (9).

The cholinergic hypothesis, on which the mechanism of medicines used in the treatment of AD, is based suggests that a reduction in ACh levels in the cortex contributes to the etiology of AD (10). Blocking the AChE and BChE, which enable this neurotransmitter to break down into choline and acetate, is an attempt to compensate for cholinergic loss, increase cholinergic transmission, and increase the decreased ACh density caused by the increase in AChE enzyme in brain cholinergic synapses and neuromuscular junctions due to AD (10). Additionally, research has shown that amyloid plaques may include cholinergic activation and protein metabolism (11). Cortical amyloid plaques expand quickly after cholinergic nuclei lesions. In a comparable manner, cognitive dysfunction and amyloidogenic metabolism in AD are triggered by a reduction in cholinergic signaling (12). Rivastigmine, galantamine, and donepezil are three groups of cholinesterase (ChE) inhibitors that have received approval from the United States Food and Drug Administration (FDA) for the treatment of AD. These ChE inhibitors are believed to enhance cognitive functions; however, their efficacy is a topic of debate (13).

Phenazines are chromatic compounds characterized by a heterocyclic structure that incorporates two nitrogen atoms, generally synthesized spontaneously by microorganisms (14). Pyocyanin, the first known phenazine, was first used as a 'blue pigment' for treating infected wounds in 1859, despite its chemical structure being identified almost a century later (15). Both natural phenazines and synthetic decoration groups show broad biological activities due to their pyrazine ring structures, and their effects on biotechnological processes are being studied (16). An extensive range of pharmacological activities, including antibacterial, antitumor, antileprosy, antitubercular, antifungal, neuroprotective, insecticide, and radical scavenging activity, have been documented for phenazine compounds since their discovery (16, 17). In addition to all these activities, we focused on the inhibitory effects of phenazine-structured compounds on ChE activities. The results showed that methylene violet 3RAX resulted in linear competitive inhibition of BChE (K_i = 0.51 ± 0.006 μM) and hyperbolic noncompetitive inhibition of AChE (K_i = 1.58 ± 0.36 μM) (18). Safranin-O (SO) induced linear competitive inhibition (Ki = 0.44 ± 0.085 μM) on human plasma BChE and hyperbolic noncompetitive inhibition (Ki = 0.69 ± 0.13 μM) on human erythrocyte AChE (19). This study explores the interactions of two essential cholinesterase enzymes with three structurally distinct phenazine-based dyes: Phenosafranine, Pyocyanin, and Janus Green B. Our Molecular Docking results showed that Janus Green B had the highest binding scores to AChE. This finding indicates that Janus Green B and the phenazine ring may be valuable in the development of novel pharmaceuticals for AD.

Material and Methods

Preparation of Ligands

The molecular structures of Safranin-O and its related compounds Phenosafranine, Pyocyanine, and Janus Green B were obtained from the PubChem database (Safranin-O: PubChem ID 33345803; Phenosafranine: ID 4764; Pyocyanine: ID 6817; Janus Green B: ID 119049). Before proceeding with docking studies, their three-dimensional conformations were energy-minimized using Avogadro software (version 1.2.0) to ensure structural stability. This process was performed using the MMFF94 force field, which optimizes molecular geometry by accounting for both electrostatic and steric interactions. Default parameters, including convergence criteria and energy thresholds, were applied to prepare the ligands for reliable docking analysis (20).

Docking Procedure

The X-ray crystal structures of human acetylcholinesterase (AChE; PDB ID: 4M0E, resolution: 2.00 Å) (21) and butyrylcholinesterase (BChE; PDB ID: 6QAA, resolution: 1.90 Å) (22)were obtained from the RCSB Protein Data Bank. Prior to docking, the structures were prepared by removing all water molecules and non-essential heteroatoms. Hydrogen atoms were added to reflect physiological protonation states, and Gasteiger partial charges were applied to both proteins. For AChE, the active site was defined based on the coordinates of the co-crystallized ligand, with the grid box centered at x =-17.27, y =-42.32, z = 25.90. Similarly, for BChE, the grid center was positioned at x = 18.99, y = 42.61, z = 40.81. In both cases, a uniform grid box size of 20 Å × 20 Å × 20 Å was

used to ensure full coverage of the active site and accommodate ligand flexibility. Docking simulations were carried out using AutoDock Vina (version 1.2.5), employing default parameters and the Lamarckian Genetic Algorithm to predict the most favorable binding poses and estimate binding affinities (23, 24). To assess the reliability of the docking results, the RMSD values of the poses that yielded the best binding scores were analyzed. Redocking was performed to validate the docking protocol using RMSD (Root Mean Square Deviation) as a pose validation metric. The RMSD value obtained for AChE (PDB ID: 4MOE) was 0.42 Å, indicating high accuracy in reproducing the crystallographic pose. For BChE (PDB ID: 6QAA), the RMSD was 2.02 Å, which is considered acceptable and supports the reliability of the docking protocol.

Analysis of Molecular Interactions

Upon completion of the docking simulations, the binding interactions between AChE, BChE, and the compounds were analyzed to identify key interaction types. Using Discovery Studio software, we visualized and carefully examined hydrogen bonds, hydrophobic interactions, and other relevant binding forces. These analyses provided valuable insights into the mechanisms driving the interaction between AChE, BChE, and the selected compounds.

Statistical Analysis

Avogadro was employed to optimize molecular geometries and ensure stable conformations prior to docking by utilizing the default MMFF94 force field for energy minimization of the compounds. The stability and energy profiles of the minimized structures are assessed using default statistical methods during this step of the process. AutoDock Vina was employed to perform docking simulations, using its standard Lamarckian Genetic Algorithm to calculate binding affinities based on both energy considerations and geometric complementarity. The docking procedure incorporated default statistical methods to evaluate the reliability and significance of the computed binding affinities. Discovery Studio, offering standard features for analyzing binding affinities and interaction frequencies, was employed to visualize the results. This software enabled the identification of key interactions, including hydrogen bonds and hydrophobic contacts, and applied default statistical analyses for providing an understanding of the distribution and importance of these interactions.

Results

Active Site-Based Docking Simulations

The analyses were conducted using the active site coordinates of AChE and BChE obtained from a previously conducted Safranin-O inhibition study (19). The docking poses of the ligands are shown within the active sites of AChE and BChE, with each ligand positioned inside the defined grid box (Figure 1). The structures illustrate how the ligands were placed within the catalytic pockets of the enzymes during the docking simulations, confirming that the targeted binding sites were appropriately defined.

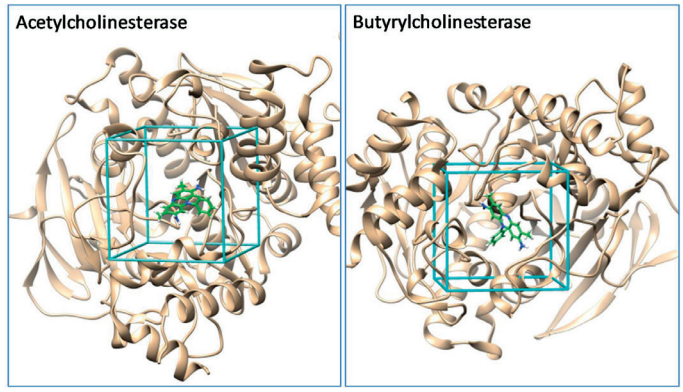


Figure 1. Binding site visualization of AChE and BChE with Safranin-O.

The docking results show how each compound fits within the active site of acetylcholinesterase (Figure 2). The binding orientations of Safranin-O, Phenosafranine, Pyocyanine, and Janus Green B are displayed to illustrate their spatial accommodation in the catalytic pocket. These visualizations support the docking scores by highlighting the ability of each ligand to interact with the active site region.

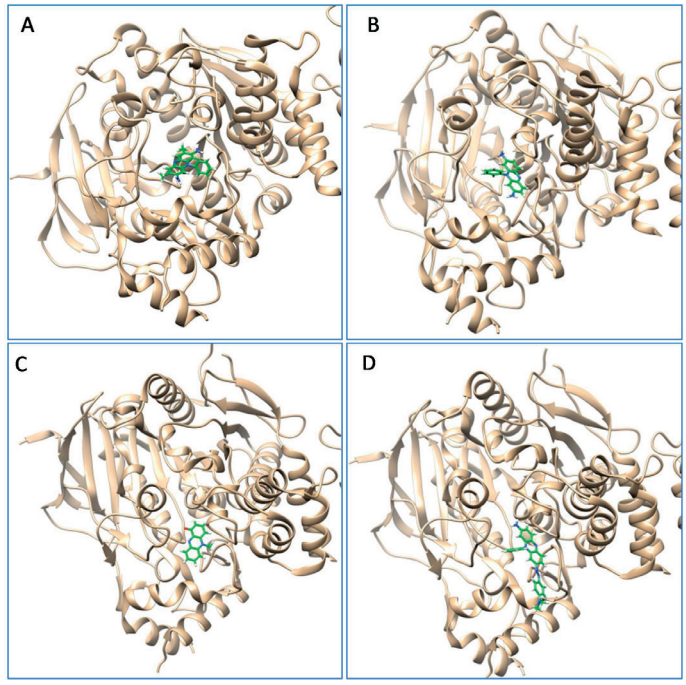


Figure 2. Docking poses of the compounds within the active site of AChE: A) Safranin-O, B) Phenosafranine, C) Pyocyanine, D) Janus Green B.

The docking results demonstrate how each compound fits into the active site of butyrylcholinesterase (Figure 3). The binding orientations of Safranin-O, Phenosafranine, Pyocyanine, and Janus Green B highlight their accommodation within the catalytic pocket, supporting the docking scores.

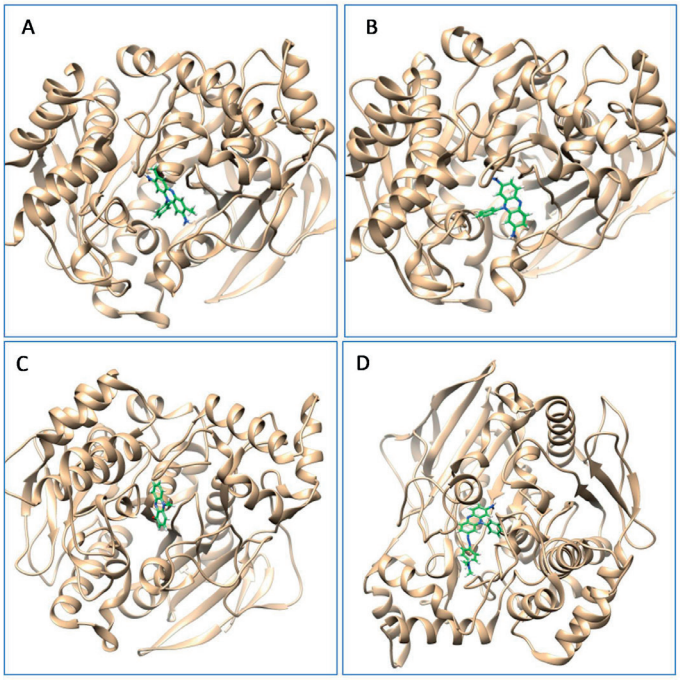
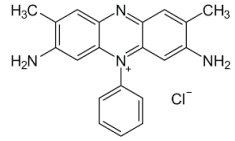
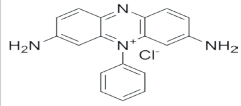
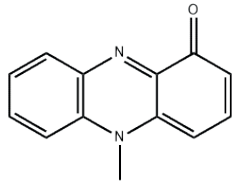
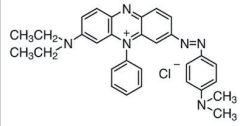


Figure 3. Docking poses of the compounds within the active site of BChE: A) Safranin-O, B) Phenosafranine, C) Pyocyanine, D) Janus Green B.

Table 1. Docking scores of compounds with AChE and BChE

Compound	Molecular Structure	Docking score for AChE (kcal/mol)	Docking score for BChE (kcal/mol)
Safranin-O		-7.6	-9.1
Phenosafranine		-8.2	-8.9
Pyocyanine		-8.3	-8.2
Janus Green B		-9.2	-8.8

Docking Results

According to the docking results, all four compounds exhibited notable binding affinities toward both acetylcholinesterase (AChE) and butyrylcholinesterase (BChE). The comparative docking scores for all compounds against both enzymes are summarized in Table 1. Janus Green B showed the strongest binding to AChE, with a docking score of –9.2 kcal/mol, indicating a favorable interaction within the enzyme's active site. For BChE, Safranin-O had the most favorable docking score at –9.1 kcal/mol, suggesting a strong potential for enzyme binding.

Analysis of Molecular Interactions

Molecular interactions revealed that Safranin-O, Phenosafranine, Pyocyanine, and Janus Green B interacted with key residues in the active site of AChE, as shown in Figure 4. Safranin-O (A) formed a hydrogen bond with HIS287 and π-alkyl interactions with TYR72, TRP286, and TYR341. Phenosafranine (B) showed hydrogen bonds with TYR124, TYR337, and PHE295, and π-alkyl interactions with TRP286, TYR341, and TYR72. Pyocyanine (C) interacted through a hydrogen bond with TYR124 and a carbon hydrogen bond with SER293, in addition to π-alkyl contacts with TRP286 and TYR341. Janus Green B (D) formed hydrogen bonds with TYR124 and TYR313, a carbon hydrogen bond with TYR341, and π-alkyl interactions with TRP286, TYR341, TYR72, and PHE337. These results suggest that all four compounds show promising binding potential to AChE, with strong and diverse interactions.

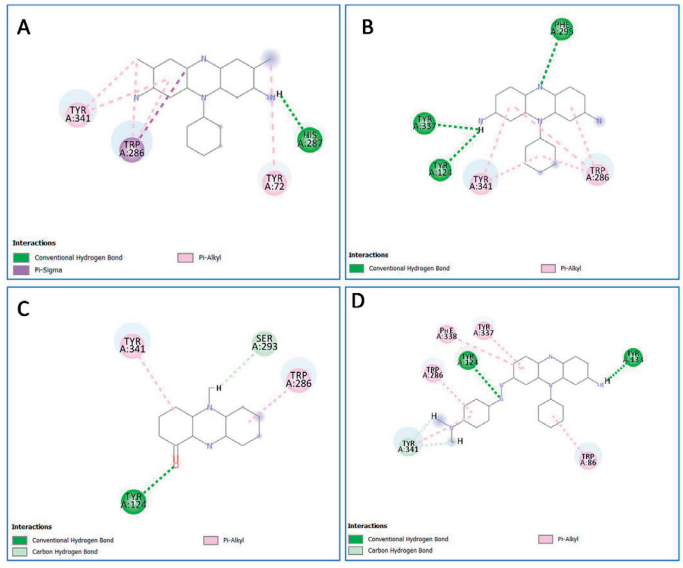


Figure 4. Molecular interactions of Safranin-O (A), Phenosafranine (B), Pyocyanine (C), and Janus Green B (D) with AChE active site

Molecular interactions revealed that Safranin-O, Phenosafranine, Pyocyanine, and Janus Green B interacted with key residues in the active site of BChE, as shown in Figure 5. Safranin-O (A) formed conventional hydrogen bonds with SER198, HIS438, and ASN83, and π-alkyl interactions with TRP82, PHE329, and LEU286. Additionally, an attractive charge interaction with ASP70 was observed. Phenosafranine (B) showed hydrogen bonds with THR120, SER79, and PRO285, along with π-alkyl interactions involving TYR332, PHE329, and ALA328. Pyocyanine (C) interacted through conventional hydrogen bonds with HIS438 and SER198, and displayed π-alkyl and alkyl

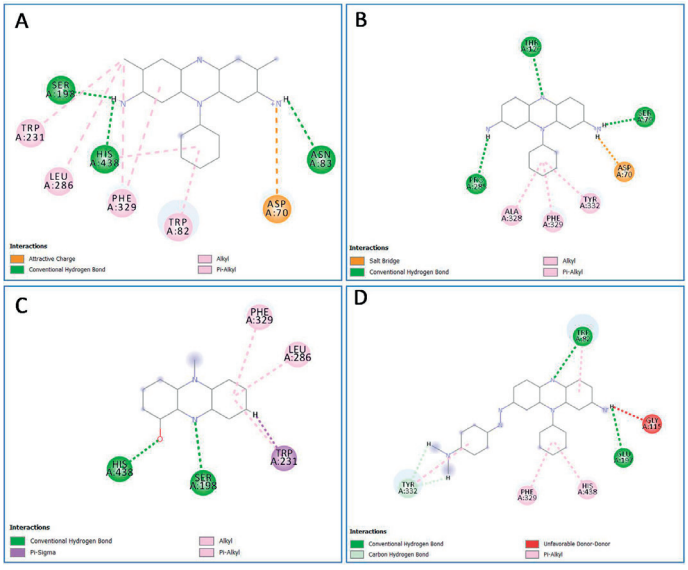


Figure 5. Molecular interactions of Safranin-O (A), Phenosafranine (B), Pyocyanine (C), and Janus Green B (D) with BChE active site

contacts with TRP231, LEU286, and PHE329. Janus Green B (D) formed hydrogen bonds with TRP82 and TYR332, a carbon hydrogen bond with GLU197, and π-alkyl interactions with TRP82, HIS438, and PHE329. Notably, an unfavorable donor–donor interaction was detected with GLY117. These findings indicate that all four compounds exhibit binding affinity towards BChE, with strong and multifaceted interactions.

Discussion and Conclusion

AD represents a significant public health issue, with its precise mechanisms and treatments remaining unidentified (25). The World Health Organization (WHO) predicts roughly 140 million cases of AD globally in 25 years (26). As the baby boom generation ages, an anticipated 10 million new diagnoses of AD are projected annually, which corresponds to almost 20 new people per minute (27). Since its identification, many hypotheses have been established for AD that involve impaired cholinergic system, Aβ accumulation, tau hyperphosphorylation, neuroinflammation, and various other pathways associated with the pathogenesis of AD (28). The association between these hypotheses and AD-related cognitive dysfunction has been well established. The cholinergic theory posits that as AD advances, ChE activity increases, hence limiting acetylcholine levels in the brain. Since cholinergic transmission is effective in both long-term memory and learning, any disruption in this system can have profound consequences (29, 30).

Therefore, cholinesterase inhibitors that will stabilize acetylcholine levels are in a critical position in the treatment of this pathology. Although ChE inhibitors cannot completely fully remediate AD, they can help patients manage symptoms and prolong the time to more intensive care (30, 31). The fact that AChE and BChE appear to have comparable functions in the pathophysiology of AD does not mean that they are equivalent targets for therapies against AD. Clinical results show that as AD progresses dramatically, AChE activity levels drop by up to 85-90%, and BChE, which is found in trace amounts in healthy individuals, increases due to plaques and tangles formed as the AD progresses. As a result, the BChE:AChE ratio could fluctuate from 0.2 to 11, representing a 22-fold increase. This alteration in the activity levels of AChE and BChE enzymes can be interpreted as an important target and strategy in drug designs to increase ACh levels, not only AChE inhibition but also BChE inhibition (32, 33).

Guillozet et al. suggested that BChE may be important in the conversion of Aβ from its non-pathogenic form to its pathogenic form related to neuritic tissue degeneration and AD (34). An *in vivo* study by Greig et al. presented the beneficial effects of BChE inhibition on learning and memory due to increased ACh levels and decreased Aβ levels (32). Their effect on cholinergic networks and amyloid plaques causes BChE inhibitors to be deserving of investigation in AD-related drug designs. Cationic phenothiazine derivative compounds, which have various pharmacological properties and clinical uses, are also of interest in AD-targeted drug research (35).

Since its discovery, numerous natural and modified synthetic phenazine ring compounds have been identified in all shades of the rainbow, and as mentioned in the introduction, a wide range of pharmacological activities has been recognized for phenazine compounds. Many studies have been presented for phenazine compounds and their pyrazine rings, which are of therapeutic importance for many diseases (17). The WHO 2019 Model List of Essential Medicines at *list.essentialmeds.org* includes four pyrazines (amiloride, bortezomib, paritaprevir, and pyrazinamide) and one phenazine (clofazimine). Common drug-target interactions for biologically active pyrazines have been demonstrated in computational studies (36). In the studies we conducted in our laboratory, we targeted the 'cholinergic hypothesis' and focused on the effects of phenazine-based compounds on cholinesterases. In our recent studies, we characterized the inhibition kinetics of methylene violet 3RAX (18) and Safranin-O (19) on cholinesterases. In this current study, the interactions of four phenazine-based dyes-Safranin-O, Phenosafranine, Pyocyanine, and Janus Green B with, two key cholinesterase enzymes, AChE and BChE enzymes were investigated via their binding energies and the potential inhibitory effects of these molecules were evaluated. The molecular docking data indicated that all four drugs revealed significant binding energies, confirming their potential as cholinesterase inhibitors. Janus Green B showed the highest affinity for AChE among the tested compounds, with a docking score of-9.2 kcal/mol. This implies that the compound's extended aromaticity and capacity to establish a variety of interactions, such as multiple hydrogen bonds and -alkyl contacts within the catalytic gorge of AChE, may contribute to a strong and stable binding. After the reference molecule Safranin-O, Phenosafranine showed

the best binding score with BChE (-8.9 kcal/mol), supported by a complex interaction network including hydrogen bonding with catalytically critical residues (e.g. THR120, SER79, and PRO285), hydrophobic -alkyl interactions and a significant electrostatic attraction with TYR332, PHE329, and ALA328. Particularly, TRP286 and TYR341 in AChE and TRP82 and PHE329 in BChE were consistently engaged in -interactions across all ligands, underlining their central role in stabilizing the ligand within the active site. This is consistent with prior structural analyses showing the significance of these aromatic residues in substrate accommodation and inhibitor binding. However, the binding affinity and interaction patterns of the ligands were observed to be influenced by subtle differences in the substituents of the ligands, despite the same central structures. In the active compartment, Pyocyanine, which showed moderate scores for both enzymes, formed fewer hydrogen bonds, potentially indicating a trade-off between steric compatibility and electronic distribution. From a therapeutic perspective, the strong binding profiles of Safranin-O and Janus Green B indicate their potential as lead compounds for the further design and optimization of dual AChE/BChE inhibitors.

Although the phenazine-based dyes examined in this study demonstrated promising binding affinities *in silico*, some of them (Pyocyanin, Janus Green B) are known to cytotoxic effects at higher concentrations. Although phenazine-based dyes demonstrate favorable binding affinities toward cholinesterase enzymes, it is crucial to emphasize that their biological behavior is highly dose-dependent. Janus Green B and other compounds are recognized as redox-active agents. Although low concentrations may produce beneficial inhibitory effects on target enzymes (37), higher doses have been linked to cytotoxicity, increased reactive oxygen species production, and mitochondrial dysfunction in neuronal and non-neuronal cell lines. Consequently, the therapeutic applicability of these molecules is contingent upon the identification of safe and effective concentration ranges, as they may function as a double-edged sword. In order to verify their potential as drug candidates, additional dose-response studies, such as IC_{50} and cytotoxicity analyses in pertinent neuronal models, are essential. Nevertheless, Phase II clinical trials for Alzheimer's disease have been initiated for certain redox-active dyes, including methylene blue (MethB), a phenothiazine derivative, as a result of their multitargeted effects, which include antiaggregatory activity on tau and Aβ and cholinesterase inhibition (38, 39). Research has demonstrated that various phenothiazine compounds, including thionine and toluidine blue, function as effective inhibitors of cholinesterases. Furthermore, these compounds have a beneficial impact on the phosphorylation of tau and the metabolism of APP (40-42). These precedents indicate that phenazine-based structures may continue to demonstrate potential as pharmacological leads when employed at optimized, non-toxic concentrations.

Considering the complex factors involved in Alzheimer's pathology, such as cholinergic dysfunction and oxidative stress, repurposing these redox-active dyes may provide a dual benefit in regulating enzyme activity and reducing oxidative damage.

In conclusion, this study highlights the cholinesterase inhibitory potential of various novel phenazine-based dyes, with Janus Green B and Phenosafranine standing out as a notably promising candidate. The results establish the groundwork for future studies focused on identifying and altering these scaffolds for the progress of neuroprotective drug development.

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