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Modulation of Human Acetylthiocholine Esterase Activity by Novel Fused Pyrimidine Derivatives: *In vitro*, Theoretical and ADMET Studies

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Abstract: Pyrimidine compounds have medicinal and biological activities as previously reported. In this work, two novel fused pyrimidine compounds were synthesized, fused pyrazolo–pyrimidine compound was synthesized by cyclization of 5-amino-4-cyano-1-phenyl pyrazole with propionic acid in the presence of $POCl_3$, and the other fused pyrrole–pyrano-pyrimidine compound was synthesized by cyclization of ethyl(*E*)-N-(3-cyano-4-(4-(dimethylamino)phenyl)-7-methyl-4,5,6,7-tetrahydropyrano[2,3-*b*] pyrrole-2-yl) formimidate with hydrazine hydrate, in methanol. These fused pyrimidine compounds were characterized by FT-IR and ^{1}H NMR. The effect of these compounds was studied on the activity of the human neurotransmission enzyme acetylthiocholine esterase AChE. Results indicated that these compounds significantly inhibited AChE activity at concentrations of 10^{-11} M. Michalis-Menton showed mixed noncompetitive inhibition of AChE activity. In conclusion, newly synthesized compounds could be promising derivatives for enhancing cholinergic neurotransmission. Among the other derivatives, derivative 4 formed H-bond interactions with key amino acid residues Tyr334, and Asp72, whereas the other electrostatic interactions formed with Tyr334, Phe330, Ile287, Tyr121, Arg289, Trp279, Gly335, and Phe288. In the case of derivatives 9, similar binding interactions with active pockets of 2ACE were observed due to the high homology of the binding site residues. In addition, we examined ADMET properties with the help of online databases to search for possible drug similarity of synthesized compounds 4 and 9 and revealed that both molecules were compatible with Lipinski's five rules.

Keywords: Fused pyrazolo–pyrimidine, AChE activity, Molecular docking, Noncompetitive inhibition, ADMET.

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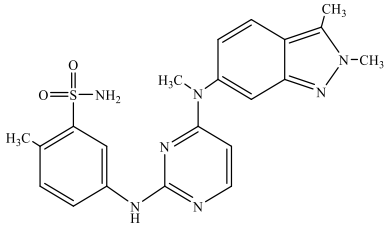
1. INTRODUCTION

Acetylcholinesterase is known to be distributed in nervous tissue such as the brainstem, cerebellum, and peripheral and autonomic nervous systems. Skeletal muscle also contains AChE with distribution patterns seemingly related to the type of muscle (fast versus slow twitch) and their specific function. The presence and function of AChE on red blood cells are less commonly known. Blood group antigens reside on the outer lipid bilayer of red blood cells for convenient antibody recognition. In the same regard, AChE is also present in red blood cell membranes (1).

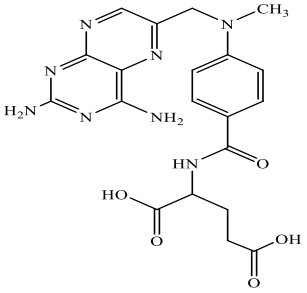
Alzheimer's disease (AD) is associated with memory and thinking impairment, behavioral problems, and disturbance in daily living activities. AD is common in old people due to irreversible neuronal loss. The deficiency of acetylcholine (ACh) in synapses of the cerebral cortex is one of the important sufferers of AD and can be treated by the inhibition of AChE that hydrolyzes ACh into choline and acetate (2).

Fused pyrimidine rings are among a wide range of nitrogen heterocyclics that have been studied due to their pharmaceutical activities to produce biologically important molecules (3-5). The pyrimidine ring is fused into different heterocyclic (6,7) and exhibits several activities such as antimicrobial, antitumor, antimalarial, antihypertensive, vasodilator, and anti-allergic (8,9). The observed activity may be due to the presence of fused pyrimidine moiety. The chloro, amine, fluorine, bromine, amino-pyrimidine, 4-methoxyphenylpyridine, hydroxynaphthalene, coumarin ring, pyrazolo-pyrimidine, thino-pyrimidine, phenyl-pyrimidine scaffold presence in various positions of the aromatic ring may enhanced biological potential (10).

On the other hand, naphthyridine also represents one of the important fused nitrogen compounds with diverse biological activities, such as antibacterial, anticancer, anti-inflammatory, and anti-ureolytic activity (11-14). During the last decade, there was a large interest in fused nitrogen heterocycles to synthesize novel pyridopyrimidine and naphthyridine derivatives. Those novel compounds were characterized using *IR Candida albicans*. Most fused pyrimidine has been used and licensed by the FDA (15) to treat some cancer types. Pazopanib (A) 'trade name votrient and methotrexate (B) has a pyrimidine core that is considered a cancer therapy drug (16) leading to the synthesis of new fused bicyclic and tricyclic pyrimidine analogs and their effect on cancer cells (17).



(A)



(B)

Figure 1: Fused pyrimidine compounds licensed by FDA. (A) Pazopanib and (B) methotrexate.

Herein, we synthesized new pyrimidine derivatives to explore their potential as inhibitors of human AChE activity. These compounds were characterized using Furious transfer infrared FT-IR and proton nuclear magnetic resonance ^{1}H NMR. The inhibition activity of the compounds was studied *in vitro* in human serum.

2. EXPERIMENTAL SECTION

2.1. Materials

All chemicals were purchased from Sigma-Aldrich and Fluka companies. ^{1}HNMR spectra were recorded on a Bruker, Ultra Shield 400 Mhz, spectrometer (Switzerland) using DMSO-d_6 and $CDCl_3$ as a solvent with tetramethylsilane (TMS) as an internal standard (Iran Polymer & Petrochemical Institute). All reactions were carried out with the thin layer chromatography technique (TLC) and revealed by a mixture of n-hexane and ethyl acetate (3: 2) as pure eluents.

2.2. Synthesis of Fused Pyrimidines

In this study, we used previously prepared compound 2 listed in Table 4 to synthesize compounds 3 and 4. The previously synthesized compound **7** was used to prepare compound **9**, see Table 1.

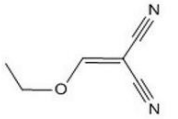
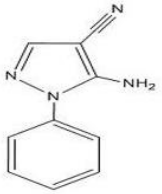
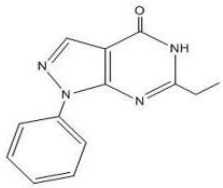
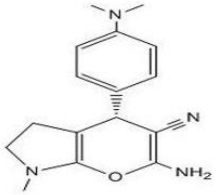
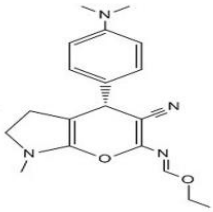
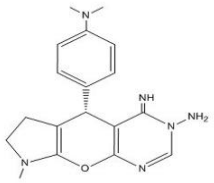
2.2.1. Synthesis of compound 2: 2-(Ethoxy methylene) malononitrile (20)

A weight of 0.66 g, 0.01 mole of redistilled malononitrile, 1.48 g, 0.01 mol of triethyl orthoformate, and 2.04 g, 0.02 mole of acetic anhydride was stirred and heated to 110 °C until the reaction had subsided and the temperature dropped to 95 °C. Distillation was used to remove impurities from the clear yellow reaction mixture until the temperature reached 115 °C. Then a vacuum distillation (100 °C, 15 mm) was used to remove the rest of the solvent. The product was cooled down to 80 °C and then refluxed 1.0 g of charcoal and 35 mL of absolute ethanol for 5 minutes. After filtering and washing with 3 mL of hot absolute ethanol, the filtrate was cooled on ice until crystallization was completed and recrystallized from ethanol to purify the white crystalline material and then dried to constant weight *in vacuo* to obtain the compound **2**, with a yield: 89% (1.8 g) and mp: 55-57 °C.

2.2.2. Preparation of compound 3: 5-Amino-4-cyano-1-phenyl pyrazole (21)

A mixture of compound **2** (1.22 g, 0.01 mole) and phenylhydrazine (1.08 g, 0.01 mole) in ethanol (40 mL) was refluxed for 1 h. The precipitate was separated by cooling, filtered, and purified by recrystallization from ethanol/water to give a light yellow compound **3**, with a yield of 88% (1.75 g) and mp: 134-136 °C.

Table 1: A library of synthesized compounds.

No.	Chemical structure	Name of the compound
2		2-(ethoxymethylene)malononitrile (18)
3		5-Amino-4-cyano-1-phenyl pyrazole
4		6-ethyl-1-phenyl-1,5-dihydro-4H-pyrazolo[3,4-*d*] pyrimidin-4-one
7		2-amino-4-(4-(dimethylamino)phenyl)-7-methyl-4,5,6,7-tetrahydropyrano[2,3-*b*]pyrrole-3-carbonitrile (19)
8		ethyl(*E*)-N-(3-cyano-4-(4-(dimethylamino)phenyl) -7-methyl-4,5,6,7-tetrahydropyrano[2,3-*b*]pyrrol-2-yl)formimidate
9		5-(4-(dimethylamino)phenyl)-4-imino-8-methyl-5,6,7,8-tetrahydropyrrolo[3',2':5,6]pyrano[2,3-*d*]pyrimidin-3(4*H*)-amine

2.2.3. Synthesis of compound 4: 6-Ethyl-1-phenyl-1,5-dihydro-4H-pyrazolo[3,4-d] pyrimidine-4-one

A mixture of compound **3** (2.67 g, 0.01 mole) was dissolved in propionic acid (5 mL), then quickly added 0.2 mL of $POCl_3$ and refluxed for 12 h. After the mixture cooled, a mass of white precipitate was observed. To neutralize the acid, fused K_2CO_3 was added until no bubble occurred. The mixture was filtered and washed with ethanol, dried, and then recrystallized from ethanol to give a pale-yellow compound **4** with a yield of 68% (1.35 g) and mp: 101-103 °C.

2.2.4. Synthesis of compound 7: 2-Amino-4-(4-(dimethylamino) phenyl)-7-methyl-4,5,6,7-tetrahydropyrano[2,3-b] pyrrole-3-carbonitrile

In a typical procedure, equimolar amounts of 4-(dimethylamino) benzaldehyde (1.49 g, 0.01mole), malononitrile (0.66 g, 0.01 mole) and N-methyl-2-pyrrolidinone (0.99 g, 0.01 mole) were mixed with tetraethyl ammonium bromide (10 mole %) in 15 mL, 90 % of ethanol and refluxed with stirring for 95 minutes. After the reaction was completed, the mixture was cooled to room temperature and poured into ice to obtain the crude products. The products were recrystallized as 1,4-dioxane to give compound **7** with a yield of 92% (1.85 g) and mp: 178-180 °C.

2.2.5. Synthesis of compounds 8: Ethyl(E)-N-(3-cyano-4-(4-(dimethylamino) phenyl)-7-methyl-4,5,6,7-tetrahydropyrano [2,3-b]pyrrol-2-yl) formimidate

A mixture of compound **7** (2.96 g, 0.01 mole), triethyl orthoformate (1.48 g, 0.01 mole), and 16 mL of acetic anhydride was reflexed for 5 h. Under vacuum, the solvent was removed and the product was recrystallized from benzene to give brown-colored compound **8** with a yield of 77% (1.46 g) and mp: 162-164 °C.

2.2.6. Synthesis of compound 9: 5- (4-(dimethylamino) phenyl) -4-imino-8-methyl-5,6,7,8 tetrahydro pyrrolo [3',2':5,6] pyrano [2,3-d] pyrimidine-3(4H)-amine

A mixture of the compound **8** (3.52 g, 0.01 mole) in 25 mL methanol, a solution of hydrazine hydrate (0.50g, 0.01mole), or ethylene diamine (0.60 g, 0.01 mole), or phenyl hydrazine (1.08 g, 0.01 mole) was stirred for 1 hour and left overnight. The next day, the mixture was filtered, dried, and recrystallized from 1,4-dioxane to give yellow-colored compound **9** with a yield of 80% (1.63 g) and mp: 261-263 °C.

2.3. The Activity of Human AChE

2.3.1. Acetylcholinesterase (AChE) assay

The activity of acetylcholine esterase (AChE) was studied according to Ellman et al method (22) with a slight modification. Acetylthiocholine iodide (ASChI, 34 µL, 0.06 M) was added to 50 µL of 5,5-dithiobis [2-nitrobenzoic acid] (DTNB) and 2.25 mL of sodium phosphate buffer (pH=7.3, 0.2 M) and finally, 10 µL of human serum was added to the mixture and vortexed well. The changes in absorbency were assayed before and after adding the substrate at 430 nm for 2 minutes.

AChE activity (µmole/ 2 min/mL) = ΔA / 2 * d.f

*2.3.2. Effect of pyrimidine derivatives (Compounds **4** and **9**) on AChE activity*

The effect of each derivative (compounds **4** and **9**) on the AchE was also studied according to the modified Ellman method. Briefly, a stock of the synthesized compounds (0.01M) was freshly prepared and used to prepare a series of different concentrations (10^{-2}, 10^{-3}, 10^{-5}, 10^{-7}, $10^{-9}$and 10^{-11} M) of dimethylsulfoxide (DMSO). A volume of 0.25 mL of pyrimidine derivatives was added to 50 µL DTNB (0.001 M), followed by the addition of 10 µL of human serum, mixed well, and 2 mL of the mixture was withdrawn to be mixed with 34 µL of ASChI substrate (0.06 M). The absorbance was assessed before and after adding the substrate at 430 nm for 2 minutes.

The inhibition percentage % of the enzyme activity was determined according to the equation:

$$\% \text{ Inhibition} = 100 - \frac{\text{The activity in the presence of an inhibitor}}{\text{The activity in the absence of an inhibitor}} \times 100 \quad \text{(Eq. 1)}$$

2.3.3. Type of inhibition (23)

To study the type of inhibition, different concentrations of AChTI (substrate) (0.02, 0.04, 0.06, and 0.08 M) were used against a constant concentration of inhibitors. The enzyme activity was assayed using the Linewer-Burk equation in the presence and absence of the compounds, and then the type of inhibition, inhibitor constant Ki, maximum velocity Vmax, and Michalis-Menton constant K_m were determined.

2.4. Docking Study

A molecular docking study was carried out using the Autodock 4.2 program (24), while Discovery Studio Visualizer (25) was used to select the best binding mode with the receptor and 3D interaction poses. The 3D structures of AChE (PDB ID: 2ACE) were obtained from the Protein Data Bank (www.rcsb.org), followed by separating the co- crystallized ligands and water molecules. Then polar hydrogens were added. Finally, the 3D structures of the tested compounds were optimized using Gaussian 03 software with the semi-empirical AM1 method.

2.5. Estimation of Pharmacokinetic and Toxicological Properties

Absorption, distribution, metabolism, excretion, and toxicity (ADMET) refers to the characteristic properties of a drug that express its pharmacokinetics in the human body. Lipinski's rule of 5 states (26) that absorption or permeability is more likely when there are no more than five hydrogen bond donors and acceptors, no molecular weight greater than 500 g/mol, and octanol/water partition coefficient greater than five. In this study, we evaluated the absorption, distribution, metabolism, and excretion properties of two synthesized molecules using the SwissADME online database (27) and screened them according to the Lipinski 5 rule. We also examined the toxicity risk assessment properties of the compounds with the help of the ProTOX-II online database (28) and OSIRIS Property Explorer (29).

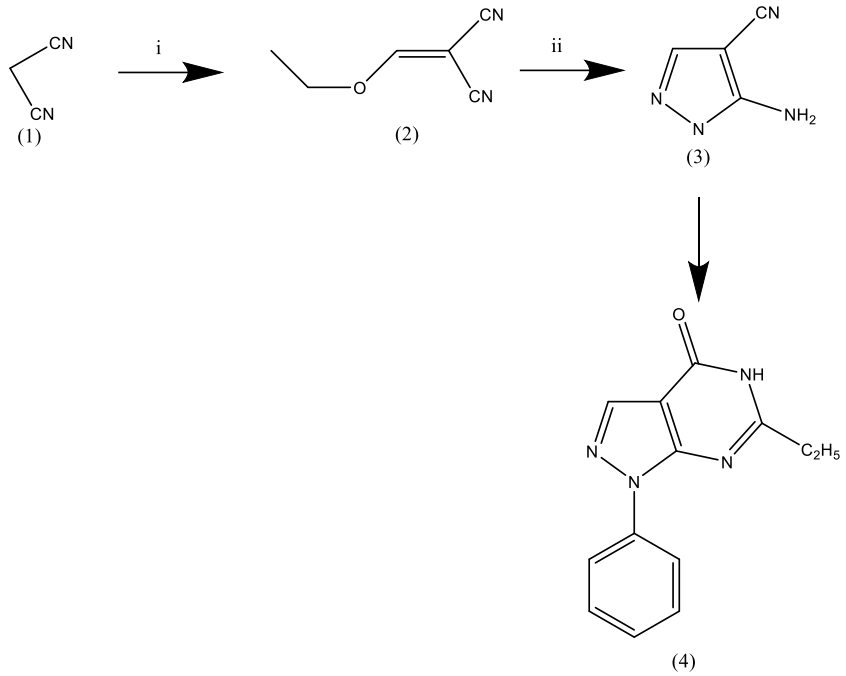
3. RESULTS AND DISCUSSION

3.1. Synthesis of Compounds 4 and 9

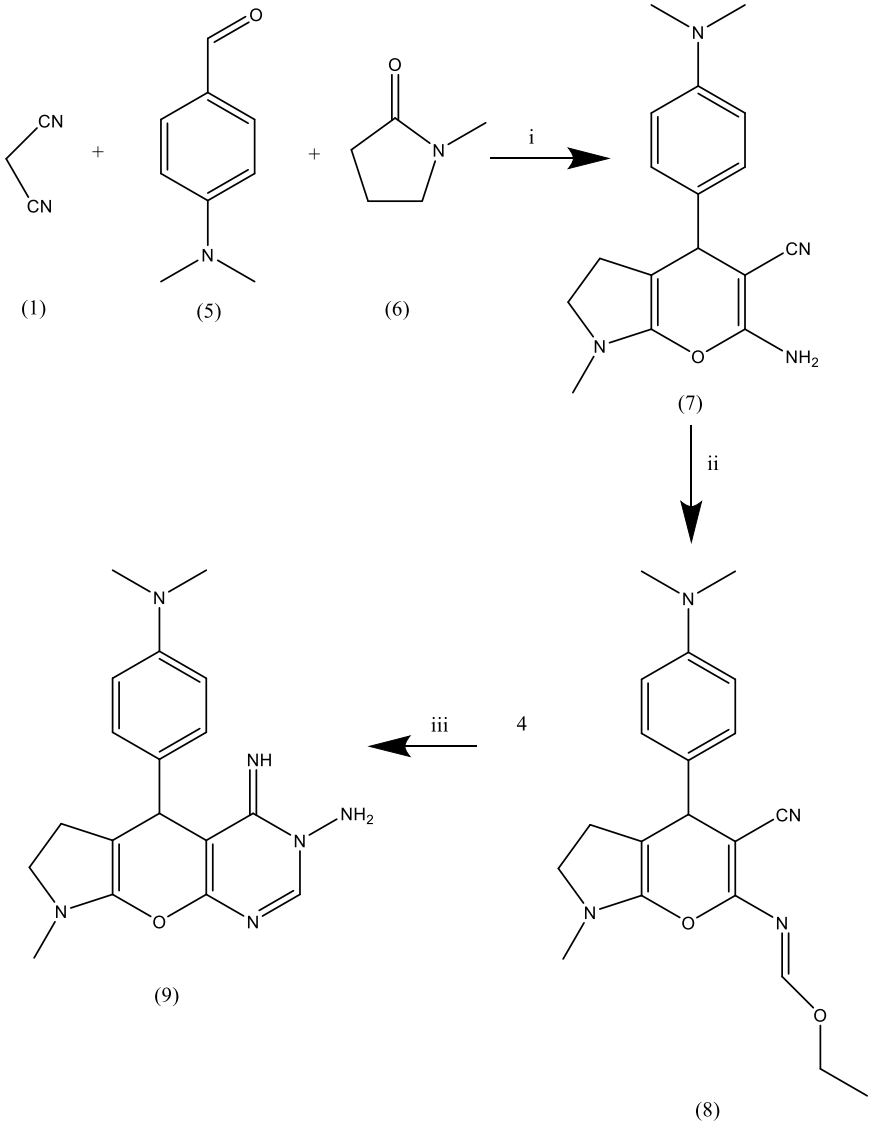
Previous studies mentioned that a good yield of compound **3** 5-amino-4-cyano pyrazole (30,31) could be obtained by the standard addition of phenylhydrazine to an unsaturated ethoxymethylene compound **2**. Compound **4** was synthesized by treating compound **3** with some aliphatic carboxylic acids such as acetic acid, formic acid, and propionic acid respectively in the presence $POCl_3$ as shown in Scheme (1).

3.2. Characterization by FT-IR and ^{1}H NMR

The intermediate compounds **3** and **7** were well characterized using FT-IR and ^{1}HNMR. The FT-IR spectra of the compound **3** in Figure (1a), showed stretching bands at 3329 and 3431 cm^{-1} for symmetrical and unsymmetrical (NH_2), 3109 cm^{-1} for (C-$H_{arom.}$), 2931-3061 cm^{-1} for (C-$H_{aliph.}$), 2222 cm^{-1} for (CN), 1637 cm^{-1} for (C = N) and 1599 cm^{-1} for (C=C) with the disappearance of stretching bands from the ether group. The FT-IR spectra of **4** in Figure (1b) showed bands of (NH_2) stretching at 3329 cm^{-1}. Although 3431 cm^{-1} and (C≡N) at 2222 cm^{-1} were not recognized, the (NH) stretching at 3155 cm^{-1}, ($CH_{arom.}$) at 3103 cm^{-1}, ($CH_{aliph.}$) at 2816-2943 cm^{-1}, (C=O) at 1685 cm^{-1}, (C=N) at 1604 cm^{-1}, and (C=C) at 1554 cm^{-1} were observed.



Scheme 1: Synthesis of compound **4**. The conditions used were (**i**) Triethylorthoformate/acetic anhydride, 140 °C,4 h. (**ii**) Phenyl hydrazine/EtOH,78 ºC, 2 h. (**iii**) Propionic acid/ $POCl_3$ / K_2CO_3,120 °C, 18 h.



Scheme 2: Synthesis of compound **9**. The reaction conditions were (**i**) (C_2H_5) 4N + Br /EtOH, 78 °C, 1h. (**ii**) Triethylorthoformate/acetic anhydride,140 °C, 5h. (**iii**) $NH_2NH_2.H_2O$/ MeOH, room temperature for 2h.

Compound **7** was added to triethylorthoformate in acetic anhydride to form an imidoformate derivative **8**, then compound **8** was added to hydrazine hydrate for 1 hr 2° to form fused pyrimidine derivative [9], Scheme (2). The FTIR spectra of the compound **7** in Figure 1c showed stretching bands symmetrical and asymmetrical at 3335 and 3433 cm^{-1} for NH_2, 3213 cm^{-1} for aromatic C-H stretching, 2860 - 3076 cm^{-1} for aliphatic C-H stretching, 2208 cm^{-1} for C≡N stretching, 1610 cm^{-1} for N-H bending, 1562 cm^{-1} for C=C, band at 1234 cm^{-1} for asymmetrical C-O-C and band at 1072 cm^{-1} for symmetrical C-O-C. For compound **9**, a band of C≡N, but bands of NH_2 stretching at 3319 and 3416 cm^{-1}, N-H at 3192 cm^{-1}, aromatic C-H at 3122 cm^{-1}, aliphatic C-H at 2810-3072 cm^{-1}, C=N at 1602 cm^{-1}, and a band C=C at 1550 cm^{-1}, Figure 1d.

The ^{1}H NMR spectrum of compound **3** in Figure (2e) showed signals at δ = 6.69 ppm (s, 2H, NH_2), δ = 7.42-7.54 ppm (m, 5H, ArH) and δ = 7.78 ppm (s, 1H, N=CH). For compound **4**, signals at δ = 2.14 ppm (s, 3H, CH_3), δ = 7.36-8.05 ppm (m, 5H, ArH), δ = 8.27 ppm (s, 1H, N=CH) and δ = 12.34 ppm (s, 1H, NH). The ^{1}HNMR spectrum of compound **7** in Figure 2f showed signals at δ = 2.28 ppm (s, 3H, NCH_3), δ = 2.40 ppm (t, 2H, CH_2), δ = 2.59 ppm (t, 2H, NCH_2), δ = 2.82 ppm (s, 6H, $N(CH_3)_2$), δ = 4.45 ppm (s, 1H, CH), δ = 6.34 ppm (s, 2H, NH_2), δ = 6.58-7.24 ppm (m, 4H, ArH).

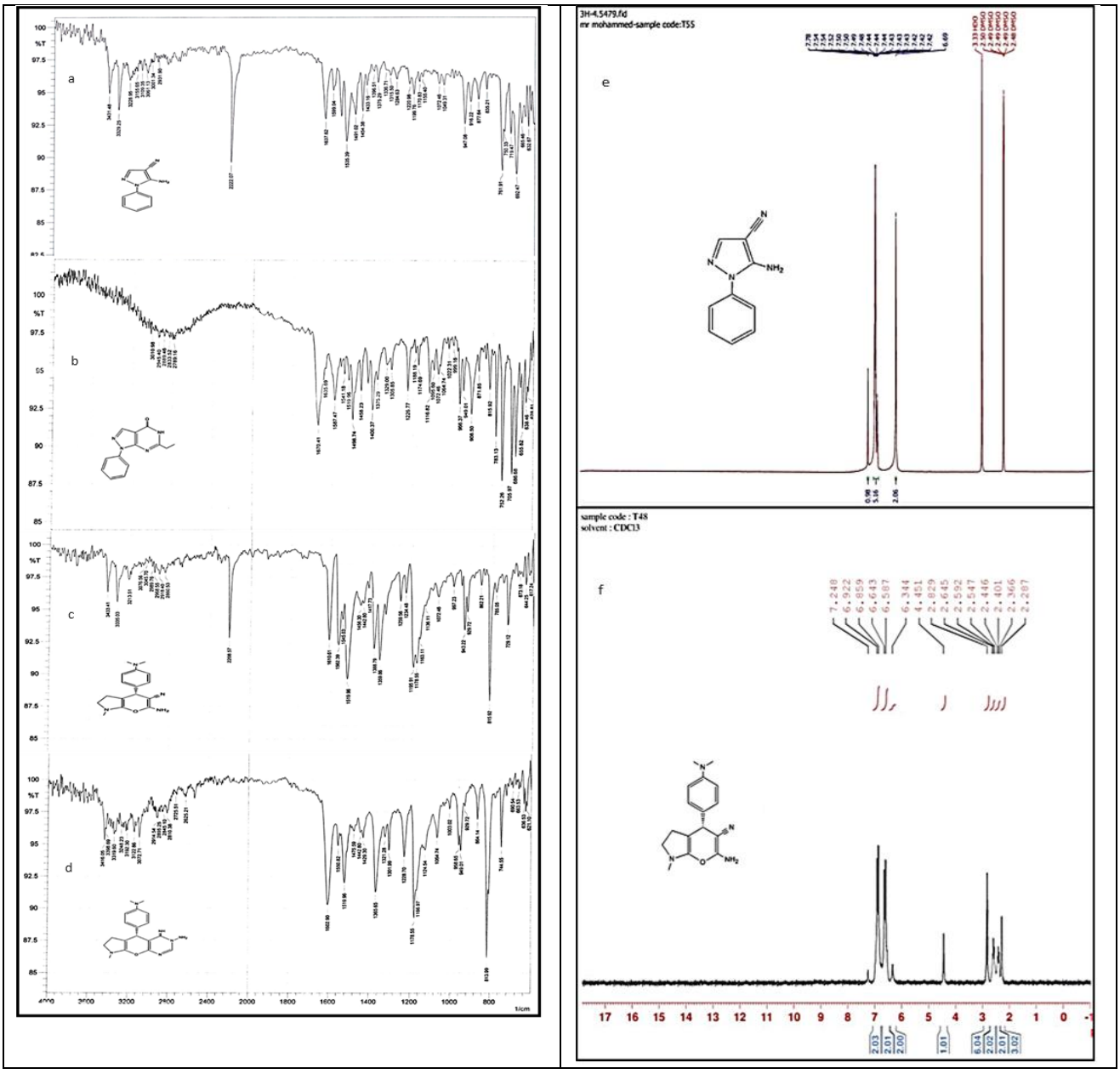


Figure 2: Characterization of the prepared compounds. The FTIR spectra of compounds **3**, **4**, **7**, and **9** are shown in a-d. The ^{1}H NMR of compounds **3** and **7** are shown in e and f.

3.3. Effect of Pyrimidine Derivatives (Compund 4 and 9) on AChE Activity

Clinically, acetylcholinesterase inhibitors (AChEI) are used to treat various diseases, including Myasthenia gravis, Glaucoma, Lewy body dementia, and Alzheimer's disease (32).

Treatment is believed to reduce symptoms by improving cholinergic function and increasing the

amount of acetylcholine in cholinergic synapses. The active site must be reversibly bound to the active site of the enzyme to be a successful inhibitor, as irreversible binding can result in severe consequences, including death (33). In this regard, in many nations, the synthesis of fused pyrimidine compounds and investigating microbiological and toxicity tests are commonly useful.

The activity of AChE was estimated before and after adding the compounds **4** and **9** using a series of concentrations (10^{-2}, 10^{-3},10^{-5},10^{-7},10^{-9},10^{-11}) M of and different concentrations of ASChI (0.02, 0.04, 0.06 and 0.08) M) Figure 2a. In this paper, the effect of compounds **4** and **9** on human AChE activity against DMSO as a blank solution was analyzed, Figure 2b. Pyrimidine derivatives **4** and **9** showed inhibitory effects on enzyme activity compared with the normal values of AChE (2.63 µmol/2 min/mL), Figure 2b, Table 2.

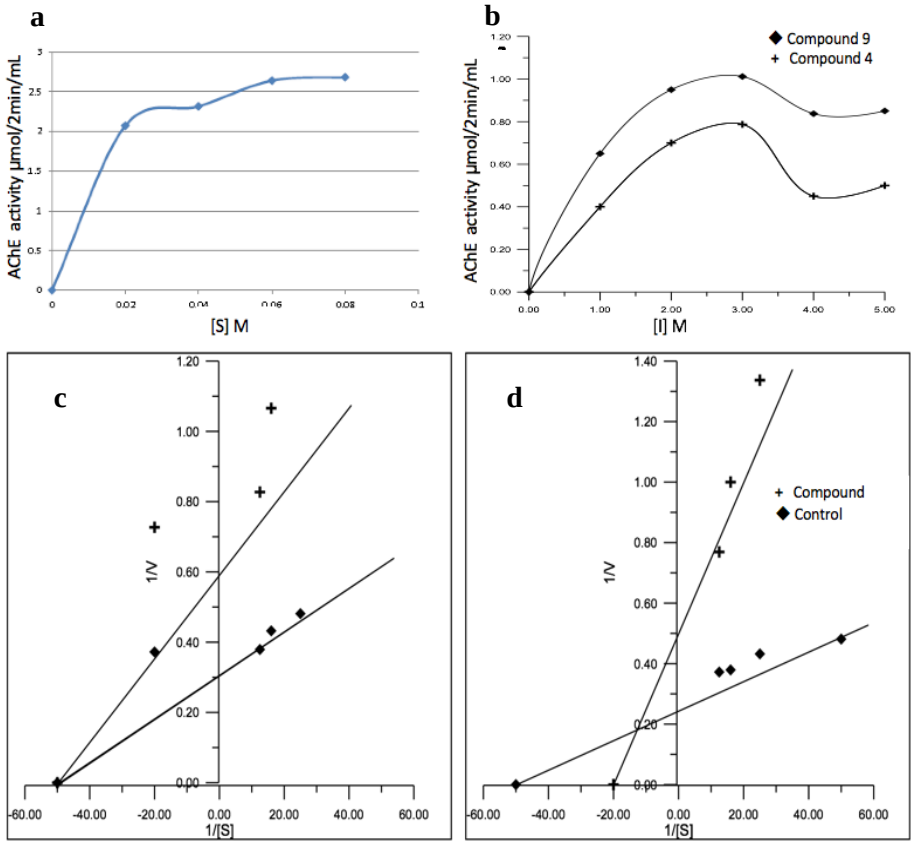


Figure 3: The activity of human AChE at different concentrations of ASChI before and after adding compounds **4** and **9**. a) The enzymatic activity of free AChE, b) The enzymatic activity of AChE after adding compounds **4** and **9**. The Lineweaver-Burk plots of AChE in the presence of the maximum concentration of compounds **4** and **9** are shown in c and d.

Table 2: Effect of pyrimidine (compounds **4** and **9**) on AChE activity.

Compounds	**Inhibitor (M)**	**AChE μ_{mol}/2min/ml**	**% inhibition**
Control	zero	2.630	-
Compound **4**	10^{-3}	0.500	80.98
	10^{-5}	0.450	82.88
	10^{-7}	0.780	70.07
	10^{-9}	0.700	73.38
	10^{-11}	0.400	84.79*
Compound **9**	10^{-3}	0.850	67.68
	10^{-5}	0.837	68.18
	10^{-7}	1.012	61.60
	10^{-9}	0.950	63.88
	10^{-11}	0.650	75.29*

* Maximum inhibition concentration in each compound.

Table 2 indicated that the highest significant inhibition% of compound **4** (84.79%) and compound **9** (75.29%) were at (10^{-11}) M concentrations, which could be attributed to the presence of more than one nucleophile side in both compounds. Compound **9** has (NH_2), dimethyl aniline group linked to anthracenyl showed good inhibition of AChE activity, whereas the phenyl ring attached to imidazole [1,2-

a] pyridine in the compound **4** may compete with the substrate ASChI and cause properly orient to fit the active site of the enzyme. The type of inhibition and kinetic parameters (Km, Vmax, and Ki) at different substrate concentrations were determined using the Linweaver-Burk plot, Figure 2c and 2d), and Table 3.

Table 3: Kinetic properties of AChE with and without pyrimidine compounds.

Sample	Inhibitor (M)	K_m (M)	V_{max} (μ_{mol}/mL/2min)	K_i (M)	Inhibition type
Control	Zero	0.02	5	-	-
Compound **4**	10^{-7}	0.02	2.5	3×10^{-8}	Non-competitive
	10^{-11}	0.02	2.22	2.1×10^{-12}	Non-competitive
Compound **9**	10^{-7}	0.05	1.92	9.2×10^{-8}	Mix
	10^{-11}	0.05	1.81	8.3×10^{-12}	Mix

Results in Table 3 showed that K_m values varied depending on the type of inhibition. The higher Km value and the lower Ki value refer to the highest affinity of the inhibitor to fit into the active site cleft of the enzyme. Accordingly, compounds **4** and **9** have the highest affinity to bind the enzyme at 10^{-11} M, where the lowest Ki values. This assumes the kinetics of a tight-binding inhibitor. The results also showed that compound **9** have mixed-type inhibition, which can be attributed to the structure of the inhibitor that makes a conformational change after binding to –SH, -COOH, imidazole group of Ser, His, Glu in AChE, which located in the active center of the enzyme or essential in determining the active conformation of the enzyme molecule. On the other hand, the non-competitive inhibition of compound **4** is a classical model of inhibitor that binds to another site at enzyme molecule and causes conformational change locking the enzymatic activity.

The amide group in the pyrimidine ring and the hetero aromatic ring contributed to the inhibition of acetyl and butyl cholinesterase. Compared to the unsubstituted pyrazole compounds, the substitution of pyrazole with the aryl ring increases the inhibition potency of the compounds. Now, it has been shown that a large number of Sp^2 carbons, and therefore the π orbitals, increases molecular recognition by the AChE enzyme, as the active site of AChE is composed of many aromatic amino acids (34). Importantly, the affinity toward the active site could be affected by several factors, such as size, three-dimensional structure, the existence of groups that easily bind noncovalently to groups within or near the active site, etc. The position in space could be attributed as a result of such a good-fit orientation such as the covalent bonding and hydrogen bonding with the serine residue. A previous study (35) reported a rational design of 5H-thiazolo[3,2-a] pyrimidine derivatives that acted as AChE inhibitors binding to the active site of the human AChE substratum domain. Studies of molecular analysis led to the discovery of some pyrimidine derivatives, such as 3H-thiazole substituted with 3H [3,2-a], where their biological activity was fully in line with the proposed binding. The replaced 5H-thiazole [3,2-a] pyrimidine derivatives could improve the structure of enzyme inhibitors into novel therapeutic agents for serious neurodegenerative diseases.

Another study (36) developed a sequence of 6H-benzo[c]chromen-6-one and 7,8,9,10-tetrahydrobenzo[c]chromen-6-one derivatives in a variety of plant-derived nutritional products. The biological activity was tested as a potential inhibitor of acetylcholine esterase and butyrylcholine esterase. However, it did not show an inhibitory effect on cholinesterase enzymes and suggested that compounds based on the inhibition of cholinesterase enzymes should be generated with benzo [c] chromene-6-one.

A new series of some novel 1,2,3,4-tetrahydropyrimidine condensed pyrazinamide has been developed and evaluated as inhibitors of acetyl and butyl cholinesterase (AChE and BuChE) (37,38) reported a straightforward two-stage synthesis and biological evaluation of novel racemic benzochromene pyrimidinones as non-hepatotoxic, acetylcholine esterase inhibitors and found significantly lower inhibition of hAChE compared to EeAChE, IC50 values ranging from 1279 to 3657 nM. Compound 3Bb was the most effective inhibitor.

The kinetic mechanism of inhibition of hAChE by compound 3Bb was investigated using classical double reciprocal plots of Lineweaver-Burk to gain insight into the mode of inhibition. Analysis of this graph revealed the interception of the lines above the x-axis, suggesting that 3Bb is capable of interacting with both the free and the acylated enzyme, thus acting as a mixed-type hAChE inhibitor. The constants of inhibitor dissociation Ki (the constant of complex dissociation enzyme-inhibitor) and K'i (the constant of complex dissociation enzyme-inhibitor-substrate) are calculated at 0.38 and 1.12 μM, respectively.

A sequence of novel 1,2,3,4-tetrahydropyrimidines of biological interest has been synthesized (37). Compound libraries were prepared using p-toluenesulfonic acid as an active catalyst compared to Lewis acid. The results showed that all synthesized compounds were active against acetyl and butylcholinesterase enzyme activities. Anis et al (39) prepared a new hybrid molecule type pyrazolo[4,3-e]-1,2,4-triazole [1,5-c] pyrimidines derivatives. The anti-acetylcholine esterase activity of compounds was evaluated, and results indicated significant activities (IC_{50}= 1.73–39.86 μM). The

dihydrobenzimidazopyrimidine derivatives were analyzed against acetylcholinesterase (AChE) and exhibited effective inhibitory activity at 46.8 nM and 42.5 nM IC_{50} (40).

3.4. Molecular Docking

In the present work, an in silico study was performed using molecular docking simulation to test the capability of some synthesized compounds as potential AChE inhibitors. Target compounds **4** and **9** and Donepezil were docked as ligands with the active pocket of AChE (PDB ID: 2ACE) to achieve favorable conformation, with the maximum number of interactions and minimal free energy, as shown in Figs. 4, 5, and 6. The study findings including binding energies and types of interactions are shown in Table 4.

Figure 4 shows 2D and 3D Donepezil interactions as a ligand with target 2ACE, including H-bond, π- π, and van der Waals interactions with residues of Tyr121, Trp279, Tyr334, Phe284, and Leu282 amino acids at the active site of AChE with a binding energy -8.6 kcal/mol and distances ranging from 2.68 to 3.23. The docking simulation of compounds **4** and **9** demonstrated stronger electrostatic interactions (van der Waal's, π−π stacking, and H-bond) with lower docking energies -11.4, -10.6 kcal/mol respectively as shown in Fig. 4, 5 & 6. Among the other derivatives, derivative **4** formed H-bond interactions with key amino acids residues Tyr334, and Asp72, whereas the other electrostatic interactions formed with Tyr334, Phe330, Ile287, Tyr121, Arg289, Trp279, Gly335, and Phe288. In the case of derivative **9**, similar binding interactions with the active pockets of 2ACE were observed due to the high homology of the binding site residues. The considerable number of various binding interactions with amino acid residues of the active pocket in the target protein, as well as favorable binding energies, suggests that these compounds could be used as clinically effective inhibitors for the AChE enzyme.

Table 4: Docking results obtained for synthesized coumarin derivatives with acetylcholinesterase (PDB ID: 2ACE).

Compound	Ligand moiety	Site	Interaction	E (kcal/mol)
4	NH_2	N TYR 334 (A)	H-bond acceptor	-12.4
	NH	O ASP 72 (A)	H- bond acceptor	
	C=O	N GLY 335 (A)	H- bond donor	
	6-ring	TYR 334 (A)	Pi-Pi	
	C=C	ILE 287 (A)	Pi-Alkyl	
9	NH_2	N ASP 72 (A)	H- bond acceptor	-11.6
	NH	O PHE 330 (A)	H- bond acceptor	
	C=C	N ASP 72 (A)	Pi- Anion	
	6-ring	TYR 334 (A)	Pi-Alkyl	
		TRP 279 (A)	Pi-Alkyl	
Donepezil	$-OCH_3$	NH TYR 121 (A)	H- bond acceptor	-8.6
		O PHE 330 (A)	H- bond acceptor	
	6-ring	N PHE 284 (A)	H- donor	
		TRP 279 (A)	Pi-H bond	
	C=C	ASP 285 (A)	Pi-Pi	
		LEU 282 (A)	Pi-Alkyl	

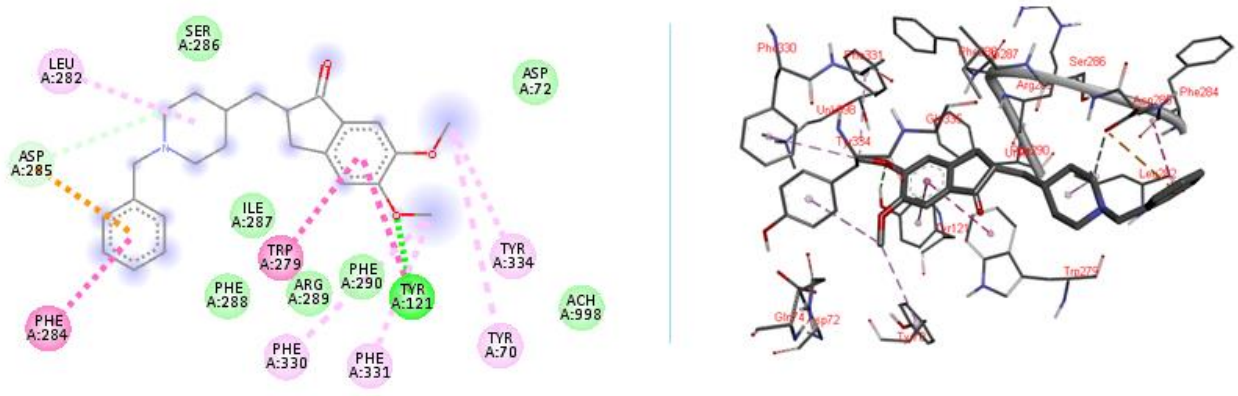


Figure 4: The receptor-ligand interactions on 2D and 3D-Diagram of Donepezil on the catalytic site of AChE enzyme.

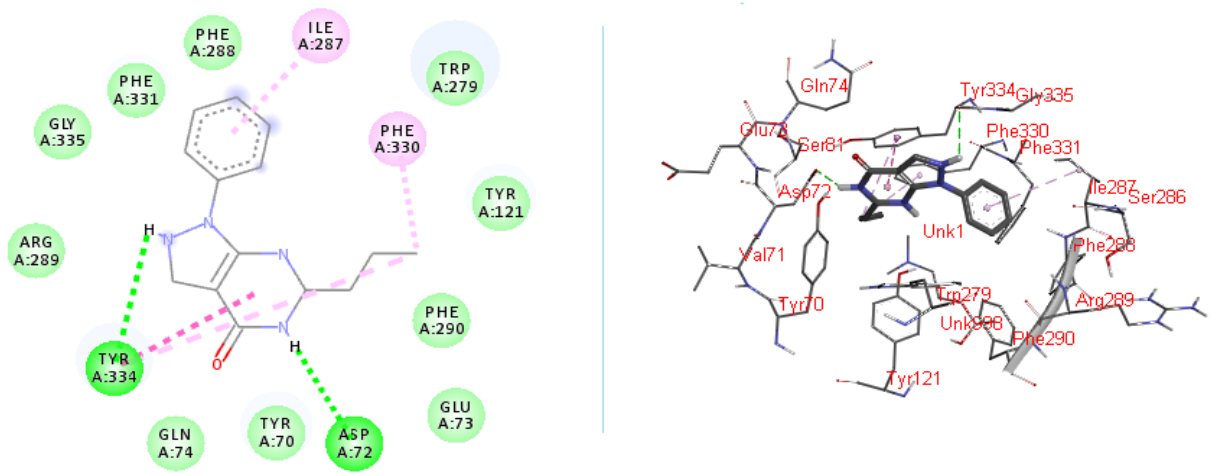


Figure 5: The receptor-ligand interactions on 2D and 3D-Diagram of compound **4** with the catalytic site of AChE enzyme.

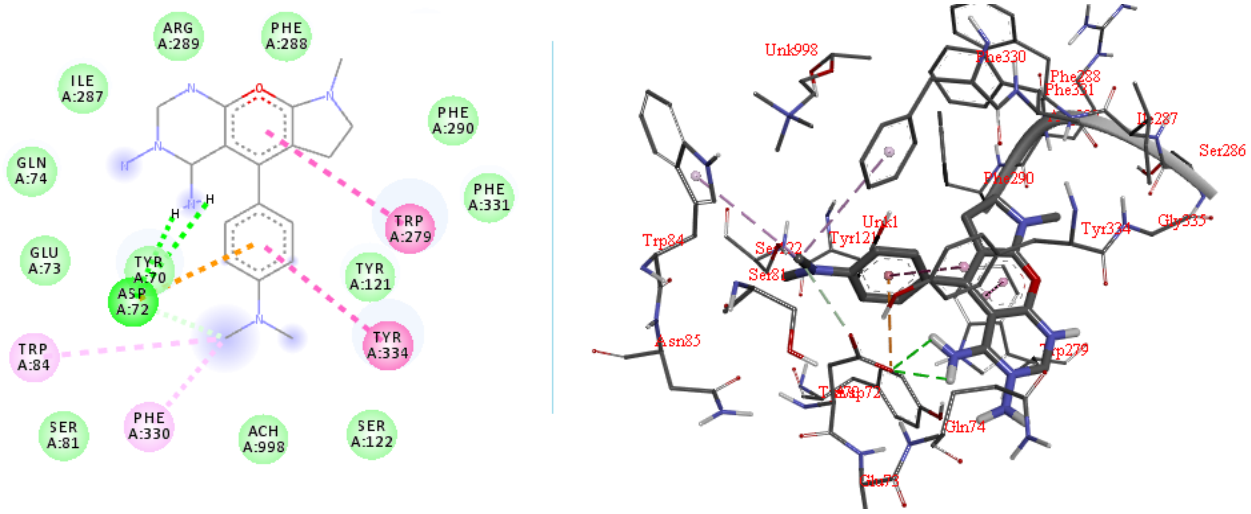


Figure 6: The receptor-ligand interactions on 2D and 3D-Diagram of compound **9** with the catalytic site of AChE enzyme.

3.5. Pharmacokinetic and Toxicological Properties

Both compounds have high gastrointestinal absorption. It shows that these compounds can be easily consumed in the gastrointestinal tract when taken by oral administration. Both compounds can cross the blood-brain barrier and cannot be used as substrates of P-glycoprotein. The red dot in the yolk area indicates that compounds **4** and **9** may remain in the brain unaffected by P-glycoprotein. According to the BOILED-Egg data model, compounds **4** and **9** have excellent drug capability for both blood-brain barrier penetration and gastrointestinal absorption, Figure 7. Although compound **4** is an inhibitor of CYP1A2, it is a non-inhibitor of the enzymes CYP2C19, CYP2C9, CYP2D6, and CYP3A4 enzymes. While Compound **9** is the non-inhibitor of CYP1A2, it is the inhibitor of CYP2C19, CYP2C9, CYP2D6, and CYP3A4 enzymes. A cytochrome P450 non-inhibitor indicates that the molecule will not interfere with the biotransformation of the substance (drug) metabolized by cytochrome P450. Both compounds comply with Lipinski's rule of 5, see Table 5. When the toxic properties of the compounds were evaluated, it was determined that Compound **4** had hepatotoxic and carcinogenic properties but was mutagenic, immunotoxic, cytotoxic, irritant, and inactive. Compound **9** is inactive in terms of hepatotoxic and carcinogenic, mutagenic, immunotoxic, cytotoxic, and irritant. When all these properties are evaluated, it is recommended to carry out further research on the drug properties of compounds **4** and **9**.

Table 5: The pharmacokinetic properties of compounds **4** and **9**.

Properties	4	9
Molecular weight[a]	240.26 g/mol	352.43 g/mol
Number of atoms	30	50
Heavy atoms	18	26
Rotatable bonds	2	5
H-Bond acceptors	3	4
H-Bond donors	1	0
Molar refractivity	69.26	105.73
TPSA (Å^2)	63.57	61.09
Log $P_{o/w}$	2.20	2.83
GI absorption	High	High
BBB permeant	Yes	Yes
P-gp substrate	No	No
CYP1A2 inhibitor	Yes	No
CYP2C19 inhibitor	No	Yes
CYP2C9 inhibitor	No	Yes
CYP2D6 inhibitor	No	Yes
CYP3A4 inhibitor	No	Yes
Log K_p (cm/s)	-6.72	-6.50
Lipinski	Yes, 0 violation	Yes, 0 violation
Toxicity class[a]	4	4
Predicted LD_{50}	1000 mg/kg	1200 mg/kg
Hepatotoxicity	Active	Inactive
Carcinogenicity	Active	Inactive
Immunotoxicity	Inactive	Inactive
Mutagenicity	Inactive	Inactive
Cytotoxicity	Inactive	Inactive
MMP[b]	Inactive	Inactive
Irritant	Inactive	Inactive

[a]The toxicity class consists of six numbers. (Number 1 means toxic; number 6 means non-toxic.)
[b]MMP: Mitochondrial Membrane Potential

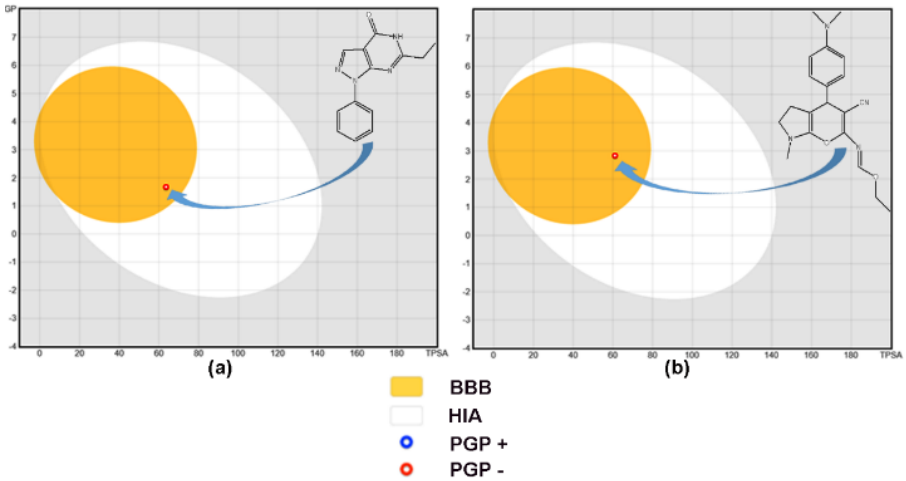


Figure 7: The BOILED-Egg models of compounds **4** (a) and **9** (b).

4. CONCLUSION

In the present study, a new family of multitarget molecules has been designed to be able to interact with AChE. These compounds were well characterized and showed significant substitutions predominantly at (NH_2) and dimethyl aniline group linked to anthracenyl. These substituted molecules showed a good inhibitory effect on AChE activity, whereas a phenyl ring attached to imidazole [1,2-a] pyridine was studied to improve the inhibitory of AChE activity. The data of this research suggest that these molecules are promising leads for the development of novel multitarget-directed ligands of MTDL with good inhibitory potency of AChE, which is presently missing in the therapeutic arsenal. The strong interactions with amino acid residues of the active pocket in the target protein as well as favorable binding energies suggest that these compounds could be used as clinically effective

inhibitors for the AChE enzyme. When the predicted pharmacokinetic and toxicological properties (ADMET) of the synthesized compounds are evaluated, it is suggested that they may be good drug candidates due to their low toxicity class and compatibility with the Lipinski rules, and further *in vivo*/*in vitro* studies are recommended.

5. CONFLICT OF INTEREST

The authors have nothing to declare.

6. ACKNOWLEDGMENTS

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7. ETHICS APPROVAL AND CONSENT TO PARTICIPATE

7.1. Ethical Approval

Research ethics was approved by the ethics committee of Mustansiriyah University (Iraq).

7.2. Human and Animal Rights

Animals were not used in this investigation. The procedures were in accordance with the standard ethical statement authorized by Mustansiriyah University. The authors have obtained permission from the participants to do the in *vitro* study.

8. AUTHOR CONTRIBUTION

The authors have contributed equally to this manuscript.

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