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Design, Synthesis, and AChE Inhibitory Activity of New Benzothiazole-Piperazines

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ABSTRACT

In the current study, 14 new benzothiazole-piperazine compounds were designed to meet the structural requirements of acetylcholine esterase (AChE) inhibitors. The target compounds were synthesised in three steps. Structures of the newly synthesised compounds (**7-20**) were confirmed using IR, 1H-NMR, 13C-NMR, and HRMS methods. The inhibitory potential of the compounds on AChE (E.C.3.1.1.7, from electric eel) was then investigated. Among the compounds, **19** and **20** showed very good activity on AChE enzyme. Kinetics studies were performed to observe the effects of the most active compounds on the substrate–enzyme relationship. Cytotoxicity studies, genotoxicity studies, and theoretical calculation of pharmacokinetics properties were also carried out. The compounds **19** and **20** were found to be nontoxic in both of the toxicity assays. A good pharmacokinetics profile was predicted for the synthesised compounds. Molecular docking studies were performed for the most active compounds, **19** and **20**, and interaction modes with enzyme active sites were determined. Docking studies indicated a strong interaction between the active sites of AChE enzyme and the analysed compounds.

Keywords: Alzheimer disease; acetylcholinesterase; benzothiazole; piperazine; docking.

Alzheimer's disease (AD) is a progressive and fatal neurodegenerative disorder characterised by memory loss and behavioural disturbances.[1-4] The worldwide prevalence of this tragic public problem has been estimated to reach 106.2 million by 2050.[4-6] Various pathological hallmarks, such as accumulation of β amyloid in senile plaques, hyper-phosphorylated neurofibrillary tangles of tau protein, and loss of cholinergic activity in certain parts of brain, have been shown to be responsible for AD.[7–10] Currently, only the non-competitive N-methyl-D-aspartate receptor antagonist, memantine, and the cholinesterase inhibitors, tacrine, donepezil, rivastigmine, and galantamine, have been approved by the US Food and Drug Administration for the treatment of AD.[11-13] Among cholinesterase inhibitors, galantamine and donepezil selectively inhibit acetylcholinesterase (AChE), whereas rivastigmine and tacrine non-selectively inhibit both AChE and butyrylcholinesterase (BChE).[14]

The brain cholinergic system is vital for learning and memory consolidation and is known to be disrupted in AD.[8,10,15,16] The synaptic cholinergic transmission is regulated by AChE and BChE enzymes that rapidly hydrolyse acetylcholine (ACh).[17] AChE shows more hydrolytic activity than BChE does. Hence, AChE inhibitors are preferred in the treatment of AD to keep ACh levels normal.[18] Donepezil is the most favourable AChE inhibitor since it gives a relatively positive response in AD treatment. Furthermore, compared to other AChE inhibitors, donepezil has some advantages, such as blood–brain barrier permeability, non-hepatotoxicity, the least side efficacy, and consumption once daily.[19-20]

Previous studies[21-31] have shown that AChE possesses a 20 Å-long, narrow gorge, which contains five separated ligand-binding sites: 1) the catalytic triad, including Ser 203, His 447, and Glu 334, which are located at the bottom of the gorge and directly participate in the catalytic cycle;[21] 2) the oxygen anion hole containing Gly 121, Gly 122, and Ala 204 and has a role in the arrangement of hydrogen bond donors that stabilise the tetrahedral enzyme-

substrate complex;[22,23] 3) the catalytic anionic site (CAS), a region where Trp86, Glu202, and Tyr337 are situated. This site is responsible for the orientation and stabilisation of the trimethylammonium group of ACh by forming cation-π interactions.[24-27] 4) The acyl pocket covers two phenylalanine residues, 295 and 297, which bind the acetyl group of ACh.[28] 5) The peripheral anionic site (PAS) comprises residues Tyr 72, Tyr 124, Trp 286, and Asp 74, which are located at the entrance of the narrow gorge.[29-31]

Tacrine and galantamine bind to PAS, whereas some tacrine dimers, donepezil and its analogue BYYT-25, bind simultaneously to both PAS and CAS.[32-36] Studies suggest that AChE inhibitors should bear a core ring system that interacts with PAS, a basic centre that binds to CAS, and a linker, such as –O–, CH_2, CONH, and CONH(CH_2)n, between the core ring system and the basic centre to fulfil structural requirements.[37–39] For example, AChE inhibitors donepezil and BYYT-25 contain an indanone core ring, methylene or oxygen linkers, and benzylpiperidine or benzylpyrrolydine basic centres. The dimethoxyindanone moiety of donepezil binds to the PAS, whereas the benzylpiperidine moiety interacts with the CAS.[33-35] BYYT-25 shows similar interactions with CAS and PAS.[36]

On the basis of the above information, we designed a new series of benzothiazole–piperazine derivatives (**7-20**) that have the aforementioned structural requirements (**Figure 1**). Benzothiazole is an important ring system in the drug discovery studies of AD. There are several benzothiazole compounds that show potential therapeutic effects against AD.[40-46] Furthermore, sabeluzole, a benzothiazole agent, delays the clinical progression of AD.[47,48] Thus, we selected benzothiazole as a core ring system. Structural similarity of benzothiazole with donepezil and BYYT-25 was increased by substitution with methoxy at the C5 and C6 positions. The basic centre was established by bioisosterically replacing piperidine with piperazine because numerous piperazine compounds have been reported as cholinesterase inhibitors.[49-53]

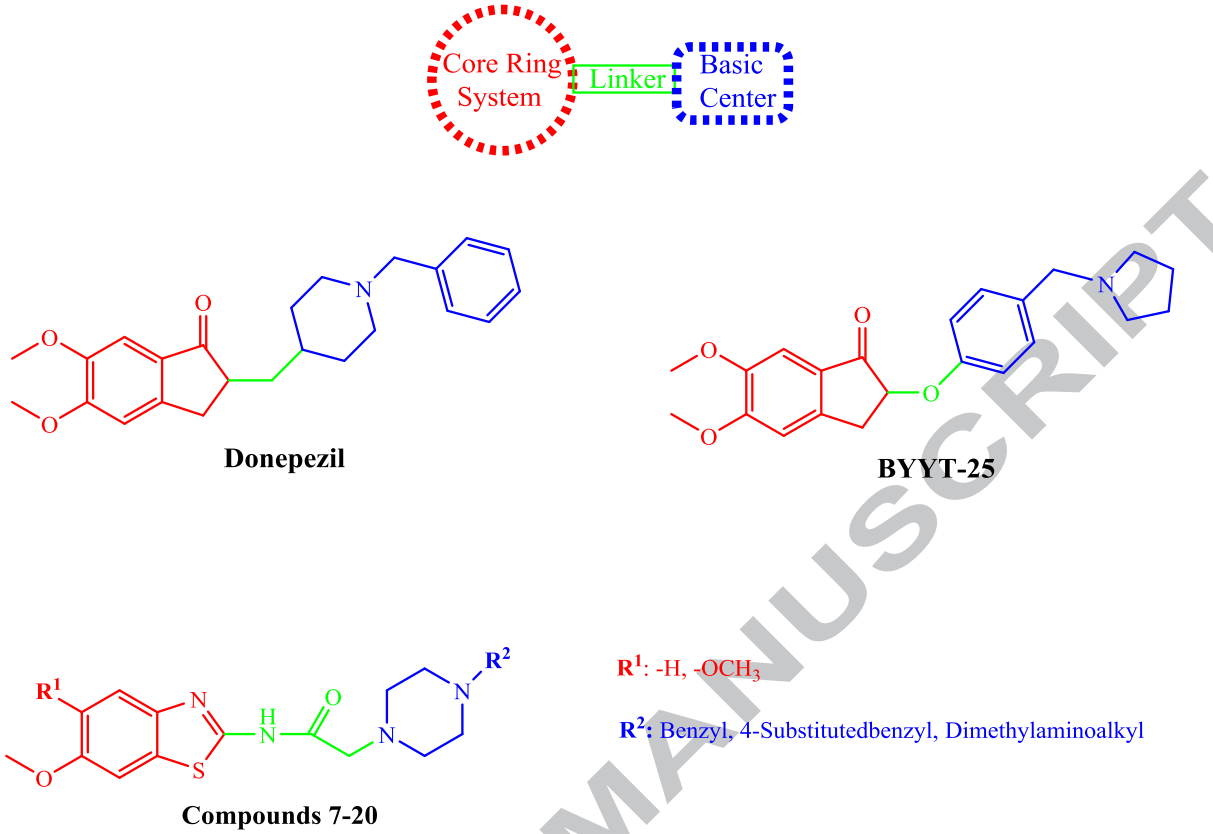
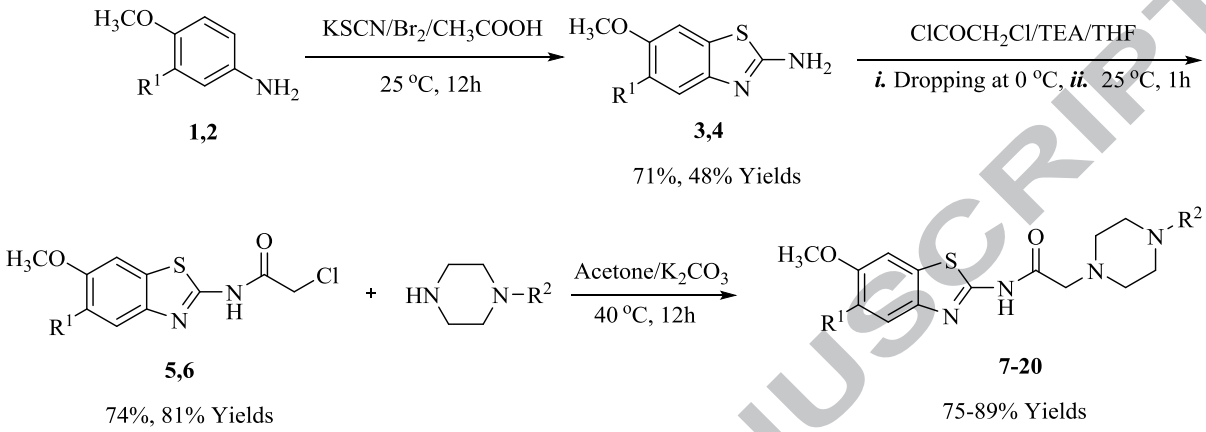


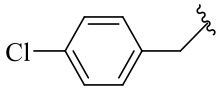
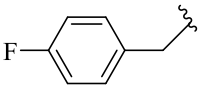
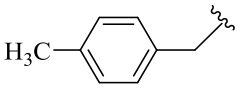
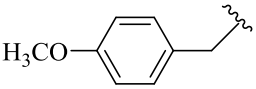
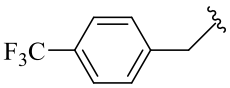
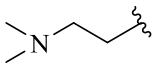
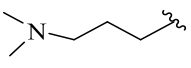
Figure 1: Structural requirements in design of the compounds **7-20**.

In the present study, we synthesized novel compounds investigate their AChE inhibition potential. The compounds **7-20** were synthesized as shown in **Scheme 1**. In the first step, 2-aminobenzothiazole derivatives (**3** and **4**) were prepared via reaction of appropriate aniline (**1** and **2**), potassium thiocyanate, and bromine. In the second step, 2-chloro-N-(benzothiazol-2-yl)acetamide derivatives (**5** and **6**) were synthesized via acetylation reaction by using chloroacetyl chloride. Finally, substitution reaction between corresponding 1-substituted piperazines and 2-chloro-N-(benzothiazol-2-yl)acetamide derivatives (**5** and **6**) yielded the target compounds **7-20**. The structures of the newly synthesized compounds (**7-20**) were elucidated using IR, ^{1}H-NMR, ^{13}C-NMR, and HRMS methods.[54]

Assessment of the compounds **7-20** as AChE (E.C.3.1.1.7, from electric eel) inhibitors was carried out using the *in vitro* modified Ellman's spectrophotometric method.[55] We used donepezil as the reference drug in the enzymatic activity for the above process. Synthesized

compounds **7-20** along with donepezil were tested at 10^{-3}–10^{-9} M concentrations. The IC_{50} values obtained for these compounds are presented in **Table 1**.



Compound	R^1	Compound	R^1	R^2
7	-H	**14**	$-OCH_3$	
8	-H	**15**	$-OCH_3$	
9	-H	**16**	$-OCH_3$	
10	-H	**17**	$-OCH_3$	
11	-H	**18**	$-OCH_3$	
12	-H	**19**	$-OCH_3$	
13	-H	**20**	$-OCH_3$	

Scheme 1. Synthesis pathway of the compounds **7-20**.

Table 1. IC_{50}±SD (μM) values against AChE (E.C.3.1.1.7, from electric eel) and some physicochemical parameters of compounds **7-20** used in prediction of ADME profiles.

Comp.	MW	logP	tPSA	nON	nOHNH	Vol	Vio	DLS	AChE IC_{50}±SD (μM)
7	430.96	4.07	57.70	6	1	372.00	0	1.66	0.2196±0.012
8	414.51	3.56	57.70	6	1	363.40	0	1.57	0.2435±0.016
9	410.54	3.84	57.70	6	1	375.03	0	1.16	0.3259±0.019
10	426.54	3.45	66.93	7	1	384.01	0	1.27	0.3965±0.024
11	464.51	4.29	57.70	6	1	389.76	0	1.16	0.2742±0.015
12	377.51	2.02	60.94	7	1	349.77	0	1.75	0.0946±0.006
13	391.54	2.30	60.94	7	1	366.57	0	1.73	0.1167±0.009
14	460.99	3.66	66.93	7	1	397.55	0	1.79	0.1297±0.014
15	444.53	3.15	66.93	7	1	388.94	0	1.69	0.1754±0.009
16	440.57	3.43	66.93	7	1	400.57	0	1.32	0.2148±0.011
17	456.57	3.04	76.17	8	1	409.56	0	1.52	0.2519±0.014
18	494.54	3.88	66.93	7	1	415.31	0	1.37	0.1977±0.016
19	407.54	1.61	70.17	8	1	375.31	0	1.76	0.0462±0.004
20	421.57	1.89	70.17	8	1	392.11	0	1.76	0.0576±0.002
Donepezil	379.50	4.10	38.78	4	0	367.89	0	1.76	0.0287±0.005

MW: Molecular weight; **log P**: log octanol/water partition coefficient; **tPSA**: Total Polar Surface Area; **nON**: number of Hydrogen acceptors; **nOHNH**: number of Hydrogen donors were calculated using Molinspiration Calculation of Molecular Properties toolkit. **DLS**: Drug likeness Model Score was calculated using MolSoft 2016 Drug-Likeness and molecular property prediction toolkit.

All the compounds showed remarkable AChE inhibition activity. At 10^{-6} M concentration, the inhibition rate of all the compounds was more than 50%. Compounds **12**, **13**, **19**, and **20** also showed more than 50% inhibition at 10^{-7} M concentration. Compounds **7**, **19**, and **20** showed more than 95% enzyme inhibition at 10^{-3} M concentration and approximately 90% enzyme inhibition at 10^{-4} M concentration. Regarding AChE inhibitory

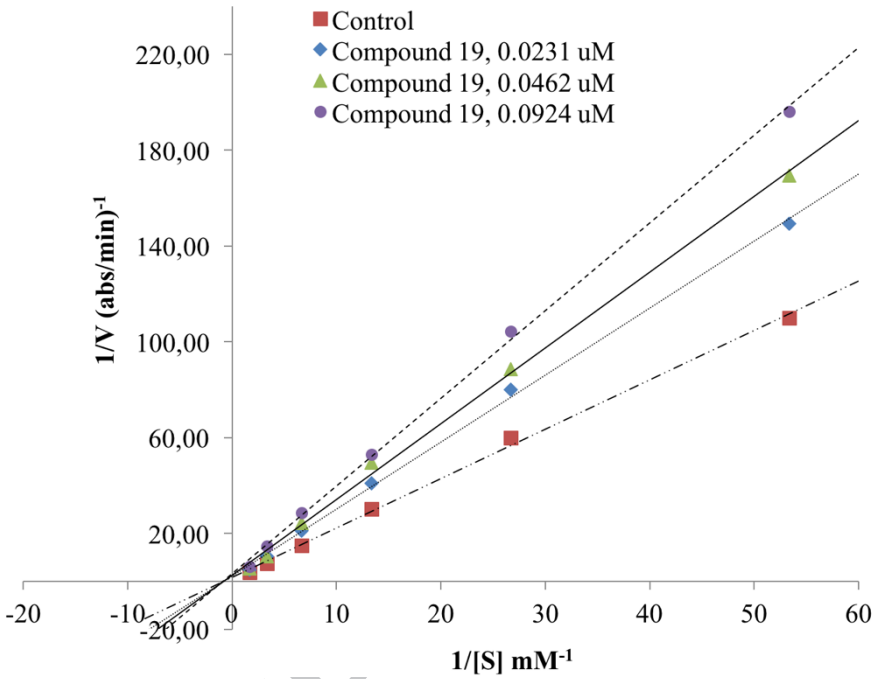
activity, all of the 6-methoxybenzothiazole derivatives (**7-13**) were less potent than the 5,6-dimethoxybenzothiazole compounds (**14-20**) were. Compounds containing the dimethylaminoethyl (**12** and **19**) and dimethylaminopropyl (**13** and **20**) moieties at the fourth position of piperazine showed stronger inhibition than did the compounds **7-11** and **14-18** that carry benzyl substitutions at the same position. Compounds **19** and **20**, which possess these two structural features, were found to be the most active derivatives in the series. The IC_{50} values of donepezil, compound **19,** and compound **20** were found to be 0.0287, 0.0462, and 0.0576 µM, respectively. These data indicate that the AChE inhibition potential of compounds **19** and **20** is close to that of donepezil. The other compounds (**7-18**) also have good IC_{50} in the range of 0.0942–0.3965 µM. As a result, all the newly synthesized compounds turned out to be potent inhibitors of AChE with a low micromolar range of IC_{50}.

The mechanism of AChE inhibition was investigated via enzyme kinetics by using the Ellman's spectrophotometric method.[56] Linear Lineweaver-Burk graphics were used to observe the type of inhibition. Further, we analyzed the enzyme kinetics by recording substrate-velocity curves in the absence and presence of the most potent compound **19** (IC_{50}=0.0462 µM). In each case, initial velocity measurements were obtained at different substrate (ATC) concentrations ranging from 600 µM to 18.75 µM. The *Ki* (intercept on the x-axis) values of the compounds **19** and **20** were calculated from the secondary plot of the 1/V versus concentrations of compounds.

The graphical analyses of steady-state inhibition data for the compounds **19** and **20** are presented in **Figures 2** and **3**. Very similar effects of these compounds on enzyme kinetics were observed. In the figures, the lines cross neither x- nor y-axis at the same point. Different *Vmax* and *Km* values were obtained for various concentrations that are presented along with the *Ki* values in **Table 2**. Thus, the Lineweaver–Burk plot reveals that compounds **19** and **20**

are typically mixed AChE inhibitors, which show significant similarity to donepezil.[57,58] The result also indicates that both these compounds interact with both CAS and PAS of AChE.

(**A**)



(**B**)

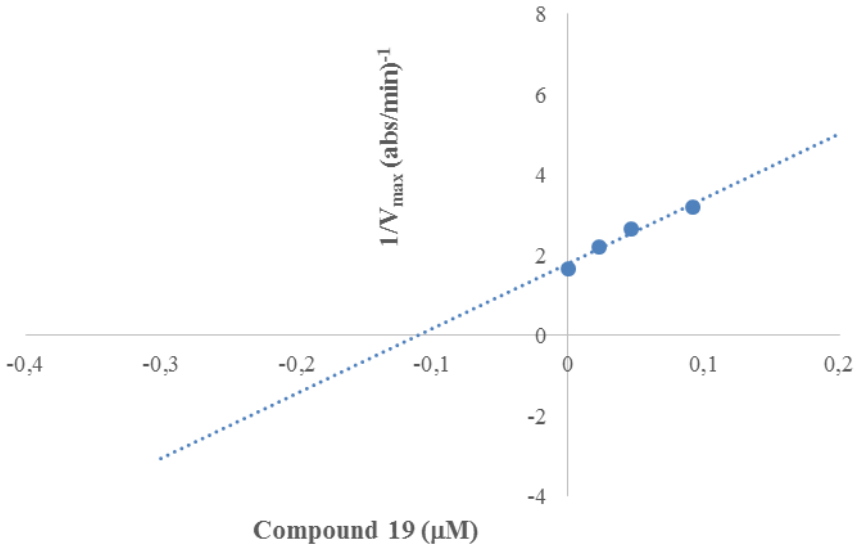
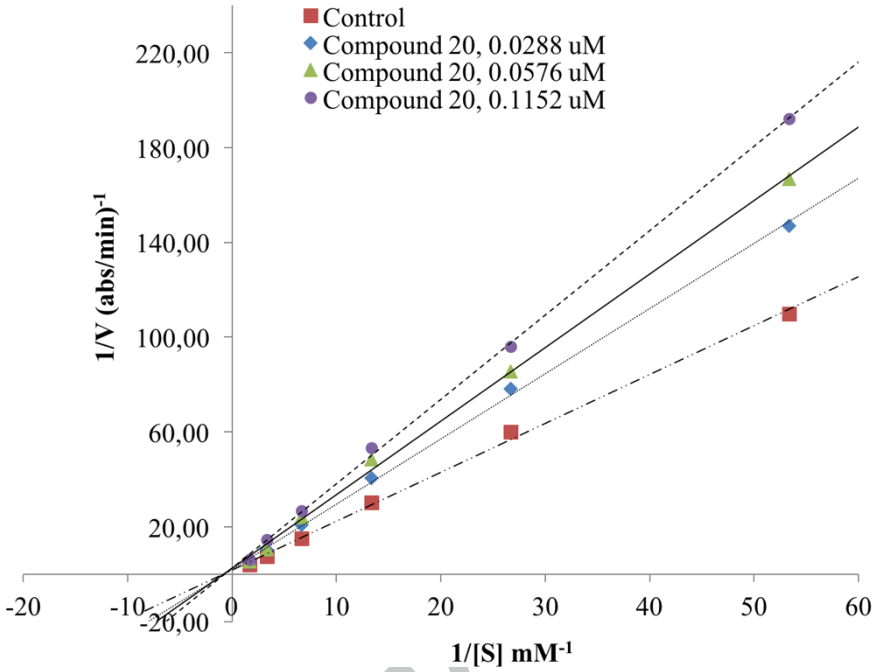


Figure 2. (**A**): Lineweaver-Burk plot for the inhibition of AChE (E.C.3.1.1.7, from electric eel) by compound **19** at different concentrations of substrate (ATC). (**B**): Secondary plot for calculation of steady-state inhibition constant (K_i) of compound **19**.

(**A**)



(**B**)

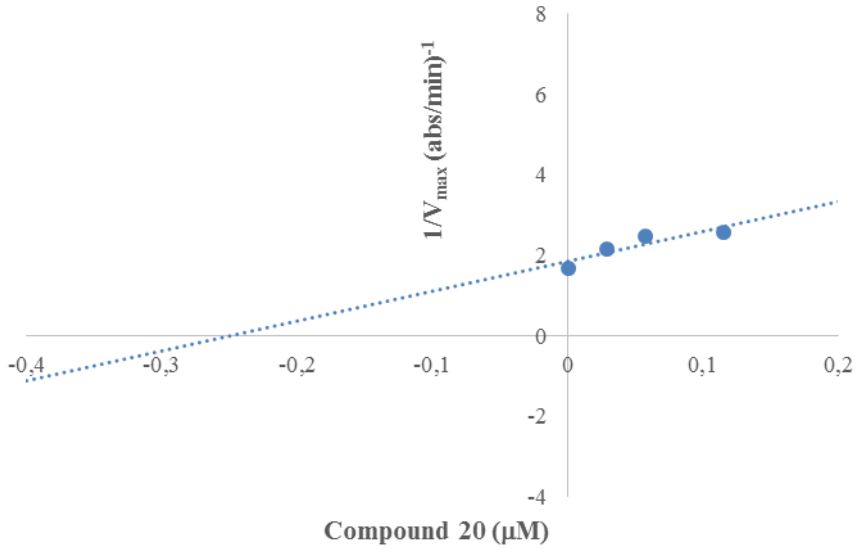


Figure 3. (**A**): Lineweaver-Burk plot for the inhibition of AChE (E.C.3.1.1.7, from electric eel) by compound **20** at different concentrations of substrate (ATC). (**B**): Secondary plot for calculation of steady-state inhibition constant (K_i) of compound **20**.

Table 2. V_{max}, K_m and K_i values of compounds **19** and **20.**

Comp.	Concentration (µM)	Vmax $(abs/min)^{-1}$	Km (mM)	Ki (µM)
19	**0.0231**	0.46	1.27	0.11
	0.0462	0.37	1.19	
	0.0924	0.31	1.15	
20	**0.0288**	0.47	1.28	0.25
	0.0576	0.40	1.25	
	0.1152	0.39	1.38	
Control		0.60	1.23	

Toxicity is the main reason for the failure at all stages of the new drug development process. The major part of safety-related attrition occurs at preclinical phases while predicting preclinical safety liabilities earlier in the drug development process. This strategy enables the design and/or selection of improved drug candidates that have more possibilities to become commercialized drugs.[59] Therefore, we used the MTT cell viability assay to determine cytotoxicity against NIH/3T3 mouse embryonic fibroblast cell lines (ATCC CRL1658), which is recommended by ISO (10993-5, 2009).[60]

Results from the cytotoxicity evaluation of the most active compounds **19** and **20** are presented in **Table 3.** IC_{50} values of donepezil, compound **19,** and compound **20** against NIH/3T3 cells were found to be 316 µM, ≥1000 µM, and 100 µM, respectively. IC_{50} values of compounds **19** and **20** against NIH/3T3 cells are higher than their IC_{50} values against AChE. Thus, it can be stated that these compounds are nontoxic at their effective concentrations against AChE. Furthermore, donepezil showed approximately 3-fold lower cytotoxicity than compound **19**. Thus, cytotoxicity test findings enhanced the importance of compounds **19** and **20** as AChE inhibitors.

Table 3. IC_{50}±SD (μM) value of compounds **19**, **20** and donepezil against NIH/3T3 cell line.

	19	**20**	**Donepezil**
$IC_{50\ (\mu M)}$	≥ 1000	100.17±16.49	316.42±21.26

Identification of mutagenic properties of new compounds is essential for safety, and thus, new drug candidates should be examined using the models of genotoxicity, such as the Ames test.[61] In the current study, the Ames assay was performed to investigate the genotoxicity of compounds **19** and **20**. In Ames ^{MPF} assay, more than 25 positive wells were observed with positive controls and negative control wells also showed less than eight positive wells in the presence and absence of S9 with TA98 and TA100, which complied with the requirements for the validation of the Ames ^{MPF} and also as described in previous studies.[62] Genotoxicity results are presented in **Table 4**.

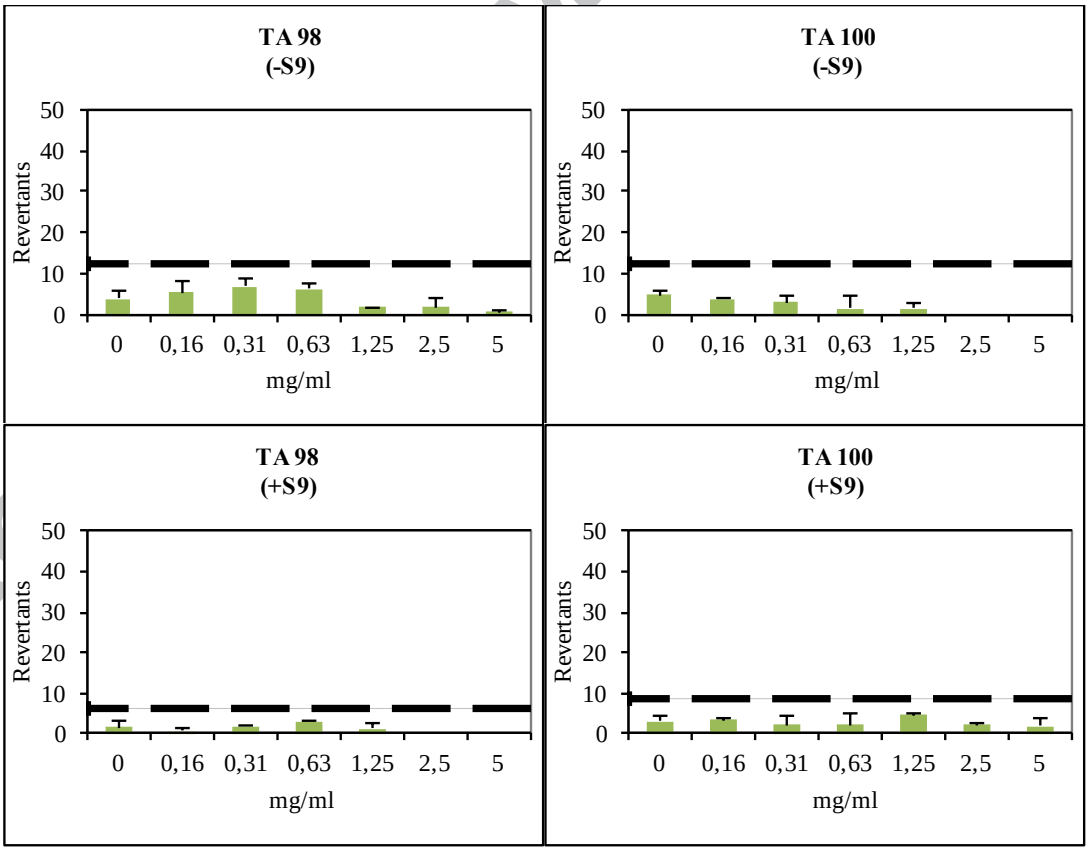
Table 4. The AMES ^{MPF} results of the compounds.

Comp.	**Concentration (mg/mL)**	**REVERTANTS Fold increase (over baseline)**			
		TA 98		**TA 100**	
		S9+	**S9-**	**S9+**	**S9-**
19	0.156	0.22	0.87*	0.78	0.66
	0.3125	0.65	1.14	0.55	0.49*
	0.625	0.97	1.03	0.55	0.27*
	1.25	0.43	0.33	1.09*	0.27***
	2.5	0.00*	0.33	0.55	0.00***
	5	0.00*	0.11*	0.47	0.00***
20	0.156	0.28	0.94	0.98	0.79
	0.3125	1.40***	0.47	0.60	0.90
	0.625	0.56	1.04*	0.53	0.69
	1.25	0.84	0.52	1.36***	1.32***
	2.5	1.02	1.15*	1.36***	0.32*
	5	1.49***	0.89	1.43***	0.05***

* t test *p* value (unpaired 1-sided) < 0.05 *** t test *p* value (unpaired 1-sided) < 0.001

Compound **19** showed a baseline of 3.08 and 6.13 against TA98 with and without S9, respectively. Fold inductions over baseline did not reach values more than 1.5 and statistically different results did not reveal any dose–response tendency. According to these findings, compound **19** did not show any mutagenicity against TA98 (**Figure 4**). Compound **19** was found to show a baseline of 4.28 and 6.10 with and without S9 against TA100, respectively. Mentioned-fold increases over the baseline according to the criteria were not determined with compound **19** and significant results did not reach these values and did not show any dose-response tendency. Compound **19** was also found to be non-mutagenic against TA100 in the presence or absence of metabolic activation (**Figure 4**).

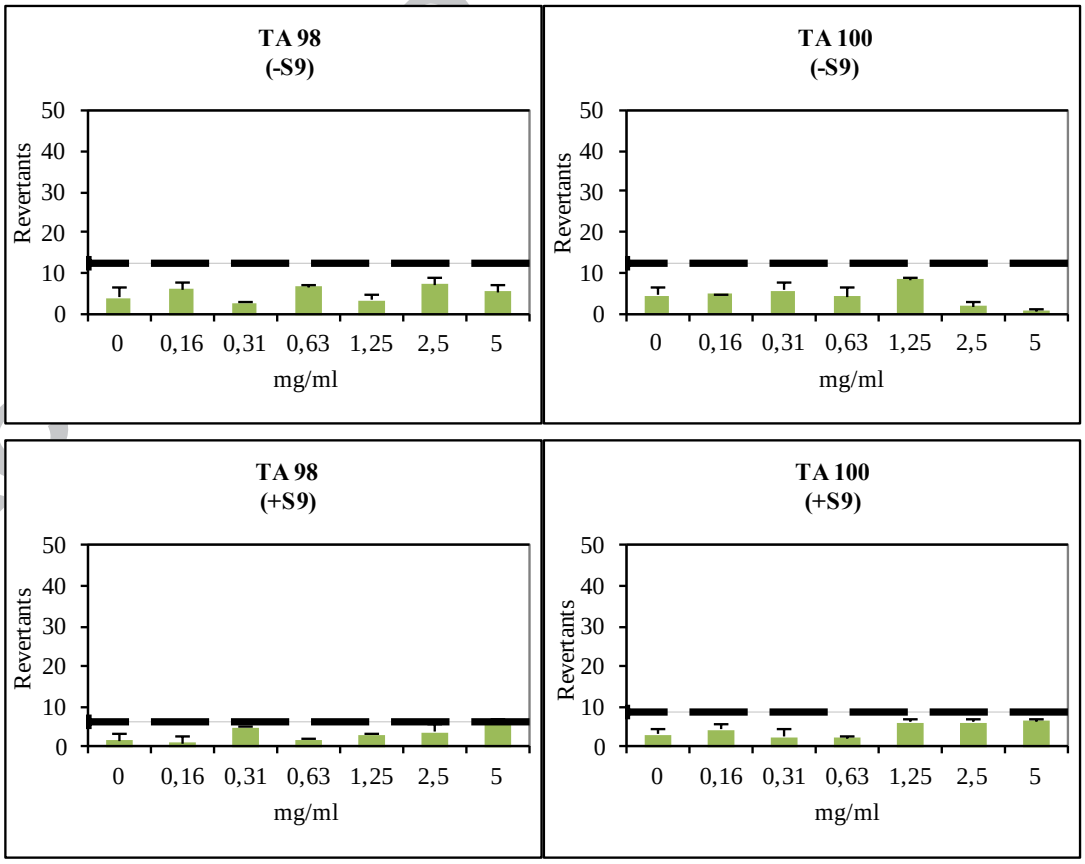
Figure 4. Dose-response curve of compound 19 against TA98 and TA100 in the presence and absence of S9 according to AMES ^{MPF} test.



* t test p value (unpaired 1-sided) < 0.05 with a fold-induction over baseline > 2; -------: 2-fold induction over baseline

Compound **20** showed a baseline of 6.40 with TA98 in the absence of S9 and a baseline of 3.58 in the presence of S9. Although there were significant differences observed, they did not reach the mentioned values above the baseline and did not show any dose–response tendency. Therefore, compound **20** was classified as non-mutagenic against TA98 in the presence or absence of metabolic activation (S9) (**Figure 5**). Compound **20** had a baseline of 6.32 with TA100 in the absence of S9 and 4.42 in the presence of S9. Furthermore, fold inductions above the baseline were less than 1.5 in each concentration of the compound and the significantly different results obtained did not show any dose–response tendency. Therefore, compound **20** was not genotoxic against TA100 with or without metabolic activation (**Figure 5**).

Figure 5. Dose-response curve of compound **20** against TA98 and TA100 in the presence and absence of S9 according to AMES [MPF] test.



* t test p value (unpaired 1-sided) < 0.05 with a fold-induction over baseline > 2; -------: 2-fold induction over baseline

On the basis of the results of the Ames ^{MPF} assay, the compounds were classified as negative. Further, cytotoxicity and genotoxicity findings strongly suggested compounds **19** and **20** to be AChE inhibitors.

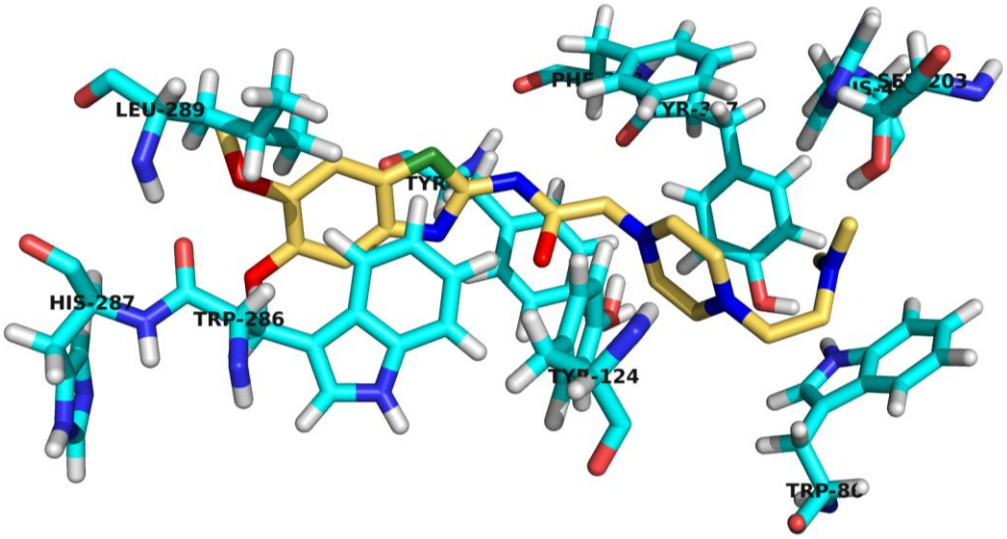
High pharmacological activity and low toxicological effects are not enough for a compound to become a drug candidate. A good pharmacokinetics profile is also very important for the new drug candidates that should be evaluated earlier in the process of drug development. In recent years, significant developments in combinatorial chemistry have made the estimation of absorption, distribution, metabolism and excretion (ADME) relatively easy.[63] ADME properties of the newly synthesized compounds (**7-20**) were calculated using online Molinspiration property program.[64] This program provides the data based on Lipinski's rule, which assesses the ADME properties of new compounds and is important for the optimization of a biologically active compound. The rule emphasizes that an orally active drug should not possess more than one violation.[65] Drug-likeness score (DLS) was also calculated for all the compounds (**7-20**) and donepezil based on the Molsoft's chemical fingerprints mode consisting of 5K of marketed drugs from World Drug Index (positives) and 10K of carefully selected non-drug compounds (negatives).[66] In the software, DLS score was found to be between 0 and 2, suggesting good pharmacokinetics for drug candidates.

The theoretical calculations of ADME parameters (molecular weight (MW), log P, topological polar surface are (tPSA), number of hydrogen donors (nON) and acceptors (nOHNH), and volume) and DLS are presented in **Table 1** along with the violations of Lipinski's rule. According to these data, all compounds (**7-20**) confirm the Lipinski's rule by causing no violation. Furthermore, the same DLS (1.76) was obtained for the most active compounds (**19** and **20**) and donepezil. This suggests that newly synthesized compounds may have a good pharmacokinetics profile, which further strengthens their biological importance.

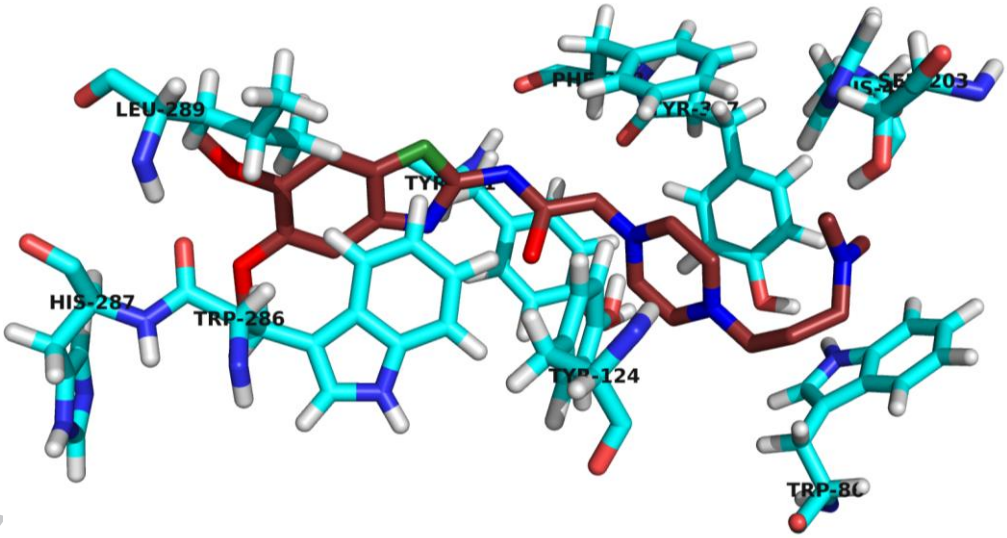
Docking of compounds 19 and 20 into the active site of AChE was carried out separately by using AutoDock Vina software[67] to visualize the possible interactions. The 3D structure of human AChE (*h*AChE, PDB ID:4EY7), which includes E2020 (donepezil) as a ligand, was retrieved from the Protein Data Bank server (www.rcsb.org). The docking poses for both the compounds are shown in **Figure 6**. According to the docking results, compounds **19** and **20** showed compatibility with the gorge and interaction with both CAS and PAS. They interact with Trp86, Tyr124, Ser203, Trp286, His287, Leu289, and Tyr341. Benzothiazole structure settles down PAS and forms a π-π interaction with the indole ring of Trp286. The methoxy substituents provide polar interactions with amino group of Trp286, His287, and Leu289 by forming hydrogen bonds. The carbonyl of amide moiety creates a hydrogen bond with the amino group of Tyr124. In the CAS, a hydrogen bond is formed between the nitrogen of the dimethylamino group and carbonyl of Ser203. The dimethylamino group and Trp86 also interact with cation-π. The ethyl/propyl group and terminal dimethyl group intensify the binding with the active site via Van der Waals interactions. All of these interactions enable to explain the proper binding with the active region of AChE.

Realizing chemical structures of the existing drugs and constituting the structural requirements for intrinsic pharmacological activity is an important approach in designing novel compounds. On the basis of this strategy, we assessed 14 new benzothiazole analogues as AChE inhibitors in the present study. Pharmacological, toxicological, and ADME studies indicated the relative potency of compounds **19** and **20** against rest of the compounds. Molecular docking studies suggested possible interactions between these compounds and AChE. Consequently, all these data may pave the way for the researchers to synthesize similar compounds possessing enhanced pharmacological profile.

Figure 6. Three dimensional models of compounds **19** (**A**) and **20** (**B**) in the active site of *h*AChE.



(**A**) Compound **19** in *h*AChE active site



(**B**) Compound **20** in *h*AChE active site

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54. In the IR spectra, significant stretching bands belonging N-H and C=O groups were observed between 3130-3283 cm^{-1} and 1680-1699 cm^{-1}, respectively. In the ^{1}H-NMR spectra, piperazine protons gave two broad singlets between 2.30 and 2.57 ppm. -CH_2 protons of acetamide moiety were recorded as a singlet between 3.23-3.36 ppm. In the 5,6-dimethoxysubstituted benzothiazole, H_4 and H_7 were observed as singlet at 7.45-7.62 ppm and 7.21-7.30 ppm, respectively. In the 6-methoxysubstituted benzothiazole, H_4, H_5 and H_7 peaks were recorded at 7.53-7.63, 6.95-7.03 and 7.47-7.56 ppm, respectively. N-H proton of acetamide gave a singlet at about 11.80 ppm. In the ^{13}C-NMR spectra, C=O of acetamide were observed at about 169 ppm. All aromatic and aliphatic carbons were recorded at expected values and also fluorinated derivatives had coherent carbon-fluorine coupling constant. HRMS also performed and all measured mass and isotope scores were compatible with calculated values.
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Highlights

- ✓ New benzothiazole-piperazines were designed based on structural requirements for AChE inhibitors.
- ✓ Compounds **19**-**20** indicated significant inhibition on AChE (E.C.3.1.1.7, from electric eel).
- ✓ These compounds did not display cytotoxicity and genotoxicity.
- ✓ A good pharmacokinetics profile and drug likeness score were predicted for the synthesized compounds.
- ✓ Interaction modes between AChE and compounds **19** and **20** were determined by docking studies.

Design, Synthesis, and AChE Inhibitory Activity of New Benzothiazole-Piperazines

Fourteen new benzothiazole-piparazines were designed and synthesized in order to investigate their AChE inhibition potency. Characterized compounds were subjected to pharmacological, toxicological, ADME and docking studies.