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Research Article

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Binding affinity and Molecular modeling study of substituted thiazoles on 5-HT_6 receptor for the enhancement of cognitive functions in Alzheimer's disease

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ABSTRACT

The potential effectiveness of 5-HT_6 receptor active agents as cognitive enhancers has been the focal point of research in Alzheimer's disease (AD). Serotonin 5-HT_6 receptors are distributed in brain regions affiliated with memory and learning making them promising, novel targets for (CNS)-mediated diseases such as Alzheimer's disease (cognitive function). A group of previously reported compounds was selected and tested in vitro for the binding affinity to 5-HT_6 receptors. The binding affinity of these compounds was tested using in vitro radioligand binding assay using cryopreserved membrane expressing target receptors as grounds for the assay and the use of Methiothepin and Haloperidol as references. Data was analyzed by calculating the total binding percentage, which was performed for the references and tested compounds. It was found that the tested compounds displayed promising activity as 5-HT_6 antagonists and partial dopamine 2 agonists. The binding mode of tested compounds was studied by molecular docking using SB-271046 as reference. The tested compounds binds with Arg 86 and Lys 14 aminoacid residues which proves the rationality of the developed models. These results may be helpful in designing novel and potential 5-HT_6 ligands.

Key words: Substituted thiazoles, 5-HT_6 receptors, Alzheimer's disease, molecular modeling study.

INTRODUCTION

Alzheimer's disease (AD) is a widespread medical condition that requires attention, as its prevalence worldwide is astonishing; approximately 26 million individuals were diagnosed with Alzheimer's disease [1]. It is a chronic neurodegenerative disease that usually starts slowly and gets worse by time. The cause of Alzheimer's disease is poorly understood. About 70% of the risk is believed to be genetic with many genes usually involved, other risk factors include a history of head injuries, depression, or hypertension [2]. In persons ageing 65 years or more, the chance of developing AD doubles every half-decade and individuals whose ages are more than 85 years were found to be diagnosed with AD [3]. The oldest hypothesis for AD causes, on which most currently available drug therapies are based, is the cholinergic hypothesis which proposes that AD is caused by a reduced synthesis of the neurotransmitter acetylcholine [4]. The cholinergic hypothesis did not gain widespread support, largely because

medications intended to treat acetylcholine deficiency have not been very effective. Other cholinergic effects have also been proposed, for example, initiation of large-scale aggregation of amyloid leading to generalized neuroinflammation [5, 6]. On the other hand, four out of five medications currently used to treat the cognitive problems of AD are acetyl cholinesterase inhibitors as a result of the reduction in the cholinergic neurons activity that is a well-known feature of Alzheimer's disease. Therefore, extensive medical and drug development research have been taking place globally, for the management and treatment of AD.

Furthermore, 5-hydroxytryptamine 6 (5-HT_6) receptor was discovered in the 1990s. These serotonin 5-HT_6 receptors are distributed in brain regions affiliated with memory and learning. The use of potent antagonists displays a promising elevation in acetylcholine and glutamate-mediated neurotransmission evidently improving the cognitive function as seen in preclinical tests, effects of 5HT_6 receptor agonists on memory have also been recently identified [7, 8]. The researchers' interest of such receptor has raised recently, either on its agonist or antagonist effects [9]. The receptor studies captured the attention and become one of the most successful therapeutic targets, from anxiety, depression, schizophrenia, obesity to learning and memory disorders [7]. Moreover, 5-HT_6 receptor agonists have boosting effects in learning and memory that were revealed in numerous animal model based studies, using a number of structurally unrelated compounds [10]. The first drug discovered as a 5-HT_6 receptor antagonist was an Arylsulfonyltryptamine analogue. It was used as a structural base to determine the general structural requirements for binding to 5-HT_6 receptors [11]. Many studies revealed an increase in the cholinergic and glutamatergic neurotransmitters by blocking the 5-HT_6 receptor [12, 13]. 5-HT_6 antagonists had effects in cognition improvement in number of rat models [14]. Moreover, many compounds have been developed and are currently undergoing clinical trials –phase I and II clinical trials- for the purpose of enhancing cognitive impairment in AD. Recent efforts indicate the potential effectiveness of 5-HT_6 receptor agonists and antagonists as cognitive enhancers for Alzheimer patients [15]. Many compounds that act as 5-HT_6 receptor antagonist were examined for their efficacy tolerability by patients that suffer from AD in cases ranging from mild to moderate conditions [16]. Moreover, in a study performed in 2011, three month old male Wister rats with scopolamine induced episodic memory defects were subjected to selective 5-HT_6 antagonist compounds -CMP X and CMP Y- and the reference 5-HT_6 antagonist GSK-SB742457 (Figure 1) resulting in improvement of the memory deficits. It was also found that the use of acetyl cholinesterase inhibitor (AChEI) –donepezil– with 5-HT_6 receptor antagonist compounds gives an additive cognition enhancement in cognitively defected rats [17].

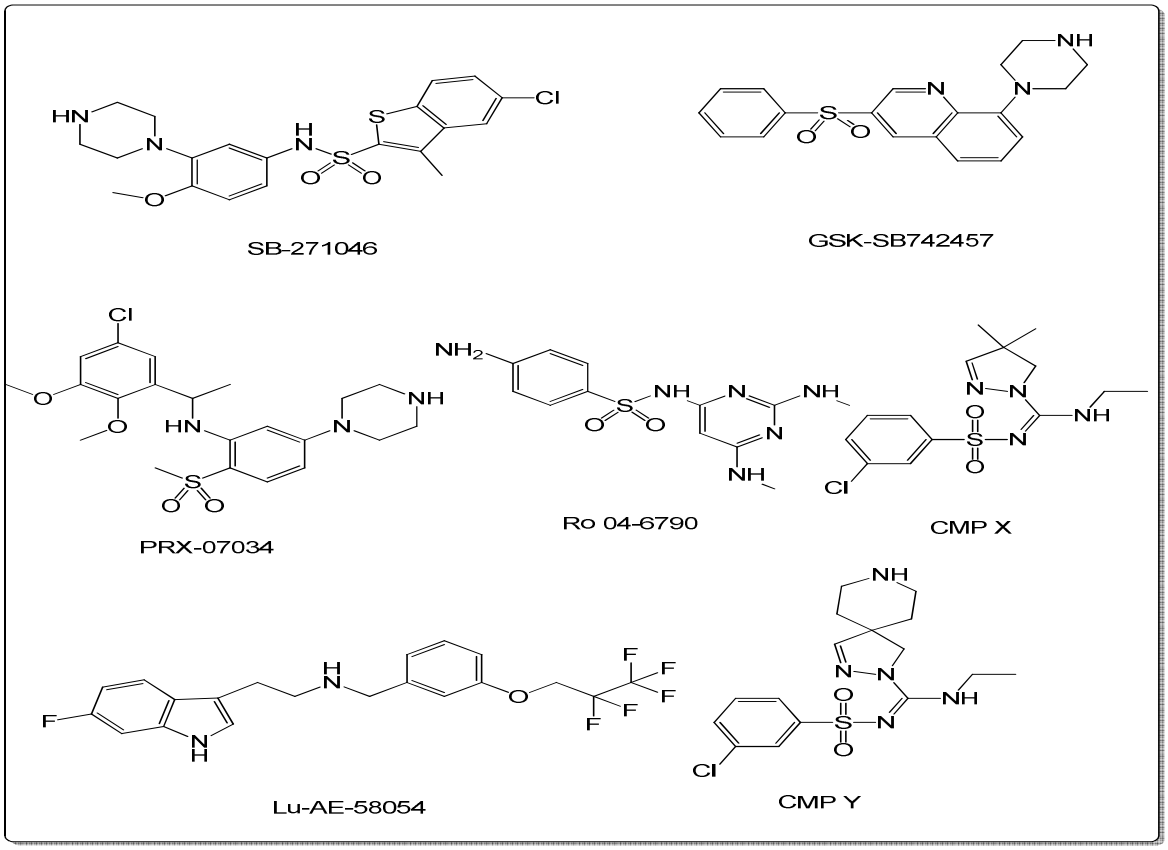


Figure (1) Compounds with potential in the cognitive Impairment improvement

PRX-07034, another 5-HT_6 receptor antagonist (Figure 1), was studied in rats raised in social isolation that developed behavioral changes familiar to the symptoms seen in schizophrenia demonstrating this drug's effect in repairing the impairment in rats' cognition. Further examples of 5-HT_6 receptor antagonists include Ro 04-6790 (Figure 1), which displayed improvement in isolation raised but not group-housed reversal learning controls in the water maze. The study of Ro 04-6790 also showed an effect on mature rats subjected to chronic intermittent phencyclidine as well as drug-naive 18-month-old rats, an improvement of object discrimination was observed [18]. When sub-chronic phencyclidine was administered to induce cognitive impairment in rats in a study conducted on Lu AE58054 (Figure 1), the administration of the potent 5-HT6 receptor antagonist Lu AE58054 produced notable reversal of such cognitive impairment in the test known as novel object recognition test, which provided proof that it could be useful in reversing cognitive impairment [19]. Consequently, the previous observations among many others generated the hypothesis that 5-HT_6 receptor antagonists can be considered promising agents for targeting cognitive disorders, i.e. Alzheimer's disease (AD) and schizophrenia.

A diversity of chemical classes as 5-HT_6 receptor agonists and antagonists were studied, the latter includes bisaryl sulfonamides and sulfones, indoles and indazoles, azaindoles and azaindazoles, benzofuran, benzothiophenes, benzimidazoles, thienopyrrols and pyrazolotriazines [10]. One recent approach in the development of potential compounds was focused on utilizing the strategy of molecular modeling-assisted design, in which designed, multiple ligands were obtained from. These ligands that target both 5-HT_6 and Dopamine 2 receptors, antagonizing the former and agonizing the latter, have proved their effectiveness in rats as anxiolytics and antidepressants [20].

Based on the above mentioned data, a structure-based study was performed to select some compounds that may have the potential of binding to 5-HT_6 receptor. Twenty compounds were selected to be tested *in vitro* for the activity and binding to 5-HT_6 receptor, as either agonists or antagonists. Their structures, i.e. pharmacophore, are thought to have good binding potential to this receptor, which might contribute to the enhancement of cognitive functions in Alzheimer's disease [8].

The compounds under investigation belong to the acetamido- and propanamido- thiazole analogues (Figure 2), within were tested *in vitro* which provided evidence of having notable biological activities, i.e. antitumor activities on various cancer cell lines [21]. Furthermore, the fact that they have structure similarity to the pharmacophore of 5-HT_6 receptor active drugs [8], might indicate potential activities on Central Nervous System (CNS) diseases, i.e. Alzheimer's. In order to determine whether this hypothesis is accurate or not, *in vitro* testing for the compounds is a necessity to analyze their efficacy on 5-HT_6 receptor.

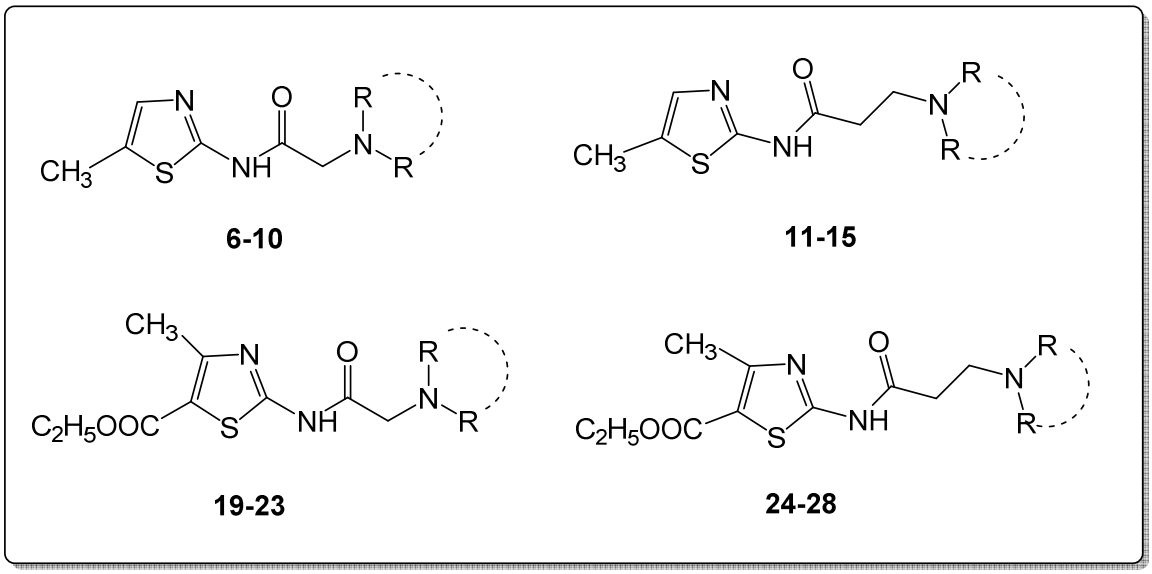


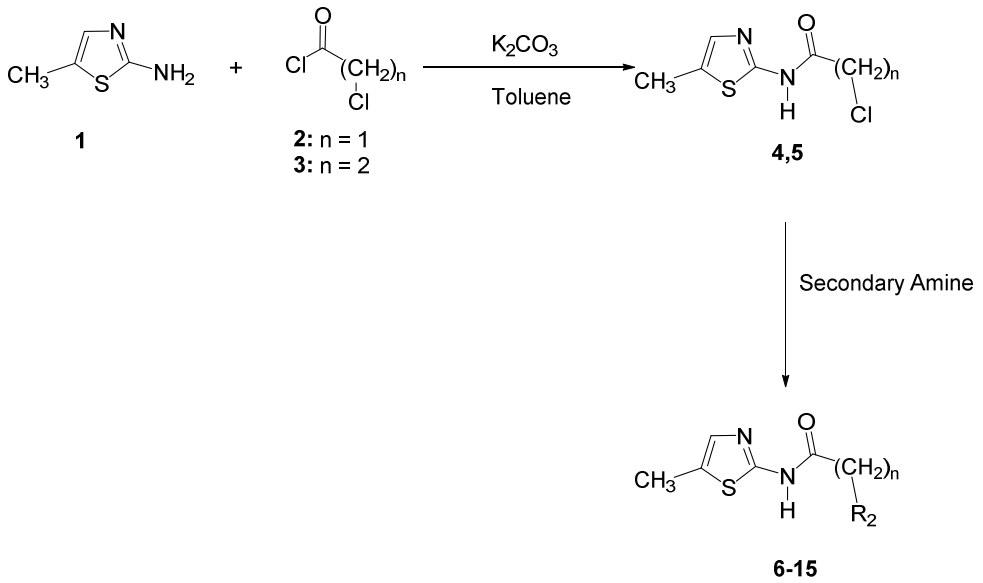
Figure (2) Structures of the compounds under investigations

However, the structure similarity to the pharmacophore of other drugs that bind to 5-HT_6 receptor was the core reason behind testing these compounds on the *in vitro* binding of the receptor, for their binding might enhance the cognitive functions in AD, as previously mentioned [8]. Therefore, as an initial step and prior to testing, the compounds were synthesized according to the reported procedure [21].

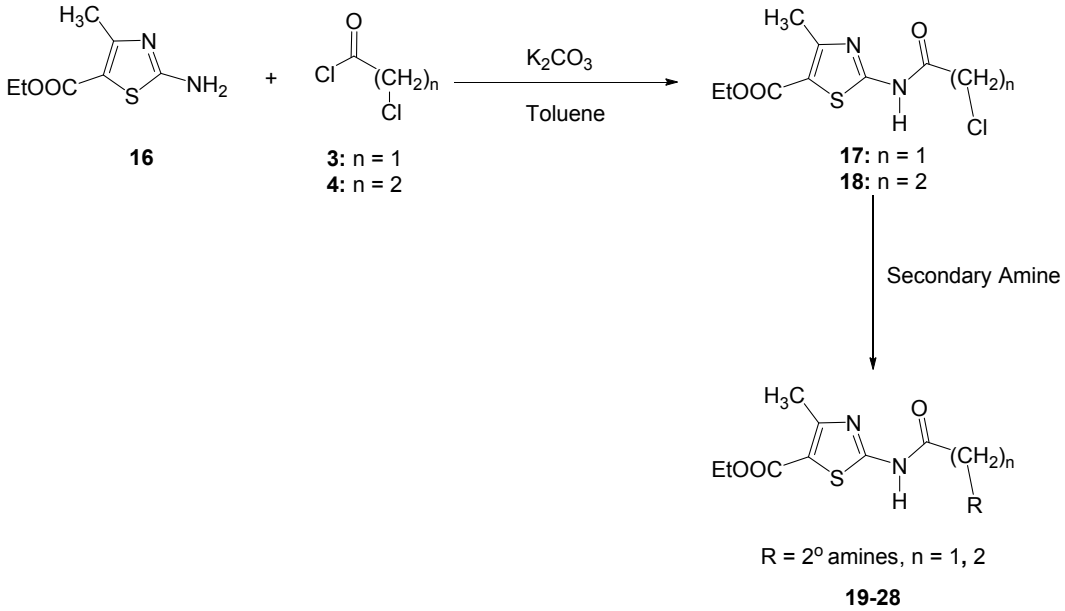
EXPERIMENTAL SECTION

Chemistry

The synthetic strategy to prepare the target compounds **6-15**, **19-28** are outlined in Schemes 1,2.

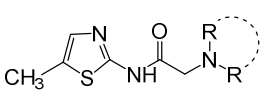


Scheme 1: Synthesis of compounds 6-15

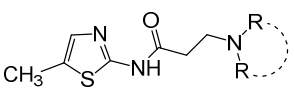


Scheme 2: Synthesis of compounds 19-28

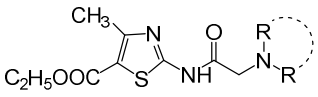
The 2-amino function of 2-amino-5-methyl-thiazole (**1**), ethyl 2-amino-4-methyl-thiazole-5-carboxylate (**16**) were acylated with either 2-chloroacetyl chloride (**2**) or 3-chloropropionyl chloride (**3**) and potassium carbonate in dry toluene to yield 2-[2-chloroacetamido or 3-chloropropanamido]- 5-methyl-thiazole (**4,5**), ethyl 2-[2-chloroacetamido or 3-chloropropanamido]-4-methyl-thiazole-5-caroxylate (**17, 18**). The target compounds **6-15** and **19-28** were obtained by the reaction of the 2-chloroacetamido or 3-chloropropanamido derivatives **4, 5**, **17, 18** with variety of secondary amines in dry toluene (Table 1). All the ^{1}H-NMR, ^{13}C-NMR, and other spectrophotometric data of the synthesized compounds were previously documented [21].



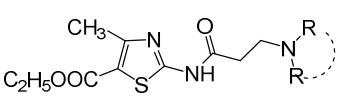
6-10



11-15



19-23



24-28

Table 1. Physicochemical properties of the newly synthesized compounds 6-15, 19-28

Compound	R	n	Solvent	Yield %	Mp ^{0}C
6	$N(CH_3)_2$	1	Ethanol	33	98-9
7	piperidino	1	Ethanol	63	137-8
8	morpholino	1	Ethanol	52	121-3
9	N-methyl-piperazino	1	Ethanol	44	103-5
10	N-phenyl-piperazino	1	Ethanol	63	164-7
11	$N(CH_3)_2$	2	Ethanol	54	63-6
12	piperidino	2	Ethanol	75	82-4
13	morpholino	2	Ethanol	59	118-9
14	N-methyl-piperazino	2	Ethanol	69	78-80
15	N-phenyl-piperazino	2	Ethanol	66	176-9
19	$N(CH_3)_2$	1	Ethylacetate	55	201-3
20	piperidino	1	Ethanol	61	130-2
21	morpholino	1	Ethanol	54	152-5
22	N-methyl-piperazino	1	Ethylacetate	57	125-7
23	N-phenyl-piperazino	1	Ethylacetate	59	98-101
24	$N(CH_3)_2$	2	Pet.ether	54	163-5
25	piperidino	2	Ethylacetate	68	99-101
26	morpholino	2	Ethanol	48	149-51
27	N-methyl-piperazino	2	Ethanol	40	138-40
28	N-phenyl-piperazino	2	Pet.ether	49	87-9

[a] *Analysed for C,H,N; results were within ±0.4 % of the theoretical values for the formulae given*

Radioligand binding assay for 5-HT6 receptor

Stock solutions of the compounds under investigations in concentration of 10^{-2} Mol were prepared; the compounds were dissolved in dimethyl sulfoxide (DMSO) with weights of 1 mg or more. A series of solution dilutions were prepared and transferred to 96-well microplates in assay buffers by an automated pipetting system. The reference compound methiothepin mesylate salt was utilized to evaluate non-specific binding with a final concentration of 10 µM in the mix of the assay. Total binding evaluation was accomplished using de-ionized water. A cryopreserved membrane expressing $5HT_6$ receptor at 37°C was used as grounds to which this ligand binding assay was performed. The previously prepared microplates were covered with a tape for the sealing, and incubated for 60 minutes at 37°C in circulated air incubator after mixing on orbital shaker for 5 minutes at 250 rpm. To end the reaction, a vacuum manifold and 96-well pipettor were used, rapid filtration technique was utilized filtering the mix onto GC/C filter mate presoaked with 0.3% polyethylene imine for half an hour, followed by ten consecutive quick washes with 300 µl 50 mM Tris buffer at a temperature of 4°C with a pH of 7.4. In an air forced fan incubator, the filter mates were left to dry overnight at 37°C. Melting ascintillator of solid origin on filter mates in 100°C for 5 minutes was used. For one hour the plates were set to equilibrate and at roughly 30% efficiency, radioactivity was measured [20, 22]. In the previously mentioned method, the following were used: i) Automated pipetting system for the use with the microplates (EpMotion 5070; Eppendrof, Germany) or CyBi Felix (CyBio AG, Germany), ii) 96-wells microplates (Greiner Bio-One, Germany), iii) 96-wells pipetting station Rainin Liquidator (MettlerToledo, Switzerland), iv) Incubation of plate in circulated air incubator ST-2 (Pol-EkoAparatura, Poland), v) Mixing on orbital shaker (DOS-10S, Elmi, Lithuania),vi) Rapid filtration by using automated harvester system Harvester-96 MACH III FM (Tomtec, USA), vii) filtermates dried in forced air fan incubator CLW 32 STD (Pol-EkoAparatura, Poland), viii) Radioactivity in MicroBeta2 scintillation counter (PerkinElmer,USA), ix) Data fitting by Prism 5 (GraphPad Software). The source of the receptor is 5-HT_6 (CHO-K1), with a radioligand final concentration/kd of [^{3}H] LSDm (2.5/2 nM). For the nonspecific binding,10 µM methiothepin was utilized and the assay buffer is 50 mM Tris, 10 mM $MgCl_2$, 0.5 mM EDTA at pH 7.4. Finally, the incubation period was 60 minutes at 37°C [20,22] (Table 1).

In vivo testing of Beta amyloid aggregates

Labeling the compounds was performed Using Zhuang method [23]. The Cui method was used for the introduction of the three radioiodinated ligands, [^{125}I] **1**, [^{125}I] **2**, and [^{125}I] **3**, they were injected into healthy mice for biodistribution experiments [24]. The average weight of mice was 22 g. Diluted [^{125}I] **9**, [^{125}I] **14**, or [^{125}I] **27** solutions in saline solution (100 lL) were injected into the tail vein of mice. The organ of interest were removed and weighed after sacrificing the mice. The automatic c-counter (WALLAC/Wizard 1470, USA) was utilized to count the radioactivity. The percentage dose per gram of wet tissue was calculated by a comparison of the tissue counts to suitably diluted aliquots of the injected material (Table 2).

Molecular Docking study

The homology model adopted by Hao was used for docking [25]. The template protein (PDB code: 2RH1 chain A, obtained from the Protein Data Bank high resolution (2.4 Å) crystal structure of human β2-adrenergic G protein-coupled receptor was employed to generate the 3D protein structure [26]. The docking studies were carried out using the MOE program 2009.10. The ligand is built in an incremental fashion, where each new fragment is added in all possible positions and conformations. All the molecules for docking were sketched in the MOE and minimized The 3D coordinates of the active sites were taken. All water molecules were removed and the protein was modified to dock inhibitor and also hydrogens were added. The active site was defined with a distance of 6.5 Å around the co-crystallized ligand. Formal charges were assigned to all the molecules and the lowest energy conformer of SB-271046 (global-minima) was docked into the selected binding domain. The enzyme structure was subjected to refinement protocol in which constraints on the enzyme were gradually minimized with the molecular mechanical forcefield 'AMBER' until the root mean square gradient was 0.01 kcal/molÅ. The energy-minimized structure was next used for molecular dynamics studies. For each ligand examined, energy minimizations were performed using 1000 steps of the steepest descent, followed by conjugate gradient minimization to a root mean square energy gradient of 0.01 kcal/molÅ [27, 28].

RESULTS AND DISCUSSION

In the *in vitro* experiment, the compounds were tested in (10^{-11} - 10^{-6} M) concentrations using Methiothepin and Haloperidol as reference compounds, which were included on the same microplate that had the tested compounds. The affinities to the receptors ($5HT_6$ and D_2) were tested by using radioligand binding assay [20, 22]. Compounds **9, 14** and **27** exhibited significant results, they exhibited higher binding affinity towards the receptors more than the references used where compound **9** had a Ki value of 0.3±0.1 nM for $5HT_6$ and 0.9±0.4 nM for D_2, compound **14** had a Ki value of 0.7±0.2 nM for $5HT_6$ and 1.4±1 nM for D_2, and compound **27** had a Ki value of 0.8±0.2 nM for $5HT_6$ and 1.6±0.5 nM for D_2 (Table 1), the rest of compounds were found active. The three compounds have Ki values that are less than 3 nM, indicating promising results and higher binding affinities. The affinity value for the $5HT_6$ reference compound Methiothepin was 4.1±0.7 nM and the value for the D_2 reference compound Haloperidol was 4.0±0.3 nM. Based on these data, functional assays were performed on these compounds on receptors $5HT_6$ and D_2 as well as muscarinic receptor M1 and hERG channel. For compounds **9, 14** and **27** , the percent inhibition of control agonist response at 1.0E-06M for the antagonism of D_2 are 100%, 99%, 100% and 45%, 46%, 48% for the D_2 agonism as well as 99%, 98%, 97% for the $5HT_6$ antagonism respectively, while showing no effect for the agnosim of $5HT_6$. The percent activity at 1.0E-06 M for compounds **9, 14** and **27** at the M1 receptor are 30%, 29%, 31% and 11%, 12%, 14% for the hERG channel respectively.

Table (1) Radioligand affinity results of the active compounds to 5-HT_6 and D_2 receptors*

Compound	Ki, nM	
	5-HT_6	D_2
9	0.3 ± 0.1	0.9 ± 0.4
14	0.7± 0.2	1.4± 1.0
27	8± 0.2	1.6 ± 0.5
Methiothepin	4.1 ± 0.7	-
Haloperidol	-	4.0 ± 0.3

**Data expressed as the mean ± SD of two independent experiments in duplicate.*

The accumulation of Beta amyloid (Aβ) fragments in the brain is one of the characteristics in Alzheimer disease. Amyloid plaques usually referred to the accumulated clusters of beta amyloid that are caused either by over production of beta amyloid or an error in mechanism of its clearance. The oxidative stress and neurotoxicity in the brain caused by Aβ plaques lead to reduction of acetylcholine level, and turn on the inflammatory responses that damage neurons of the brain [29]. By using ^{125}I-labelled compounds, *in vitro* and *in vivo* examination studies on Aβ (1- 40) and Aβ (1- 42) aggregates of amyloidogenesis in Alzheimer patients have been performed [30]. The bio-distribution of the labeled compounds [^{125}I] **9**, [^{125}I] **14**, and [^{125}I] **27** was measured in addition to the organs uptake and clearance of each compound were calculated. All the three compounds exhibited significant binding affinity against Aβ aggregates. The study result showed that compound **27** presented the highest brain uptake (3.41% ID/g at 2 min) and rapid clearance from the brain (0.56% ID/g at 2 min), while compound **9** showed lower brain uptake (3.23% ID/g at 2 min) however it is still considered a good result with promising pharmacokinetic properties (Table 2).

Table (2) Biodistribution of the tested compounds after IV injection in healthy mice

Comp.	Kd nM	Kd for aggregates of AB (1-40) nM	Kd for aggregates of AB (1-42) nM	Brain uptake %ID/g at 2 minutes	Brain clearance %ID/g at 2 minutes	Hepatic clearance %ID/g at 2 minutes	IC_{50} ng/ml
9	0.04	0.9	0.8	3.23	0.49	0.54	1.02
14	0.09	0.12	0.9	3.39	0.53	0.63	1.01
27	0.1	0.14	1.03	3.41	0.56	0.66	1.03

All the compounds showed significant binding affinity against Aβ aggregates with Ki value ranges

The homology model adopted by Hao was used for docking [25]. Docking of studied compounds reveals their modes of binding affinity with the amino acids. The binding modes were evaluated and validated against SB-271046 as reference with selective 5-HT_6 activity. The reference has high affinity with Arg 86, Lys 19 *via* both hydrogen bonding and cationic arene interactions, especially mentioned its piperazine counterpart Figure (3). This reveals that modification of ligand at those specific sites can improve the inhibitory activity of the receptor. Hence the process of docking can be regarded as a key aspect in reforming the correlation between calculated and observed binding affinities in effect to develop an effective novel compound. Putting the selected candidates into consideration, it was found that compound **9** gave a side chain acceptor with Lys14 *via* piperazine nitrogen with 1.6 A and 88% in addition to with Ile19, Tyr18, Glu17, by 11% (1,96 A) , 16% (1,96) and 25 % (1.75) Figure (4a). Compound **14** showed a side chain acceptor link with Asp 48% (2.01A) *via* piperazine nitrogen and a cationic arene interaction with Arg 23 and Lys14 through its thiazole part Figure (4b). Compound **27** formed a bifurcated link with Asn 53 *via* N of thiazole and carbonyl oxygen by (36%, 1.2 A), (36 and 0.9 A) respectively. In addition to a hydrogen bond with Gln 79 by (14%. 1.3A), moreover a cationic-arene interaction with Arg 86 and Lys 14 with thiazole ring was diagnosed. The smaller the Ki, the greater the binding affinity and the smaller amount of medication needed in order to inhibit the activity of that enzyme. All the 3 compounds under study have shown smaller Ki values even better than standard drug which could be interpretated by modeling by showing similar binding profiles to specific 5HT_6 (SB-271046) antagonists especially mentioned Arg 86 and Lys 19 aminoacid residues. The piperazine and thiazole moieties of tested compounds play a major role in their binding to 5-HT_6 homology model and hence to their specific activity (Figure 5, 6).

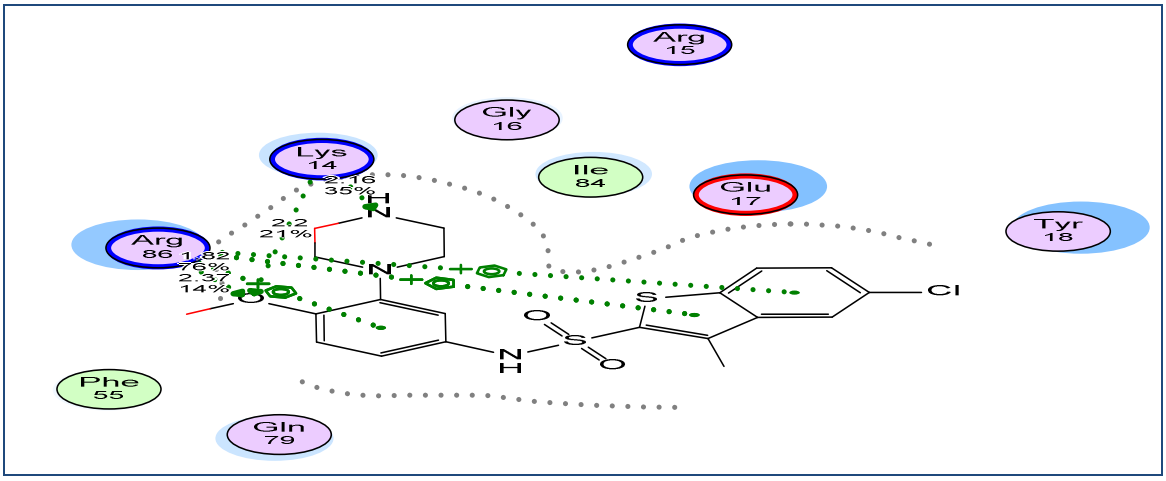


Figure (3): 2D binding mode and residues involved in the recognition for SB- 271046 (reference compound)

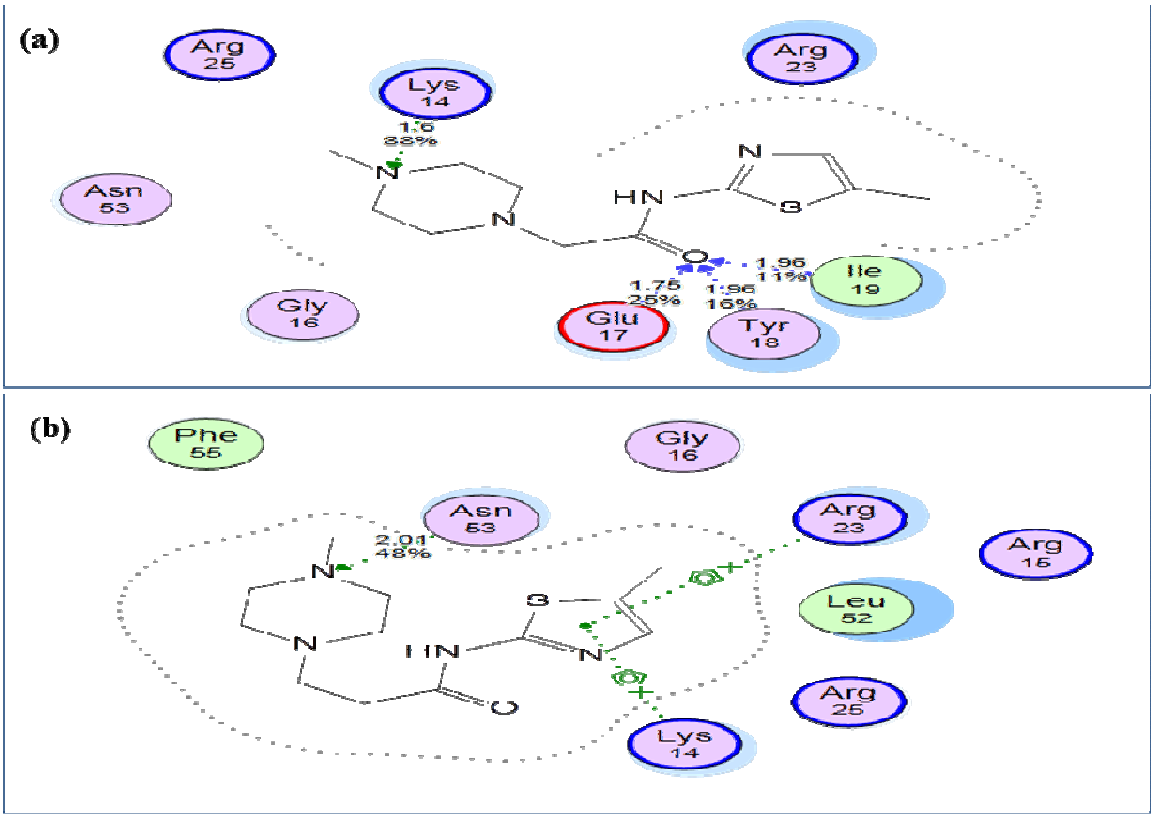


Figure (4): 2D binding mode and residues involved in the recognition for compound 9 (a) and compound 14 (b)

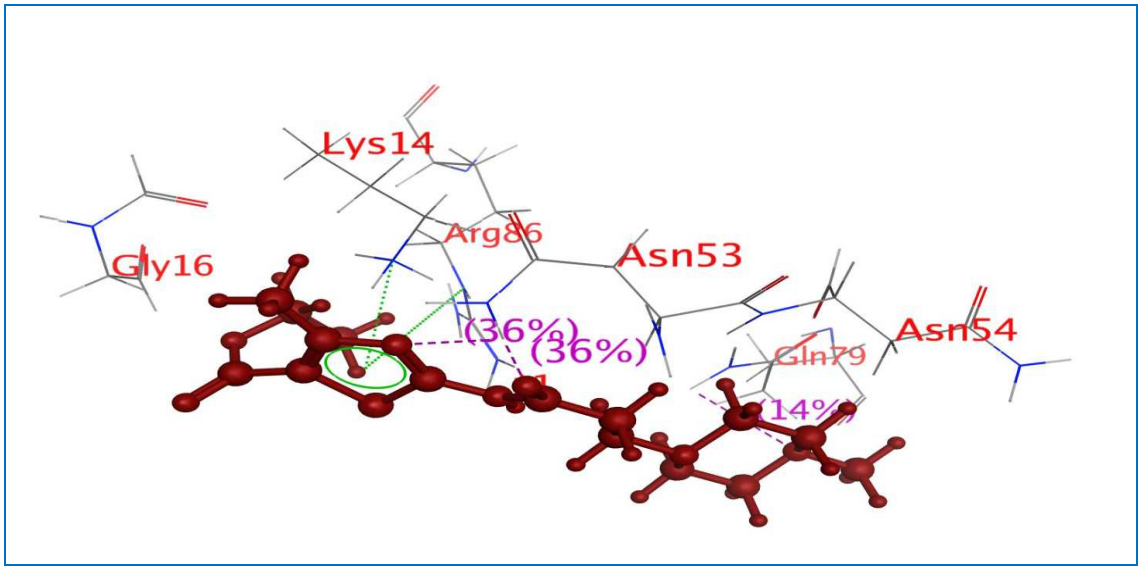


Figure (5): 3D binding mode and residues involved in the recognition for compound 27

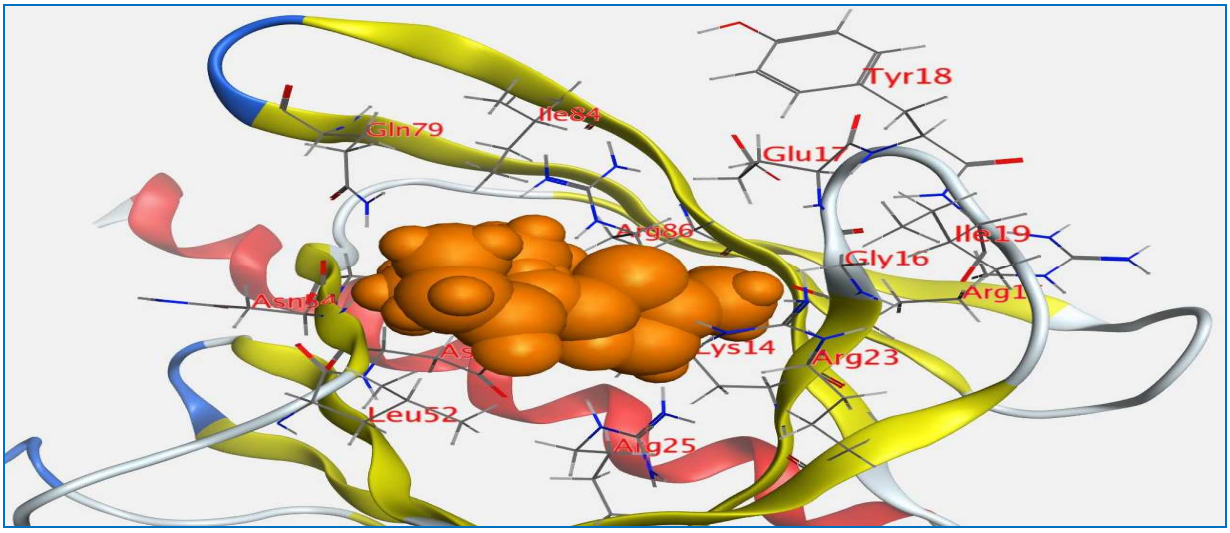


Figure (6): The aligned conformation of compound 27 in the binding pocket

From the mentioned data, it was concluded that three of the synthesized acetamido and propanamido- thiazole analogues showed promising activity as $5HT_6$ antagonists and D_2 antagonists and partial agonists. They also showed excellent brain uptake and rapid clearance which encourages further investigation and development to reach the optimal agents that can be used for Alzheimer's disease. A similar binding profiles to specific $5HT_6$ (SB-271046) antagonist especially mentioned Arg 86 and Lys 19 aminoacid residues could attribute the selected compounds activity. The piperazine and thiazole moieties of tested compounds play a major role in their binding to 5-HT_6 homology model. The pharmacophoric elements of tested compounds need further investigation and development to reach the optimal 5-HT_6 activity and used as cognitive enhancement agent.

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