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Investigation of potent inhibitors of cholinesterase based on thiourea and pyrazoline derivatives: Synthesis, inhibition assay and molecular modeling studies

Amara Mumtaz[a,*,1], Abdul Majeed[a,1], Sumera Zaib[b], Shafiq Ur Rahman[b], Saba Hameed[a], Aamer Saeed[c], Hummera Rafique[d], Ehsanullah Mughal[d], Aneela Maalik[e], Izhar Hussain[f], Jamshed Iqbal[b,f,*]

[a] Department of Chemistry, COMSATS University Islamabad, Abbottabad Campus, Abbottabad 22060, Pakistan
[b] Centre for Advanced Drug Research, COMSATS University Islamabad, Abbottabad Campus, Abbottabad 22060, Pakistan
[c] Department of Chemistry, Quaid-i-Azam University Islamabad, Pakistan
[d] Department of Chemistry, University of Gujrat, Gujrat, Pakistan
[e] Department of Chemistry, COMSATS University Islamabad, Islamabad Campus, 45550-Islamabad, Pakistan
[f] Department of Pharmacy, COMSATS University Islamabad, Abbottabad Campus, Abbottabad 22060, Pakistan

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ABSTRACT

Owing to the desperate need of new drugs development to treat Alzheimer's ailment the synthesis of 1-aroyl-3-(5-(4-chlorophenyl)-1,2,4-triazole-3-thioneaminylthioureas **(2–6)** starting from (4-amino-5-(4-chlorophenyl)-4*H*-1,2,4-triazole-3-thiol) **(1)** and synthesis of 1-(3-(4-aminophenyl)-5-phenyl-4,5-dihydro-1*H*-pyrazol-1-yl)-2-(4-isobutylphenyl)propan-1-one (**7–9**) starting from 2-(4-isobutylphenyl)propanehydrazide **(a)** with the cyclization with substituted chalcones **(c-e)** was carried out. To check the biological potential of the synthesized compounds, all were subjected to acetylcholinesterase (AChE) and butrylcholinesterase (BChE) inhibition assays. The most potent and selective inhibitor for the acetylcholinesterase was compound **7** having an inhibitory concentration of 123 ± 51 nM, whereas, compound **6** was found as selective inhibitor of butyrylcholinesterase (BChE) with an IC_{50} value of 201 ± 80 nM. However, the compounds **1** and **2** were found as dual inhibitors i.e. active against both acetylcholinesterase as well as butyrylcholinesterase.

1. Introduction

Despite the way that it has been more than a century since the dynamic, neurodegenerative turmoil known as Alzheimer's ailment (AD) was first officially portrayed in the scientific writings; still there is much more we need to know about this overwhelming malady [1]. The actual reason and succession of occasions prompting sickness and development has escaped scientists regardless of the recognizable proof of various elements which are reason of AD [2–4]. Current pharmacological medicines for AD may offer some help for the outstanding psychological indications. Over a century has gone since the first report of Alzheimer, and the infection has developed up to great extent [5]. Alone in the United States that it was consider that AD is assessed to influence an aggregate of 5.2 million Americans among them 96% are age 65 and more. Globally, it is evaluated that more than 24 million individuals are experiencing AD [6]. According to world health organization WHO, AD is sixth driving reason for death over all ages in the United States. With the time this number is increasing [7]. Generally, AD is supposed to be the reason for neurodegeneration and neuronal decay in the brain. Several neurotic signs of AD have been distinguished and observation of these characteristics has prompted a few theories in endeavors to clarify the basic reason for this disease. In any case, the correct reason and time allotment of occasions prompting AD stays unclear, and it is likely multifactorial and includes an unpredictable cluster of components [8–16].

Acetylcholine (ACh) is a neurotransmitter that under normal and neurodegenerative conditions helps in the modulation of function of memory while cholinesterases (ChEs) is a class of enzymes that play a

* Corresponding authors at: Department of Chemistry, COMSATS University Islamabad, Abbottabad Campus (A. Mumtaz). Centre for Advanced Drug Research, COMSATS University Islamabad, Abbottabad Campus, Abbottabad 22060, Pakistan (J. Iqbal).
E-mail addresses: amaramumtaz@ciit.net.pk (A. Mumtaz), drjamshed@ciit.net.pk (J. Iqbal).
[1] Equal contribution.

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significant role in the cholinergic signaling and regulation of Ach. [17,18] AChE and BChE simultaneously play a vital role in the regulation of ACH level, its synaptic hydrolysis and in terminating its neurotransmitter action. In order to re-establish the neurotransmitter level is done by using inhibitors of cholinesterase that function by suppressing the ChE enzymes which results in both the level increase and duration of the neurotransmitter action [19]. The ChE inhibitors (ChEIs) are the first generation of drugs for the treatment of Alzheimer's disease, glaucoma and myasthenia gravis and being used for the treatment of many other neuromuscular disorders. It has a limitation, with the increase in the level of Ach, symptoms of these diseases increase. Thus, search for new ChEIs is highly appreciated for further advancement in the treatment of such diseases [5,20].

In our efforts to contribute to the development of novel compounds that may be useful in the treatment of neurodegenerative disorders such as Parkinson's disease (PD) or Alzheimer's disease (AD), we are focusing on the synthesis of derivatives of thioureas and pyrazolines. Thioureas derivatives are famous for their broad spectrum of activities which include antifungal, antibacterial, anticancerous, insecticidal activities [21,22]. Coumarin substituted thioureas [23] and 1-butanoyl-3-arylthiourea [24] showed moderate to excellent activity as cholinesterase inhibitor. On the other hand, derivatives of pyrazolines are famous for their analgesic, anti-inflammatory activities [25]. Pyrazoline substituted sulfonamide derivatives showed inhibition against AChE at the nanomolar concentrations [26]. 1-*N*-substituted thiocarbamoyl-3-phenyl-5-thienyl-2-pyrazoline derivatives were synthesized by Gulberk *et al* and on exposure to acetylcholinesterase and butrylcholinesterase enzymes they were found very active [27]. Similarly, indolopyrazoline derivatives were synthesized by Sridevi *et al* and found excellent inhibitors of acetylcholinesterase [28]. Keeping in mind the inhibitory activities of thioureas and pyrazolines as acetylcholinesterase inhibitors, we are focusing on the synthesis of ibuprofen substituted pyrazolines and thioureas of 5(4-chlorophenyl)-1,2,4-triazole-3-thioneamine [29] as new compounds with potential activity as cholinesterase inhibitors.

2. Results & discussion

2.1. Chemistry

Synthesis of 1-aroyl-3–5-(4-chlorophenyl)-1,2,4-triazole-3-thioneaminylthioureas **(2–6)** and 1-(3-(4-aminophenyl)-5-phenyl-4,5-dihydro-1H-pyrazol-1-yl)-2-(4-isobutylphenyl)propan-1-one **(7–9)** were carried out staring from their common precursor hydrazide (Table 1). For the synthesis of 1-aroyl-3–5-(4-chlorophenyl)-1,2,4-triazole-3-thioneaminylthioureas **(2–6)** reaction of **1** [29] with substituted benzoylisothiocyanate prepared *in situ* was carried out to get **2–6** (Scheme 1).

In the FT-IR data of the compounds **(2–6)** structures were confirmed by the appearance of a stretching band at 2923–3001 cm^{-1} indicating the presence of C—H group, the stretching bands at 1578–1673, 1584–1600 cm^{-1} showed the presence of the aromatic rings while the stretching band at 3409–3489 cm^{-1} showed the presence of N—H groups. In ^{1}H NMR data of the compounds **(2–6)** presence of two singlets at 10.32–10.75 ppm and 9.98–10.19 ppm confirmed the presence of two NH groups which are the characteristic signal and confirm the structure of thiourea group. In ^{13}C NMR peaks at 165.0–195.0 ppm and signal at 147.1–164.7 ppm confirmed the presence of C═O and C═S groups respectively. [30]

Synthesis of 1-(3-(4-aminophenyl)-5-phenyl-4,5-dihydro-1*H*-pyrazol-1-yl)-2-(4-isobutylphenyl)propan-1-one **(7–9)** was carried out by condensation of ibuprofen hydrazide **(a)** with substituted chalcones **(b-d)** [31] (Scheme 1). In the FT-IR data of the compounds **(7–9)** a stretching band at 2955–3110 cm^{-1} confirmed the presence of C—H group, the stretching bands at 1428–1442, 1551–1595 cm^{-1} for the aromatic rings while the stretching band at 3288–3355 cm^{-1} showed the presence of C—N groups and stretching at 1698–1711 cm^{-1} confirmed the presence of C—O bond. In ^{1}H NMR data of the compounds **(7–9)** presence of chiral center of pyrazoline ring and NH_2 groups were confirmed by the presence of doublet of a doublet at a range of 3.87–3.96 ppm and a singlet at 2.73–4.23 ppm respectively. Similarly, in ^{13}C NMR peak at 173.3–194.8 ppm confirmed the presence of carbonyl carbon. [31]

Table 1
Physical data of compounds **(2–9)**.

Sr. No.	M.P (°C)	R_f^a	Yield (%)
1	198–200	0.2	81
2	190–192	0.6	83
3	204–206	0.5	72
4	220–222	0.4	78
5	201–203	0.5	83
6	207–209	0.4	82
7	227–229	0.4	76
8	203–205	0.5	77
9	Semisolid	0.5	80

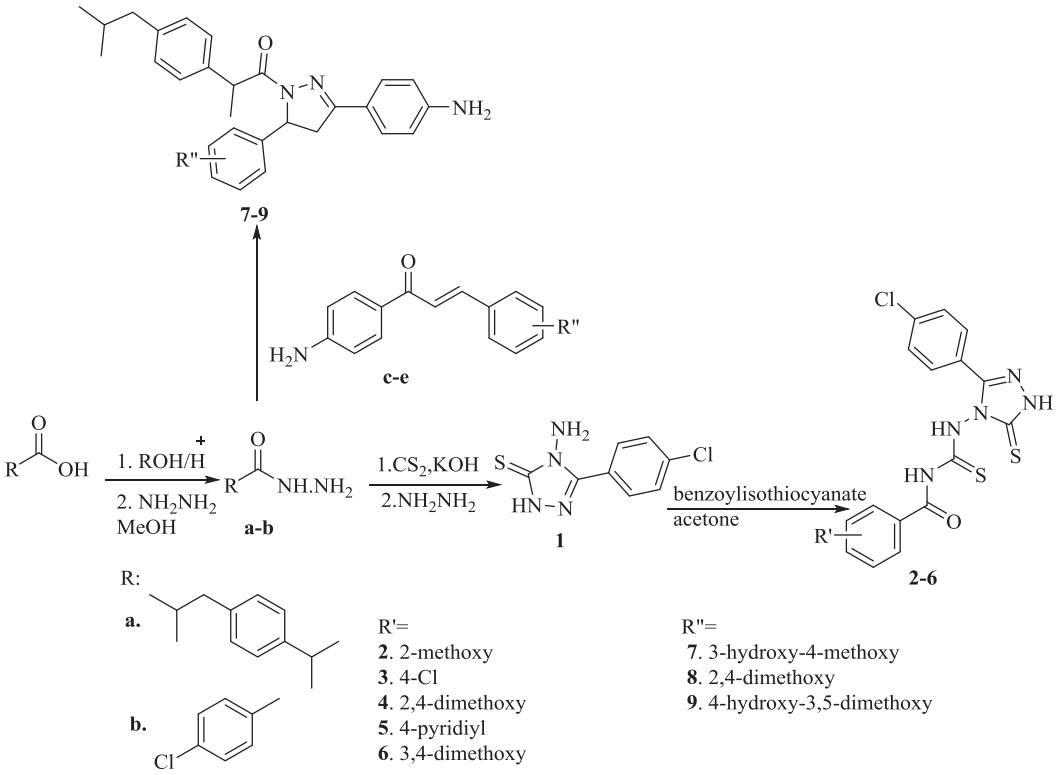
2.2. Biological assay

2.2.1. Cholinesterase inhibitory activity

The thiourea moiety with different derivatives like butanoyl and acetyl arylthioureas [24,32], were screened against cholinesterases. Moreover, the similar studies were carried out against coumarin linked thioureas [23] and halogenated thioureas [33] and results suggested that synthesized analogues were potent inhibitors of cholinesterases. Therefore, 1,2,4-triazole-3-thioneaminyl substituted thioureas were designed and synthesized **(1–8)** and tested for their potential inhibition against two isozymes of cholinesterase family, acetylcholinesterase and butyrylcholinesterase. Dual inhibitors of both the isozymes were also identified among screened compounds. The key objective of the study was to explore the most potent and selective inhibitors of acetylcholinesterase and butyrylcholinesterase. The inhibition data obtained from the enzymatic analysis was presented in Table 2. The donepezil and galanthamine were taken as reference standards during the assay. The inhibitory concentration obtained for the donepezil was 32.1 ± 3.1 nM for the acetylcholinesterase and 6414 ± 342 nM for butyrylcholinesterase, whereas, IC_{50} value of galanthamine against acetylcholinesterase was 622 ± 11 nM and that of butyrylcholinesterase was found as 878 ± 37 nM.

2.2.2. Structure-activity relationship

The most potent and selective inhibitor for the acetylcholinesterase was **7** having an inhibitory concentration of 123 ± 51 nM, however, compound **6** was found as selective inhibitor of butyrylcholinesterase with an IC_{50} value of 201 ± 80 nM. However, the compounds **1** and **2** were chosen as dual inhibitors of acetylcholinesterase as well as butyrylcholinesterase. These compounds exhibited significant inhibition against acetylcholinesterase along with good inhibitory activity against butyrylcholinesterase and our findings were like previously reported data [23]. Figs. 1 and 2 represented the detail structure activity relationship of active scaffolds and substituents attached. From the Table 2, it was noticed that compounds **4** and **8** showed no significant inhibition against any of cholinesterase. From the substituted pyrazolines, the compound **8**, 1-(3-(4-aminophenyl)-5-(2,4-dimethoxyphenyl)-4,5-dihydro-1*H*-pyrazol-1-yl)-2-(4-isobutylphenyl)propan-1-one showed very less inhibition towards acetyl and bytyrylcholinesterase. Similar pattern was observed by compound **4,** *N*-((3-(4-chlorophenyl)-5-mercapto-4*H*-1,2,4-triazol-4-yl)carbamothioyl)-2,4-dimethoxybenzamide, which belongs to 5–4-chlorphenyl-1,2,4-triazole-3-thione amine substituted thioureas. However, rest of the derivatives were found as either selective inhibitors of acetylcholinesterase/ butyrylcholinesterase or have shown dual inhibition



Scheme 1. Synthesis of thiourea (2–6) and pyrazoline (7–9) derivatives.

Table 2
Cholinesterase inhibition efficacy and anti-proliferative activity of synthetic derivatives.

Code	AChE	BChE	Anti-proliferative activity (HeLa)
	IC_{50} (nM) ± SEM/% inhibition		
1	139 ± 12	377 ± 21	62.5%
2	375 ± 55	148 ± 33	36.8%
3	26.2%	5.8%	32.2%
4	24.3%	23.6%	28.5%
5	513 ± 48	255 ± 19	19.7%
6	27.1%	201 ± 80	21.1%
7	123 ± 51	26.4%	21.2%
8	17.9%	12.2%	18.6%
Donepezil	32.1 ± 3.1	6414 ± 342	–
Galanthamine	622 ± 11	878 ± 37	–
Cisplatin	–	–	89.3%

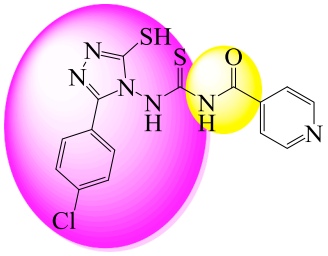
towards both the enzymes. The Fig. 1 showed the detail structure activity relationship of 5-(4-chlorophenyl)-2,4-dihydro-3*H*-1,2,4-triazole-3-thione amine, 5–4-chlorphenyl-1,2,4-triazole-3-thione amine substituted thioureas, in which **1**, **2** and **5** were dual inhibitors, however, the selective inhibition towards the butyrylcholinesterase was exhibited by **6**. Fig. 2 presented the substituted pyrazolines derivatives and their inhibition potential towards both the enzymes.

The results depicted that the parent compound of thioureas, **1** (4-amino-5-(4-chlorophenyl)-4*H*-1,2,4-triazole-3-thiol) [29] exhibited dual inhibition towards AChE and BChE with an IC_{50} values of 139 ± 12 and 377 ± 21 nM, respectively. Upon addition of substituent on the main compound, different patterns were observed in the inhibition. The addition of 2-methoxy benzamide group exhibited similar pattern like parent compound and showed inhibition for both the enzymes. However, upon substitution of similar group at position 3 and 4 give rise to a selective and potent inhibitor of butyrylcholinesterase. But when the substitution of same group (methoxy) at position 2 and 4 was noticed, the compound has not shown any inhibition towards acetylcholinesterase and butyrylcholinesterase. Therefore, it may be suggested that the attachment of methoxy group at position 3 was the contributing factor for inhibition. The only compound with different substituent in thioureas was **5**, having isonicotinamide group attached to the parent ring. The substituent gave rise to a dual inhibitor and showed inhibitory activity of 513 ± 48 and 255 ± 19 nM, for acetylcholinesterase and butyrylcholinesterase, respectively.

When the structures of substituted pyrazolines, (**7** and **8**) were investigated, it was observed that 3-hydroxy-4-methoxyphenyl group in compound **7** was the important factor towards the inhibition profile of the compound. The compound was noted as potent and selective inhibitor of AChE. The compound **8,** having 2 and 4-methoxy phenyl was found as inactive and showed less than 50% inhibition towards acetylcholinesterase and butyrylcholinesterase. The results showed that the attachment of different substituents at ortho, para and meta position at the main moiety has a significant impact and contribution towards inhibitory profile of acetylcholinesterase and butyrylcholinestase. The red and yellow colors in Fig. 2 are the positions at which the groups vary, and these groups were found to be responsible for the inhibitory activity of the synthesized derivatives.

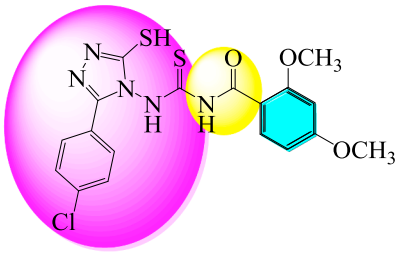
2.2.3. Molecular docking analysis

To justify the experimental results, the docking analysis was carried out against the potent and selective inhibitors of acetylcholinesterase as well as butyrylcholinesterase. The *in vitro* results clarify that compound **7** was potent and selective inhibitor of acetylcholinesterase, whereas, compound **6** was selective as well as potent inhibitor of butyrylcholinesterase. However, some dual inhibitors were found and docking analysis was carried out against those dual inhibitors, **1** and **2** in the active site of both the receptors. To investigate the binding poses of selected compounds, the protein structures were selected from protein databank. In case of acetylcholinesterase, the crystal structure of *Tetronarce californica* was selected and downloaded [34] because electric eel structures for AChE are only available at low crystallographic resolutions (> 4 Å) and the vesicular acetylcholine transporter of electric ray (*Torpedo californica*) is structurally related to the vesicular



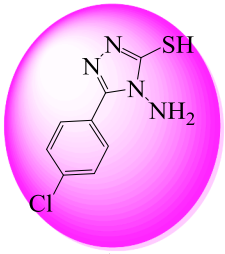
5

AChE (IC_{50} ± SEM) = 513 ± 48 nM
BChE (IC_{50} ± SEM) = 255 ± 19 nM



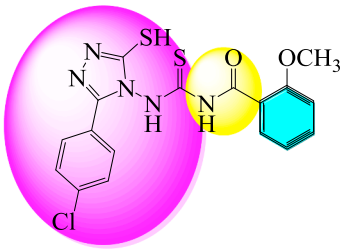
4

AChE = 24.3%
BChE = 23.6%



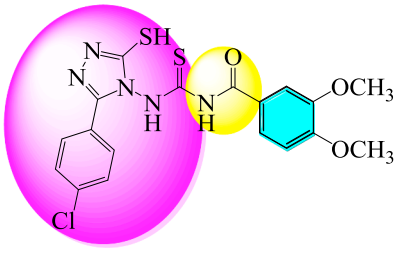
1

AChE (IC_{50} ± SEM) = 139 ± 12 nM
BChE (IC_{50} ± SEM) = 377 ± 21 nM



2

AChE (IC_{50} ± SEM) = 375 ± 55 nM
BChE (IC_{50} ± SEM) = 148 ± 33 nM



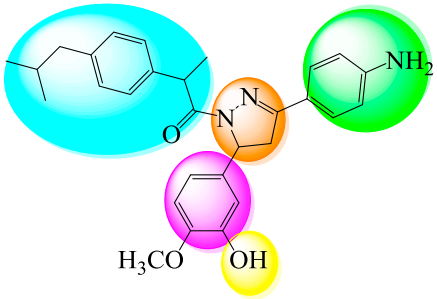
6

AChE = 27.1%
BChE (IC_{50} ± SEM) = 201 ± 80 nM

Fig. 1. Structure activity relationship of synthetic compounds.

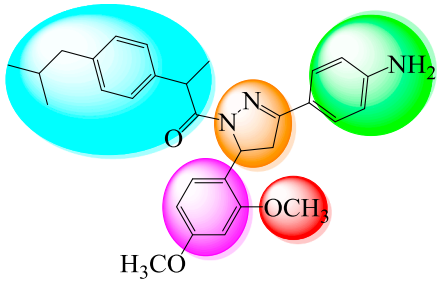
monoamine transporters of mammals, therefore structure of electric ray was chosen. For butyrylcholinesterase, the x-ray structure of human butyrylcholinesterase was selected and downloaded [35] because structures of equine BChE are currently not available. Fig. 3 (a, b) depicts the overlap of all the selected compounds docked inside the active site of AChE and BChE, respectively.

To validate the docking studies, the co-crystallized ligands of acetylcholinesterase and butyrylcholinesterase were docked inside the active site after extraction from the respective receptor. The re-docking of co-crystallized ligands (E2020 for 1EVE and THA for 4BDS) were carried out by root mean square deviation of 1.32 and 1.04 Å for acetylcholinesterase and butyrylcholinesterase, respectively. The active



7

AChE (IC_{50} ± SEM) = 123 ± 51 nM
BChE = 26.4%



8

AChE = 17.9%
BChE = 12.2%

Fig. 2. Structure activity relationship of synthetic compounds.

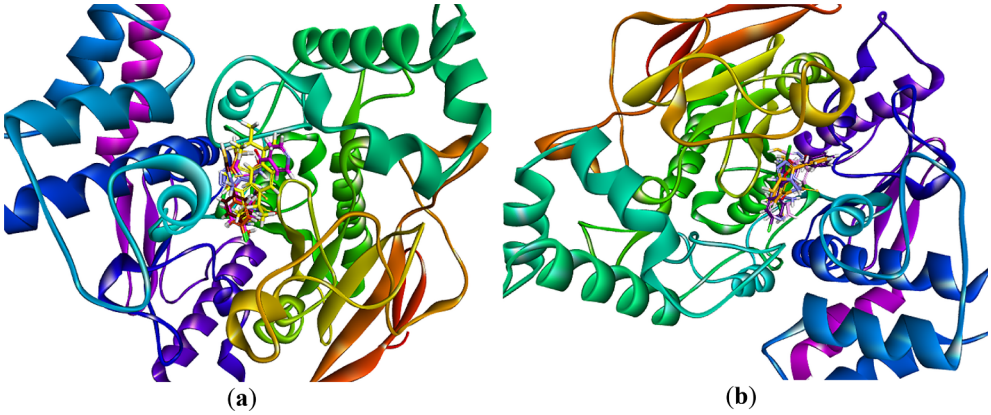


Fig. 3. The overlap of all the docked inhibitors (**7** (yellow), **2** (red), **5** (pink) and **1** (brown)) inside the active site of acetylcholinesterase (a) and inhibitors (**6** (orange), **2** (purple), **5** (pink) and **1** (green)) inside the butyrylcholinesterase (b).

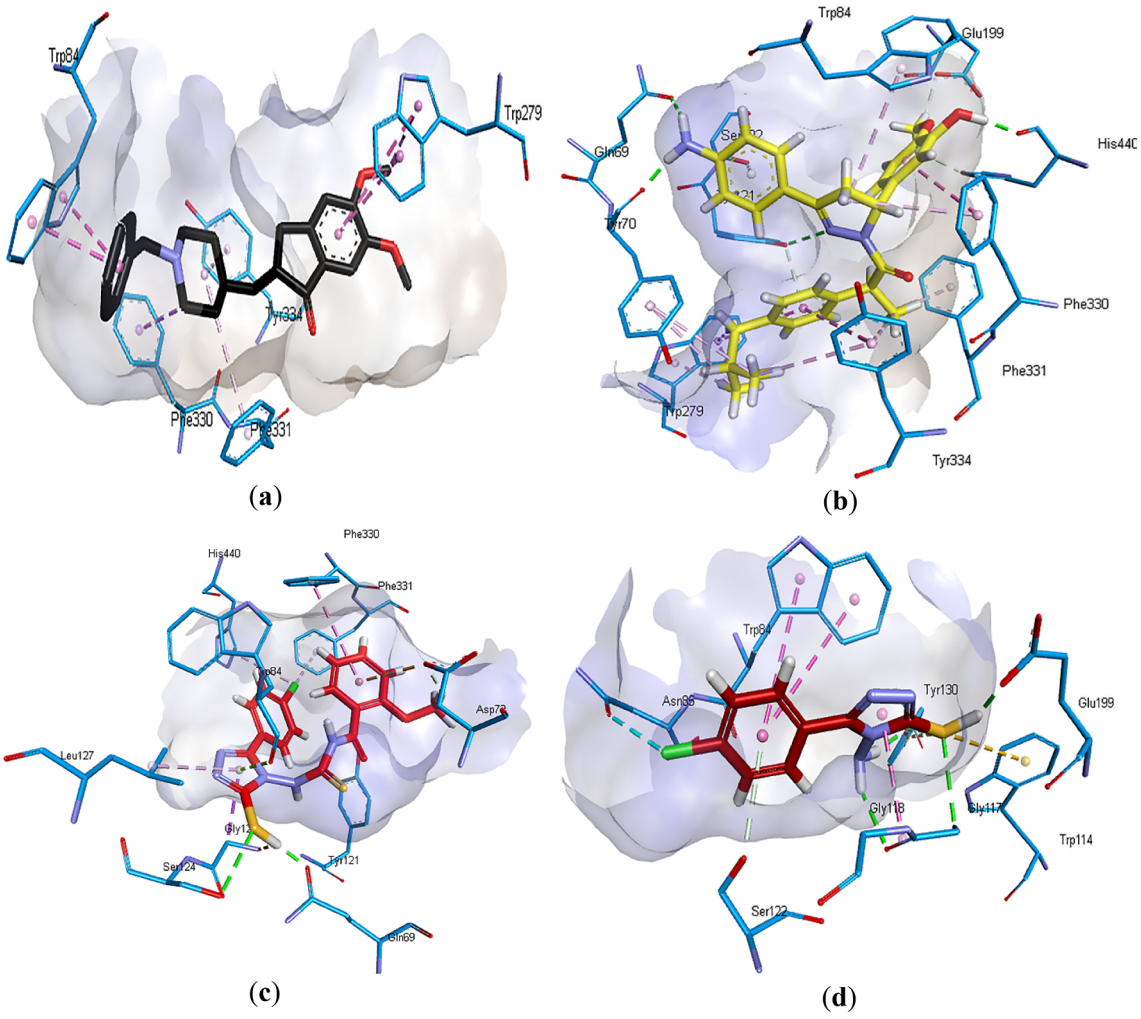


Fig. 4. 3D interaction diagrams of (**a**) cognate ligand (E2020) (**b**) selective inhibitor **7**, of AChE along with dual inhibitors (**c**) compound **2** and (**d**) compound **1** inside the 1EVE.

pockets were selected after analysis of binding interactions of co-crystallized ligands within the active site. The 3D interaction diagrams of co-crystallized ligand, selective inhibitor and dual inhibitors of acetylcholinesterase were shown in Fig. 4. While, the 3D interaction diagrams of co-crystallized ligand, selective inhibitor and dual inhibitors of butyrylcholinesterase was shown in Fig. 5.

The detailed analysis of acetylcholinesterase co-crystallized ligand and the inhibitors showed that Tyr334, Trp114 and Trp84 are the most important residues showing interaction with the inhibitors within the active site. The cognate ligand E20, 1-benzyl-4-[(5,6-dimethyoxy-1-indanon-2-yl)methyl]piperidine presented the same interactions and our selective inhibitor, **7** was making π-π interactions with Tyr84. In addition to π-π interactions, the hydrogen bonds were noticed between active site amino acid residues and compound. The dual inhibitors, **2**

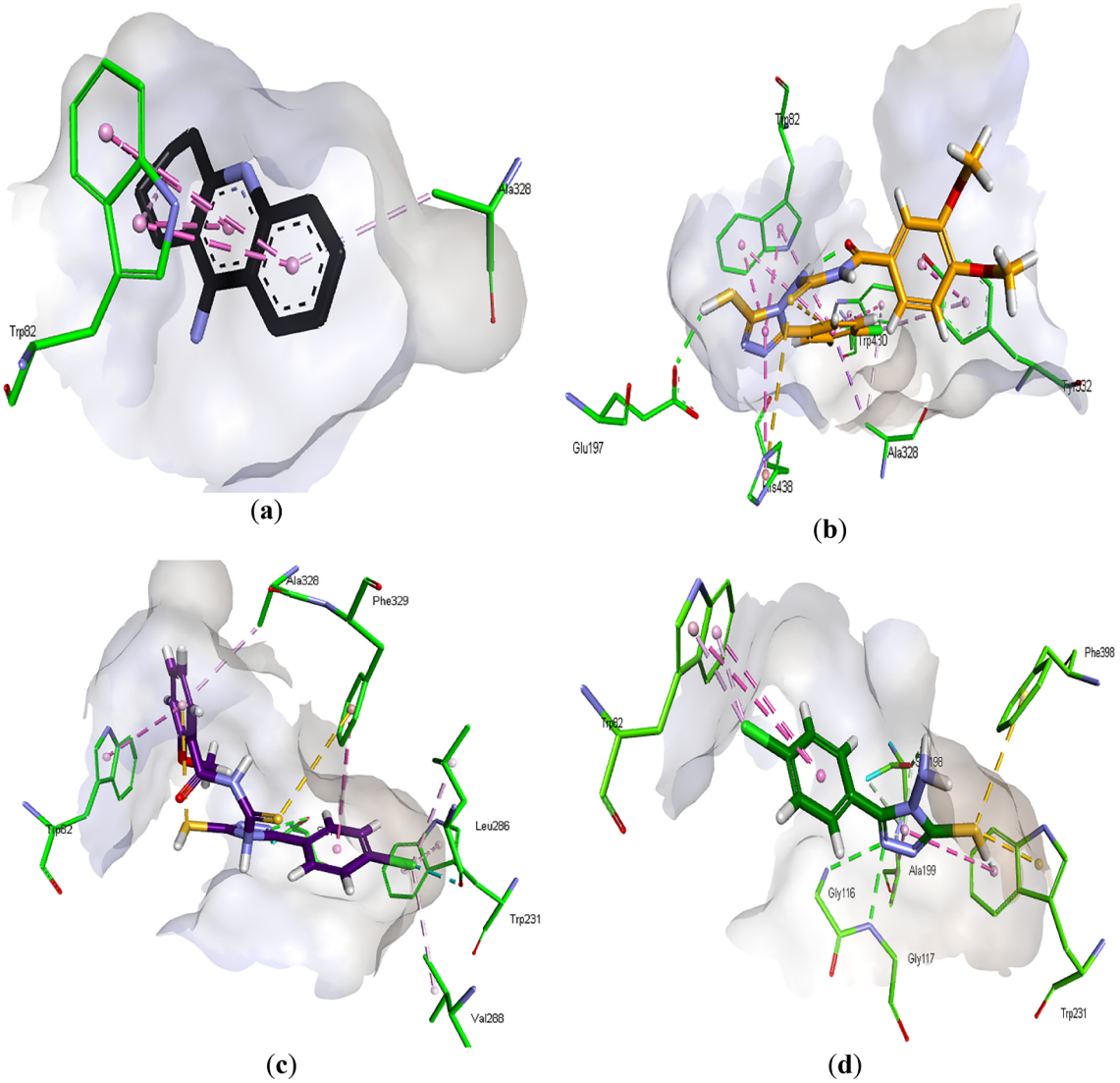


Fig. 5. 3D interaction diagrams of (**a**) cognate ligand (THA) (**b**) selective inhibitor compound **6,** of BChE along with dual inhibitors (**c**) compound **2** and (**d**) compound **1** inside the 4BDS.

and **1** were making the hydrogen bonds and π-π interactions with important residues. The interactions analysis suggested that the selected compounds showed best fit interaction inside the pocket of acetylcholinesterase. However, the bond between the selective inhibitor of acetylcholinesterase, compound **7** was noticed as hydrogen bonding in addition to π-π interactions with Phe330. Tyr334 showed similar π-π interactions with isobutylphenyl group of the compound **7.** Moreover, His440 was hydrogen bonded with methoxy group. Trp279 was showing pi-alkyl linkage with isobutyl moiety. The bond distance showed by Trp84 with E2020 was 2.78 Å and that of our compound **7** was 2.93 Å. Similarly, the dual inhibitors showed the interactions with active site residues. In the compound **2** having 2-methoxy group, the phenyl ring was showing π-π interactions with amino acid Phe330, His440, Phe331 and 5-mercapto group was showing π-π interactions. Similar interactions were noticed, when compound **1** was considered. The residue Trp84 showed hydrogen bonds along with π-π interactions with the 4-chlorophenyl part of the compound. The selected compounds showed the binding interactions necessary for the inhibition of acetylcholinesterase within the active site.

When butyrylcholinesterase active site residues and interaction analysis of selected compounds were investigated, it was noticed that the compounds exhibited most of the interactions with Trp82, along with His438 and Tyr332 and are responsible for the inhibitory behavior towards butyrylcholinesterase that were coherent with previous studies [23]. However, the bond distance of some of the important residues and their type of interactions are explained here. The co-crystallized ligand, tacrine, showed the hydrogen bonding with Trp82 at the bond distance of 2.98 Å. The tacrine showed π-π interactions with Trp82 and hydrogen bond with Ala328. However, the selective inhibitor of butyrylcholinesterase, compound **6** showed the same interaction with Trp82 at bond distance of 3.14 Å. The dual inhibitors, **2** and **1** exhibited 3.64 and 3.99 Å, respectively with Trp82. In addition to π-π interactions, hydrogen bonding was noticed, and the residue involved in the formation of hydrogen bond with docked compounds were Phe398, Trp231, Gly116, Ala328, Leu286, Val288 and Tyr332. All the compounds showed hydrogen bonds. The formation of strong hydrogen bonding suggested that these contribute well towards the inhibition profile and our selected compounds showed these bonding. The interaction analysis suggested that the compounds were found in best fitted poses inside the active site of butyrylcholinesterase and therefore, the investigated interactions are responsible for the inhibition profile of the compounds.

2.2.4. *The physicochemical properties of synthesized derivatives*

In silico physicochemical properties and drug-likeness of the screened derivatives were evaluated by MedChem Designer 3.0 [36].

Different characteristics are responsible for categorizing the synthetic compound into either a drug or a chemical. The parameter used to check out the drug-likeness was Lipinski's rule of 5, according to it, Mol. Wt. must be less than 499 Dalton, calculated octanol/water partition coefficient not more than 5 (lipophilicity), the no. of H-bond donors must be less than 5 and the no. of H-bond acceptors can be equal of less than 10 [37]. The literature reports that drugs for central nervous system must have lower level of lipophilicity and Mol. Wt. should be lower than 500. Moreover, low hydrogen bond and little number of rotatable bonds are also required for a compound to act as a drug [38]. Therefore, we evaluated the compounds for these parameters and evaluated the distribution of octanol-water and partition coefficients (S + log D), coefficient of octanol-water partition (S + log P), and number of hydrogen bond donors (HBD), hydrogen bond acceptor (MNO) and topological polar surface area (TPSA). Any drug with the polar surface area of 60–140 $Å^2$, is supposed to be sufficiently bioavailable [39]. Octanol-water partition helps to estimate the level of hydrophobicity and hydrophilicity of molecule. The synthetic derivatives showed significant contribution towards the physicochemical parameters. All the compounds exhibited less than 100 $Å^2$. Therefore, it may be suggested that all the derivatives can be penetrated easily if used as drugs. The compounds with Mol. Wt. less than 500, M log P value lesser than 5 are considered as orally bioavailable with favorable absorption, distribution, metabolism and excretion (ADME) profile [40,41]. The derivatives have Mol. Wt. less than 500 and bear lesser than 10 rotatable bonds. The physicochemical parameters of the synthetic derivatives are given in Table 3.

2.2.5. In vitro anti-proliferative activity against HeLa cervical carcinoma cells

The anti-proliferative activity of **(1–8)** against cervical carcinoma cells (HeLa) was tested by MMT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay [42,43]. The %age inhibition of the synthetic derivatives was assessed at 100 μM (Table 2). The positive standard used was cisplatin and it exhibited 89.3% inhibition against cells at same concentration. The screened compounds presented lower cytotoxicity than that of cisplatin and these derivatives revealed lower anti-proliferative activity as compared to cisplatin towards the cervical HeLa cells. The compounds exhibited %age inhibition against cancer cells in the range of 18.6 – 62.5%. The compound **1**, (4-amino-5-(4-chlorophenyl)-4*H*-1,2,4-triazole-3-thiol) [29] was found to have more anti-proliferative activity than other derivatives with the percentage inhibition of 62.5%. All the other compounds presented less than 50% inhibition towards HeLa cell lines.

2.3. Materials & Methods

Capillary tubes were used for recording melting point by using electrothermal melting point apparatus, model SMP 10 digital melting point apparatus OE/Digital (08–09) 169/1 DMPA 09–01. ^{1}H NMR was recorded on 300 MHz spectrophotometer by using deuterated DMSO as solvent, while the tetramethylsilane (TMS) was used as internal reference. The chemicals and reagents used in the study were of analytical grade and purchased from Merck (Germany) and Sigma Aldrich (USA).

Table 3
Physicochemical parameters of synthetic compounds.

Codes	M log P	S + log P	S + log D	Mol. Wt.	MNO	TPSA	HBDH
1	2.389	1.997	1.348	226.688	4	56.730	2
2	3.670	3.216	1.803	419.914	7	81.070	2
4	3.154	3.221	1.887	449.940	8	90.300	2
5	2.678	2.662	1.223	390.875	7	84.730	2
6	2.643	3.329	1.943	449.940	8	90.300	2
7	4.854	5.831	5.828	485.630	6	88.150	3
8	4.533	6.004	6.004	499.657	6	77.150	2

2.4. Experimental

2.4.1. General procedure for the synthesis of 5-(4-chlorphenyl)-1,2,4-triazole-3-thione amine substituted thioureas (2–6)

For the synthesis of thiourea (2–6), acid chloride of substituted acid was prepared by refluxing the substituted benzoic acid with thionyl chloride (1.5 eq) for 2 hr. In another two neck round bottom flask potassium thiocyanate (KSCN) (1 eq) was suspended in 5 mL of acetone. Acid chloride was added in it and stirred for 35 min. After adding triazole amine **(1)** [29] dissolved in 5 mL of acetone (dry) into reaction mixture and refluxed for one hour. Upon completion, the reaction mixture was poured in ice cold water; obtained solid precipitates were passed through filter paper, washed and purified by using suitable solvent [30].

2.4.1.1. N-((3-(4-chlorophenyl)-5-mercapto-4H-1,2,4-triazol-4-yl) carbamothioyl)-2-methoxybenzamide (2).

M.P (°C): 190–192; Color: yellow; yield (%): 83; R_f (*n*-Hexane: Ethyl acetate, 7:3): 0.6; FT-IR (ύ, cm^{-1}): 3489 (NH), 2923 (C—H), 1564, 1496 (C—H arom), 1423 (C—H), 1145 (C—O),744 cm^{-1}; ^{1}H NMR (300 MHz, DMSO-d_6)δ: 10.75 (s, 1H, NH), 10.08 (s, 1H, NH), 7.95 (d, J = 8.4 Hz, $2H_{Ar}$), 7.76 (dd, J = 7.6 Hz, $1H_{Ar}$), 7.60 (d, J = 8.7 Hz, $2H_{Ar}$), 7.54 (td, J = 3.5 Hz, $1H_{Ar}$), 7.53 (td, J = 3.0 Hz, $1H_{Ar}$), 7.19 (d, J = 7.6 Hz, $1H_{Ar}$), 3.91 (s, 3H, OCH_3), ^{13}C NMR (75 MHz, DMSO-d_6) 165.0, 164.7, 157.5, 137.1, 133.2, 131.7, 130.9, 129.9, 129.8, 122.1, 120.0, 112.5, 56.3.

2.4.1.2. 4-Chloro-N-((3-(4-chlorophenyl)-5-mercapto-4H-1,2,4-triazol-4-yl)carbamothioyl)benzamide (3).

M.P (°C): 204–206; Color: yellow; yield (%): 72; R_f (*n*-Hexane: Ethyl acetate, 7:3): 0.5; FTIR (ύ, cm^{-1}): 3201, 3167 (=C—H), 3001 (C—H), 1733 (C=O), 1707, 1689(C=O), 1649, 1592 (C—H arom), 1500 (C=C), 1496 (C—H arom), 1101 (C—O) cm^{-1}; ^{1}H NMR (300 MHz, DMSO-d_6) δ: 10.73 (s, 1H, NH), 9.98 (s, 1H, NH), 8.16(d, J = 8.7 Hz, $2H_{Ar}$), 7.95 (d , J = 8.4 Hz, $2H_{Ar}$), 7.72 (d, J = 8.7 Hz, $2H_{Ar}$), 7.58 (d, J = 7.6 Hz, $2H_{Ar}$); ^{13}C NMR (75 MHz, DMSO-d_6) δ: 166.9, 161.6, 140.6, 138.2, 132.7, 131.6, 130.0, 129.9, 129.8, 129.2, 128.7, 127.3.

2.4.1.3. N-((3-(4-chlorophenyl)-5-mercapto-4H-1,2,4-triazol-4-yl) carbamothioyl)-2,4-dimethoxybenzamide (4).

M.P (°C): 220–222; Color: yellow; yield (%): 78; R_f (*n*-Hexane: Ethyl acetate, 7:3): 0.4; FTIR (ύ, cm^{-1}): 3167 (=C—H), 3201, 3001 (C—H), 1707, 1689(C=O), 1649, 1592 (C—H arom), 1500 (C=C), 1496 (C—H arom), 1101 (C—O), cm^{-1}; ^{1}H NMR (300 MHz, DMSO-d_6) δ: 10.76 (s, 1H, NH), 10.03 (s, 1H, NH), 7.95 (d , J = 8.4 Hz, $2H_{Ar}$), 7.74 (d, J = 7.8, $2H_{Ar}$), 7.62 (d, J = 7.6 Hz, $1H_{Ar}$), 7.10 (d, J = 8.7 Hz, $1H_{Ar}$), 6.92 (s, $1H_{Ar}$), 3.91 (s, 3H, OCH_3), 3.88 (s, 3H, OCH_3), ^{13}C NMR (75 MHz, DMSO-d_6) δ: 195.5, 158.8, 155.0, 147.5, 134.9, 131.2, 125.3, 124.8, 118.6, 116.5, 65.5.

2.4.1.4. N-((3-(4-chlorophenyl)-5-mercapto-4H-1,2,4-triazol-4-yl) carbamothioyl)isonicotinamide (5).

M.P (°C): 201–203; Color: yellow; yield (%): 83; R_f (*n*-Hexane: Ethyl acetate, 7:3): 0.5; FTIR (ύ, cm^{-1}): 3201, 3167 (=C—H), 3001 (C—H), 1707, 1649, 1689(C=O), 1592 (C—H)arom, 1500 (C=C), 1496 (C—H)arom, 1101 (C—O) cm^{-1}; ^{1}H NMR (300 MHz, DMSO-d_6) δ: 10.34 (s, 1H, NH), 10.19 (s, 1H, NH), 7.95 (d , J = 8.4 Hz, $2H_{Ar}$), 7.76 (d, J = 7.6 Hz, $2H_{Ar}$), 7.60 (d, J = 8.7 Hz, $2H_{Ar}$), 7.49 (d, J = 7.6 Hz, $2H_{Ar}$); ^{13}C NMR (75 MHz, DMSO-d_6) δ: 185.5, 160.0, 156.8, 137.5, 132.9, 129.2, 127.2, 123.1.

2.4.1.5. N-((3-(4-chlorophenyl)-5-mercapto-4H-1,2,4-triazol-4-yl) carbamothioyl)-3,4-dimethoxybenzamide (6).

M.P (°C): 207–209; Color: yellow; yield (%): 82; R_f (*n*-Hexane: Ethyl acetate, 7:3): 0.4; FTIR (ύ, cm^{-1}): 3201, 3167 (=C—H), 3001 (C—H), 1698, 1688 (C=O), 1623, 1592 (C—H arom), 1500 (C=C), 1496 (C—H arom), 1101 (C—O) cm^{-1}; ^{1}H NMR (300 MHz, DMSO-d_6) δ: 10.32 (s, 1H, NH), 10.02 (s, 1H, NH), 7.89 (d , J = 8.4 Hz, $2H_{Ar}$), 7.71 (d, J = 7.6 Hz, $1H_{Ar}$), 7.60 (d, J = 8.7 Hz, $2H_{Ar}$), 7.49 (d, J = 7.0 Hz, $1H_{Ar}$), 7.19 (d, J = 2.8 Hz,

$1H_{Ar}$), 3.91 (s, 3H, OCH_3), 3.90 (s, 3H, OCH_3); ^{13}C NMR (75 MHz, DMSO-d_6) δ: 197.6,147.7, 147.1, 137.0, 127.6, 125.8, 124.0, 122.3, 119.4, 65.2, 56.5.

2.4.2. General procedure for the synthesis of 1-(3-(4-aminophenyl)-5-phenyl-4,5-dihydro-1H-pyrazol-1-yl)-2-(4-isobutylphenyl)propan-1-one (7–9):

Prepared chalcones [31] were dissolved in ethanol and refluxed for 7 hr with ibuprofen hydrazide in the presence of piperidine as a catalyst.

2.4.2.1. 1-(3-(4-aminophenyl)-5-(3-hydroxy-4-methoxyphenyl)-4,5-dihydro-1H-pyrazol-1-yl)-2-(4-isobutylphenyl)propan-1-one (7). M.P (°C):227–229; Color: white; yield (%): 76; R_f (*n*-Hexane: Ethyl acetate, 7:3): 0.4; FTIR (ύ, cm^{-1}): 3355, 2995, 1509, 1438, 1155; ^1H NMR (300 MHz, DMSO-d_6) δ: 7.97 (d, *J* = 8.1 Hz, $2H_{Ar}$), 7.80(d, *J* = 7.5 Hz, $2H_{Ar}$), 7.68 (d, *J* = 7.1 Hz, $1H_{Ar}$), 7.60 (s, $1H_{Ar}$), 7.59 (d, *J* = 2.4 Hz, $1H_{Ar}$), 7.51 (d, *J* = 8.1 Hz, $2H_{Ar}$), 7.37 (d, *J* = 7.5 Hz, $2H_{Ar}$), 6.61 (d, *J* = 4.7 Hz, 2H, CH), 4.01(q, 1H, *J* = 6.7 Hz, $CHCH_3$), 3.96 (dd, *J* = 4.7 Hz, 1H, CH), 3.78 (s, 3H, OCH_3), 2.73 (s, 2H, NH_2), 1.83 (d, *J* = 5.5, 3H, CH_3CH), 1.31 (m, *J* = 5.6, $CHCH_3$), 0.84 (d, *J* = 6.1, CH_3); ^{13}C NMR (75 MHz, DMSO-d_6) δ: 173.3, 171.4, 169.1, 168.4, 159.9, 159.6, 154.0, 140.3, 144.1, 139.7, 139.2, 136.6, 132.5, 130.1, 129.6, 129.1, 129.0, 127.5, 127.4, 127.3, 126.1, 56.1, 55.8, 46.1, 30.1, 23.6, 22.6.

2.4.2.2. 1-(3-(4-aminophenyl)-5-(2,4-dimethoxyphenyl)-4,5-dihydro-1H-pyrazol-1-yl)-2-(4-isobutylphenyl)propan-1-one (8). M.P (°C): 203–205; Color: brown; yield (%): 77; R_f (*n*-Hexane: Ethyl acetate, 7:3): 0.5; FTIR (ύ, cm^{-1}): 3444, 3378, 3110, 1677, 1655, 1595, 1499, 1428, 1163, ^1H NMR (300 MHz, DMSO-d_6) δ: 7.97 (d, *J* = 8.1 Hz, $2H_{Ar}$), 7.80 (d, *J* = 7.5 Hz, $2H_{Ar}$), 7.53 (s, $1H_{Ar}$), 7.51 (d, *J* = 8.1 Hz, $2H_{Ar}$), 7.37 (d , *J* = 7.5 Hz, $2H_{Ar}$), 7.13 (d, *J* = 8.4 Hz, $1H_{Ar}$), 6.95 (d, *J* = 8.1 Hz, $1H_{Ar}$), 6.09 (d, *J* = 4.7 Hz, 2H, CH), 4.01 (s, 2H, NH_2), 3.81 (dd, *J* = 4.7 Hz, 1H, CH), 3.78 (s, 3H, OCH_3), 1.84 (d, *J* = 4.7, 3H, CH_3CH), 1.49 (m, *J* = 5.1, $CHCH_3$), 0.81 (d, *J* = 6.2, CH_3); ^{13}C NMR (75 MHz, DMSO-d_6) δ: 173.3, 171.4, 169.1, 168.4, 140.3, 139.7, 139.2, 129.6, 129.1, 127.5, 127.4, 127.3, 56.2, 44.7, 30.1, 25.6, 24.4, 22.6, 19.9, 18.7.

2.4.2.3. 1-(3-(4-aminophenyl)-5-(4-hydroxy-3,5-dimethoxyphenyl)-4,5-dihydro-1H-pyrazol-1-yl)-2-(4-isobutylphenyl)propan-1-one (9). M.P (°C):oil; Color: darkbrown; yield (%): 80; R_f (*n*-Hexane: Ethyl acetate, 7:3): 0.5; FTIR (ύ, cm^{-1}): 3341, 3288, 3009, 1551, 1465, 1128; ^1H NMR (300 MHz, DMSO-d_6) δ: 8.32 (bs, IH, OH), 7.98 (d, *J* = 8.3 Hz, $2H_{Ar}$), 7.84(d, *J* = 7.7 Hz, $2H_{Ar}$), 7.57 (d, *J* = 7.9 Hz, $2H_{Ar}$), 7.43 (d , *J* = 7.4 Hz, $2H_{Ar}$), 7.11 (s, $2H_{Ar}$), 6.55 (d, *J* = 4.9 Hz, 2H, CH), 4.23 (s, 2H, NH_2), 4.00 (q, 1H, *J* = 6.7 Hz, $CHCH_3$), 3.88(s, 6H, OCH_3), 1.46 (d, *J* = 5.3, 3H, CH_3), 1.33(m, 3H), 0.81 (d, *J* = 4.7, 6H, CH_3); ^{13}C NMR (75 MHz, DMSO-d_6) δ: 195.3, 186.5, 162.9, 159.9, 149.7, 149.2, 139.5, 136.6, 132.5, 131.2, 131.0, 130.2, 129.1, 127.5, 127.4, 127.3, 126.1, 119.7, 116.5, 116,3, 56.1, 55.8, 46.1, 39.3, 27.6, 25.2, 23.4, 22.9, 21.7.

2.5. Cholinesterase inhibition assay

In vitro inhibition potencies of synthesized derivatives were determined by spectrophotometric method established by Ellman [44] with little modifications [45]. A mixture consisting of 60 µL phosphate buffer (KH_2PO_4/ KOH, having pH 7.7), a 10 µL test compounds (end concentration of DMSO was 1%) and 10 µL enzyme (0.015 unit/ well for AChE & BChE). The mixture was incubated at 37 °C for 10 min for preincubation. After this, 10 µL substrate (acetylthiocholine chloride or butyrylthiocholine chloride, 1 mM) were added to respective AChE or BChE reaction mixtures to start the reaction. Later, 10 µL of 0.5 mM DTNB was added as coloring reagent. After this incubation at 37 °C for 20 min was carried out and later on measurments were taken at 405 nm with plate reader (Bio-Tek ELx 800™, USA). All the experiments were carried out in triplicate and the results were calculated as percentage inhibition values. The compounds which exhibited above 50% inhibition against cholinesterases were further checked by 7–8 dilutions to get the IC_{50} values by GraphPad PRISM (USA).

2.6. Molecular docking studies

The crystal structures of AChE (1EVE) [34] and BChE (4BDS) [35] were taken from Protein databank. The structures were prepared by MOE builder tool [46]. Afterwards the energy minimization and protonation of downloaded proteins was carried out by MOE [46]. The structures of compounds were prepared by MOE builder tool and minimization was done using MMFF94x forcefield [47]. LeadIT (BioSolveIT GmbH, Germany) [48] was used to perform the docking studies of prepared ligands inside the respective receptors. After docking studies, the HYDE assessment was used to inspect the possible interactions of ligands with proteins [49]. Visualization was carried out by Discovery Studio Visualizer [50].

2.7. Cell viability assay

The cytotoxic potential of the test derivatives was investigated in human cervical adenocarcinoma cells HeLa by MTT based cell viability assay as described earlier [42,43]. Briefly, the cells were cultured (2.5×10^4/mL) in a volume of 90 µL in each well of a 96-well plate and kept in incubator at 37 °C in 5% CO_2. After an overnight incubation, the dosing of compounds (100 µM) was done and further incubated for overnight on the same conditions. The media was poured out and 10 µL MTT reagent was added to well to crystallize the viable cells. The plate was kept at 37 °C and 5% CO_2, for 4 hr followed by the addition of 100 µL of the reagent (1:1 solution of 50% isopropanol and 10% SDS in solution). The plate was further incubated for 30 min at RT and the OD was observed at 570 nm wavelength using microplate reader (Bio-Tek ELx 800™, Winooski, USA). The assay was carried out thrice and the results were calculated as percentage inhibition.

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Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bioorg.2019.103036.

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