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New cholinesterase inhibitors for Alzheimer's disease: structure activity relationship, kinetics and molecular docking studies of 1–Butanoyl–3–Arylthiourea derivatives

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Running Title: Arylthiourea derivatives as cholinesterase inhibitors

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Abstract

Highly progressive neurodegenerative disorder generally known as Alzheimer's disease (AD), is a type of dementia, which is very common in elderly. The most common symptoms may include loss of memory along with disturbed behavioral and cognitive functions. Until now, only 4 cholinesterase (ChE) inhibitors are approved by FDA for symptomatic treatment of AD. Aroyl thiourea derivatives are well known bioactive organic molecules containing carbonyl and thiocarbonyl functional groups. Here, total 14 different thiourea derivatives **(3a–3n)** were synthesized and characterized by NMR, FTIR and X–ray crystallographic techniques. The synthesized compounds displayed varying inhibition activities on both acetylcholineterase (AChE) and butyrylcholinesterase (BuChE) enzymes. Among all compounds, **3b** and **3e** were potent inhibitors of AChE (IC_{50} ± SEM = 8.92 ± 1.03 μM) and BuChE (IC_{50} ± SEM = 6.96 ± 0.961 μM) respectively. Enzyme kinetic studies showed that **3b** exhibited uncompetitive binding with AChE while **3e** demonstrated a mixed inhibition of BuChE. Molecular docking studies on AChE showed that **3b** got binding interaction with Trp86 and Tyr337 while **3e** showed binding affinity with Trp82 and His438 when docked with BuChE. The obtained results indicated that these thiourea derivatives could be considered as potential candidates to treat AD.

Keywords: Thiourea/ Acetylcholinesterase/ Butyrylcholinesterase/ Alzheimer's disease

1 Introduction

Among highly progressive neurodegenerative diseases, Alzheimer's disease (AD), that is a type of dementia frequently seen in elderly [1]. Every year, about 5 million people worldwide have been diagnosed with AD where it is expected that by 2050 this number might be raised remarkably to one among every eighty five individuals [2, 3]. As AD is closely associated with aging, it might have potential to alter mood and behavior that badly affect functional competences which may lead to the loss of cognitive and memory functions [4]. Likewise, AD exerts financial burden that further adds more suffering for patient's family [5]. In spite of modern scientific development, till now there is no effective therapy to alleviate AD symptoms [6]. AD patients are characterized by neurotransmitter imbalance at synaptic terminal due to the loss of cholinergic neurons associated with memory functions [7]. AD displays extracellular accumulation of insoluble amyloid β (Aβ) proteins, creating plaques and intracellular deposition

of neurofibrillary tangles (NFTs) resulting from hyperphosphorylated tau proteins [8]. These pathological signs trigger different neurotoxic pathways that result into synaptic malfunctioning and neuronal loss [9]. Moreover, hereditary factors, oxidative stress, mitochondrial dysfunction, as well as calcium and hormonal imbalance may also take part in AD progression. Numerous enzymes are involved in the pathological process of AD e.g. lipooxygenases, secretases, glycogen synthase kinase–3 (GSK3) and more specifically cholinesterases (ChEs). The inhibition of these enzymes could play a role in stagnation of AD [10, 11].

Cholinesterases including acetylcholinesterase (AChE) and butyrylcholinesterase (BuChE) are found in mammalian brain [12]. Both enzymes normalize the concentration of acetylcholine (Ach) in a variable capacity by deactivating neurotransmitter at synaptic cleft. These enzymes have equal potential to disintegrate Ach expeditiously with 10,000 molecules in each second [13]. The integrated course of signal transmission at neuromuscular juncture occurs particularly in nanosecond including Ach liberation, its diffusion across synaptic gap, interaction with nicotinic acetylcholine receptors (nAChRs) and lastly, hydrolysis by AChE that terminates Ach–mediated neurotransmission [14].

The crystal structure of these enzymes describes an active hydrophobic gorge that is deep-rooted (20Å) and receives Ach. This active site is further categorized into anionic and esteratic sub-sites [15]. The positively charged quaternary amine of Ach binds to anionic sub-site while its hydrolysis into choline and acetate occurs after binding with esteratic spot present on the surface of both enzymes [16]. The amino acid sequence in AChE and BuChE is similar up to 65% regardless of being encoded by distinct genetic material present on human chromosomes 7q22 and 3q26 correspondingly [17]. Furthermore, the deep–rooted gorge at the bottom of both enzymes accommodates catalytic triad which consists of three amino acids to interact with varied substrates. In acyl pocket of AChE, two amino acids Phe295 and Phe297 generally obstruct substrate interaction caused by aromatic ring that bulge out into gorge. In contrast, the amino acids in acyl pocket of BuChE are replaced by low molecular weight Valine (Val) and Leucine (Leu) thus providing more space and permit the binding of large molecules. Functional dissimilarity among AChE and BuChE is related to specific location and their ability to interact with neuroactive peptides [18]. AChE is primarily located in neurons and hydrolyzes 80% ACh, whereas, BuChE present in non-neuronal cells which is less substrate–specific and responsible for the residual activity [19].

Human AChE (*h*AChE) is comprised of a permanent core with 534 amino acids while its flexible C–terminal and N–terminal peptide chains are composed of variable 14, 26 or 40 and 60–66 amino acids respectively [20]. AChE mainly works at neuromuscular junction where it is involved in termination of impulse transmission at synaptic region through hydrolysis of Ach into choline and acetate moieties [21]. The role of BuChE in regulating Ach levels has generally been disregarded. Though, it is a viable sign that AChE and BuChE mutually contribute in controlling Ach levels that is critical in evolution and advancement of AD [22]. Furthermore, it is evident that BuChE protects AChE from natural toxins by distracting and reacting simultaneously with these molecules [23].

Latest efforts for an efficient treatment of AD is particularly related to improve cholinergic function, either by utilizing cholinergic receptor agonists or ChE inhibitors obtained from natural and synthetic source. Aroyl thiourea derivatives are well known bioactive organic molecules containing carbonyl and thiocarbonyl functional groups. The presence of sulfur and oxygen atoms inducts hard and soft donor sites for metal complexation and finds numerous applications like extraction of precious metals such as platinum and gold [24, 25]. Thiourea moiety contains different sites for hydrogen bonding, which has made them suitable as organo–catalyst. Moreover, these thiourea derivatives can serve as basic precursor for the synthesis of variety of heterocycles [26, 27]. Synthesis of aryl thiourea derivatives, their coordination chemistry and biological aspects have been described previously [24, 28-30]. Moreover, the role of thiourea derivatives in therapeutic, their utilization in polymer synthesis and corrosion resistance are also evident in previous reports [31, 32]. Moreover, metal containing bis–thiourea found applications in optoelectronics [33].

Here we report a new series of thiourea derivatives as cholinesterase inhibitors, which can be of great importance to treat AD with potent and selective inhibitors of AChE and BuChE.

2 Experimental methods

2.1 Materials

All chemicals, reagents and organic solvents were commercially purchased and utilized without further purification. Furthermore, the enzymes (AChE and BuChE) and their substrates acetylthiocholine chloride and butyrylthiocholine chloride were purchased from sigma–Aldrich Germany.

2.2 Chemistry

For melting point determination, a digital Gallenkamp (MPD.BM 3.5) instrument was employed. The ^{1}H NMR and ^{13}C NMR spectra were recorded with the help of Bruker (AM–300) set at 300 MHz frequency. FTIR studies of thiourea derivatives were conducted on FTS (3000 MX) spectrometer.

2.3 General synthesis of lead compound (1–butanoyl–3–mesitylthiourea (3a–n))

A novel series of thiourea derivatives (**3a–n**) was synthesized from 1–butanoyl isothiocyanate. The latter was produced by adding butanoyl chloride to a solution of potassium thiocyanate in presence of dry acetone for 30 min. After cooling, different aromatic substituted amines were incorporated to get various derivatives of 1–butanoyl–3–arylthiourea (see supplementary material).

2.4 Biochemical assay

2.4.1 AChE and BuChE inhibitory assay

A previously reported assay was followed to determine the inhibition potential of thiourea derivatives against AChE and BuChE enzymes [34]. Briefly, the test compound (10 µL) was mixed with assay buffer (60 µL) with pH 7.7 in a 96 well plate. A 10 µL from specified units of enzymes (AChE or BuChE) were added into the mixture. After 10 min of incubation at 37^{o}C the absorbance was measured at 405 nm and considered as pre–incubation reading. Lastly, a 10 µL of specific substrate for each particular enzyme and 10 µL of coloring agent (2–Nitro benzoic acid) were added into a 96 well plate. The plate was kept in incubator for at least 20 min. Post–incubation absorbance was recorded at 405 nm employing Bio–Tek microplate reader (ELx–800 USA). The absorbance data was further used to estimate inhibitory concentration (IC_{50}) for each potential inhibitor (≥50%) after suitable dilutions.

2.4.2 Kinetics Study

The mode of inhibition of test compounds **3b** and **3e** versus AChE and BuChE was determined through enzyme kinetic studies carried out at different concentrations of substrate (0, 0.25, 0.5, 1 and 1.5 mM) and inhibitor (0, 0.5, 1, 2 µM). The change in initial velocity of reaction was

calculated at each concentration with the help of double–reciprocal graph to obtain information about the type of inhibition.

2.5 In silico studies

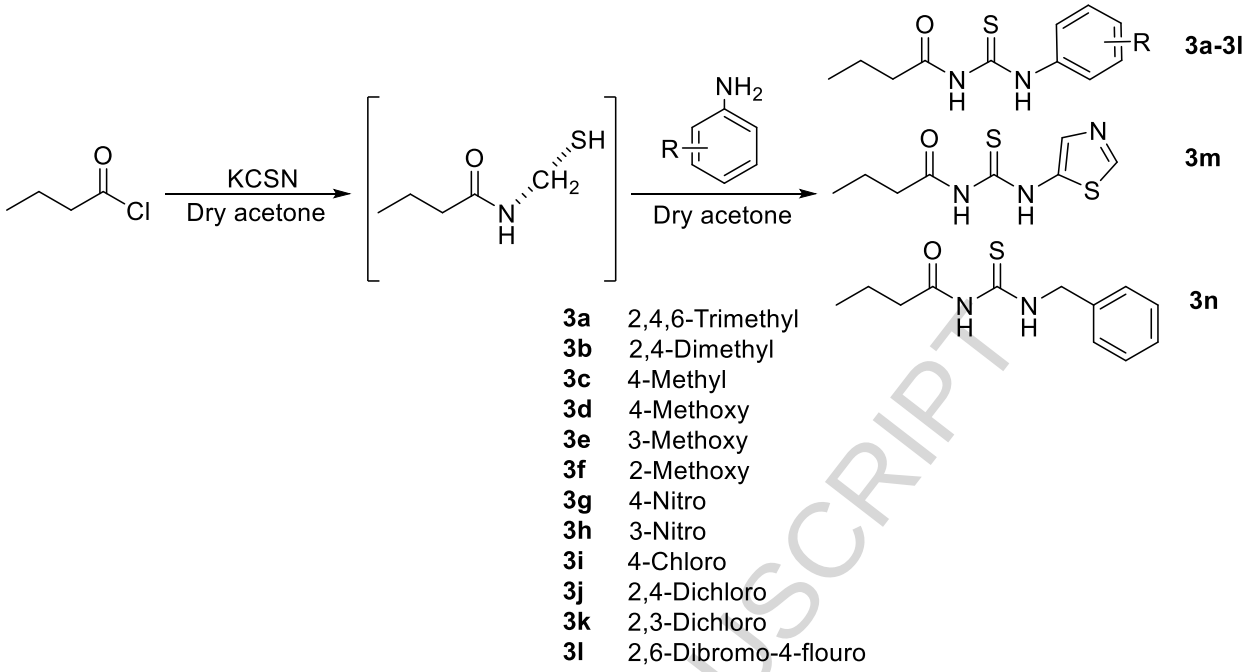
2.5.1 Molecular Docking

Initially 3D structures of selected compounds were drawn and protonated with the help of molecular operating environment (MOE) [35]. Energy minimization of selected compounds was performed with the help of MMFF94x force–field through adjustment of hydrogen atoms [36]. The crystal structure of *h*–AChE (PDB ID 4BDT) and BuChE (PDB ID 4BDS) were selected as prototype for docking studies. Active site on each receptor was carefully chosen around 6.5Å radius of co-crystallized ligand. The solvent handling and amino acid flip parameters were set as default. By making use of LeadIT program, the best 50 scoring docked posed were nominated for further analysis [37]. For visual analysis, the Discovery studio visualizer v4 was employed [38]. The mode of binding of docked poses was evaluated with the help of HYDE assessment tool of LeadIT [37]. Binding free energy (ΔG) for each pose was determined to explore the degree of interaction with receptor. The minimum energy value reflects high stability and affinity of molecules to bind with receptor.

3 Results and discussion

3.1 Synthesis scheme of thiourea derivatives

The aromatic ring in synthesized intermediate 1–butanoyl–3–arylthiourea was further utilized to prepare different derivatives by substituting various functional groups on aromatic ring have been shown in scheme 1 below.



Scheme 1 Synthetic pathway of novel thiourea derivatives

3.2 Characterization of thiourea derivatives

3.2.1 NMR analysis

The ^{1}H NMR spectrum showed two types of methyl (–CH_3) groups, i.e. –CH_3 on acyl group appeared at 2.35 ppm while another –CH_3 attached with aromatic ring was slightly shielded. In ^{1}H NMR, it was found that –NH proton when positioned among phenyl ring and C=S bond got maximum de–shielded effect and produced a single peak at 12 ppm (Figure 1(a)). This effect was due to intra-molecular hydrogen bonding. When –NH proton located in between C=O and C=S, it was appeared to be less de–shielded and gave signal in the range of 10–11 ppm [25, 39]. Furthermore, the presence of signals between 7 and 8 ppm was due to aromatic ring proton.

In ^{13}C NMR, a signal of carbonyl carbon was found in the range of 172–175 ppm while C=S showed a sharp peak at 180 ppm (Figure 1(b)). Aromatic carbon gave signal between 120–140 ppm while the signal of ipso carbon was weak in intensity because of NOE effect.

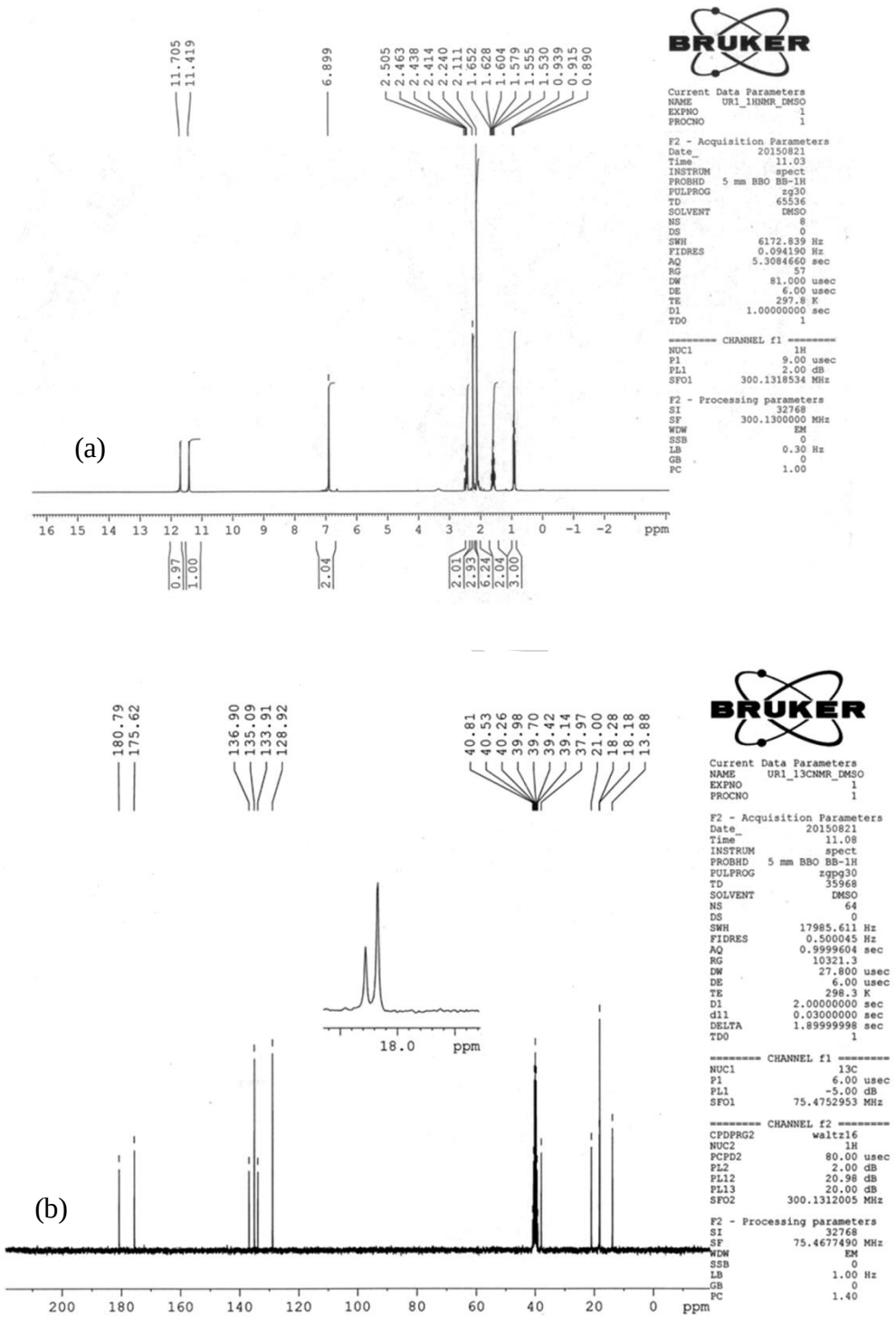


Figure 1 (a) ^{1}H NMR spectrum of 1–butanoyl–3–mesitylthiourea (b) ^{13}C NMR spectrum of compound 1–butanoyl–3–mesitylthiourea

3.2.2 FTIR analysis

In FTIR spectrum, a broad peak appeared at 3200 cm^{-1} was due to hydrogen bonding among –NH and oxygen atom of carbonyl group present in thiourea derivatives. The strong peak in functional group region (1600–1700 cm^{-1}) was due to the presence of carbonyl group while broad peak at 3000 cm^{-1} was due to Ar–H stretching vibration. The presence of C=S bond was confirmed with sharp and highly intense peaks in the region of 1050 cm^{-1} and 1250 cm^{-1} (Figure 2).

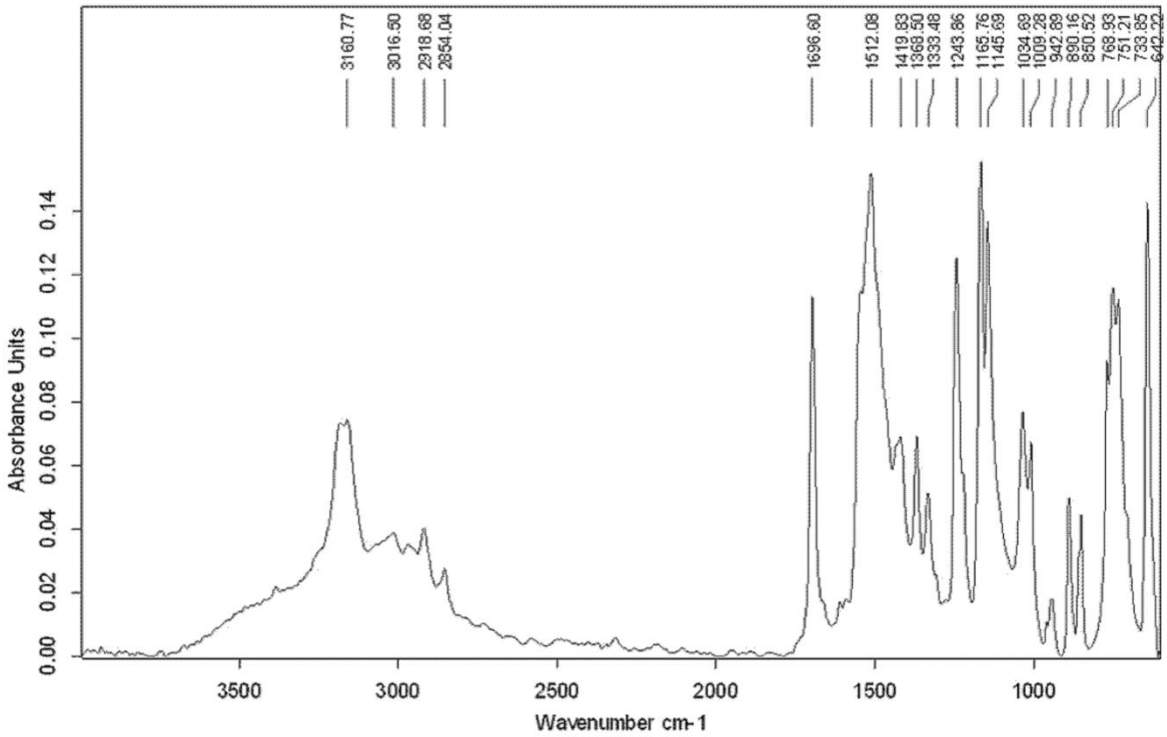


Figure 2 FTIR spectra of synthesized thiourea derivative 1–butanoyl–3–mesitylthiourea

3.2.3 X-ray crystallography

The crystal structure of intermediate 1–butanoyl–3–arylthiourea displayed monoclinic system with vector length a = 10.0387(4) Å, b = 15.3739(5) Å and c = 19.3798(8) Å and bond angles $\alpha = 90^{o}$, $\beta = 97.442(3)^{o}$ and $\gamma = 90^{o}$ with Z value equals to 8. There are two molecules in the asymmetric unit (Figure. 3 (a)), which possesses very similar geometries. There were only minor differences in the torsion angles of the mesityl ring and the propyl group. In the crystal,

the molecules were connected by N–H...O and N–H...S hydrogen bonds with the chain running along a-axis (Figure. 3 (b)).

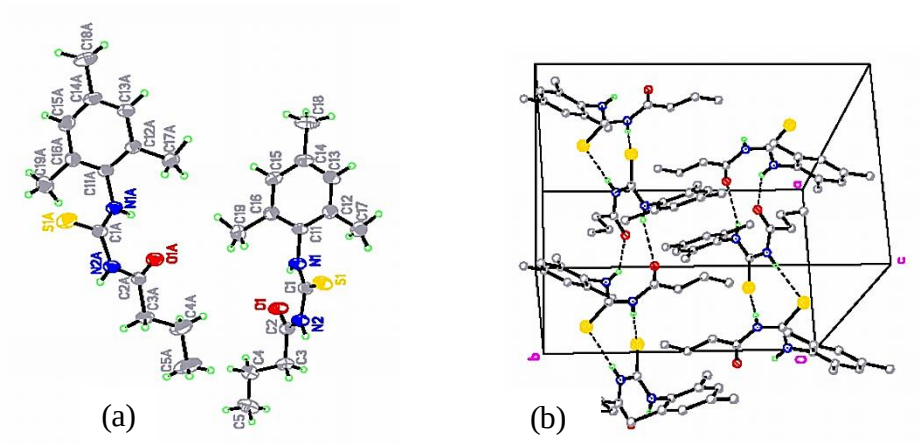


Figure 3 (a) Crystal structure of the 1–butanoyl–3–mesitylthiourea. Anisotropic displacement ellipsoids are drawn at the 50% probability level. (b) Crystal packing of 1–butanoyl–3–mesitylthiourea viewed along a–axis with intermolecular hydrogen bonds as dotted lines. H atoms not involved are omitted.

3.3 Biochemical assays

3.3.1 Enzyme inhibition studies

The enzymes (AChE and BuChE) inhibition studies of thiourea derivatives were performed by a previously reported method with minor variations [34]. After initial screening, the inhibitors (≥50 % inhibition) were further investigated to determine their IC_{50} values. All synthesized molecules exhibited inhibitory values in the range of 8–158 µM and 6–150 µM against AChE and BuChE respectively. The data was obtained in triplicate and IC_{50} of inhibitors was calculated by non–linear regression analysis.

The phenyl ring in thiourea derivatives with the exception of **3n** (1–butanoyl–3–benzylthiourea), was undergone controlled electrophilic and nucleophilic substitutions. In comparison to *para– (p–)* and *meta– (m–)* positions, an *ortho– (o–)* position was found to be more favorable to inhibit AChE. In that context compound **3b** (1–butanoyl–3–(2, 6–dimethylphenyl) thiourea), having a methyl substitution at *o–* position, was produced excellent inhibition against AChE with IC_{50} ± SEM = 8.92 ± 1.03 µM. The compound **3b** showed 2.5 fold greater inhibition in comparison to neostigmine i.e. IC_{50} ± SEM = 22.2 ± 3.21 µM used as standard inhibitor. In case of electron withdrawing substituents, a *p*–position was found to be more favorable as confirmed

by IC_{50} value in case of **3i** (1–butanoyl–3–(4–chlorophenyl)thiourea) i.e. IC_{50} ± SEM = 11.7 ± 3.78 µM. As for as BuChE is concerned, compound **3e** ((1–butanoyl–3–(3–methoxyphenyl) thiourea) having an electron donating group at *m*–position, was found to be an excellent inhibitor with IC_{50} ± SEM = 6.96 ± 0.961 µM. The SI between AChE and BuChE was calculated which showed high selectivity of **3b** against AChE with SI = 0.073 while compound **3e** displayed selectivity against BuChE with the highest SI (5.568) as compared with the series (see Table 1).

Table 1 The activities of potential inhibitors against AChE and BuChE enzymes

Code	AChE	BuChE	Selectivity index
	IC_{50} (µM) ± SEM		(SI)
3a	58.9 ± 3.56	43.9 ± 6.25	1.341
3b	**8.92 ± 1.03**	121 ± 27	0.073
3c	42.5 ± 3.56	36.6 ± 5.83	1.16
3d	49.1 ± 5.34	74.1 ± 8.09	0.662
3e	34.3 ± 8.82	**6.96 ± 0.961**	5.568
3f	96.6 ± 7.92	75.8 ± 13.6	1.274
3g	29.7 ± 4.25	68.1 ± 5.94	0.436
3h	26.4 ± 2.32	64.5 ± 4.12	0.409
3i	11.7 ± 3.78	16.2 ± 2.19	0.722
3j	42.7 ± 5.34	15.5 ± 2.77	2.754
3k	158.3 ± 15.7	150.1 ± 11.2	1.054
3l	40.3 ± 2.33	23.1 ± 2.09	1.744
3m	10.4 ± 1.858	36.5 ± 3.37	0.284
3n	132.5± 9.42	113.1 ± 14.5	1.171
Neostigmine	22.2 ± 3.21	49.6 ± 6.31	0.447

The initial screening of compounds was done at 800 µM against AChE and BuChE enzymes

Selectivity index = IC_{50}(*h*AChE)/ IC_{50} (*h*BuChE)

3.3.2 Kinetic models

The kinetic studies of the most potent inhibitors **3b** and **3e** against AChE and BuChE were performed at various concentration of substrate (0, 0.25, 0.5, 1 and 1.5 mM) and inhibitors (0, 0.5, 1.0, 2.0 µM). The initial velocity of reaction at each concentration was determined to observe the type of inhibition and presented as double–reciprocal plot of inhibition kinetics (Figure 4). The data showed that **3b** produced uncompetitive enzyme inhibition while compound **3e** expressed as mixed inhibitor of BuChE enzyme

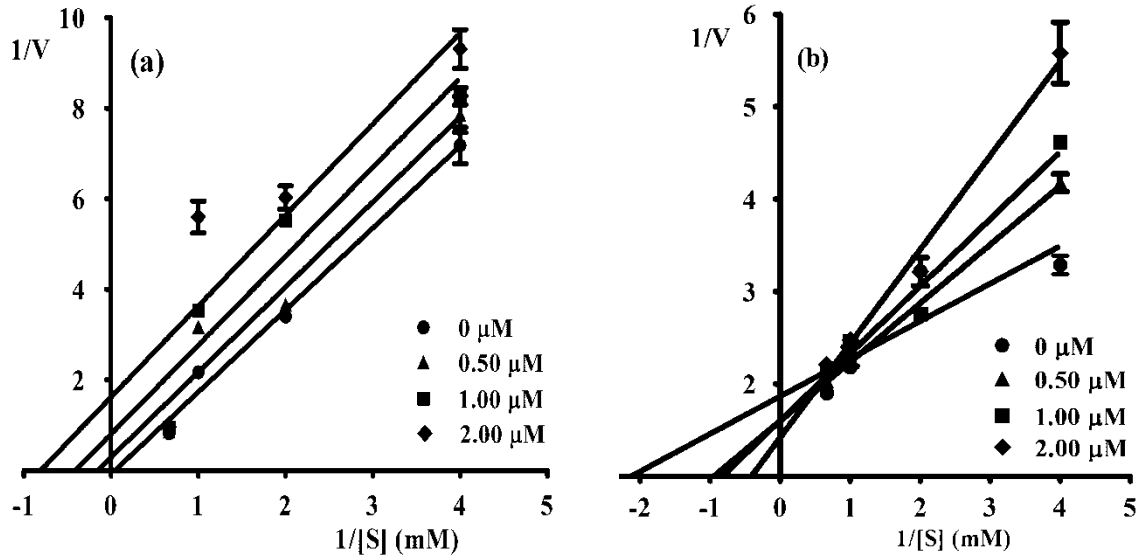


Figure 4 A Double–reciprocal graph shows inhibition kinetics of AChE and BuChE. (a) an uncompetitive inhibition of AChE after incubation with 3b (b) a mixed inhibition of BuChE by 3e

3.4 Molecular docking

Enzyme–inhibitor interactions were evaluated with the help of molecular docking where binding free energy (ΔG) was recorded at each possible position. The lowest ΔG value predicts the stability of molecule with the highest affinity for receptor interaction [35, 37-38].

The predicted interaction of **3b** with the active site of AChE revealed that it was entrenched in a remarkable group of amino acids with aromatic ring including Trp86 and Tyr337 (Figure 5). The compound **3b** was docked with AChE with its lowermost active site available whereas **3b was** laid parallel to Trp86 and Tyr337. A central aromatic ring of F–4 was fronting Trp86 and Tyr337 for creating π–π stacking. Hydrogen attached to N3 atom making hydrogen bonding with –OH group of His447 and another hydrogen bond also formed between hydrogen atom attached to N6 and the –OH group of Tyr337.

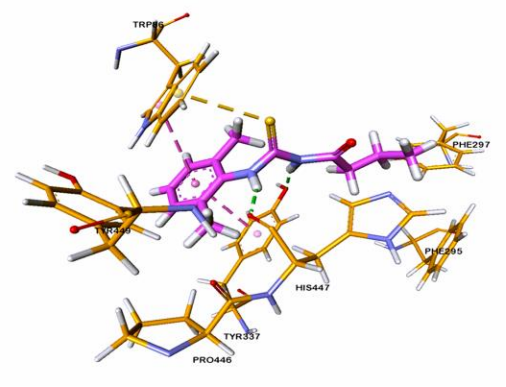


Figure 5 Possible binding mode of 3b (pink colored) and AChE (gold colored)

Alternatively, the type of binding interaction between inhibitor **3e** and BuChE was different from interaction among **3b** and AChE due to a difference of amino acid chain in active site of BuChE (Figure 6). The chain was composed of Ala199, Glu197, Gly115–117, His438, Leu286, Phe329, Phe398, Trp82 and Trp231 amino acid residues. The leading interactions were in accordance with previous reports [40]. For instance, aromatic π–π stacking occurred between Trp82 and hydrogen bonding with carbonyl group of His438. Molecular docking has also revealed some other important interactions such as aromatic π–π stacking between Trp82 as well as hydrogen bonding with Gly116 and Ala199 and carbonyl group of His438.

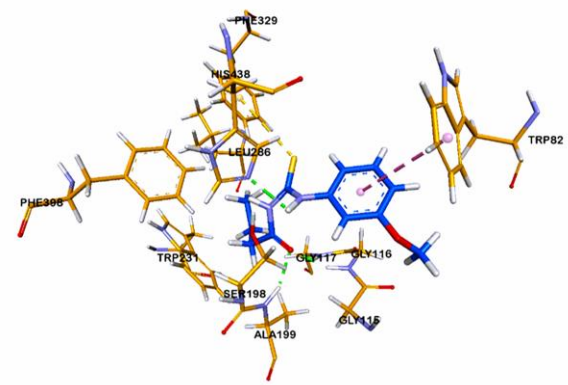


Figure 6 Expected binding mode of 3e (Blue colored) in active site of BuChE (gold coloured)

4 Conclusions

In summary, we have successfully synthesized new derivatives of thiourea and tested biochemically at cholinesterases (AChE and BuChE) to evaluate their inhibition potential. All these compounds proved to be cholinesterase inhibitors to a variable extent. Compound **3b** was established as the most potent and selective inhibitor of AChE (IC_{50} ± SEM = 8.92 ± 1.03 μM) while **3e** showed maximum inhibition (IC_{50} = 6.16 ± 0.98 μM) with highest SI value (5.568)

against BuChE. Furthermore, *in silico* studies confirmed the results obtained from *in vitro* experiments by explaining molecular interaction that may provide an insight into the enzyme–inhibitor binding interaction.

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Conflict of interest

The authors declare no competing financial interests.

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