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# 3,4-Dihydroquinazoline derivatives inhibit the activities of cholinesterase enzymes

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**Keywords:** Alzheimer′s disease; cholinesterase inhibitors; 3,4-dihydroquinazolines; molecular docking study; kinetic study

**Abstract:** A series of 3,4-dihydroquinazoline derivatives consisting of the selected compounds from our chemical library on the diversity basis and the new synthetic compounds were in vitro tested for their inhibitory activities for both acetylcholinesterase (AChE, from electric eel) and butyrylcholinesterase (BChE, from equine serum) enzymes. It was discovered that most of the compounds displayed weak AChE and strong BuChE inhibitory activities. In particular, compound **8b** and **8d** were the most active compounds in the series against BChE with $IC_{50}$ values of 45 nM and 62 nM, as well as 146- and 161-fold higher affinity to BChE, respectively. To understand the excellent activity of these compounds, molecular docking simulations were performed to get better insights into the mechanism of binding of 3,4-dihydroquinazoline derivatives. As expected, compound **8b** and **8d** bind to both catalytic anionic site (CAS) and peripheral site (PS) of BChE with better interaction energy values than AChE, in agreement with our experimental data. Furthermore, the non-competitive/mixed-type inhibitions of both compounds further confirmed their dual binding nature in kinetic studies.

Alzheimer's disease (AD) is a fatal progressive neurodegenerative disorder that has no cure to date. It is the cause of 60 to 70% of senile dementia.[1] Although the complete etiology of AD is still not clear, several pathological events play an important role in the onset and progress of this disease such as amyloid-β (Aβ) oligomerization and plaques formation,[2] oxidative stress,[3] decreasing levels of acetylcholine (ACh),[4] and τ-protein aggregation.[5] Among these conditions, low levels of ACh appear to be a critical element in the development of cognitive and neurodegenerative disorders in AD patients.[6] In the synapses, acetylcholinesterase (AChE, EC 3.1.1.7) and butyrylcholinesterase (BChE, EC 3.1.1.8) are mainly responsible for the hydrolysis of ACh into choline and acetic acid, an essential process allowing for the control of the cholinergic transmission.[7-8] In a healthy brain, AChE predominates and BChE is considered to play a minor role in regulating ACh levels. On the other hand, it was found that BChE activity increases in CNS during the development of AD, while AChE activity decreases at the same time.[9-10] Therefore, targeting AChE and BChE may be one promising approach in treating AD. To date, several cholinesterase inhibitors such as tacrine (withdrawn),[11] donepezil,[12] rivastigmine[13] and galantamine[14] have been approved for the treatments of AD.[15] Although they offered some cognitive improvements in AD, several adverse drug reactions such as nausea, diarrhea, dizziness,

and vomiting have also been associated with cholinesterase inhibitors.[16] Therefore, new safer cholinesterase inhibitors with minimal adverse effects and/or multi-target activity are urgently warranted.[17-18]

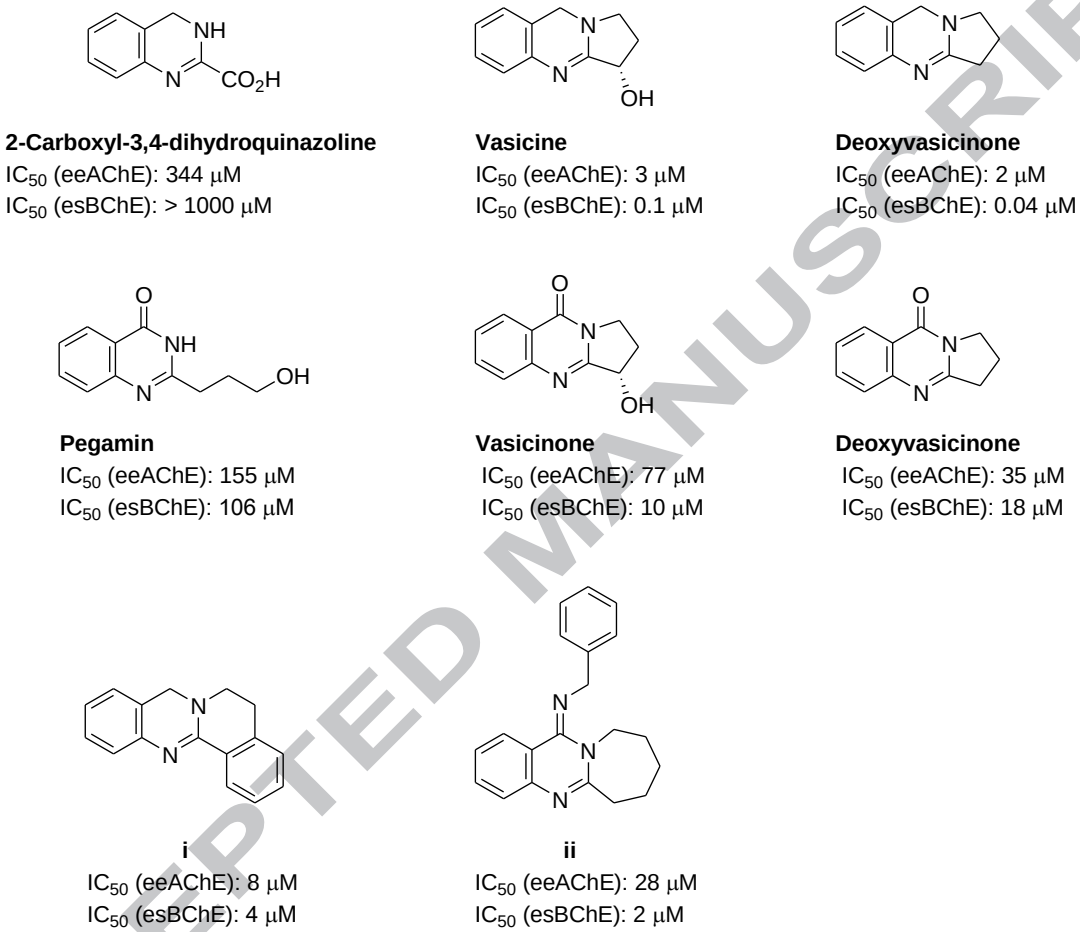
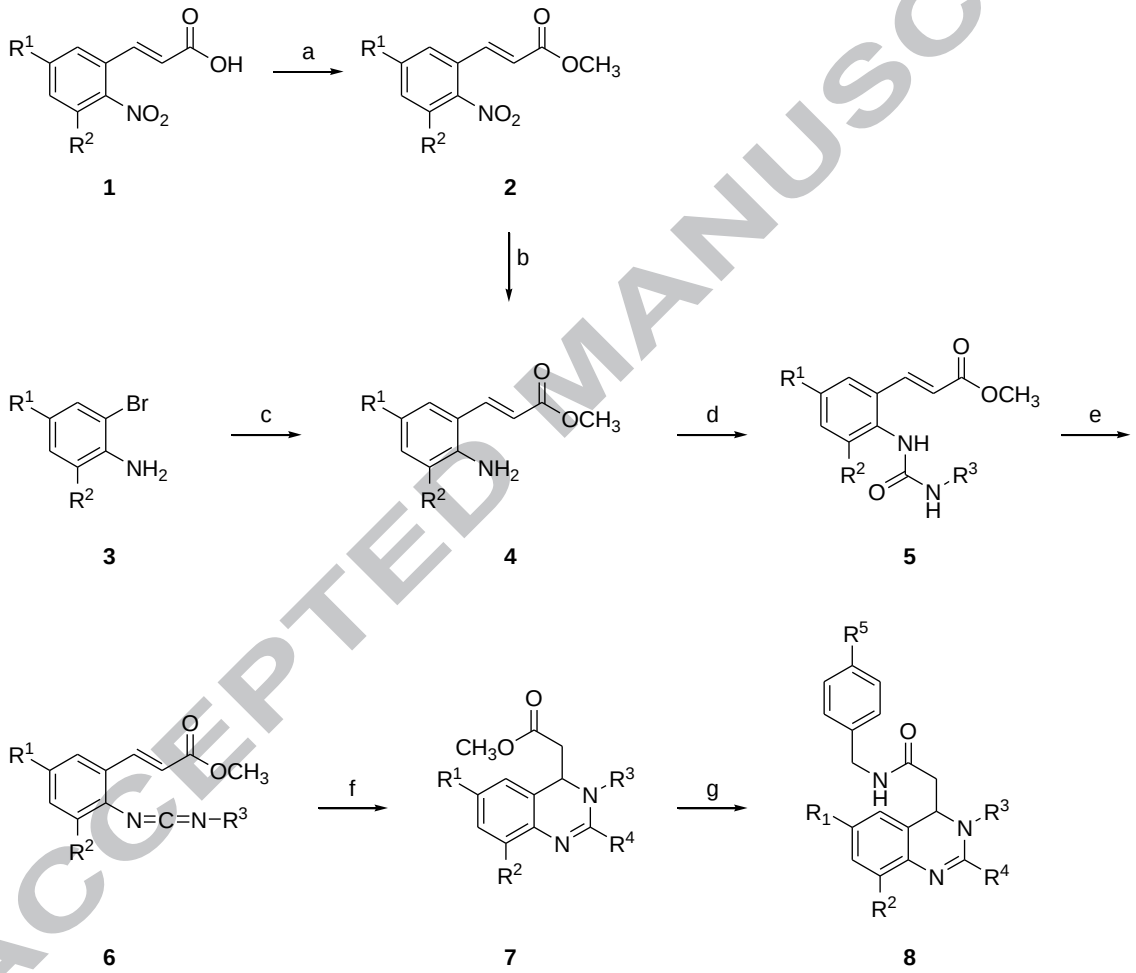



**Figure 1.** 3,4-Dihydroquinazoline derivatives showing anticholinesterase activities, wherein eeAChE is eel AChE and esBChE is equine serum BChE.

3,4-Dihydroquinazoline scaffold characterizes well-known alkaloids showing different types of biological activities such as inhibition of trypanothione reductase (TryR),[19] anticonvulsant[20] and antidepressant[21] activities, T-type calcium channel blocking activity,[22] and especially anticancer activity.[23] In addition, the moderate or strong inhibitory activities of 3,4-dihydroquinazoline derivatives towards AChE and BChE were also identified as shown in Figure 1.[24-25] Based on this anticholinesterase activity, a series of 3,4-dihydroquinazoline derivatives,

which were originally identified as potent T-type $Ca^{2+}$ channel blockers by our group,[22-23] were selected from our in-house compound library (for compound **7a**-7**b** and **8a-8f** in Table 1) or newly synthesized (for compound **8g-8l** in Table 1), and evaluated for their inhibitory activity towards the ChEs. To better understand the enzyme binding mode and inhibition mechanisms, molecular modeling and kinetic studies were performed, making the design of better ChE inhibitors possible.




**Scheme 1**. Reagents and conditions: (a) $H_2SO_4$, MeOH, 70 ℃, 12 h, >99%; (b) Zn, $NH_4Cl$, MeOH, reflux, 70 ℃, 12 h, 98%; (c) $Pd(OAc)_2$, methyl acrylate, (*o*-tol)$_3$P, $Et_3N$, ACN, reflux, 48 h, 23-70%; (d) $R^3$-NCO (commercially available or *in situ* prepared), toluene, rt to 100 ℃, 3 h, 40-70%; (e) $PPh_3.Br_2$, $Et_3N$, $CH_2Cl_2$, 0℃, 3 h, 40-55%; (f) amine ($R^4$-$NH_2$), toluene, rt, 1 h, 25-96%; (g) $R^5$-$BnNH_2$, TBD, 40℃, 12 h, 40-80%.

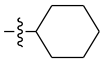
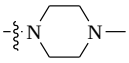
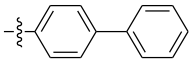
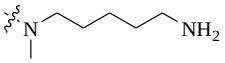
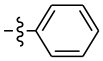
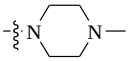
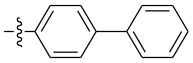
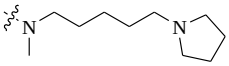
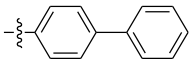
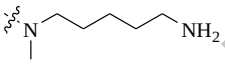
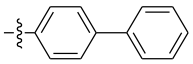
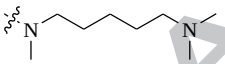
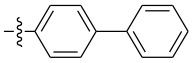
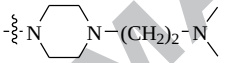
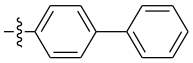
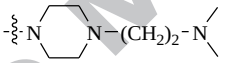
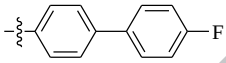
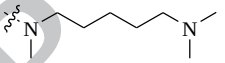
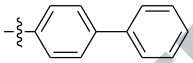
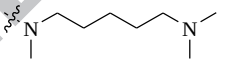
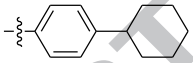
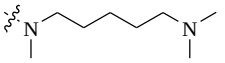
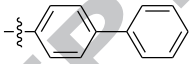
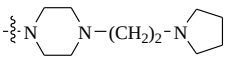
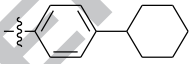
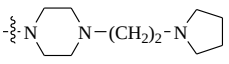
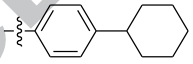
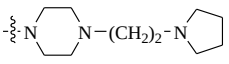
New F-substituted 3,4-dihydroquinazoline derivatives (**8g-8l**) were prepared using Heck reaction and the procedure described previously by our group as shown in Scheme 1[22-23]: 2-

Bromoaniline (**3**) was coupled with methyl acrylate under Heck reaction condition [$Pd(OAc)_2$, (*o*-tol)$_3$P, $Et_3N$] to provide the intermediate **4** in 23-70% optimized yields, which was coupled with isocyanate ($R^3$-NCO: commercially available or *in situ* prepared from the corresponding carboxylic acid $R^3$-$CO_2H$ via Curtius rearrangement) to provide a urea compound **5**. The dehydration of **5** with $Ph_3P{\cdot}Br_2$ and $Et_3N$ provided a carbodiimido compound **6**. Subsequently, the coupling reaction of **6** with amine compound ($R^4$-$NH_2$) afforded the 3,4-dihydroquinazoline ester compound **7** as a racemic mixture. The treatment of **7** with $R^5$-benzylamine and 1,5,7-triazabicyclo[4.4.0]dec-5-ene (TBD) as a catalyst under solvent-free condition afforded the final amide compound (**8**).

The spectrophotometric Ellman's test[26] for the *in vitro* inhibition assay of AChE from electric eel and BChE from equine serum was followed as previously described.[27] The $IC_{50}$ values for AChE/BChE inhibitions are summarized in Table 1, together with those of donepezil used as reference compound. According to AChE inhibitory activity results, some of the compounds displayed moderate anti-AChE activity ($IC_{50}$ values of 1.2 to 8.0 μM). Among them, compound **8h** showed good activity ($IC_{50}$ = 1.2 μM) against AChE but much less than donepezil ($IC_{50}$ = 0.020 μM), a selective AChE inhibitor.[28] Interestingly, ester compound **7a-~~b~~** and amide compound **8a** containing short phenyl (or *c*-hexyl) ring and 4-methylpiperazine ring at $R^3$ and $R^4$ positions exhibited low activity ($IC_{50}$ = >10 μM). The noticeable structure-activity relationship could not be established for AChE inhibitions due to a variety of $IC_{50}$ values of compounds as shown in Table 1. In respect to BChE inhibition, most of compounds except **7a** and **8a** generally displayed remarkable selective behavior against BChE and exhibited broad $IC_{50}$ values ranging from 6.1 to 0.045 μM against BChE (Table 1). In particular, compound **8b** and **8d** provided the best $IC_{50}$ values of 0.045 and 0.062 μM, respectively, while showing $IC_{50}$ values of 6.6 and 10 μM for AChE inhibition, revealing 146- and 161-fold selectivity for BChE over AChE, respectively. Through a brief analysis and structural comparison, we found that the inhibitory activity against BChE depends mainly on the properties of the substituent at 2-position of 3,4-dihydroquinazoline scaffold, that are: the linker

length (**8a** vs. the other compounds) and the flexibility (**8g-8i**: 2-aminoethyl-piperazine ring vs. **8j-8l**: 1,5-pentanediamine chain) and the type of terminal amine (**8b** and **8d** vs. **8c**).

**Table 1**. Inhibitory activities of 3,4-dihydroquinazoline derivatives (**7** and **8**) against electric eel acetylcholinesterase (eeAChE) and equine serum butyrylcholinesterase (esBChE).[a]

| Entry | $R^1$ | $R^2$ | $R^3$ | $R^4$ | $R^5$ | eeAChE | esBChE |
|---|---|---|---|---|---|---|---|
| | | | | | | $IC_{50}$ (μM) | $IC_{50}$ (μM) |
| **7a** | H | H |  |  | - | ≥10 | >10 |
| **7b** | H | H |  |  | - | ≥10 | 0.14±0.01 |
| **8a** | H | H |  |  | H | >10 | >10 |
| **8b** | H | H |  |  | H | 6.6±0.8 | 0.045±0.004 |
| **8c** | H | H |  |  | H | 4.1±0.5 | 5.3±0.1 |
| **8d** [b] | H | H |  |  | H | ≥10 | 0.062±0.002 |
| **8e** | H | H |  |  | F | ≥10 | 2.6±0.2 |
| **8f** | H | H |  |  | $OCH_3$ | >10 | 6.1±0.4 |
| **8g** | H | H |  |  | F | 6.5±0.2 | 0.24±0.04 |
| **8h** | F | F |  |  | F | 1.2±0.4 | 0.38±0.02 |
| **8i** | H | H |  |  | F | 4.4±0.6 | 0.40±0.09 |
| **8j** | H | H |  |  | F | >10 | 1.47±0.47 |
| **8k** | H | H |  |  | F | 8.0±1.6 | 2.13±0.69 |
| **8l** | H | F |  |  | F | >10 | 2.04±0.53 |
| **Donepezil** [c] | | | | | | 0.020±0.002 [d] | 2.3±0.1 [d] |

[a] Values represent the mean of two/three independent experiments; [b] Tested as dihydrochloride salt; [c] Positive control for acetylcholinesterase inhibitory assay; [d] Data taken from reference [28].

With respect to the inhibitory activity against AChE/BChE inhibitions, it can be concluded that the different activities of 3,4-dihydroquinazoline compounds against AChE and BuChE may be resulted from the different hydrophobic gorges of active center in two enzymes. At the base of the gorge in AChE, the binding of the substrate is represented by two phenylalanine residues (Phe288,

and Phe290) whose aromatic residues protrude into the gorge. In BuChE, these residues are replaced by two smaller amino acid residues Leu286 and Val288 as shown in Figure 2.[29] This change of amino acid sequence creates a larger space within the deepest area of the gorge of BuChE.[30-31] Therefore, higher BuChE inhibitory activities of tested compounds may be related to the larger active site gorge of BuChE than that of AChE.[32]

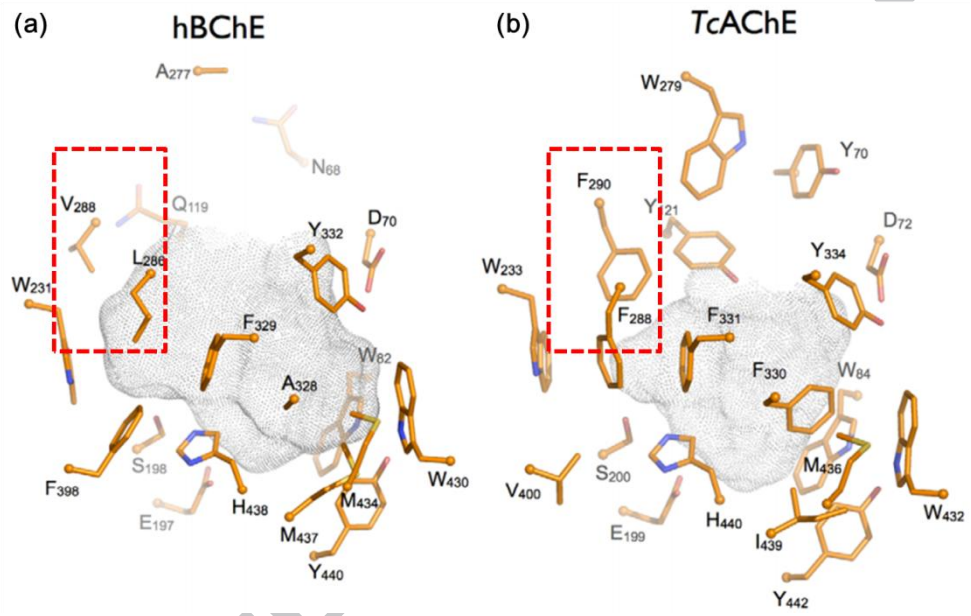



**Figure 2**. Active site gorges of (a) *H. sapiens* BChE (hBChE; PDB code 1P0I) and (b) *Torpedo californica* AChE (*Tc*AChE; PDB code 1EA5). The dotted red box indicates two important amino acids at the base of the gorge in two enzymes, respectively. This figure was taken from reference [29].

To detect and better understand the main interaction underlying the ChEs inhibition, molecular docking studies were performed on the two most active compound **8b** and **8d,** as well as donepezil as a reference, by using the recently crystallized structure of hAChE (PDB code: 4EY7)[31] and hBChE (PDB code: 4TPK)[33] based on the findings that the overall identity of 88 and 90% resulted for eeAChE and hAChE, and esBChE and hBChE, respectively. Moreover no significant difference was detected for the amino acid sequences within the active sites of enzymes from human and non-human sources.[34] The docking studies were performed using Molegro Virtual Docker (MVD) 2013.6.0.1 for Windows.[35] We identified one cavity with volume (171 Å$^3$ for 4EY7

and 399 $Å^3$ for 4TPK) containing ligand from the x-ray crystallized structure of each ChE by the same software, which can automatically detect cavities from protein surfaces. The putative active site of the enzyme was defined to include residues within a 15 Å radius to this cavity. The docking wizard of 2013.6.0.1 was used to dock compound **8b** and **8d**, which were used as two protonated forms and arbitrary (*R*)–enantiomer because both (*R*)– and (*S*)–enantiomer were found to exert similar biological activities against T-type $Ca^{2+}$ channel and cancer cells,[36] for both docking validation and data comparison in the presence of $H_2O$. The most stable docking pose was selected according to the best MolDock score conformation predicted by the MVD scoring function and inserted into Table 2. The MolDock score of **8b** and **8d** with hAChE was found to be -152.4 and -160.4 kcal/mol, respectively, and was lower than that (-202.7 kcal/mol) of donepezil as a selective hAChE inhibitor, suggesting a lower binding-affinity of these compounds at hAChE enzyme than donepezil (Table 2). In the case of hBChE, however, the MolDock score of **8b** and **8d** with hBChE was found to be -195.4 and -199.5 kcal/mol, respectively, two values higher than that (-133.8 kcal/mol) of donepezil. The calculated MolDock scores of the **8b** and **8d** suggested their potent inhibition and selectivity towards BChE over AChE, which was almost consistent with the experimental data (Table 2 and Table S1 in Supplementary content).

**Table 2**. The inhibitory concentration ($IC_{50}$), calculated MolDock score and selectivity index (S.I.) of the two most active compound **8b** and **8d** investigated against AChE and BChE

| Compound | AChE | | BChE | | Selectivity index [b] |
|---|---|---|---|---|---|
| | $IC_{50}$ (μM) | MolDock Score [a] (PDB 4EY7) | $IC_{50}$ (μM) | MolDock Score [a] (PDB 4TPK) | |
| **8b** | 6.6 | -152.4 | 0.045 | -195.4 | 146 |
| **8d** | ~10 | -160.4 | 0.062 | -199.5 | 161 |
| **Donepezil** [c] | 0.02 | -202.7 | 2.3 | -133.8 | 0.009 |

[a] Unit = kcal/mol; [b] Selectivity index (S.I.) is the BChE selectivity index defined as $IC_{50}$ eeAChE/$IC_{50}$ esBChE ratio; [c] Reference compound as a selective AChE inhibitor.

The proposed 2D binding mode of best pose for each compound in the active site of each

ChE was calculated using both MVD's Pose Viewer utility. The overall interactions between the amino acids in active site gorge of each ChE with the proposed pose for each compound were illustrated in Figure 3 and 4, respectively. The active site of AChE involves two sites: the peripheral anionic site (PAS), which corresponds to the peripheral site (PS) for BChE, and the catalytic site. This peripheral site (P-site) contains a number of aromatic amino acid residues as well as an aspartate residue that is able to interact with cationic substrates and guide them down the gorge to the catalytic triad. The catalytic site of AChE consists of two subsites: the esteratic site and the anionic site. In the esteratic site, a catalytic triad consisting of Glu334, His447 and Ser203 forms a planar array. In the anionic site, the Trp86 binds trimethylammonium group of ACh. In the overall catalytic site, the substrate is well positioned to be hydrolyzed into acetic acid and cholin.[37-38]

In the case of AChE, compound **8b** exhibited steric interactions with Tyr72 (PAS), Asp74 (PAS), Tyr124 (PAS: non-favorable), Trp286 (PAS), Phe295 (acyl binding pocket), Phe297 (acyl binding pocket), Phe338, and Tyr341 (PAS: non-favorable) in the active site of AChE. It also formed five hydrogen bonds between the nitrogen atom of 3,4-dihydroquinazoline ring with Tyr124 (PAS, 2.17 Å), between the nitrogen atom of 3,4-dihydroquinazoline ring with water (3.42 Å), and between the nitrogen and oxygen atom of amide and water953 (3.20 and 3.25 Å), between the oxygen atom of amide and water728 (3.27 Å) [Figure 3(a)]. Compound **8d** showed steric interactions with Tyr72 (PAS), Asp74 (PAS), Leu76, Tyr124 (PAS), Trp286 (PAS), Ser293 (non-favorable), Phe295 (acyl binding pocket: favorable & non-favorable), Phe297 (acyl binding pocket: non-favorable), Phe338, Tyr341 (PAS: non-favorable), and His447 (catalytic triad) in the active site of AChE. It also formed five hydrogen bonds between the oxygen atom of amide with Tyr341 (PAS, 3.29 Å) and water954 (2.60 Å), and between the nitrogen atom of 3,4-dihydroquinazoline ring with Phe295 (3.00 Å), between two nitrogen atoms of 3,4-dihydroquinazoline ring with water728 (2.84 and 2.96 Å) [Figure 3(b)].

With respect to the docking with BChE enzyme, compound **8b** exhibited steric interactions

with Asp70 (PS), Trp82 (CAS), Thr120 (CAS), Gly116 (oxyanion hole), Pro285, Met437, and Tyr332 (PS) in the active site of BChE. It also formed two hydrogen bonds (2.15 and 3.52 Å, respectively) between the nitrogen atom of 3,4-dihydroquinazoline ring with Thr120, and between the hydrogen atom of pyrrolidine ring with water736 [Figure 4(a)]. Compound **8d** exhibited the steric interactions with Asn68 (PS), Asn83, Trp82 (CAS), Thr120 (CAS), Pro285, Leu286 (acyl binding site), and His438 (catalytic triad) in the active site of enzyme. Furthermore, it formed two hydrogen bonds between the terminal hydrogen atom of side chain at 2-position of 3,4-dihydroquinazoline ring with His438 (catalytic triad, 2.61 Å), and between nitrogen atom of 3,4-dihydroquinazoline ring with water736 (3.20 Å) [Figure 4(b)]. The overall binding models showed that two compounds were able to interact with both catalytic anionic site (CAS) and peripheral site (PS) residues in active site of BChE.

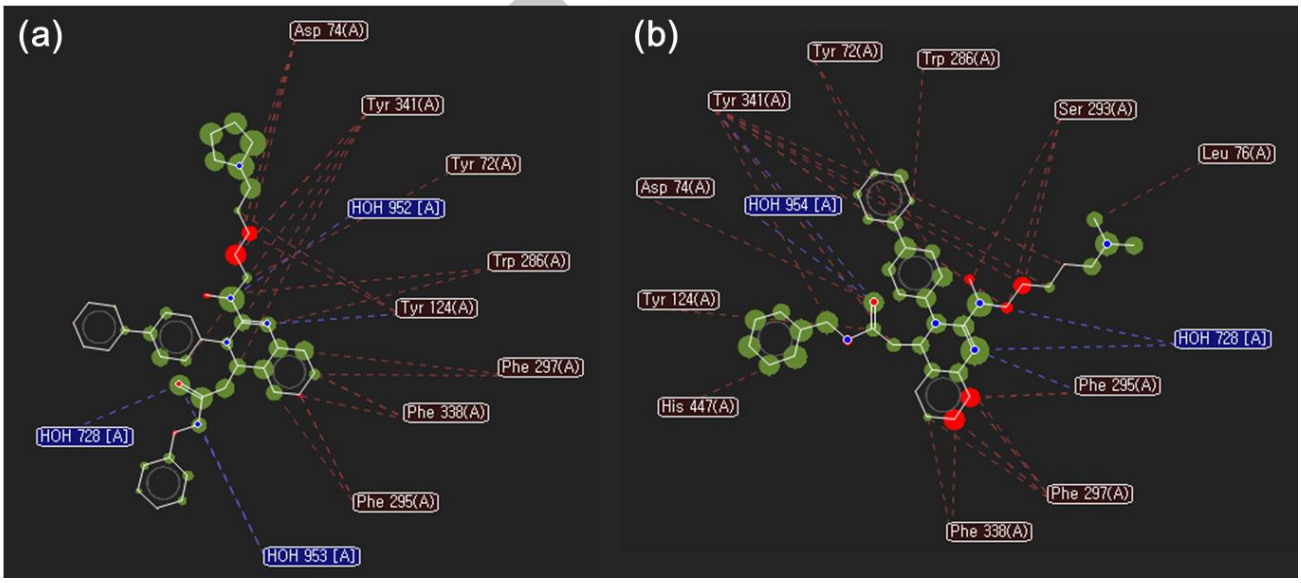



**Figure 3**. (a) and (b) are the proposed 2D binding mode of **8b** and **8d**, respectively, with hAChE (PDB ID: 4EY7). Hydrogen bonds and steric interactions are shown in blue and red dotted lines, respectively. The green and red sphere centered at each atom visualizes the strength of the favorable and non-favorable (clash) interactions, respectively, for this specific atom with amino acid residues in the active site.

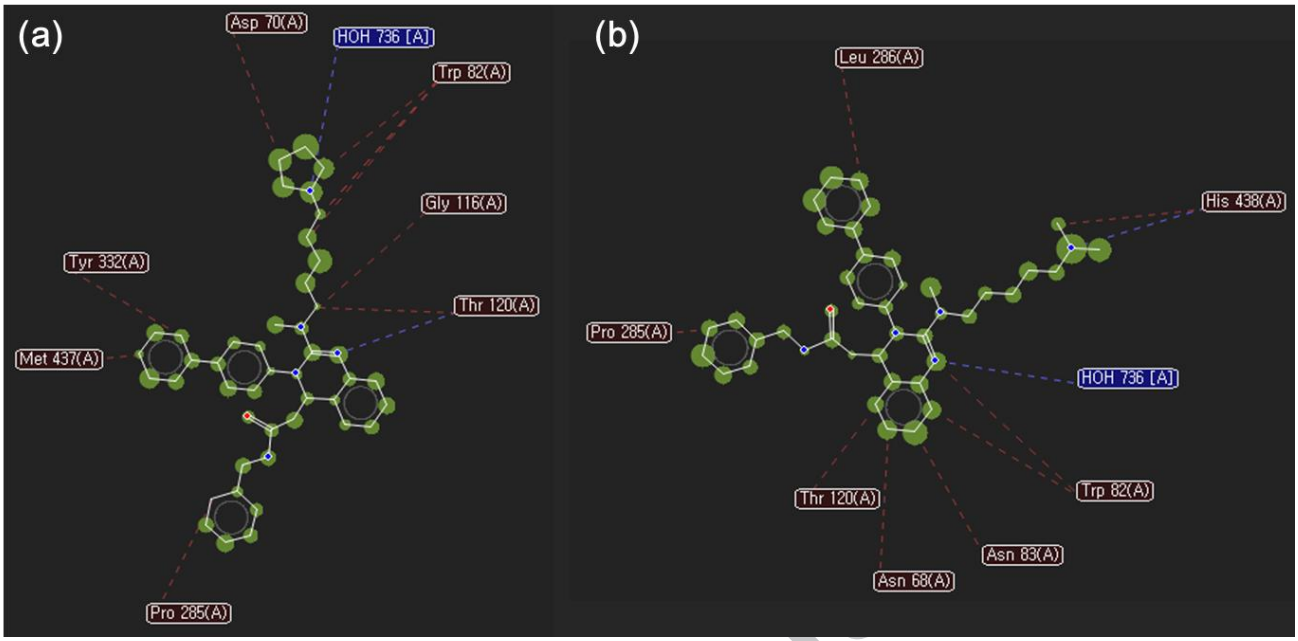



**Figure 4**. (a) and (b) are the proposed 2D binding mode for **8b** and **8d**, respectively, with hBChE (PDB ID: 4TPK). Hydrogen bonds and steric interactions are shown in blue and red dotted lines, respectively. The green sphere centered at each atom visualizes the strength of the favorable interactions for this specific atom with amino acid residues in the active site.

To gain further insight into the mechanism of action on BChE of the potent compound **8b** and **8d**, Lineweaver–Burk double reciprocal plots were generated (Figure 5).[39] The interception of the lines in the Lineweaver–Burk plot at the x-axis with both increased slope (decreased $V_{max}$) and intercepts (higher $K_m$) at increasing concentration of the inhibitor, which indicated a non-competitive/mixed-type inhibition with a $K_i$ value equal to 29±1 and 51±3 nM, respectively. Based on their kinetic studies, we concluded that both compounds might be able to interact with both the catalytic active site (CAS) and peripheral site (PS) of BChE, which was also consistent with the docking results.

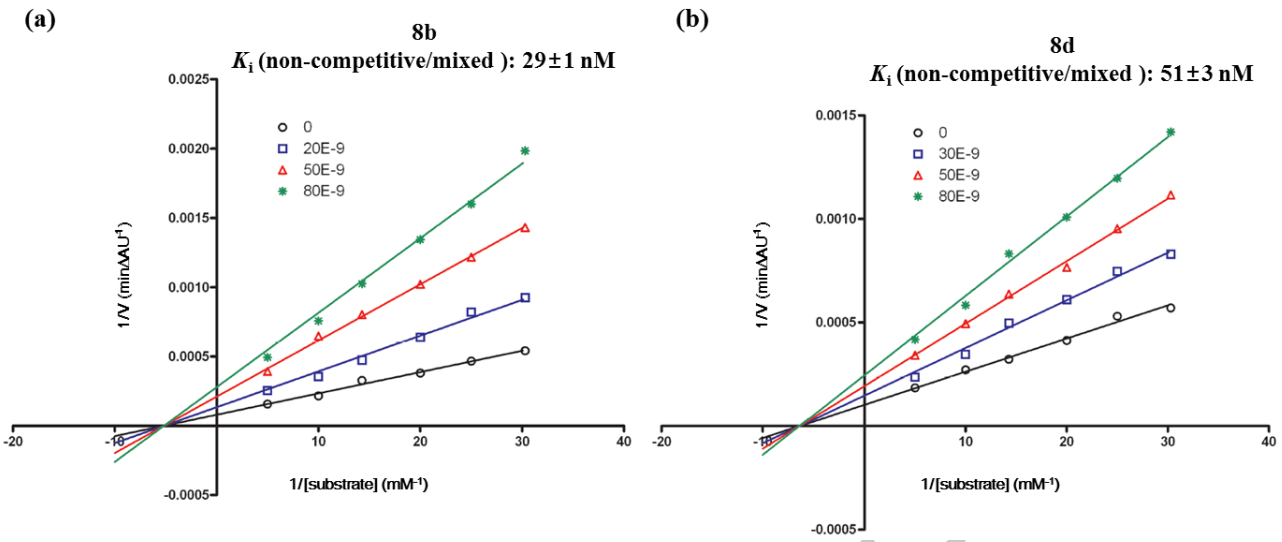



**Figure 5**. Lineweaver–Burk plot of inhibition kinetics of (a) **8b** and (b) **8d**: reciprocals of enzyme activity (esBChE) versus reciprocals of substrate concentration in the presence of different concentrations of inhibitor.

In conclusion, in this study a series of 3,4-dihydroquinazoline derivatives was selected from our compound library and evaluated for its ChE inhibitory activity. These compounds showed good levels of inhibition against BChE, while inhibiting AChE with less potency. In particular, compound **8b** and **8d** exhibited the highest BChE inhibition. Our molecular docking also revealed that both of compounds would bind both CAS and PS residues in the active site of BChE, which implicates that these compounds could act as dual binding site (DBS) inhibitors. In addition, kinetic analysis also indicated that both of compounds might be non-competitive/mixed-type inhibitors against BChE, in accordance to the docking data. Considering these overall results, our findings could be extended to design and develop a new selective BChE inhibitor containing 3,4-dihydroquinazoline scaffold as potential agents for the treatment of AD.

## Acknowledgments

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complexes was studied using both MVD's Pose Viewer utility.

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## Graphical Abstract

**3,4-Dihydroquinazoline derivatives inhibit the activities of cholinesterase enzymes**

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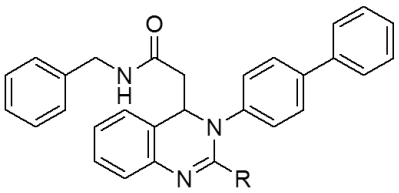


**8b:** R = -N(CH$_3$)(CH$_2$)$_5$N(CH$_2$)$_4$
IC$_{50}$ = 45 nM against BChE; S.I. = 146

**8d:** R = -N(CH$_3$)(CH$_2$)$_5$N(CH$_3$)$_2$·2HCl
IC$_{50}$ = 62 nM against BChE; S.I. = 161