Overcome the occurrence, symptoms and progression of AD [3,4]. Preserving acetylcholine (ACh) levels would be an effective way to preserve the function in the areas of the brain related to memory and learning. This neurological disorder is also associated with the presence of amyloid β-peptide (Aβ) deposits and neurofibrillary tangles in the brain [2]. The enhancement of cholinergic neurotransmission by preserving acetylcholine (ACh) levels would be an effective way to overcome the occurrence, symptoms and progression of AD [3,4]. Accordingly, the inhibition of acetylcholinesterase (AChE) which is responsible for the metabolic breakdown of ACh has been regarded as one of the most promising approaches [5]. Therefore, anti-AChE drugs such as donepezil, rivastigmine, galantamine and tacrine, were developed for treatment of AD [6]. Among the anti-AChE drugs, tacrine is associated with hepatotoxicity thus it is rarely used [7]. On the other hand, donepezil and rivastigmine which are commonly used in the early-to-moderate stages of AD often present adverse effects and are not completely effective [8]. However, clinical trial studies revealed that galantamine shows promising pharmacological profile and clinically relevant neuroprotective effects in AD [9]. Therefore, design of more effective anti-AChE drugs with low side effects and better pharmacokinetics properties is an urgent need in the field of AD pharmacotherapy.

Previous studies on the structure and function of AChE revealed that this enzyme has two binding sites; catalytic anionic site (CAS) and peripheral anionic site (PAS) [10]. It was proposed that PAS could promote the deposition and aggregation of Aβ in the brain [11]. Accordingly, the multi-binding inhibitors which can inhibit catalytic activity of AChE and perturb the self-assembly of Aβ could be more effective agents for the management of AD [12]. For example, the dual-binding mode of donepezil with AChE has been demonstrated by X-ray crystallography and docking studies. While the hydrophobic aromatic part (5,6-dimethoxyindan-1-one) of:

**Indolinone-based acetylcholinesterase inhibitors: Synthesis, biological activity and molecular modeling**

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**ARTICLE INFO**

**Abstract**

A series of indolinone-based compounds bearing benzylypyridinium moiety was designed as dual-binding inhibitors of acetylcholinesterase (AChE). The target compounds 3a–u were synthesized by condensation of oxindole and pyridin-4-carbalehyde, and subsequent N-benzylation. The anti-cholinesterase activity evaluation of synthesized compounds revealed that most of them had very potent inhibitory activity against AChE, superior to standard drug donepezil. Particularly, 2-chlorobenzyl derivative 3c was the most potent compound against AChE with IC50 value of 0.44 nM, being 32-fold more potent than donepezil. Also, most of compounds were more potent than standard drug donepezil against butyrylcholinesterase (BuChE). Docking study revealed that the hydrophobic aromatic part (indoline) of representative compound 3c binds to the PAS and the N-benzylpyridinium residue binds to the CAS of AChE.
donepezil binds to the PAS, the N-benzyl piperidine residue binds to the CAS of AChE [13,14]. Accordingly, it was found that the presence of functionalized amine group such as benzyl piperidine, benzylamino, phenylpiperazine, and anilino moieties contribute to inhibitor activity by interacting with the catalytic site of the AChE. On the other hand, a ligand that is rich in aromatic groups, may engage to favorable stacking interactions with PAS [15]. Numbers of aromatic and heteroaromatic rings were found in AChE inhibitors as PAS binding scaffolds [16,17]. Recently, we introduced benzofuranone-based AChE-inhibitors containing benzylpyridinium moiety (Fig. 1) [18a]. In continuation of our previous efforts in order to find new AChE inhibitors [18], in this work we describe indolinone-based compounds bearing benzylpyridinium moiety as dual-binding inhibitors of AChE.

2. Results and discussion

2.1. Chemistry

The oxindole derivatives 3a–u were synthesized via the route outlined in Scheme 1. In the first step, (E)-3-(pyridin-4-ylmethylene)indolin-2-one (2) were synthesized using oxindole (1) and pyridin-4-carboxaldehyde in the presence of p-toluenesulfonic acid (PTSA) as a catalyst. Several acid catalysts and solvents were screened for this reaction but the best results were obtained in the presence of PTSA in refluxing toluene. In this reaction, the (E)-isomer was the major product which further crystallized from acetonitrile to obtain the pure (E)-2. The chemical shift of the vinylic proton could be used to assign the configuration of product. In the (E)-geometry of compound 2, the vinylic proton would be shifted downfield due to deshielding effect of carbonyl group [19]. According to the literature reports, the vinylic hydrogen in (E)-isomers is appeared at 7.6–8.0 ppm [20]. The target compounds 3a–u were easily prepared by the reaction of proper benzyl bromide or chloride with compound (E)-2. Accordingly, the reaction mixture was stirred in dry acetonitrile without catalyst, at 60–70 °C for 6–24 h. On cooling, the product was precipitated as a solid which was separated, washed with diethyl ether or n-hexane and recrystallized from ethanol—water. The 1H NMR data of final compounds 3 revealed that the (E)-geometry of compounds have been preserved based on the downfield chemical shifts of vinylic proton (δ > 7.9 ppm).

The IC50 values of test compounds against AChE revealed that compounds 3b, 3c, 3e, 3g, 3i–m showed very potent inhibitory activity (IC50 values = 0.44–12.8 nM) superior to standard drug donepezil. Among them, 2-chlorobenzyl derivative 3c was the most potent compound against AChE, with IC50 value of 0.44 nM. This compounds was about 32-fold more potent than donepezil. Moreover, 2-fluoro and 2-bromo analogs (compounds 3b and 3e, respectively) with IC50 values ≤1.46 nM showed high activity against AChE. The comparison of un-substituted compound 3a with ortho- or meta-substituted analogous 3b–m demonstrated that introduction of halo, methyl and methoxy group at 2- or 3-position of N-benzyl pendent residue significantly improved the anti-AChE activity. The 2-chloro substituent had the most impact on the AChE inhibition of designed compounds. In contrast, introduction of different substituents on the para-position of benzyl group diminished the inhibitory activity against AChE (3n–r vs. 3a). As shown by compound 3r, the 4-nitro group more significantly decreased the activity. Interestingly, the insertion of second chlorine atom on ortho or meta positions of 4-chlorobenzyl derivative 3o resulted in more potent compounds 3s and 3t. While, in the case of 2- or 3-chlorobenzyl derivatives (compounds 3c or 3i, respectively), introduction of second halogen decreased the anti-AChE activity as observed with compounds 3g, 3h, 3s, and 3t. The displacement of halogen atom (Br, Cl and F) on benzyl group dramatically affects the anti-AChE activity. The order of activity was as follow: 2-halo > 3-halo > 4-halo.

The observed IC50 values of target compounds against BuChE revealed that all compounds with the exception of 3g and 3r were more potent than standard drug donepezil. The anti-BuChE activity of the most potent compound 3d was 6 times higher than that of donepezil. Most of substituted benzyl compounds were more potent than unsubstituted analog 3a against BuChE. These results showed that substitution on benzyl group had often positive effect on anti-BuChE activity. The highest activity was observed with 2-methyl analog. However, 3-fluoro, 4-methoxy and 4-nitro substituents decreased the inhibitory activity against BuChE.

As calculated in Table 1, the most active compound against AChE (compound 3c) showed very high selectivity for this enzyme (SI = 3113). Moreover, other potent compounds 3b and 3e had high selectivity for AChE (SI > 842).

2.2. Docking studies

In order to gain functional and structural insight into the binding mode of the compounds, molecular docking simulation was performed using Autodock Vina software. To confirm the
To get better insight to the concluded SAR, the best pose of both more and less active compounds (3c and 3r, respectively) were also overlaid and shown in Fig. 4. As illustrated in Fig. 4, the binding modes are similar in a way that two $\pi-\pi$ interactions were observed with Trp84 and Phe330. However, substitution on the para position of compound 3r was not tolerated due to the steric hindrance with the residues in the bottom of active site (CS). To get rid of the steric hindrance, the orientation of (4-nitrobenzyl)pyridinium fragment of compound 3r was changed, leading to weak $\pi-\pi$ interactions. These findings could explain the lower potency of 4-substituted benzylpyridinium compounds.

To gain further insight to the mechanism of action of these series of compounds on AChE, a kinetics study was performed on the most active compound 3c. Graphical analysis of reciprocal Lineweaver–Burk plot revealed that this compound has shown mixed-type inhibition on AChE (Fig. 5). The type of inhibition is in agreement with the proposed binding mode of these compounds in the active-site gorge of AChE. Plotting of the slopes vs. concentration of 3c gave an estimation of inhibition constant, $K_i$ of 1.14 nM (Fig. 6).

### 2.5. ADMET prediction

The ADMET (absorption, distribution, metabolism, excretion and toxicity) properties of the target compounds were predicted using admetSAR web-based application [21]. The predicted ADMET data were included in the Supplementary material. Based on the predicted values for BBB penetration, all compounds might be able to pass through blood brain barrier and penetrate into the CNS and therefore, are considered as CNS active compounds. Moreover, all the compounds may not show either acute toxicity according to the calculated LC$_{50}$ values nor mutagenic effect with respect to the AMES test data.
3. Conclusion

In conclusion, we have described indolinone-based compounds bearing benzylpyridinium moiety as dual-binding inhibitors of AChE. The target compounds 3a–u were synthesized by condensation of oxindole (1) and pyridin-4-carbalehyde to obtain (E)-3-(pyridin-4-ylmethylene)indolin-2-one (2), and subsequent N-benzylization with benzyl halides. The anti-cholinesterase activity evaluation of synthesized compounds revealed that most of them had very potent inhibitory activity against AChE, superior to standard drug donepezil. Particularly, 2-chlorobenzyl derivative 3c was the most potent compound against AChE, with IC50 value of 0.44 nM. This compound was about 32-fold more potent than donepezil. Kinetics study revealed that the hydrophobic aromatic part (indoline) of representative compound 3c binds to the PAS and the N-benzylpyridinium residue binds to the CAS of AChE. Kinetics study with compound 3c demonstrated that this compound has mixed-type inhibition on AChE. The favorable in silico ADMET properties of designed compounds with in vitro anti-cholinesterase potential of compounds make them as new lead compounds for further optimization in the field of AD pharmacotherapy.

4. Experimental protocols

4.1. Chemistry

All commercially available reagents were purchased from Merck AG or Aldrich and used without further purification. Melting points were measured on the Buchi Melting point B-540. FT-IR spectra were run on a Bruker, Equinox 55 spectrometer (KBr disks). Mass spectra of the products were obtained with an HP (Agilent technologies) 5937 Mass Selective Detector. 1H NMR spectra were recorded on a Bruker 500 MHz NMR instrument. Elemental analyses were carried out by a CHN-Rapid Heraeus elemental analyzer. The results of elemental analyses (C, H, N) were within ±0.4% of the calculated values.

4.1.1. Synthesis of (E)-3-(pyridin-4-ylmethylene)indolin-2-one (2)

A mixture of oxindole (1, 1 mmol), pyridine-4-carbaldehyde (1 mmol), and PTSA (1 mmol) was refluxed in dry toluene for 3 h. After completion of the reaction (monitored by TLC), the solvent was evaporated under vacuum. Then, sodium carbonate solution (30% (15 mL)) was added and extracted with chloroform (3 × 15 mL). The organic phase was dried (Na2SO4) and the solvent was evaporated under vacuum to obtain yellow solid. The solid was recrystallized from ethanol-water (1:1) to give pure compounds 3a–u.

4.1.2. General procedure for the preparation of compounds 3a–u

To a mixture of (E)-3-(pyridin-4-ylmethylene)indolin-2-one (2, 1 mmol) in dry acetonitrile (5 mL), proper benzyl bromide or chloride (1.5 mmol) was added and the mixture was stirred at 60–70 °C for 6–24 h. Then, the mixture was cooled and the precipitated solid was filtrated off and washed with diethyl ether or n-hexane. The product was recrystallized from ethanol-water (1:1) to give pure compounds 3a–u.

4.1.2.1. (E)-1-[(Benzyl)-4-((2-oxoindolin-3-ylidene)methyl)pyridinium bromide (3a)

Red solid; yield 92%; mp 224–226 °C; IR (KBr, cm−1): 3430, 1696, 1634; 1H NMR (500 MHz, DMSO-d6) δ:
5.97 (s, 2H, H-a), 11.00 (s, 1H, NH), 9.09 (d, J = 6.3 Hz, 2H, H-b), 8.29 (s, 1H, H-vinyl), 7.78–7.85 (m, 2H, Ar H3,4), 7.79 (br s, 2H, Ar H3,4), 7.36–7.31 (m, 3H, Ar H4,6,8–H), 7.07 (t, J = 7.8 Hz, 1H, H-5), 6.89 (d, J = 7.8 Hz, 1H, H-7), 6.21 (s, 2H, –CH2N+). Anal. Calc'd for C21H17BrN2O4 (348.27): C, 57.55; H, 3.68; N, 9.59. Found: C, 57.32; H, 3.32; N, 9.80.

4.12.7. (E)-1-(2-Chloro-6-Fluorobenzyl)-4-((2-oxoindolin-3-ylidenemethyl)pyridinium chloride (3g). Red solid; yield 92%; mp 261–263°C; IR (KBr, cm−1): 3360, 1695, 1632; 1H NMR (500 MHz, DMSO-d6) δ: 10.97 (s, 1H, NH), 9.05 (d, J = 5.7 Hz, 2H, H-a), 8.72 (d, J = 5.7 Hz, 2H, H-b), 8.00 (s, 1H, H-vinyl), 7.76 (d, J = 7.5 Hz, 1H, H-4), 7.3–7.61 (m, J = 7.7 Hz, 1H, Ar H3,4), 7.51 (d, J = 9.1 Hz, Ar H3), 7.44 (t, J = 7.9 Hz, 1H, Ar H4), 7.80 (s, 1H, H-vinyl), 7.04 (t, J = 7.5 Hz, 1H, H-5), 6.88 (d, J = 7.7 Hz, 1H, H-7), 6.42 (s, 2H, –CH2N+). MS m/z (%) 368 ([M + 2]+, 4), 366 (M1, 11), 222 (100), 221 (48), 194 (29), 184 (144), 162 (107), 24 (89), 51 (12). Anal. Calc'd for C21H16ClF6N4O (401.26): C, 62.86; H, 3.77; N, 6.98. Found: C, 62.58; H, 3.97; N, 7.26.

4.12.8. (E)-1-(2,6-Dichlorobenzyl)-4-((2-oxoindolin-3-ylidenemethyl)pyridinium chloride (3h). Red solid; yield 89%; mp 187–189°C; IR (KBr, cm−1): 3372, 1686, 1633; 1H NMR (500 MHz, DMSO-d6) δ: 10.92 (s, 1H, NH), 9.00 (d, J = 5.9 Hz, 2H, H-a), 8.72 (d, J = 5.9 Hz, 2H, H-b), 7.98 (s, 1H, H-vinyl), 7.77 (d, J = 7.7 Hz, 1H, H-4), 7.70 (d, J = 7.3 Hz, 2H, Ar H3,5), 7.66–7.63 (m, 1H, Ar H4), 7.39–7.35 (m, 1H, H-6), 7.06 (t, J = 7.7 Hz, 1H, H-5), 6.84 (d, J = 7.7 Hz, 1H, H-7), 6.13 (s, 2H, –CH2N+). Anal. Calc'd for C21H16Cl2N4O (417.72): C, 60.38; H, 3.62; N, 6.71. Found: C, 60.62; H, 3.85; N, 6.35.

4.12.9. (E)-1-(3-Chlorobenzyl)-4-((2-oxoindolin-3-ylidenemethyl)pyridinium bromide (3i). Red solid; yield 91%; mp 225–227°C; IR (KBr, cm−1): 3175, 1695, 1633; 1H NMR (500 MHz, DMSO-d6) δ: 10.87 (s, 1H, NH), 9.20 (d, J = 6.6 Hz, 2H, H-a), 8.72 (d, J = 6.6 Hz, 2H, H-b), 7.95 (s, 1H, H-vinyl), 7.75 (d, J = 7.5 Hz, 1H, H-4), 7.73 (s, 1H, Ar H2), 7.55–7.48 (m, 3H, Ar H3,5,6), 7.33 (t, J = 7.5 Hz, 1H, H-6), 7.05 (t, J = 7.5 Hz, 1H, H-5), 6.85 (d, J = 7.5 Hz, 1H, H-7), 7.31 (s, 1H, Ar H6), 7.22 (s, 2H, –CH2N+). 13C NMR (DMSO-d6, 125 MHz) δ: 166.3, 149.9, 144.5, 142.8, 133.6, 132.0, 131.6, 131.3, 130.1, 128.6, 128.4, 128.1, 123.2, 121.9, 121.8, 110.2, 60.8. Anal. Calc'd for C20H15Cl2N5O (358.25): C, 56.71; H, 3.02; N, 7.45. Found: C, 56.60; H, 3.01; N, 7.43.

4.12.10. (E)-1-(2-Bromomethyl)-4-((2-oxoindolin-3-ylidenemethyl)pyridinium chloride (3j). Red solid; yield 86%; mp 183–185°C; IR (KBr, cm−1): 3395, 1702, 1633; 1H NMR (500 MHz, DMSO-d6) δ: 10.97 (s, 1H, NH), 9.27 (d, J = 6.5 Hz, 2H, H-a), 8.73 (d, J = 6.5 Hz, 2H, H-b), 7.98 (s, 1H, H-vinyl), 7.90 (s, 1H, Ar H2), 7.74 (d, J = 7.5 Hz, 1H, H-4), 7.64–7.60 (m, 2H, Ar H3,5), 7.40 (t, J = 7.5 Hz, 1H, Ar H3), 7.32 (t, J = 7.5 Hz, 1H, H-6), 7.03 (t, J = 7.5 Hz, 1H, H-5), 6.85 (d, J = 7.5 Hz, 1H, H-7), 5.87 (s, 2H, –CH2N+). Anal. Calc'd for C20H15Br2N5O (427.72): C, 58.97; H, 3.77; N, 6.55. Found: C, 58.60; H, 3.54; N, 6.86.

4.12.11. (E)-1-(3-Methylbenzyl)-4-((2-oxoindolin-3-ylidenemethyl)pyridinium chloride (3k). Red solid; yield 85%; mp 225–227°C; IR (KBr, cm−1): 3419, 1706, 1633; 1H NMR (500 MHz, DMSO-d6) δ: 10.94 (s, 1H, NH), 9.22 (d, J = 6.5 Hz, 2H, H-a), 8.71 (d, J = 6.5 Hz, 2H, H-b), 7.97 (s, 1H, H-vinyl), 7.75 (d, J = 5.5 Hz, 1H, H-4), 7.39 (s, 1H, Ar H2), 7.35–7.32 (m, 3H, Ar H3,4,5), 7.24 (br s, 1H, H-6), 7.03 (t, J = 5.5 Hz, 1H, H-5), 6.86 (d, J = 5.3 Hz, 1H, H-7), 5.80 (s, 2H, –CH2N+), 3.21 (s, 3H, –CH3). 13C NMR (DMSO-d6, 125 MHz) δ: 166.3, 149.5, 144.0, 137.8, 136.3, 135.3, 134.7, 130.2, 128.8, 128.5, 127.8, 125.9, 123.1, 121.88, 121.81, 110.2, 62.8, 20.8. Anal. Calc'd
for C$_2$H$_4$SN$_2$O (362.85): C, 72.82; H, 5.28; N, 7.72. Found: C, 73.02; H, 5.54; N, 7.89.

4.1.2.12. (E)-1-(3-Methoxybenzyl)-4-((2-oxoindolin-3-ylidene)methyl)pyridinium chloride (3f). Red solid; yield 90%; mp 223–225 °C; IR (KBr, cm$^{-1}$): 3417, 1709, 1633; $^1$H NMR (500 MHz, DMSO-d$_6$): $\delta$: 11.02 (s, 1H, NH), 9.31 (d, J = 5.3 Hz, 2H, H-a), 8.72 (d, J = 5.3 Hz, 2H, H-b), 8.01 (s, 1H, H-vinyl), 7.76 (d, J = 7.0 Hz, 1H, H-4), 7.60 (s, 1H, Ar H$_e$), 7.38–7.26 (m, 3H, 1H, Ar H$_g$, 7.16 (t, J = 7.0 Hz, 1H, H-6), 7.02–6.99 (m, 1H, H-5), 6.87 (d, J = 7.0 Hz, 1H, H-7), 5.84 (s, 2H, –CH$_2$N), 3.76 (s, 3H, –OCH$_3$). $^{13}$C NMR (DMSO-d$_6$ 125 MHz): 166.3, 149.4, 143.8, 142.7, 135.3, 131.9, 130.9, 130.7, 130.3, 128.6, 127.7, 126.2, 123.2, 121.9, 121.8, 110.2, 62.4, 55.2. Anal. Calcd for C$_2$H$_9$ClN$_2$O$_2$ (378.85): C, 69.75; H, 5.05; N, 7.39. Found: C, 69.95; H, 5.40; N, 7.05.

4.1.2.18. (E)-1-(4-Nitrobenzyl)-4-((2-oxoindolin-3-ylidene)methyl)pyridinium chloride (3r). Red solid; yield 89%; mp 150–152 °C; IR (KBr, cm$^{-1}$): 3394, 1700, 1633; $^1$H NMR (500 MHz, DMSO-d$_6$): $\delta$: 10.93 (s, 1H, NH), 9.26 (d, J = 6.3 Hz, 2H, H-a), 8.77 (d, J = 6.3 Hz, 2H, H-b), 7.98 (s, 1H, H-vinyl), 7.72–7.83 (m, 4H, Ar H$_{2,5,6,8}$), 7.62 (d, J = 7.5 Hz, 1H, H-4), 7.33 (br s, 1H, H-6), 7.04 (br s, 1H, H-5), 6.86 (d, J = 7.5 Hz, 1H, H-7), 6.03 (s, 2H, –CH$_2$N$^+$). Anal. Calcd for C$_2$H$_9$ClN$_2$O (393.82) C, 64.05; H, 4.09; N, 10.67. Found: C, 64.31; H, 4.21; N, 10.39.

4.1.2.19. (E)-1-(3,4-Dichlorobenzoyl)-4-((2-oxoindolin-3-ylidene)methyl)pyridinium chloride (3s). Red solid; yield 90%; mp 235–237 °C; IR (KBr, cm$^{-1}$): 3387, 1711, 1634; $^1$H NMR (500 MHz, DMSO-d$_6$): $\delta$: 10.65 (s, 1H, NH), 8.70 (d, J = 5.4 Hz, 2H, H-a), 7.61 (d, J = 5.4 Hz, 2H, H-b), 7.54 (s, 2H, Ar H$_2$, H-vinyl), 7.34 (d, J = 7.7 Hz, 1H, H-7), 7.23–7.26 (m, 2H, H-4, H-6), 6.87 (d, J = 7.7 Hz, 1H, H-7), 6.82 (t, J = 7.7 Hz, 1H, H-5), 5.82 (s, 2H, –CH$_2$N$^+$). Anal. Calcd for C$_2$H$_9$Cl$_2$N$_2$O$_2$ (417.72) C, 60.38; H, 3.62; N, 6.71. Found: C, 60.18; H, 3.95; N, 6.49.

4.1.2.20. (E)-1-(2,4-Dichlorobenzoyl)-4-((2-oxoindolin-3-ylidene)methyl)pyridinium chloride (3t). Red solid; yield 94%; mp 171–173 °C; IR (KBr, cm$^{-1}$): 3421, 1701, 1633; $^1$H NMR (500 MHz, DMSO-d$_6$): $\delta$: 10.95 (s, 1H, NH), 9.10 (d, J = 5.2 Hz, 2H, H-a), 8.74 (d, J = 5.2 Hz, 2H, H-b), 7.99 (s, 1H, H-vinyl), 7.81–7.87 (m, 2H, H-4, Ar H$_b$), 7.63–7.59 (m, 2H, Ar H$_g$), 7.32–7.35 (m, 1H, H-6), 7.05 (t, J = 7.5 Hz, 1H, H-5), 6.87 (d, J = 6.8 Hz, 1H, H-7), 5.79 (s, 2H, –CH$_2$N$^+$). MS m/z (%) 385 [M + 4$^+$], 313 [M + 2$^+$], 191, 381 [M$^+$], 30, 222 (100), 194 (56), 166 (24), 159 (95), 144 (87), 89 (38), 63 (34), 51 (30). Anal. Calcd for C$_2$H$_9$Cl$_2$N$_2$O$_2$ (417.72) C, 60.38; H, 3.62; N, 6.71. Found: C, 60.18; H, 3.45; N, 6.91.

4.1.2.21. (E)-1-(4-Chloromethylbenzoyl)-4-((2-oxoindolin-3-ylidene)methyl)pyridinium chloride (3u). Red solid; yield 92%; mp 121–123 °C; IR (KBr, cm$^{-1}$): 3371, 1699, 1632; $^1$H NMR (500 MHz, DMSO-d$_6$): $\delta$: 10.95 (s, 1H, NH), 9.22 (d, J = 6.3 Hz, 2H, H-a), 8.69 (d, J = 6.3 Hz, 2H, H-b), 7.96 (s, 1H, H-vinyl), 7.75 (d, J = 7.5 Hz, 1H, H-4), 7.56 (d, J = 8.5 Hz, 2H, Ar H$_g$), 7.32 (t, J = 7.5 Hz, 1H, H-5), 7.04–6.99 (m, 3H, H-2, H-5, Ar H$_g$), 6.85 (d, J = 7.5 Hz, 1H, H-7), 5.77 (s, 2H, –CH$_2$N$^+$), 3.75 (s, 2H, –CH$_2$Cl). $^{13}$C NMR (DMSO-d$_6$, 125 MHz): 166.3, 159.9, 149.4, 143.8, 142.7, 135.2, 131.9, 130.7, 128.6, 128.5, 128.1, 121.3, 121.3, 121.7, 114.5, 110.1, 62.4, 55.2. Anal. Calcd for C$_2$H$_9$ClN$_2$O (397.30) C, 66.51; H, 4.57; N, 7.05. Found: C, 66.80; H, 4.25; N, 7.34.

4.2. In vitro inhibition studies on AChE and BuChE

The spectrophotometric method of Ellman [23] was used to assess the inhibitory activity of the target compounds toward AChE and BuChE. Acetylcholinesterase (AChE, E.C. 3.1.1.7, Type V-S, lyophilized powder, from electric eel, 100 unit), butyrylcholinesterase (BuChE, E.C. 3.1.1.8, from equine serum), and butyrylthiocholine iodide (BTC) were purchased from Sigma-Aldrich. 5,5'-Dithiobis-(2-nitrobenzoic acid) (DTNB), potassium dihydrogen phosphate, dipotassium hydrogen phosphate, potassium dihydrogen phosphate, sodium hydrogen carbonate, and acetyltihiocholine iodide were obtained from Fluka. The reaction took place in a final mixture of 3 mL phosphate buffer (0.1 M, pH = 8.0), 100 μL of DTNB.
solution, 100 μL of 2.5 unit/mL enzyme solution (AChE) and 100 μL of each tested compounds. The above mixture was pre-incubated for 10 min prior to adding 20 μL of substrate (acetylthiocholine iodide). Changes in absorbance were detected at 412 nm for 6 min and the IC₅₀ values were determined graphically from inhibition curves. Five different concentrations for each compound were tested to obtain the range of 20%–80% enzyme inhibition. All experiments were performed in triplicate at 25 °C on a UV Unico Double Beam Spectrophotometer. The same method was also taken for BuChE inhibition assay.

4.3. Kinetics study

To obtain estimates of the inhibition constant Kᵢ and inhibition model of compounds, reciprocal plots of 1/v vs. 1/[s] were constructed at different concentrations of the substrate acetylthiocholine (0.14–0.69 mM) by using Ellman’s method. The experiments were performed as same as enzyme inhibition assay in triplicate. The rate of enzyme activity was measured in the presence of different concentrations of inhibitor and without inhibitor for proposed concentrations of substrate. For each experiment after adding acetylthiocholine as substrate, progress curves were monitored at 420 nm for 2 min. Then the double reciprocal plots (1/v vs. 1/[s]) were constructed using the slopes of progress curves to obtain the type of inhibition. Slopes of the reciprocal plots were then plotted against the concentration of inhibitor, to evaluate Kᵢ data. Data analysis was performed with Microsoft Excel 2003.

4.4. Molecular modeling

The crystal structure of acetylcholinesterase complexed with E2020 (code ID: 1EVE) was obtained from the Protein Data Bank. Then, the water molecules and inhibitor were removed. Further preparation of protein was performed by Autodock Tools (ver 1.5.4) [23] using default parameters and finally saved as pdbqt format. The 2D structures of ligands were generated using Maestro 17.4 [24] and then converted to 3D and pdbqt format by Openbabel (ver. 2.3.1) [24]. Docking studies were carried out using the Autodock Vina (ver. 1.1.1) program [25]. The search space was defined as a box with following parameters: size_x = 40, size_y = 40, size_z = 40 which centered on the geometrical center of co-crystallized ligand using these parameters: center_x = 2.023, center_y = 63.295, center_z = 67.062. The exhaustiveness was set to 80 and other parameters were left as default values. Finally, the most favorable conformations based on the free energy of binding were selected for analyzing the interactions between the AChE and inhibitor. All the 3D models are depicted using the Chimera 1.6 software [26].

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.ejmech.2014.01.017.

References