Synthesis, characterization, and molecular docking analysis of novel benzimidazole derivatives as cholinesterase inhibitors

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ABSTRACT

Two series of novel acetylcholinesterase (AChE) and butyrylcholinesterase (BChE) inhibitors containing benzimidazole core structure were synthesized by a four-step reaction pathway starting from 4-fluoro-3-nitrobenzoic acid as the basic compound. The structure of the novel benzimidazoles was characterized and confirmed by the elemental and mass spectral analyses as well as ^{1}H NMR spectroscopic data. Of the 34 novel synthesized compounds, three benzimidazoles revealed AChE inhibition with $IC_{50} < 10$ μM. The highest inhibitory activity ($IC_{50} = 5.12$ μM for AChE and $IC_{50} = 8.63$ μM for BChE) corresponds to the compound **5IIc** (*ethyl 1-(3-(1H-imidazol-1-yl)propyl)-2-(4-nitrophenyl)-1H-benzo[d]imidazole-5-carboxylate*). The relationship between lipophilicity and the chemical structures as well as their limited structure–activity relationship was discussed.

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1. Introduction

Alzheimer's Disease (AD), discovered by Alois Alzheimer in 1907, is described as a degenerative disease of the central nervous system (CNS) characterized especially by premature senile mental deterioration. Although the disease was once considered rare, it is now established as the leading cause of dementia and affects approximately 24% of people aged 85 years and above worldwide [1]. Despite all the major efforts, AD remains not curable although recent advances in drug therapy have challenged this view [2,3].

In the last decades, the treatment for AD has been based on the "*cholinergic hypothesis*," that it is associated with an impairment in cholinergic transmission [4]. This led to the suggestion that cholinesterase (ChE) inhibitors would probably stop a deficit in acetylcholine levels and help to reverse the memory impairments characteristic of the disease [5]. Four pharmaceutical drugs, namely donepenzil, rivastigmine, tacrine, and galantamine, are applied clinically as cholinesterase inhibitors. We were surprised that not much effort has been put into utilizing benzimidazoles in developing new AD drugs [6]. The benzimidazole nucleus is of significant importance in medicinal chemistry research due to high affinity toward a variety of enzymes and protein receptors [7,8]. Furthermore, since benzimidazole derivatives have been widely used in other areas such as anti-hypertensive [9], anti-viral [10], and anti-mycobacterial [11] agents, their pharmacokinetics are well understood. Therefore, they represent a good starting point to develop new drugs.

Based on the limited option of drugs available for the treatment of AD, we set our sights to use benzimidazole as lead compound to develop new drugs to combat AD. In this paper, we describe the synthesis and cholinesterase inhibition activity of 34 novel benzimidazole derivatives.

2. Results

The synthesis scheme is a four-step pathway leading to the formation of a variety of benzimidazole derivatives. Esterification of 4-fluoro-3-nitro benzoic acid in ethanol by reflux, with concentrated sulfuric acid as catalyst yielded the ethyl ester **1** in 75% yield. The ethyl benzoate **1** was then treated with 2-aminoethanol and N,N-Diisopropylethylamine (DIPEA) in dry dichloromethane at room temperature to yield amino compound **2**, which was reduced to the amine **3** using ammonium formate and 10% Pd/C. This phenylenediamine **3** was found to be stable at room temperature, unlike

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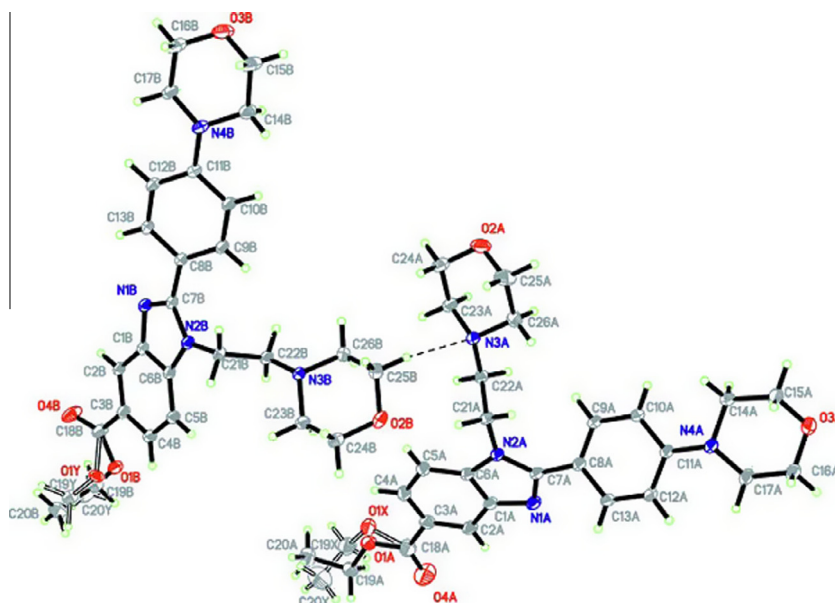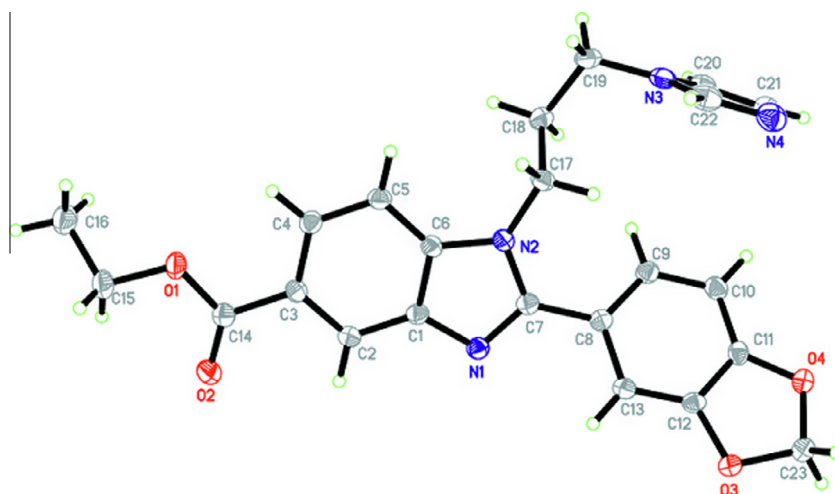
Fig. 1. ORTEP diagram of **5Ii**.

other phenylenediamine derivatives that were reported to decompose over a short period of time at room temperature [12]. The stability of the amino derivative **3** is possibly due to the substitution at 5-position which somewhat reduced the reactivity of the amino group. The structure of 3-amino ethyl benzoate **3** was confirmed by ¹H NMR and LC–MS analysis.

The phenylenediamine **3** was then refluxed with various substituted bisulfite adduct of aromatic aldehydes **4** in DMF overnight to afford benzimidazole derivatives **5** in moderate to good yields (43–90%). The structure and purity of the novel benzimidazoles were confirmed by chromatographic and spectroscopic analysis and further unambiguously ascertained by single X-ray crystallographic analysis (Figs. 1 and 2) [13,14]. Proton NMR assignment for **5Ig** (Fig. 3) and **5IIc** (Fig. 4) was shown as representation for other compounds in the **5I** series and **5II** series, respectively. Among the literature reports available for the synthesis of benzimidazoles by the reaction of phenylenediamine with acid chloride [15], aldehyde [16], and acid [17], we found that access into benzimidazole derivatives *via* this metabisulfite route is efficient, environmental friendly and afforded good to excellent yield of the benzimidazoles.

The formation of the novel benzimidazole derivatives is proposed and summarized in Scheme 1.

A total of 34 novel benzimidazole derivatives were synthesized and assayed for their cholinesterase inhibition potency by Ellman's method [18]. Results for their acetylcholinesterase (AChE) inhibition potentials are shown in Table 1. The five most potent compounds were then selected to undergo further testing for butyrylcholinesterase (BChE) inhibition activites. Table 2 showed their IC_{50} values for AChE and BChE inhibitions as well as the $\log P/\text{Clog} P$ values. Donepezil and rivastigmine were used as reference in the assays.

A simple structure–activity relationship analysis showed that the AChE inhibition potency is closely related to the substitution of the 2-position of the benzimidazole core. Comparing the two series of synthesized compounds (**5I** series and **5II** series), it can be concluded that compounds from the **5II** series in general gave better AChE inhibition activity. In our effort to improve the activity, further modification of the 4-substitution on the sodium bisulfite adduct was then carried out. We synthesized compounds with a wide range of substitution comprising electron-donating as well

**Fig. 2.** ORTEP diagram of **5III**.
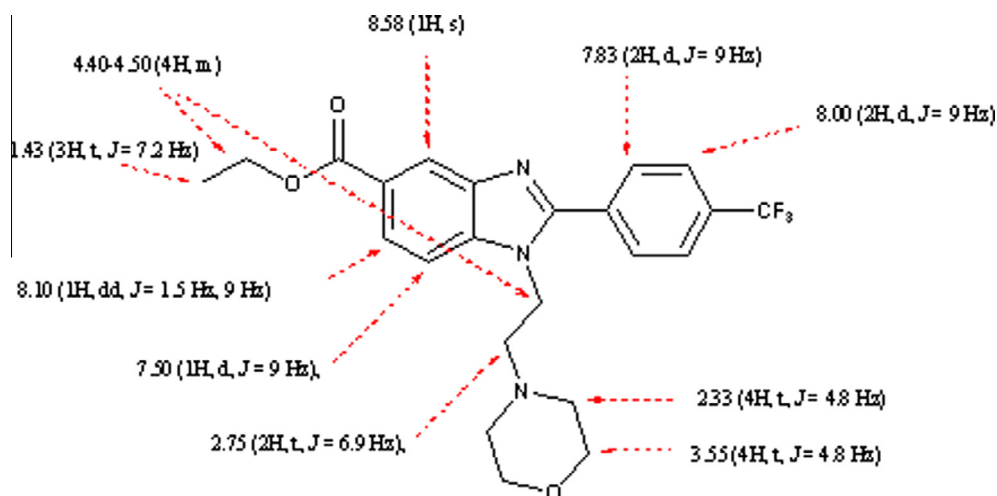
Fig. 3. Proton NMR assignment for **5Ig**.

as electron-withdrawing groups. Across the board, we observed that electron-withdrawing substituents at 4-position in the phenyl ring are important for good activities as shown by **5IIc** (–NO$_2$), **5IIg** (–CF$_3$) and **5IIn** (–COOH). All three of them, together with **5In** and **5IIo,** gave AChE inhibition activities with IC$_{50}$ values of less than 20 μM. The best inhibition was achieved by **5IIc** with IC$_{50}$ of 8.63 μM. These five compounds were selected for further testing against butyrylcholinesterase (BChE). All of them showed better inhibition activity against BChE compared to AChE as shown in Table 2 with the best IC$_{50}$ shown by **5IIc** (5.12 μM). Furthermore, their log$P$ values are also in good range for prediction of drug activity and tolerable toxicity. The slight preference for BChE inhibition could be due to the 4-position substitution on the phenyl ring of the benzimidazole derivatives. Recently published results have indicated that AChE prefers para- and meta-substitution than ortho-substitution, whereas BChE prefers para-substitution over

ortho- and meta-substitution. These results implied the steric differences in the active sites of both enzymes [19].

All five compounds were more potent than rivastigmine, however, relatively less potent on AChE as compared to Donepezil. Quite interestingly, these new compounds also have a similarly strong inhibition toward BChE as compared to AChE which implied that they are dual cholinergic inhibitors. This could be an added advantage as BChE may represent an important therapeutic target for AD.

Molecular docking analysis was performed to get better insight into mechanism of action of benzimidazoles. The active site of AChE is located at the base of ~20 Å deep and narrow gorge from the surface of the enzyme and is composed by two subsites: (1) catalytic esteratic site (CES) and (2) peripheral anionic site (PAS). The active site consists of Gln71-Tyr-Val-Asp-Thr-Leu76, Gly82-Thr-Glu84, Trp86-Asn-Pro88, Tyr121, Leu130, Tyr133, Glu199,
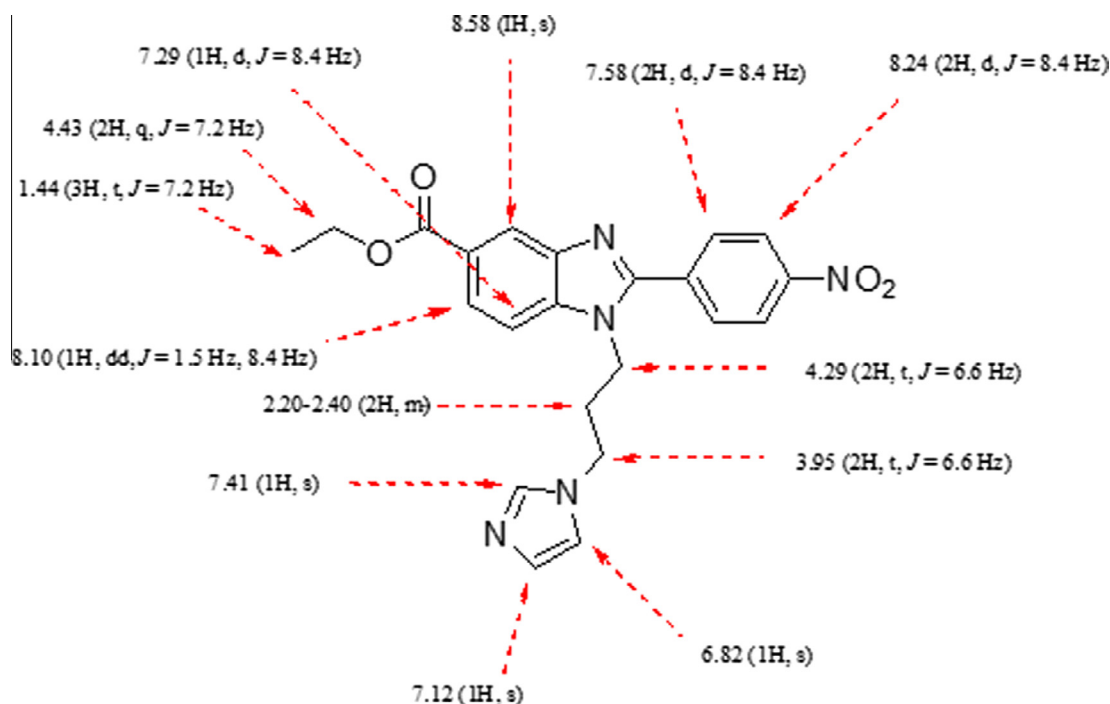
Fig. 4. Proton NMR assignment for **5IIc**.
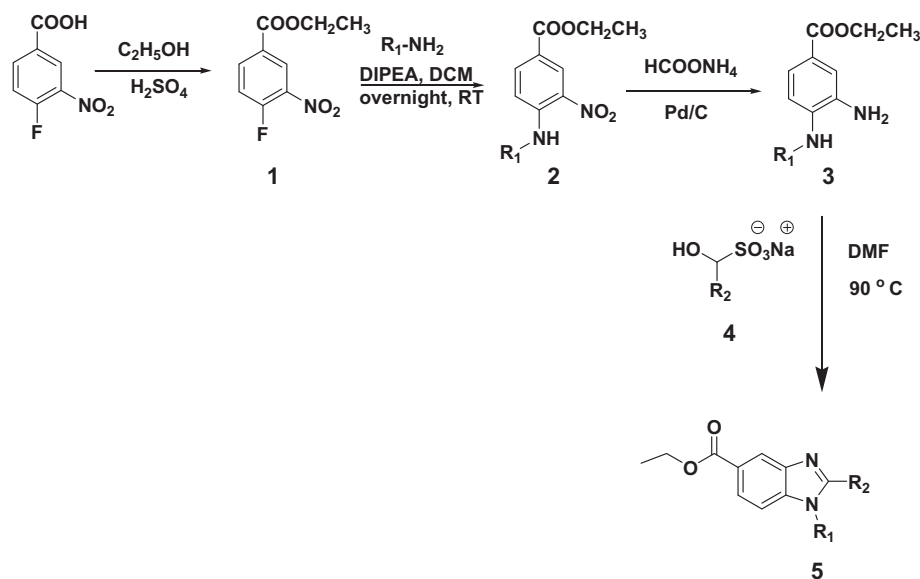
Scheme 1. Protocol for synthesis of titled compounds.

Table 1
Inhibition of AChE by the synthesized compounds.

Compound	R₁	R₂	AChE inhibition (%) at 10 μM
5Ia	4-Ethylmorpholine	Phenyl	18.54
5Ib	4-Ethylmorpholine	4-Bromophenyl	21.99
5Ic	4-Ethylmorpholine	4-Nitrophenyl	38.82
5Id	4-Ethylmorpholine	4-Hydroxy-3-methoxyphenyl	26.39
5Ie	4-Ethylmorpholine	4-Trifluoromethoxyphenyl	23.37
5If	4-Ethylmorpholine	4-Dimethylaminophenyl	14.23
5Ig	4-Ethylmorpholine	4-Trifluoromethylphenyl	29.74
5Ih	4-Ethylmorpholine	1-(4-(Piperidine-1-yl)phenyl)	15.17
5Ii	4-Ethylmorpholine	1-(4-(Morpholine-1-yl)phenyl)	22.15
5Ij	4-Ethylmorpholine	5-[(4-Fluorophenyl)-pyridine]	18.26
5Ik	4-Ethylmorpholine	4-Methoxyphenyl	15.88
5Il	4-Ethylmorpholine	Benzo[d][1,3]dioxole	27.84
5Im	4-Ethylmorpholine	1-(4-Methoxyphenyl)-4-oxo-3-phenylazetidine-2-carbaldehyde	16.08
5In	4-Ethylmorpholine	4-Carboxyphenyl	39.72
5Io	4-Ethylmorpholine	4-Hydroxyphenyl	29.31
5Ip	4-Ethylmorpholine	4-Chlorophenyl	19.12
5Iq	4-Ethylmorpholine	2,4-Dihydroxyphenyl	17.21
5IIa	N-propylimidazole	Phenyl	24.26
5IIb	N-propylimidazole	4-Bromophenyl	25.68
5IIc	N-propylimidazole	4-Nitrophenyl	55.73
5IId	N-propylimidazole	4-Hydroxy-3-methoxyphenyl	28.39
5IIe	N-propylimidazole	4-Trifluoromethoxyphenyl	26.26
5IIf	N-propylimidazole	4-Dimethylaminophenyl	20.77
5IIg	N-propylimidazole	4-Trifluoromethylphenyl	53.07
5IIh	N-propylimidazole	1-(4-(Piperidine-1-yl)phenyl)	19.78
5IIi	N-propylimidazole	1-(4-(Morpholine-1-yl)phenyl)	17.45
5IIj	N-propylimidazole	5-[(4-Fluorophenyl)-pyridine]	23.53
5IIk	N-propylimidazole	4-Methoxyphenyl	18.33
5IIl	N-propylimidazole	Benzo[d][1,3]dioxole	23.14
5IIm	N-propylimidazole	1-(4-Methoxyphenyl)-4-oxo-3-phenylazetidine-2-carbaldehyde	22.19
5IIn	N-propylimidazole	4-Carboxyphenyl	39.40
5IIo	N-propylimidazole	4-Hydroxyphenyl	58.65
5IIp	N-propylimidazole	4-Chlorophenyl	23.41
5IIq	N-propylimidazole	2,4-Dihydroxyphenyl	19.68
Rivastigmine	–	–	34.12
Donepezil	–	–	98.91

Ser200, Glu202-Ser-Ala204, Trp279, Trp286, Phe295, Phe297, Glu327, Phe330, Glu334, Tyr337-Phe338, Tyr341, Trp439, His447-Gly-Tyr449, Ile451. The mechanism of acetylcholine (ACh) hydrolysis involves trapping of ACh to PAS for better catalytic efficiency of the process followed by transfer of ACh to active site.

All the molecules were docked in the active site of AChE (pdb-1GQR, X-ray resolution = 2.20 Å). The receptor and the drug candidates were optimized before actual docking in Schrodinger Suite 2011 using standard procedure of the software. For simplicity, as a representative, we report the docking pose for the most active compound **5IIc** only.

Table 2
IC$_{50}$ values activities toward AChE and BChE for selected compounds and their log$P$ values.

Compounds	AChE inhibition IC$_{50}$ μM	BChE inhibition IC$_{50}$ μM	Selectivity Index (SI) for AChE	Lipophilicity log$P$/Clog$P$
5In	19.57	18.08	0.92	2.42/1.749
5IIc	8.63	5.12	0.59	2.50/4.030
5IIg	9.74	6.59	0.68	3.61/5.168
5IIn	16.38	11.44	0.70	2.25/4.092
5IIo	9.20	6.81	0.74	2.30/3.812
Rivastigmine	50.20	22.00	0.44	–
Donepezil	0.03	7.95	265	–

The docking analysis reveals that the benzimidazoles interact with receptor primarily due to hydrophobic and mild polar interactions. They are situated near to peripheral anionic site at the entrance of the gorge with prominent interactions with Tyr70, Asp276, Trp279, Asn280, Leu282, Ser286, and Gly335. It appears that similar to Donepezil, benzimidazoles also target the gorge connecting the active site with the surface of the enzyme, the benzimidazole moiety interacting with Trp279, and the phenyl moiety at 2-position is responsible for interaction with Ser286 and Gly335. The interaction between phenyl moiety (at 2-position) and the amino acids ser286 and Gly335 are prominent when an electron-withdrawing group (EWG) is present at *para*-position of phenyl ring. The alkyl chain at R$_1$ provides additional flexibility for interaction with the receptor, bringing the heterocyclic ring closer to Leu282 (Fig. 5).

3. Discussion

Over the years, there has not been a unanimous conclusion on the role of butyrylcholinesterase in AD. There were numerous recent reports which have shown that BChE level increases in AD patients' central nervous system [20–22]. Furthermore, for AD patients below 75 years old who were not responding to Donepezil (selective AChE inhibitor), most of them showed improvement when they were treated with Rivastigmine (dual AChE and BChE inhibitor) [23]. Although inhibition of BChE may cause potentiating side effects [24], these patients only showed some minor effects of nausea and vomiting. Therefore, BChE may represent an important therapeutic target for AD, and dual AChE and BChE inhibitors such as the novel benzimidazole derivatives described herein could
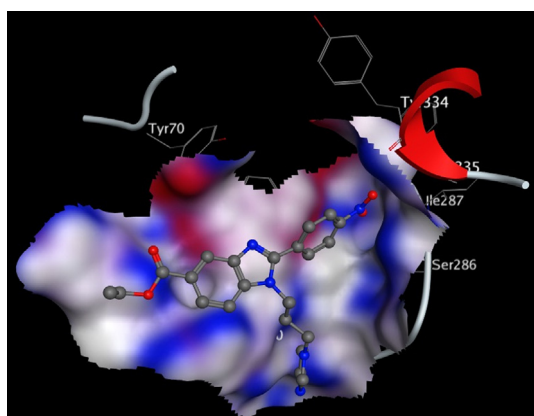
potentially be a good alternative to AD patients not responding to selective AChE inhibitors.

Encourage by the positive results, we have reported here, further modification on the 2-substituted position on the benzimidazole core as well as 4-position on the bisulfite adducts is currently in progress in our laboratory.

In summary, we have described the molecular design, synthesis, and cholinesterase inhibition activities of 34 novel benzimidazole derivatives. The preliminary bioassay data and cytotoxicity test show that some of the compounds possess moderately good AChE and BChE inhibition. The present work demonstrates that replacement of 2-position substitution on the benzimidazole core as well as 4-position on the phenyl ring moiety could potentially result in new compounds with excellent cholinesterase inhibition activity.

4. Materials and methods

4.1. Chemistry

All chemicals were supplied by Sigma–Aldrich (USA) and Merck Chemicals (Germany). Thin layer chromatography (TLC) using silica gel G was performed in the solvent system chloroform–methanol (9:1). The spots were located under short (254 nm)/long (365 nm) UV light. Elemental analyses were performed on Perkin Elmer 2400 Series II CHN Elemental Analyzer and were within ±0.4% of the calculated values. ^{1}H and ^{13}C NMR were performed on Bruker Avance 300/500 (^{1}H: 300 MHz/500 MHz, ^{13}C: 75 MHz/125 MHz) spectrometer in CDCl$_3$ using TMS as internal standard. Mass spectra were recorded on Varian 320-MS TQ LC/MS using ESI. Crystal structure analysis was carried out using *Bruker* SMART APEXII CCD area-detector diffractometer.

4.1.1. Procedure for the preparation of ethyl-4-fluoro-3-nitrobenzoate (**1**)

4-Fluoro-3-nitrobenzoic acid (5 g, 27 mmol) was refluxed in ethanol (50 mL) and concentrated H$_2$SO$_4$ (2 mL) for 8 h. After completion of reaction (as evident from TLC), the solvent was evaporated under reduced pressure. The aqueous layer was extracted with ethyl acetate (25 mL × 3). The organic layer was dried over Na$_2$SO$_4$ and concentrated under reduced pressure to yield **1** as cream-colored powder (75%).

4.1.2. General procedure for the preparation of 4-(2-substitutedamino)-3-nitro-ethyl benzoate (**2**)

Ethyl-4-fluoro-3-nitrobenzoate, **1** (0.5 g, 2.34 mmol), amine [**I**: 4-(2-Aminoethyl)morpholine; **II**: N-(3-Aminopropyl)imidazole] (2.58 mmol), and N,N-Diisopropylethylamine, DIPEA (0.49 mL, 2.78 mmol) were mixed in dichloromethane (10 mL). The reaction mixture was stirred overnight at room temperature. After completion of reaction (as evident from TLC), the reaction mixture was washed with water (10 mL × 2) followed by 10% Na$_2$CO$_3$ solution (10 mL). The organic layer was dried over Na$_2$SO$_4$ and concentrated under reduced pressure to afford **2** as brown oil/yellow solid (80–95%).

4.1.3. General procedure for the preparation of ethyl-3-amino-4-(2-substituted amino)benzoate (**3**)

4-(2-Substituted amino)-3-nitro-ethyl benzoate, **2** (1 mmol), ammonium formate (3 mmol), and Pd/C (50 mg) were mixed in ethanol (10 mL). The reaction mixture was refluxed until completion (solution turned colorless). The reaction mixture was then filtered through Celite 545. The filtrate was evaporated under reduced pressure. It was resuspended in ethyl acetate (20 mL), washed with water (10 mL × 2), dried over Na$_2$SO$_4$, and evaporated to dryness to yield **3** (60%).

Fig. 5. Compound **5IIc** docked in first active site.

4.1.4. General procedure for the preparation of sodium bisulfite addcuts of 4-substituted benzaldehyde (4a–4q)

Appropriate benzaldehyde (10 mmol) was dissolved in ethanol (20 mL). Sodium metabisulfite (15 mmol) in 5 mL water was added in portion over 5 min. The reaction mixture was stirred at room temperature for 1 h and subsequently stirred at 4 °C overnight. The precipitate formed was filtered and dried to afford sodium bisulfite adducts (55–90%).

4.1.5. General procedure for the preparation of 2-substituted benzimidazole derivatives (5Ia–5Iq and 5IIa–5IIq)

Ethyl-3-amino-4-(2-substituted amino)benzoate, **3** (1 mmol), and various sodium bisulfite adducts, **4** (1.5 mmol), were dissolved in DMF (5 mL). The reaction mixture was stirred at 90 °C under N$_2$ atmosphere for 24–48 h. After completion of reaction (evident by TLC), the reaction mixture was diluted in ethyl acetate (25 mL) and washed with water (10 mL × 3). The organic layer was collected, dried over Na$_2$SO$_4$, and evaporated under reduced pressure to afford compounds **5** in 43–90% yields.

Data for **5IIc**. Yield: 77%; ^{1}H NMR (300 MHz, CDCl$_3$): δ ^{1}H NMR: 1.44 (3H, t, J = 7.2 Hz), 2.20–2.40 (2H, m), 3.95 (2H, t, J = 6.6 Hz), 4.29 (2H, t, J = 6.6 Hz), 4.43 (2H, q, J = 7.2 Hz), 6.82 (1H, s), 7.12 (1H, s), 7.29 (1H, d, J = 8.4 Hz), 7.41 (1H, s), 7.58 (2H, d, J = 8.4 Hz), 8.10 (1H, dd, J = 1.5 Hz, 8.4 Hz), 8.24 (2H, d, J = 8.4 Hz), 8.58 (1H, s). ^{13}C NMR: 14.42, 30.89, 41.87, 43.76, 61.13, 109.30, 118.32, 122.81, 125.89, 126.11, 126.17, 129.45, 132.54, 133.27, 137.02, 138.44, 142.70, 150.01, 153.29, 166.79. ESI-MS: m/z 420.2 [M+H]$^+$. Anal. Calcd for C$_{22}$H$_{21}$N$_5$O$_4$: C, 63.00%; H, 5.05%; N, 16.70%. Found: C, 63.19%; H, 5.10%; N, 16.53%.

Data for **5IIg**. Yield: 90%; ^{1}H NMR (500 MHz, CDCl$_3$): δ ^{1}H NMR: 1.43 (3H, t, J = 7.0 Hz), 2.20–2.30 (2H, m), 3.92 (2H, t, J = 6.5 Hz), 4.27 (2H, t, J = 7.5 Hz), 4.43 (2H, q, 7.0 Hz), 6.76 (1H, s), 7.09 (1H, s), 7.28 (1H, d, J = 8.5 Hz), 7.39 (1H, s), 7.77 (2H, d, J = 8 Hz), 7.80 (2H, d, J = 8 Hz), 8.09 (1H, dd, J = 1.5 Hz, 8.5 Hz), 8.56 (1H, s). ^{13}C NMR: 14.42, 30.89, 41.93, 43.81, 61.12, 109.30, 118.29, 122.78, 125.24, 125.85, 126.10, 126.21, 129.54, 130.43, 132.49, 133.32, 137.01, 138.37, 142.67, 153.33, 166.79. ESI-MS: m/z 444.2 [M+H]$^+$. Anal. Calcd for C$_{23}$H$_{21}$N$_4$O$_2$F$_3$: C, 62.44%; H, 4.78%; N, 12.88%. Found: C, 62.44%; H, 4.78%; N, 12.89%.

Data for **5IIn**. Yield: 80%; ^{1}H NMR (300 MHz, CDCl$_3$): δ ^{1}H NMR: 1.45 (3H, t, J = 7.2 Hz), 2.20–2.40 (2H, m), 3.93 (2H, t, J = 6.6 Hz), 4.27 (2H, t, J = 6.6 Hz), 4.44 (2H, q, J = 7.2 Hz), 6.79 (1H, s), 7.12 (1H, s), 7.28 (1H, d, J = 8.4 Hz), 7.38 (1H, s), 7.52 (2H, d, J = 8.4 Hz), 7.68 (2H, d, J = 8.4 Hz), 8.09 (1H, dd, J = 1.5 Hz, 8.4 Hz), 8.58 (1H, s). ^{13}C NMR: 14.48, 29.03, 41.99, 43.85, 61.55, 109.98, 112.67, 124.13, 125.09, 128.73, 129.13, 129.96, 130.17, 131.11, 134.95, 137.66, 143.84, 151.47, 167.84, 169.11. ESI-MS: m/z 419.3 [M+H]$^+$. Anal. Calcd for C$_{23}$H$_{22}$N$_4$O$_4$: C, 66.02%; H, 5.30%; N, 13.39%. Found: C, 66.06%; H, 5.32%; N, 13.35%.

Data for **5IIo**. Yield: 76%; ^{1}H NMR (300 MHz, CDCl$_3$): δ ^{1}H NMR: 1.45 (3H, t, J = 7.0 Hz), 2.20–2.30 (2H, m), 3.88 (2H, t, J = 6.5 Hz), 4.30 (2H, t, J = 7.5 Hz), 4.43 (2H, q, J = 7.0 Hz), 6.79 (1H, s), 7.07 (1H, s), 7.28 (1H, d, J = 8.5 Hz), 7.39 (1H, s), 7.77 (2H, d, J = 8 Hz), 7.80 (2H, d, J = 8 Hz), 8.09 (1H, dd, J = 1.5 Hz, 8.5 Hz), 8.56 (1H, s). ^{13}C NMR: 14.62, 29.05, 42.01, 43.83, 62.02, 107.83, 109.87, 110.24, 111.06, 123.07, 126.59, 131.18, 137.76, 151.74, 153.40, 157.67, 167.89. ESI-MS: m/z 391.2 [M+H]$^+$. Anal. Calcd for C$_{22}$H$_{22}$N$_4$O$_3$: C, 67.68%; H, 5.68%; N, 14.35%. Found: C, 67.66%; H, 5.62%; N, 14.50%.

Data for **5In**. Yield: 89%; ^{1}H NMR (300 MHz, CDCl$_3$): δ ^{1}H NMR: 1.44 (3H, t, J = 7.2 Hz), 2.68 (4H, t, J = 4.8 Hz), 3.01 (2H, t, J = 4.8 Hz), 3.79 (4H, t, J = 4.8 Hz), 4.46 (2H, q, J = 7.2 Hz), 4.64 (2H, t, J = 4.8 Hz), 7.50 (1H, d, J = 9 Hz), 7.82 (2H, d, J = 9 Hz), 8.10–8.20 (3H, m), 8.60 (1H, s). ^{13}C NMR: 14.46, 42.78, 53.84, 57.49, 60.48, 66.60, 110.05, 112.51, 124.13, 125.09, 128.73, 129.13, 129.96, 130.17, 134.95, 143.85, 167.81, 169.04. ESI-MS: m/z 424.2

[M+H]$^+$. Anal. Calcd for C$_{23}$H$_{25}$N$_3$O$_5$: C, 65.19%; H, 6.03%; N, 9.94%. Found: C, 65.24%; H, 6.13%; N, 10.00%.

4.2. Biology

Acetylthiocholine iodide (ATCl), acetylcholinesterase (AChE, from electric eel), butyrylcholinesterase (BChE, from equine serum), S-butyrylthiocholine chloride, and 5,5′-Dithiobis(2-nitrobenzoic acid) (Ellman's reagent, DTNB) were purchased from Sigma–Aldrich (USA).

4.2.1. In vitro cholinesterase inhibition assay

Cholinesterase enzyme inhibitory potential of the test samples was determined following Ellman's method with slight modifications. Briefly, test samples and Donepezil were prepared in DMSO at the initial concentration of 1 mg/mL. The final concentration of DMSO in reaction mixture was 1%. At this concentration, DMSO has no inhibitory effect on both acetylcholinesterase (AChE) and butyrylcholinesterase (BChE) enzymes as was determined in separate prior experiments.

For acetylcholinesterase (AChE) inhibitory assay, 140 μL of 0.1 M sodium phosphate buffer (pH 8) was added to a 96 wells microplate followed by 20 μL of test samples and 20 μL of 0.09 units/mL AChE enzyme. Then, 10 μL of 10 mM 5,5′-Dithiobis(2-nitrobenzoic acid) (DTNB) was added into each well followed by 10 μL of 14 mM of acetylthiocholine iodide. After 30 min of incubation, absorbance of the colored end product was measured using Tecan Infinite 200 Pro Microplate Spectrophotometer at 412 nm. Each test was conducted in triplicate.

For butyrylcholinesterase (BChE) inhibitory assay, the same procedures were applied as AChE except for the use of enzyme and substrate, which were BChE from equine serum and S-butyrylthiocholine chloride.

Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.biorg.2013.06.008. These data include MOL files and InChiKeys of the most important compounds described in this article.

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