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Syntheses, Cholinesterases Inhibition and Molecular Docking Studies of Pyrido[2,3-*b*]pyrazine Derivatives

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Running Title:
Pyrido[2,3-*b*]pyrazine derivatives as Cholinesterases Inhibitors

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Abstract

Cholinesterases, acetylcholinesterase (AChE), and butyrylcholinesterase (BChE), have a role in cholinergic deficit which evidently leads to Alzheimer's disease (AD). Inhibition of cholinesterases with small molecules is an attractive strategy in AD therapy. The present study demonstrates synthesis of pyrido[2,3-*b*]pyrazines (**6a-6q**) series, their inhibitory activities against both cholinesterases, AChE and BChE, and molecular docking studies. The bioactivities data of pyrido[2,3-*b*]pyrazines showed 3-(3'-nitrophenyl)pyrido[2,3-*b*]pyrazine **6n** a potent dual inhibitor among the series against both, AChE and BChE with IC_{50} values of 0.466 ± 0.121 and 1.89 ± 0.05 μM, respectively. The analogues, 3-(3'-methylphenyl)pyrido[2,3-*b*]pyrazine **6c** and 3-(3'-fluorophenyl)pyrido[2,3-*b*]pyrazine **6f** were found to be selective inhibitors for BChE with IC_{50} value of 0.583 ± 0.052 μM and AChE with IC_{50} value of 0.899 ± 0.10 μM, respectively. Molecule docking studies of the active compounds suggested the putative binding modes with cholinesterases. The potent compounds among the series could potentially serves as good leads for the development of new cholinesterase inhibitors.

Keywords: Acetophenone derivatives, diaminopyridine, pyrido[2,3-*b*]pyrazines, cholinesterases, acetylcholinesterase (AChE), butyrylcholinesterase (BChE)

1. Introduction

Alzheimer's disease (AD) is recognized as most common cause of dementia which affects 36 million people worldwide. According to a world Alzheimer's report 2012, the data could projected to 115 million patients with dementia by 2050, unless an effective treatment is developed to reverse the AD symptoms (1). The severity of the AD complications put it into the loop of top ten leading causes of death and makes it imperious for chemists and pharmacists to develop efficacious drugs. The symptoms associated with AD are gradual decline in memory, weakening in language skills and other cognitive dysfunctions (2-4). Evidence showed a significant role of cholinesterases, acetylcholinesterase (AChE, EC 3.1.1.7) and butyrylcholinesterase (BChE, EC 3.1.1.8), in cognitive dysfunctions (5). These enzymes predominantly effect the hydrolysis of acetylcholine neurotransmitters at synaptic cleft which lead to severe loss of cholinergic neurons in AD patients (6). The major hallmarks associated with this neurodegenerative disorder are including; 1) deposition of γ-amyloid peptides due to excessive cleavage of amyloid proteins and 2) development of neurofibrillary tangles from aggregated tau protein, both together lead to degeneration of cholinergic neurons. The current available therapeutics to reduce AD symptoms are including the AChE inhibitors donepezil, galantamine, neostigmine and dual inhibitor rivastigmine to improve cognitive impairments in AD patients (7-9). The available remedies prove to be effective only in the early stages of disease, impels to develop new inhibitors for the management of AD (10-12).

Both cholinesterases, AChE, and BChE, differ in their distribution pattern and activities in a healthy individual and AD patient (13). The AChE is distributed in multiple cells, but mainly concentrated in cholinergic nervous system while BChE, constituted in liver, distribute in the body *via* blood plasma. Previous studies demonstrate that in AD brain the activity of AChE reduces to almost its half while the activity of BChE elevates in later stages of AD complications to compensate the hydrolysis of AChE (9, 14). The role of BChE in the deposition of β-amyloid (Aβ) has also been reported in AD brain (15). A

general comparison of both enzymes showed a 20 A° long narrow gorge deep in the protein which ends to a bottom catalytic triad where the hydrolysis of acetylcholine takes place to choline and acetic acid. The size of AChE active site gorge is relatively small (302 A°), due to 14 bulky aromatic amino acids residues, as compared to BChE gorge (502 A°) which is comprised of 8 aryl amino acids residues (16, 17). The difference in gorge size and aryl residues pattern in both cholinesterases provides a mean of selectivity for inhibitors.

The approaches utilized to counter AD symptoms *via* cholinesterases inhibition, AChE and BChE, are either selective or non-selective. Various types of molecules such as carbamates (phenothiazine derivatives, neostigmine etc.), tacrine, piperidines (donepezil) organophosphates, [1,6] or [1,8]-naphthyridine derivatives (12, 18, 19), Schiff bases (20) and purine nuelcoside (21) *etc*. have been reported as cholinesterases inhibitors. In the present study, we have synthesized a series of pyrido[2,3-*b*]pyrazines (**6a-6q**) due to their close resemblance with [1,8]-naphthyridine derivative **2** and tacrine **3** with an extra nitrogen atom at C-4 or C-5/9 position which may also participate in π-π interactions of inhibitors with the active site of enzyme during inhibition (Figure-1). Literature showed varied applications of pyrido[2,3-*b*]pyrazine type compounds in pharmaceutical field such as antituberculosis, (22) antitumor, (23, 24) antiallergic agents (25) *etc*. The synthesized pyrido[2,3-*b*]pyrazines (**6a-6q**) were evaluated as cholinesterases inhibitors against both electric eel AChE and equine serum BChE by using the standard Ellman's colorimetric assay (26).

Figure-1: Structure of pyrido[2,3-*b*]pyrazines **1**, [1,8]-naphthyridine derivative **2** and tacrine **3**

2. Results and discussion

2.1. Chemistry

To synthesize desired pyrido[2,3-*b*]pyrazines (**6a-6q**), a concise synthetic route was adopted. The layout of the synthetic route has been presented in the Scheme-1. The *α*-methyl group of the corresponding acetophenone **4** was oxidized with selenium oxide to dicarbonyl intermediate **4A** (27). In the next step, the corresponding dicarbonyl intermediate **4A** was coupled with pyridine-2,3-diamine **5** at room temperature furnishing the pyridopyrazine ring system (28). The method proved to be consistent and straight forward to assemble the pyrido[2,3-*b*]pyrazine core. The optimized reaction conditions were then deployed on other acetophenone derivatives as well as cyclohexanone **7** to afford the corresponding pyrido[2,3-*b*]pyrazines in 50-90% yield in a regioselective way. (29) The regioselectivity was observed due to difference in reactivity of the aldehyde and ketone moieties within a same molecule *i.e.* intermediate **4A**. The structures of pyrido[2,3-*b*]pyrazines (**6a-6q**) were confirmed with different spectroscopic techniques including ^{1}H and ^{13}C NMR spectroscopy, mass spectrometry (EI^{+}, ESI^{+} and HRMS), IR spectroscopy (30, 31). The X-ray crystal structures of compounds **6c** and **6f** further confirm the structure and regioselectivity in the pyridopyrazine analogues (Figure-2)..

Scheme-1: Synthesis of pyrido[2,3-*b*]pyrazine derivatives (**6a-6q**)

Figure-2. X-ray crystal structures of 3′-methyl **6c** and 3′-fluoro **6f** pyrido[2,3-*b*]pyrazines

2.2. *In vitro* inhibitory activity of pyridopyrazine analogues against AChE and BChE

The synthetic pyrido[2,3-*b*]pyrazines (**6a-6q**) were evaluated as inhibitors against electric eel AChE and equine BChE by following the Ellman's procedure (26). Bioactivities results showed that most of the compounds in the series have significant activity against both cholinesterases; AChE and BChE (Table-1). Neostigmine (IC_{50} = 22.2 ± 3.2 μM for AChE and IC_{50} = 49.6 ± 6.11 μM for BChE) and donepezil (IC_{50} = 0.032 ± 0.003 μM for AChE and IC_{50} = 6.41 ± 0.34 μM for BChE) has been used as standard cholinesterases inhibitors. For AChE, compound **6a** without any substituents showed the least inhibitory activity with an IC_{50} value of 9.65 ± 0.324 μM, while 3′-fluorophenyl **6f** and 3′-nitrophenyl **6n** pyridopyrazine analogues bearing electron withdrawing substituents displayed highest inhibitory activities among the series with an IC_{50} values of 0.899 ± 0.10 and 0.466 ± 0.121 μM, respectively. Substitution of 3′-fluoro group with chloro group slightly reduced the activity of **6h** to IC_{50} values of 1.26 ± 0.022 μM. The 4′-nitrophenyl **6m** derivatives also showed significant activity with IC_{50} values of 1.97 ± 0.02 μM. Further compounds in the series showed inhibitory activities with IC_{50} values ranging between 2.45 ± 0.31 μM – 5.88 ± 0.1.21 μM (Table-1).

For BChE, the 3′-methylphenyl derivative **6c** bearing electron denoting substituent displayed potent inhibitory activity with IC_{50} values of 0.583 ± 0.052 μM. Whereas, substituting 3′-methyl group with electron withdrawing nitro group makes a slight decline in the activity of **6n** to IC_{50} value of 1.89 ± 0.05 μM. The 4′-chlorophenyl **6g** also displayed significant inhibitory activities with IC_{50} values of 2.63 ± 0.312 μM while the other chloro (**6h, 6i**) or fluoro **6f** derivatives in the series showed weak or poor activities against BChE. Since the compound **6n** demonstrated good inhibitory activity for AChE with IC_{50} values of 0.466 ± 0.121 μM. So, it is consider as dual inhibitor for cholinesterases. The compound with bulky substituent, 4′-piperidine **6p** was found less active against AChE with an IC_{50} value 4.75 ± 0.521 μM, while moderately active against BChE with an IC_{50} value 3.91±0.621 μM. This may be due to bulky piperidine residue to accommodate at the bottom of gorge of both enzymes. A tacrine **3** (12) like compound **6q** also displayed moderate inhibitory activity against both cholinesterases (Table-1). Summatively, the inhibitory data of synthetic compounds demonstrate that the positions, electronic effect and size of the corresponding substituents influence the binding and π-π interaction of pyridopyrazine derivatives (**6a-6q**) with targeted enzymes. The active compound **6f** displayed 88 times more selectivity for AChE inhibition. Conversely, the compound **6c** showed 10 times more for BChE inhibition (32). The selectivity of the compounds towards targeted cholinesterases inhibition added an advantage to regulate the activity of AChE or BChE in AD therapy.

Table-1: *In Vitro* AChE and BChE inhibitory activities of pyrido[2,3-*b*]pyrazines (**6a-6q**).

3. Computational Studies

In the RCSB Protein Bank, X-rays structures of equine BChE are currently not available and for electric eel AChE only low crystallographic resolution (>4 Å) structures are available (33). Therefore, X-ray structures of human AChE (PDB ID 4BDT) and human BChE (PDB ID 4BDS) were used as template structures for docking. The active site gorge of AChE is considerably smaller than the gorge of BChE (16, 17). For dual AChE/BChE inhibitors, this might lead to different binding characteristics. The analysis of

AChE X-ray structures revealed multiple side chain orientations of Tyr337 depending on the co-crystallized ligands. Accordingly, in our docking study, the side chain of Tyr337 in AChE was treated flexibly. In BChE the corresponding residue Ala328 has no side chain flexibility and hence the entire active site was kept rigid.

Figure-3 (left side) showed an overlay of the best AChE inhibitor **6n** and the crystallographic inhibitor huprine W. In its preferred docking pose, compound **6n** is located at the bottom of the active site gorge and almost coplanar with residues Trp86 and Tyr337, which enables of π-π-interactions. The nitro substituent is in hydrogen bonding distance to the residue Tyr341. Two additional hydrogen bonds might be formed between two nitrogen atoms of the pyridopyrazine ring and the hydroxyl group of Tyr337. Although the large gorge area of AChE might also accept different binding conformations, the predicted binding mode resembles the co-crystallized inhibitor huprine W and is further stabilized by several well defined short range interactions.

The putative binding mode of the most potent BChE inhibitor **6c** (Figure-3, right side) in the active site of BChE differs from the pose of compound **6n** in the active site of AChE. The exchange of residue Pro446 in AChE with the larger residue Met437 in BChE prevents a coplanar orientation of the pyridopyrazine compounds in the active site of BChE. Instead, the methyl substituted phenyl substructure is directed into a lipophilic pocket formed by residues Phe329, Leu286, Val288, Trp231, and Phe398. The pyridopyrazine core fragment is in π-interaction distance to residues Trp82 and His438. The gorge area of BChE is even larger than in AChE and therefore accommodates other plausible binding conformations. However, after detailed analysis of potential short range interactions, the presented binding pose was preferred.

With the exception of inhibitor **6p**, all other pyridopyrazine derivatives presented herein are expected to display similar binding modes as the compound **6n** in AChE and **6c** in BChE. In compound **6p**, the piperidine substituent is too large to be accommodated at the bottom of the gorge of both enzymes and therefore most likely binds to the mid gorge area.

Figure-3: On the left, an overlay of the predicted binding mode of inhibitor **6n** (carbon atoms colored magenta) and the crystallographic inhibitor huprine W (carbon atoms colored yellow) in the active site of AChE (cyan) is shown. On the right, the putative binding conformation of inhibitor **6c** (carbon atoms colored green) in the active site of BChE (orange) is shown.

4. Conclusion:

The bioactivity data of pyrido[2,3-*b*]pyrazines **6a-6q** and molecular docking concluded that some of these derivatives exhibited good inhibitory activity against AChE and BChE. Compound **6n** with an IC_{50} value of 0.466 ± 0.121 μM for AChE and 1.89 ± 0.05 μM for BChE may serve as dual inhibitor. Compound **6f** showed selective inhibition for AChE with an IC_{50} value of 0.899 ± 0.10 μM and compounds **6c** displayed selective inhibition for BChE with an IC_{50} value of 0.583 ± 0.052 μM. The potential leads found in anticholinesterase assay can be used to develop new therapeutic agents for AD therapy.

Conflict of Interest

The authors have declared no conflict of interest**.**

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Supplementary Information

The supplementary information has the scanned copies of ^{1}H NMR spectra and X-ray crystallographic data of crystal structures **6c** and **6f**. CCDC number of **6c** 1033368 and **6f** 1033369, respectively, contains these supplementary crystallographic data. The data can be obtained free of charge *via* www.ccdc.cam.ac.uk/data_request/cif, or by e-mailing data_request@ccdc.cam.ac.uk, or by contacting The Cambridge Crystallographic Data Centre, 12 Union Road, Cambridge CB2 1EZ, UK; fax: +44(0)1223-336033.

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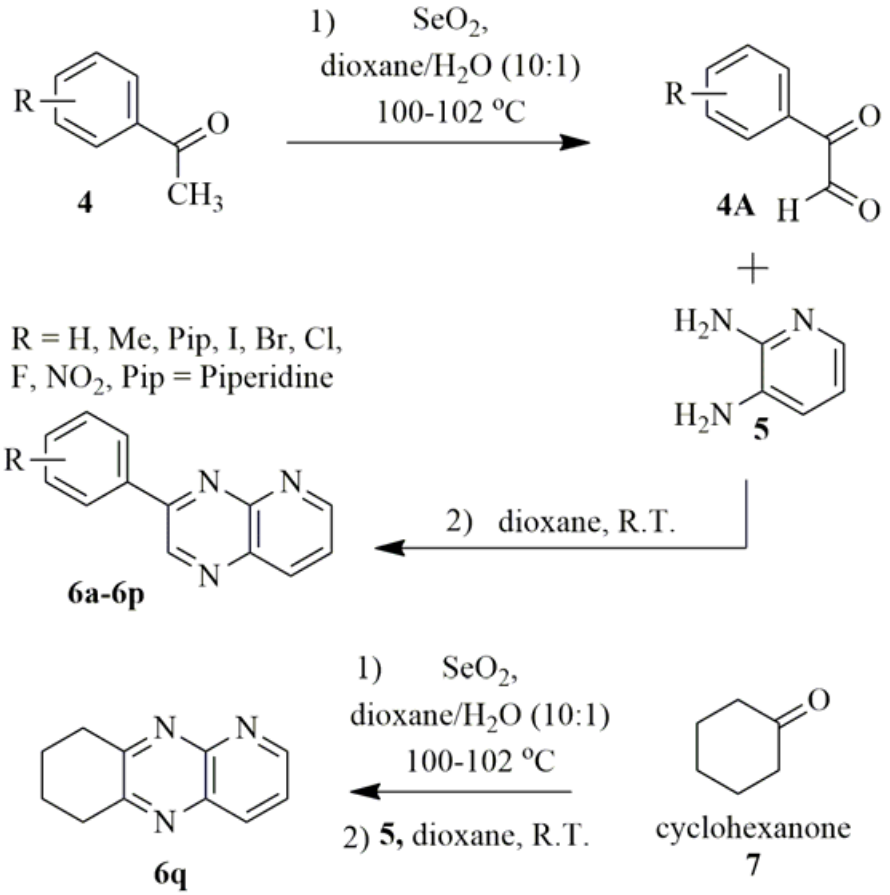
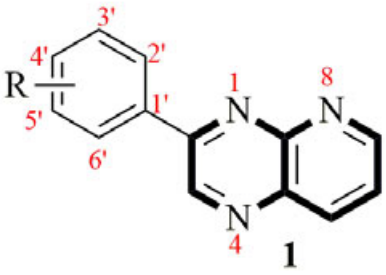
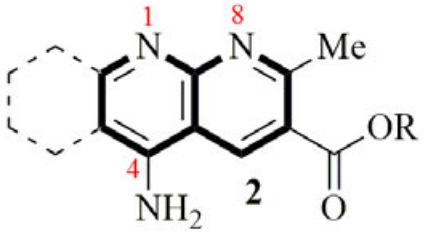




Pyrido[2,3-b]pyrazine derivaitive

R = subsituents



[1,8]-Naphthyridine derivaitive

Tacrine