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Inhibition of Butyrylcholinesterase with Fluorobenzylcymserine, An Experimental Alzheimer's Drug Candidate: Validation of Enzoinformatics Results by Classical and Innovative Enzyme Kinetic Analyses

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Abstract

Background—Selective butyrylcholinesterase (BuChE)-inhibition, increases acetylcholine (ACh) levels. In rodents, this inhibition is known to boost cognition. Also, this occurs without the typical unwanted adverse effects of acetylcholinesterase-inhibitors or AChE-Is. The novel compound, fluorobenzylcymserine (FBC), is derived from our effort to design a selective BuChE-inhibitor. Also, we wanted to check whether butyrylcholinesterase-inhibitors (BuChE-Is) possessed an edge over AChE-Is in Alzheimer's disease (AD) in terms of efficacy and/or tolerance.

Method—FBC was synthesized as reported earlier while enzymatic activity of BuChE was calculated by Ellman-technique. Molecular docking was performed using Autodock4.2. We applied classical as well as innovative analyses of enzyme-kinetics for exploring "FBC:human BuChE-interaction". The mode of inhibition and kinetic parameters were also determined.

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CONFLICT OF INTEREST

The authors declare no conflict of interest, financial or otherwise.

Results—Docking results displayed two strong interacting sites for FBC. One of these binding sites was previously identified as a deep narrow groove having polar aromatic residues while a second site was identified during this study which displayed better interaction and was lined with aliphatic and sulphur containing residues. At low concentrations of BuChE, the IC₅₀ was found to be very low *i.e.* 4.79 and 6.10 nM for 12 and 36 µg, respectively, whereas it increased exponentially by increasing the units of BuChE.

Conclusion—These analyses indicate that FBC is an interesting AD drug candidate that could provide a potent and partial mixed type of inhibition of human BuChE.

Keywords

Alzheimer's disease; anticholinesterase; butyrylcholinesterase; cymserine; docking; enzyme inhibition kinetics; fluorobenzylcymserine

1. INTRODUCTION

Changes in the levels of some inflammatory proteins, (*e.g.* C-reactive protein and TNF- α , to name a few) and lipid peroxides are common to type-2 diabetes as well as type-3 diabetes, a term coined for. Alzheimer's disease (AD) [1]. Increasing evidence suggests that an elevation in the plasma butyrylcholinesterase (BuChE)-levels and tissue levels of the same (*i.e.* in pancreas or brain) forms a bridge between these two diseases [1]. BuChE, is known to play an established part in the regulation of acetylcholine (ACh) as well as cholinergic type neurotransmission. However, it does possess non-cholinergic functions as well, *e.g.* differentiation, proliferation and also apoptosis may involve role of BuChE [2]. Similarly, ACh performs its action on subtypes of nicotinic/muscarinic-receptors. In this way it primarily acts as a 'cholinergic-neurotransmitter'. However, it is also known to act as a modulator of "nervous system:immune system-interactions" [3]. Particularly, it is the vagus nerve (efferent) which signals the cholinergic-type pathway against inflammation. This is facilitated mainly via nicotinic ACh-receptors which are present on tissue macrophages [4]. This anti-inflammatory pathway basically performs production-inhibition of relevant proteins such as TNF- α /IL-1/MIF/HMGB1. Furthermore, the pathway performs 'activation-suppression' of expression of NF- κ B [4]. ACh, is also known to play a role in the regulation of neurotransmitters like dopamine and serotonin [5]. In both the diseases *i.e.* diabetes and AD, elevated BuChE-levels may be observed. Also, BuChE efficiently hydrolyses ACh. A BuChE-induced down regulation in ACh levels can result in a type of systemic-inflammation albeit of a low-grade. This happens because of dysregulation of the aforementioned pathway. We intended to inhibit this destructive cycle of events by selectively inhibiting the catalytic activity of BuChE, while selectively inhibiting proinflammatory cytokines (*e.g.*, TNF- α) would restore ACh levels. Hence, the development of fluorobenzylcymserine (FBC) was undertaken [6]. In prior studies, we developed novel, drug-like compounds to selectively inhibit BuChE and TNF- α that appear to be well tolerated, and would be utilized to quantitatively characterize strategies for type 2 diabetes and AD therapy [7, 8].

Compromised tolerance of glucose levels leads to a decline in cognition, irrespective of age as a factor. Furthermore, an overwhelmingly large scientific literature attributes diabetes as

an underlying cause in the development of AD [9, 10]. Whereas the localization of these two disorders is significantly different from each other yet there exist a plethora of common features such as sharing of several degenerative molecular processes, biochemical and pathological features [11]. Both of these could be considered as long-lasting as well as continuing illnesses which are already existent even prior to diagnosis and/or treatment initiation. Oxidative stress can be caused due to Hyperglycemia. This may in turn be aided by the presence of important regulatory-proteins in glycosylated forms, rendering them as malfunctioning proteins. However, insulin along with its degrading protein appears to have a significant say in the progress of both the diseases. It is known that, there exists a significant presence of insulin-receptors throughout the brain. Also, parts of the brain like, the hippocampus, hypothalamus as well as entorhinal cortex possess high density of these receptors in particular [12]. Insulin displays an important impact with reference to memory in AD patients, exerts a regulatory influence on beta-amyloid as well as the precursor protein, and may regulate tau phosphorylation [13–16]. The brain is considered to be sensitive to insulin-levels, and that insulin has an established say in the maintenance of a normal physiology of the brain, and any hindrance in the insulin-signaling-pathway could have a major impact on the plasticity of the synapses and viability of the neuronal cells [15, 17].

Recent studies suggest that BuChE is also among the markers, the levels of which act as indicators of low grade inflammation. This is in harmony with its function in Alzheimer's brain. Also, authors have argued that serum BuChE levels are related to hypertriglyceridaemia as well as sensitivity to insulin among diabetes patients [18, 19]. Interestingly, the variant 'BuChE K' is observed to have a greater prevalence in patients suffering from type-2 diabetes compared to their healthy counterparts. This indicates that some significant role is played by the BuChE 3q26 (gene) in patients suffering from diabetes type-2. Referring to this, it is noteworthy that a susceptibility locus is known to exist on the chromosome 3q27 [19, 20]. The normalization of elevated BuChE levels found in AD may hence be of value in type 2 diabetes.

Amplification of residual cholinergic potential by inhibition of cholinesterases is regarded as the major treatment option for AD patients [21]. To achieve this, AD is presently treated by symptomatic-type therapy; primarily with acetyl-cholinesterase inhibitors (AChE-Is). Tacrine (Cognex) was the first ChE-I drug for AD which was approved in 1993. It was observed to be non-selective. In contrast, drugs belonging to the 2nd generation of ChE-Is, namely, donepezil hydrochloride, rivastigmine and galatamine approved subsequently by the US in 1996, 2000 and 2002, respectively, are still being used. On the other hand, some studies have raised doubts about the level of advantages obtained by the use of AD-drugs of this class. This controversy has opened new vistas of drug designing and research. The first idea was to design and synthesize a fresh category of ChE-Is which would have an edge over the routine symptomatic-type AD-drugs. The second idea was to perform targeted and precise drug-optimization for existing drug molecules utilizing the in-depth knowledge of protein-drug binding thereby reviving them. Accordingly, this study of ours has used both wet (*in vitro*) and dry (computational) approaches.

Selective butyrylcholinesterase (BuChE)-inhibition, increases acetylcholine (ACh) levels. In rodents, this inhibition is known to boost cognition. Also, this occurs without the typical unwanted effects of acetylcholinesterase-inhibitors or AChE-Is [7]. BuChE-Is thereby represent a new strategy to ameliorate AD [7, 22, 23]. This is understandable in the light of the fact that during AD progression the activity of AChE declines, in accord with the lowered available levels of ACh molecules present, whereas BuChE activity in brain get elevated [24]. The BuChE to AChE ratio hence gets altered significantly within those regions of the cortex which are challenged with AD. This ratio may range from 0.2 to 11, in healthy vs. AD subjects, respectively [22, 23]. Such a change likely modifies the normal functioning of BuChE in the nervous system of hydrolysing excess ACh to depleting already reduced levels of the neurotransmitter. Previously, we had performed extensive research in drug design and synthesis using analogues of cymserine for inhibiting the BuChE present in the brain [7]; and to move agents from the laboratory to clinical assessment [25, 26]. The scientific term “ENZONFORMATICS” was coined by Shazi Shakil and M.A. Kamal in 2012 which appeared as the affiliation of Shazi Shakil for the first time in scientific literature [27]. However, specific definition of the term was not provided. Shazi Shakil hereby defines ‘Enzoinformatics’ as “*A sub-discipline of Bioinformatics which specifically deals with computational enzyme-ligand binding studies*”.

2. MATERIALS AND METHODS

All the chemicals used in this study namely, Butyrylthiocholine iodide (or BuSch); 5,5'-dithiobis (2-nitro-benzoic acid) (or DTNB); and 1,5-bis(4-allyldimethyl-ammoniumphenyl) pentan-3-one dibromide (BW284C51) were purchased from Sigma-Aldrich (United States). The enzyme used in this study (BuChE obtained from human serum) was kindly provided by an Australian blood-bank (ARCBS/Sydney/Australia). FBC was synthesized as reported earlier [6] (Fig. 1a). Enzymatic activity of BuChE was calculated by Ellman-technique [28]. The mixture for assay consisted of BuSch (used in varied concentrations); DTNB (0.250mM) and BW284C51 (an AChE-inhibitor which was selective albeit irreversible, 0.050 mM). This mixture was finally prepared in sodium phosphate buffer (50 mM/pH=7.2). BuChE-activity was read as the absorbance at 405 nm wavelength. Varied concentrations of the substrate and FBC were used while performing the kinetic experiments. $V_{\max}$ & V_{mi} stand for maximum activity, yet of two varied sub-types. $V_{\max}$ represents the apparent-maximum BuChE-activity with reference to a specific FBC-concentration corresponding to different substrate-concentrations. The latter (V_{mi}) expresses the apparent maximum activity at a particular concentration of BuSch along with varied concentrations of FBC. FBC molecular structure was drawn using Gap-edit and geometry optimization along with energy minimization was corrected by MOPAC using RM1 semi-empirical method. Molecular docking was performed using Autodock4.2. Chi-mera1.5.2 was used for molecular visualization and analyses of the results. Bradford method was employed to measure concentration of the total protein in the serum. Absorbance was read at 595 nm wavelength, as described elsewhere [29]. GOSA (France) and PRISMv4.0 programs were used to prepare the graphs for this study. All the regression analyses described in this article were performed using the built in functions provided within the aforementioned programs.

3. RESULTS

BuChE inhibition by FBC with respect to various BuChE concentrations in human serum and reaction times are presented in Fig. (1b). The correlation coefficient (R^2) was determined as 0.928, 0.942, 0.868, 0.710 for the plot of 0.002 μ M, 0.2 μ M, 10 μ M and 20 μ M FBC, respectively (Fig. 1b(A)). The R^2 values were found as 0.840, 0.924, 0.947 and 0.949 for the plot of 0.002–20 μ M FBC, respectively (Fig. 1b(B)). Accordingly, these values were found to be 0.782, 0.551, 0.520 and 0.478 for the plot of 0.002–20 μ M FBC, respectively (Fig. 1b(C)). Poor R^2 values were noticed in case of Fig. 1b(D) which were found to be 0.825, 0.523, 0.428 and 0.421 for the plot of 0.002–20 μ M FBC, respectively. In the presence of high amount of BuChE there was no correlation as R^2 was -0.00024 , 0.0344, 0.335 and 0.381 for the plot of 0.002–20 μ M FBC, respectively (Fig. 1b(E)). The R^2 was observed as 0.92, 0.90, 0.73, 0.92 and 0.94 for BuChE of 12, 36, 60, 90 and 120 μ g protein, respectively (Fig. 1b(F)). According to polynomial second order equation for tertiary data derived from previous subfigure of **1b (F)**, the R^2 was found to be 0.887 for correlation between V_i (maximum overall comparative activity index of BuChE in the presence of FBC) and the concentration of BuChE (Fig. 1b(G)). There is a significant R^2 (0.9797) in final case of correlation plot for IC_{50} with the amount of BuChE in the assay (Fig. 1b(H)). The results of this relationship of IC_{50} indicated that there is a critical transition concentration of BuChE (~ 54 μ g). At low concentrations of BuChE, the IC_{50} was found as 4.79 and 6.10 nM for 12 and 36 μ g, respectively.

The human serum BuChE inhibition by FBC corresponding to changing concentrations of substrates and FBC at varied reaction times is demonstrated in Fig. (1c). Here, R^2 was found as 0.996, 0.999, 0.999, 0.998 and 0.994 (A); 0.999, 0.999, 0.999, 0.999 and 0.998 (B); 0.999, 0.999, 0.999, 0.999 and 0.998 (C) 0.999 for all plots (D); 0.999, 0.999, 0.999, 0.999 and 0.997 (E) for control, 0.10, 0.20, 10 and 20 μ M FBC, respectively. Reversible kinetic data of human serum BuChE inhibition by FBC in the presence of different concentration of substrates was elucidated using two types of analysis softwares (GOSA and Prism) to compare kinetic constants and mode of inhibition obtained by these packages (Fig. 2a). A plot according to the general kinetic equation of GOSA package presented in Fig. 2a(A) yields three following constants: k_s , k_i and k_{cat} as a value of 0.342 ± 0.19 mM, 0.24 μ M and 0.089 ± 0.027 au, respectively. In this analysis, the sum of squared error (SSE) and a global correlation coefficient was found as 0.00022 and 0.977, respectively. After adjusting this data in the general kinetic equation of GOSA, the obtained values of it's a, b and c, were indicative of partial mixed inhibition. Hence the data was reanalyzed according to a partial mixed type of inhibition system (Fig. 2a(B)). The values obtained for the following four kinetic constants: k_m , k_{i1} , k_{i2} and V_{max} were found to be 0.419 mM, 11.79 μ M, 16.03 μ M and 0.0885 μ M/ μ g/min, respectively. In this later analysis, the sum of squared error (SSE) and a global correlation coefficient was found as 0.00023 and 0.975, respectively.

For a comparative and more precise analysis, the reciprocal kinetic data of human serum BuChE inhibition by FBC in the presence of different concentration of substrates was evaluated by a classical Lineweaver-Burk plot Fig. 2a, where R^2 was found as 0.99, 0.98, 0.99, 0.99 and 0.95 for control, 0.10, 0.20, 10 and 20 μ M FBC respectively. The mode of inhibition displayed a mixed type of inhibition pattern. By the Dixon plot (Fig. 2a(D)), R^2

was found as 0.91, 0.95, 0.95, 0.96 and 0.96 for 0.05, 0.125, 0.20, 0.35 and 0.5 mM of BuSch. Two new types of secondary-replots (prepared from the Lineweaver-Burk plot) were constructed in the current study: (i), Log percentage slope of primary replots of Lineweaver-Burk plot versus Log concentration of the inhibitor (FBC) to obtain innovative binding constants for residual free enzyme (BuChE) and inhibitor (I_{bc} 3.16 nM; Fig. 2a(E)), (ii), Log percentage of reciprocal V_{maxapp} of primary replots of Lineweaver-Burk plot versus Log concentration of the inhibitor (FBC) to get second innovative binding constant for residual fractional butyrylated enzyme (after BuChE-BuSch complex stage) and inhibitor (αI_{bc} 7.94 nM; Fig. 2a(F)). On the other hand, a classical secondary replot of the Lineweaver-Burk plot yielded the values as 20 μ M and 8.2 μ M for K_i and αK_i , respectively (Fig. 2a(G)). The values of K_{m1} and K_{m2} (0.114 mM and 0.27 mM) were calculated from secondary-replot of slope and inverse of $V_{maxiapp}$ of primary Dixon plots versus reciprocal of BuSch, respectively (Fig. 2a(H, I)).

The R^2 in Fig. (2b) was found as A: 0.9987, 0.9964, 0.9949, 0.9875, 0.9623; B: 0.9992, 0.9986, 0.9974, 0.9935, 0.9794; C: 0.9996, 0.9995, 0.9988, 0.9976, 0.9937; D: 0.9993, 0.9995, 0.9993, 0.9988, 0.9972; E: 0.9995, 0.9996, 0.9994, 0.9991, 0.9983; F: 0.9995, 0.9995, 0.9994, 0.9993, 0.9992; G: 0.045, 0.074, 0.21 and 0.26 for control, 0.10, 0.20, 1.0 and 2.0 μ M FBC, respectively. Overall, the effect of preincubation period on the inhibition of BuChE (obtained from human serum) by FBC at 0–70 min preincubation time is illustrated in Fig. 2b(G), where it is obvious that increase in preincubation period did not decrease percentage Product maximum (P_{max}), which indicates the reversible nature of the inhibition while it became reduced with respect to an increase in the concentration of FBC (Fig. 2b(H)). This tertiary replot of plots in G was found fruitful in obtaining a new constant, $P_{maxPT50}$ (0.149 μ M). The results of molecular docking further confirmed significant binding interaction between FBC and BuChE. Importantly, FBC was found to bind to a newly predicted binding site (shown in Fig. 3a in cyan color in stick format) with a ΔG value of -9.29 kcal/mol.

4. DISCUSSION

Recently, we explored the interaction of AChE with the widely used drugs cyclophosphamide and methotrexate [30, 31] to vigorously test and hone our molecular docking expertise [30, 31]. Accordingly, our docking results revealed that there were two hotspots for FBC binding within the receptor (BuChE). Both of these interactions displayed pertinent interaction energy values. FBC was found to bind to an already predicted site (shown in Fig. 3a in violet color in stick format) with an estimated free energy of binding (ΔG) as -8.54 kcal/mol while the same was found to bind to a newly predicted binding site (shown in Fig. 3a in cyan color in stick format) during this study with a ΔG value of -9.29 kcal/mol. In Fig. 3b, FBC binding to BuChE is shown in detail at the previously reported binding site while Fig. 3c represents the same for the newly predicted binding site.

Apparent consequences of neurochemical alteration during age-related anorexia are well known, like declines in food intake and body weight in aging [32]. Authors such as Reale, *et al.*, 2004, have studied the expression of markers which were pro-inflammatory as well as anti-inflammatory present in mononuclear cells of AD-patients [33]. In this regards, it can

be assumed that the localization, active forms of that particular enzyme, sequence of amino acids in and around the active catalytic, true/pseudo substrate binding subsite as well as peripheral anionic or cationic subsite are important factors towards the prediction of functions of an unfamiliar physiological role that a specific enzyme may take on in a particular location. Likewise, influences of BuChE concentration on its inhibition by FBC is studied for the first time along with its catalytic pathway (Fig. 1b). In the presence of a high amount of BuChE there was no correlation found due to fast speed of the forward reaction (Fig. 1b(E)) while, on the other hand, there was a significant correlation (R^2 0.98) between a tertiary data for estimated IC_{50} at each concentration of BuChE (Fig. 1b(H)). The results of this relationship of IC_{50} indicated that there is a vital transition state for BuChE (~54 μ g) where either the binding potency of FBC becomes reduced due to some potential reason, such as the cohesiveness of BuChE protein folding or a hydrolysis factor some where in its structure, or that that sensitivity of BuChE drops significantly. At low concentrations of BuChE, the IC_{50} was found as 4.79 and 6.10 nM for 12 and 36 μ g, respectively, which supports its previous report of 9 nM values at 0.5 mM substrate concentration and 30 min preincubation time for human plasma BuChE [6]. As per the foundation of the Ellman assay upon which this study is based, it is understandable that BuChE molecules get complexed with BuSch. Following this, BuSch is hydrolysed to butyrate and thiocholine. Subsequently, thiocholine combines with DTNB and a detectable color is produced (as the product is colored). This occurs in a concentration- as well as time-dependent fashion.

A supportive co-regulating role of BuChE in the brain cholinergic system is very evident, and hence an assessment of the binding affinity of the inhibitor with this enzyme is pertinent. As illustrated in the Fig. (4a), a negative role is predicted for elevated levels of BuChE in dysregulating brain ACh levels in AD. This could be usefully corrected with a well tolerated BuChEI. Additionally, BuChE inhibition by a well tolerated anti-AD agent may be of indirect potential value for controlling type 2 diabetes by elevating ACh levels and, via the “cholinergic anti-inflammatory pathway”, by lowering proinflammatory cytokine levels that are known to drive the progression of AD and type 2 diabetes. Accordingly, we studied FBC interactions with human serum BuChE at various BuSch concentrations. The significant R^2 values in Fig. (3) prove smooth enzymatic reactions occur in the current study. The kinetics data of human serum BuChE inhibition by FBC in the presence of different concentrations of substrate was elucidated in detail using two types of analysis software, namely GOSA and PRISM. A plot according to the general kinetic equation of the GOSA package presented in Fig. 2a(A) yielded k_s , k_i and k_{cat} values as 0.342 ± 0.19 mM, 0.24 μ M and 0.089 ± 0.027 au, respectively. After adjusting this data in general kinetic equation, values of its a, b and c factors were obtained. The estimated values and their limits so obtained were indicative of a partial mixed inhibition system in this interaction. Hence, the data was reanalyzed as per the inhibition pattern of a partial-mixed type (Fig. 2a(B)). The value obtained for the four kinetic constants: k_m , k_{i1} , k_{i2} and V_{max} were found to be 0.419 mM, 11.79 μ M, 16.03 μ M and 0.0885 μ M/ μ g/min, respectively. According to the literature, there is no sound data to compare these kinetic values particularly for k_m or k_s , and k_{cat} or V_{max} regarding human serum BuChE. Tezcan and co-workers reported k_s values for purified human serum BuChE, human serum BuChE and sheep brain BuChE as 0.128 ± 0.012 mM, 0.11 mM and 0.14 mM, respectively [34–36].

For a comparative analysis, the reciprocal kinetic data of human serum BuChE inhibition by FBC corresponding to varied substrate-concentrations was evaluated by a classical Lineweaver-Burk plot (Fig. 2a(C)), Dixon plot (Fig. 2a(D)), secondary-replots (constructed from the Lineweaver-Burk plot) (Fig. 2a(E–G)) and secondary-replots of Dixon plot (Fig. 2a(H, I)). By these plots, the mode of inhibition seems to be that of a mixed type. The effect of the pre-incubation period on inhibition of BuChE (obtained from human serum) with FBC at 0–70 min preincubation time is illustrated in Fig. 2b(G), where it is obvious that an increase in preincubation period did not decrease percentage Product maximum ($P_{\max}$), which indicated a reversible nature of the inhibition; while the same was reduced with respect to an increase in the concentration of FBC (Fig. 2b(H)). This tertiary replot of plots in G was found fruitful in obtaining a new constant, $P_{\max PT50}$ (0.149 μ M). A significant insight in correctly elucidating the mechanism of action of a putative drug or enzyme-inhibitor is obtained from its kinetic-analyses. This is illustrated in Fig. (4b) in the light of our findings. Moreover, Yan, *et al.* have reported that a peptide mimic of amylin blocked the cytotoxicity of amyloid β and hence proposed yet another molecular link between AD and type 2 diabetes [37]. We extend both our prior studies and those of others by evaluating the inhibition of human BuChE with a small molecular weight inhibitor, FBC, that is structurally related but different from several agents on the same backbone (Fig. 1A) that are being clinically evaluated in AD. Docking results confirmed that two strong interacting sites exist in BuChE protein for binding of FBC. One of those binding sites was previously identified [38]. It was located in a deep narrow groove lined with polar aromatic residues Trp82, 430, Thr-Pro-Ser (284,285,287). It is noteworthy that the second site which was identified during this study displayed better interaction with FBC. The groove, described herein as ‘the second site’ was composed of aliphatic and sulphur containing residues *i.e.* Met 302, Asp303, Pro304, Cys400 and Pro-401 where Asp displayed H-bond interaction as well.

CONCLUSION

Enzoinformatics and enzyme kinetic analyses performed in the current study support FBC as an interesting AD drug candidate displaying a partial mixed type of inhibition of human BuChE. An increasing amount of evidence supports the hypothesis that well tolerated small molecular weight experimental drugs that selectively inhibit BuChE, such as FBC, may have therapeutic value in not only AD but also type 2 diabetes. Clearly, in vivo research - based on the favorable binding interactions demonstrated and quantitatively characterized in the present study of FBC induced inhibition of human BuChE activity - is required to determine whether the potency found in our *ex vivo* study translates to the brain, particularly to regions impacted by AD. Further such studies on FBC and analogs in the clinic, such as bisnorcymserine, phenserine and Posiphen are warranted, as is the application of Enzoinformatics to other areas of drug design.

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LIST OF ABBREVIATIONS

ACh	Acetylcholine
AChE	Acetylcholinesterase
AChE-Is	Acetylcholinesterase Inhibitors
AD	Alzheimer's Disease
APP	Amyloid- β Precursor Protein
Aβ	Amyloid- β Peptide
BuChE	Butyrylcholinesterase
BuSCh	Butyrylthiocholine Iodide
BuChE-Is	Butyrylcholinesterase Inhibitors
ChEs	Cholinesterases
ChE-Is	Cholinesterase Inhibitors
CNS	Central Nervous System
FBC	Fluorobenzylcymserine
I_{bc}	Innovative Binding Constant
K_i	Inhibition Constant
K_m	Michaelis-Menten Constant
V_{max}	Apparent Maximal Activity

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Fig. (1).

a). Chemical structures of **A:** (-)-Phenserine (AChE selective), **B:** (-)-Cymserine (semi-BuChE selective), **C:** Dihydrobenzodi-oxepine cymserine, **D:** (±)-Tetrahydrofurobenzofuran cymserine (BuChE selective), **E:** Fluorobenzylcymserine. **b).** BuChE inhibition by FBC with respect to various levels of protein concentrations and reaction times. **A,** 12 µg; **B,** 36 µg; **C,** 60 µg; **D,** 90 µg and **E,** 120 µg serum protein where □ for 0.002 µM, ▲ for 0.2 µM, ▽ for 10 µM, ◆ for 20 µM FBC. **F.** Secondary plot of data presented in A–E where □ represents for 12 µg, ▲ for 36 µg, ▽ for 60 µg, ◆ for 90 µg protein concentration. **G.** Tertiary data derived from F, where V_i is maximum overall comparative activity index of BuChE in the presence of FBC. **H.** Correlation of IC_{50} with the amount of BuChE in the assay. **c).** Demonstration of human serum BuChE inhibition by FBC in the presence of different concentrations of substrates and FBC at various reaction times. **A,** 0.05 mM BuSchI; **B,** 0.125 mM BuSchI; **C,** 0.2 mM BuSchI; **D,** 0.35 mM BuSchI and **E,** 0.50 mM BuSchI where ■ for control, △ for 0.10 µM FBC, ▽ for 0.20 µM FBC, ◇ for 10 µM FBC and ● for 20 µM FBC.

Fig. (2).

a). Reversible kinetics of human serum BuChE inhibition by FBC in the presence of different concentrations of substrates and FBC. **A**, Plot according to general kinetic equation. **B**, Plot according to partial mixed inhibition system. **C**, Lineweaver-Burk plot where ■ for control, Δ for 0.10 μM FBC, ∇ for 0.20 μM FBC, $\diamond$ for 10 μM FBC and ● for 20 μM FBC. **D**, Dixon plot where ■ for 0.05 mM BuSch, Δ for 0.125 mM BuSch, ∇ for 0.20 mM BuSch, $\diamond$ for 0.35 mM BuSch and ● for 0.50 mM BuSch. **E**, **F**, **G**, Secondary replot of Lineweaver-Burk plot. **H**, **I**, Secondary replot of Dixon plot. **b).** Demonstration of effect of preincubation period on the human serum BuChE-inhibition with FBC. **A**, 0 min preincubation time (PT). **B**, 6 min PT. **C**, 20 min PT. **D**, 34 min PT. **E**, 50 min PT. **F**, 70 min PT. **G**, Secondary replot of plots in A–F. **H**, Tertiary replot of plots in G.

Fig. (3).

a). In this figure, the drug-BuChE complex is shown after docking is completed. Ligand is bounded at two binding sites within the internalized pocket of BuChE that ACh molecules enter and become rapidly hydrolysed; one is shown in hot pink color (already predicted site) in stick format and the second in cyan-green color in stick format. BuChE binding domain is shown in surface format. **b).** In this figure the interacting residues of the BuChE binding domain are in stick format and ligand in surface and net format with violet color; where TRP82, TRP430, ASP70, THR284, PRO285, SER287, PHE329, ALA328, TYR332, TYR440, HIS438 and MET 437 are binding residues shows interaction with the ligand (violet). Moreover SER287 also has hydrogen bonding interaction with FBC. **A)** Shows front of binding site of FBC with BuChE. **B)** In this back side of interacting residues is shown. **C)** Right side of interacting point is shown and **D)** shows left side of interacting point. **c).** In this figure the interacting residues of the BuChE binding domain are in stick format and ligand in surface and net format with violet color; where ASN228, PRO230, PRO303, PRO577, VAL233, VAL294, SER235, LEU236, MET302, ASP304, TYR396, CYS400, CYS401 and TRP522 are binding residues having interaction with the ligand (cyan green). Moreover ASP304 also has hydrogen bonding interaction with FBC. **A)** Shows front of binding site of FBC with BuChE. **B)** In this back side of interacting residues is shown. **C)** Right side of interacting point is shown and **D)** shows left side of interacting point.

Fig. (4).

a). Scheme 1- Similarities between type 2 diabetes and type 3 diabetes (*e.g.*, Alzheimer's disease (AD)) reflecting a vital role for butyrylcholinesterase (BuChE) in regulating acetylcholine (ACh) levels (a key neurotransmitter that modulates the immune system via the "cholinergic anti-inflammatory pathway"). Hence, selective BuChE inhibition by a well tolerated anti-AD agent may provide a potentially valuable indirect action for mitigating type 2 diabetes by elevating ACh levels and consequently reducing proinflammatory cytokine levels which are known to drive the progression of both AD and type 2 diabetes.

Abbreviations: eNO, endothelial-nitric oxide; LP, lipid peroxides; MD, membrane damage; VC, Vaso-constriction; IS, Immune-system; HLB, high level of BuChE; LGS I, Low-Grade Systemic-Inflammation; NS, Nervous System. **b).** Scheme 2 - Understanding of interaction of enzyme (BuChE), substrate (BuSch) and the inhibitor (FBC) in current finding of a partial mixed type of inhibition system.