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Kinetics of human butyrylcholinesterase inhibition by 1,9-dimethyl-methylene blue

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Abstract: Alzheimer's disease (AD) is an irreversible and progressive neurodegenerative disorder, characterized by β-amyloid plaques, neurofibrillary tangles, and loss of cholinergic neurons. Butyrylcholinesterase (BChE) inhibition is a critical strategy for the treatment of AD since BChE causes inactivation of neurotransmitter acetylcholine and has positive effects on promoting the formation of β-amyloid fibrils. Our previous studies showed that various phenothiazine-derived compounds such as thionine and toluidine blue O (TBO) cause a potent inhibition of human cholinesterases. TBO was also found to affect amyloid precursor protein processing in-vitro and in-vivo models of AD. In this study, it was aimed to determine the inhibitory effect of 1,9-dimethyl-methylene blue (DMMB), a phenothiazine-derived compound, on human plasma BChE and explore its inhibitory mechanism. The inhibition of human BChE was assessed by the colorimetric method of Ellman using butyrylthiocholine as substrate and 0-0.375 µM of DMMB. The kinetic findings showed that DMMB acts as a linear mixed-type inhibitor of human BChE with K_i value of 23 ± 0.004 nM and α= 3.6 ± 1.6. In conclusion, DMMB, which is a potent inhibitor effective at nM level, may be helpful in designing new cholinesterase inhibitors for the treatment of AD.

Keywords: Alzheimer's disease, butyrylcholinesterase, 1,9-dimethyl-methylene blue, cholinesterase inhibition, phenothiazine.

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INTRODUCTION

There are two types of cholinesterases in all mammalian tissues: Acetylcholinesterase (AChE, EC 3.1.1.7) and butyrylcholinesterase (BChE, EC 3.1.1.8) (1). These are known as sister enzymes, but they differ from each other genetically, structurally and for enzyme kinetics. BChE (also called as pseudocholinesterase or plasma cholinesterase) is a serine hydrolase that has a toxicological and clinical importance in scavenging and detoxifying ester containing compounds like succinylcholine, aspirin, cocaine, organophosphates, carbamate pesticides, and chemical warfare agents (1-3). It can accommodate larger substrates and displays wider substrate specificity than AChE (3). AChE is mainly localized in neurons whereas BChE is primarily expressed in white matter and glia (4). Alzheimer's disease (AD) is a cerebral disorder characterized clinically by problems with memory function, cognitive decline and behavioral impairments (5). Histopathological hallmarks of the disease include extracellular aggregates of the amyloid β-peptide (Aβ), so-called amyloid plaques in the parenchyma of the brain and intraneuronal neurofibrillary tangles containing abnormally phosphorylated tau (6). Moreover, the cholinergic dysfunction is accompanied by a progressive decline in the level of neurotransmitter acetylcholine (7-9). Unfortunately, currently approved pharmacotherapies including cholinesterase inhibitors (ChEIs) and *N*-methyl D-aspartate antagonists provide only transient symptomatic benefit and there is no effective treatment to prevent or halt the disease yet (10).

Besides of the cholinergic functions, both AChE and BChE are involved in the Aβ fibril formation that increases the neurotoxicity of Aβ peptides (11). In addition to these results, several ChEIs such as tacrine, donepezil, or huperzine A have been shown

to have neuroprotective effects against amyloid-induced toxicity (12). Due to the reciprocal connections between amyloid pathology and cholinergic function, the development of new ChEIs which reduce the hallmarks of AD has attracted great attention (13). Nowadays, cationic phenothiazine-derived compounds have shown to be prominent drug candidates for the treatment of AD due to their inhibitory effects on cholinesterase activity (14, 15), Aβ pathology, and tau aggregation (16-18). Phenothiazines which are six-membered heterocyclic compounds containing nitrogen and sulfur were discovered during second half of the 19th century. The first clinical use of phenothiazines was for the treatment of malarial infections (19). These compounds can exhibit inhibitory effects on several proteins due to their chemical structure (20, 21). For instance, they can inhibit calcium-dependent proteins such as calmodulin and protein kinase C which have important roles in cellular physiology (22). Phenothiazine-structured compounds are the most commonly prescribed psychotropic drugs in the world (23). Apart from their main neuroleptic actions, these compounds also show a wide spectrum of pharmacological/biological activities such as antifungal, antiprotozoal, antiviral, antihistaminic, antibacterial, or antiemetic activities (19). They have been also suggested to destroy cancer cells by targeting various signaling pathways in vitro and in vivo, but the most outstanding mechanism is their directly damaging effect on DNA (19, 24). Among phenothiazine-structured compounds, methylene blue (MethB) is a previously known cholinesterase inhibitor (25) which has shown promising results in phase II clinical trials for the treatment of AD (26).

In a recent research performed in our laboratory, we have tested the inhibitory effects of numerous phenothiazine-structured compounds on different types of cholinesterases (15). The findings showed that toluidine blue O (TBO) and thionine (TH) are highly potent inhibitors of both human BChE and human erythrocyte AChE with K_i in the nM-μM range (15). In addition, TBO was also found to affect amyloid precursor protein processing in-vitro and in-vivo models of AD (16, 17). These results encouraged us to test whether a structurally closely related cationic phenothiazine compound, 1,9-dimethyl-methylene blue (DMMB) (Figure 1) shows an inhibitory effect on human plasma BChE.

In the present study, the inhibitory effect of DMMB and its inhibitory mechanism were determined on human plasma BChE for the first time. The kinetic results indicate that DMMB has a high inhibitory potential on human plasma BChE with a K_i value in nM range.

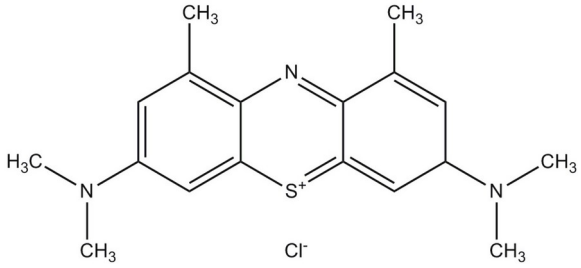


Figure 1. The chemical structure of 1,9-dimethyl-methylene blue.

EXPERIMENTAL SECTION

Chemicals

All reagent grade chemicals including butyrylthiocholine iodide (BTC), 5,5′-dithiobis (2-nitrobenzoic acid) (DTNB, Ellman's reagent), and DMMB were purchased from Sigma-Aldrich (Germany). All other chemicals were purchased from Merck or Sigma-Aldrich (Germany), if not indicated otherwise. Stock solution of DMMB (4.8 mM) was freshly prepared in methanol on the day of use.

Purification of Butyrylcholinesterase

Purification of human BChE from outdated human plasma was performed in two steps:

1. DEAE-Trisacryl anion exchange chromatography (Sigma-Aldrich)
2. Affinity chromatography on procainamide Sepharose 4B as described previously (27). (Specific activity, 44 U/mg; purification, 250 fold).

Inhibitory Potency of DMMB

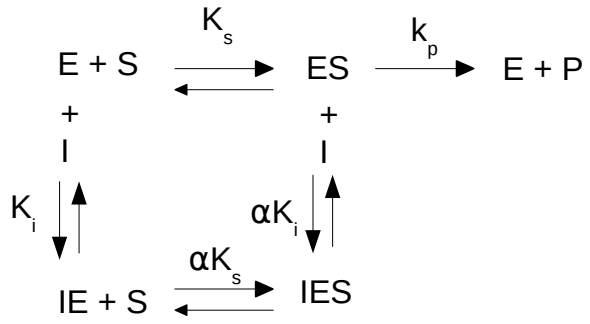
The inhibitory potential of DMMB on BChE was tested at different inhibitor concentrations (0.03125 μM, 0.0625 μM, 0.125 μM, 0.25 μM and 0.375 μM) in the presence of 0.4 mM BTC. The half-maximal inhibitory concentration (IC_{50}) value was calculated by plotting a graph of percent remaining activity versus log [inhibitor] by using GraphPad Prism 5.0.

Butyrylcholinesterase Activity Assay and Inhibition Studies

BChE hydrolysis of BTC (0.05-0.4 mM) was measured spectrophotometrically in MOPS buffer (50 mM, pH 8) at 25°C in the presence of DTNB (0.125 mM) according to the Ellman method (28). The reactions were started by adding 0.01 U/mL BChE. The rate of increase of absorbance was monitored at 412 nm on a Shimadzu UV-1601 UV-Visible spectrophotometer (Kyoto, Japan). Enzyme activity was determined according to the linear segments of the progress curves in the initial 60 sec period using the extinction coeffient of 14.2 mM^{-1} cm^{-1}. The inhibition of BChE was studied by adding 0-0.375 μM DMMB to the reaction mixture (final volume was 1.2 mL). The presence of methanol (≤1.25% (v/v)) in the reaction mixture did not affect enzyme activity (14).

Kinetic Analysis

The kinetic parameters of inhibitory activity of DMMB on human BChE was evaluated at 5 different concentrations of BTC and 6 different concentrations of DMMB. The initial rate data were analyzed according to a simplified rapid equilibrium model for linear mixed-type inhibition (Scheme I; β = 0). The corresponding rate equation, Dixon equation and the [S]-dependence of the observed slope in Dixon plots of 1/V versus [I] at constant [BTC] are shown in Equations I, II, and III, respectively (29). The kinetic parameters inactivation rate constant (K_i) and (α) were calculated from Dixon slope replots using Equation (III) (29).



Scheme I. Rapid equilibrium model for linear mixed-inhibition.

E: enzyme; S: substrate; I: inhibitor; ES: enzyme-substrate complex; IE: inhibitor-enzyme complex; IES: inhibitor-enzyme-substrate complex; P: product; K_s: the dissociation constant for ES complex; K_i: the dissociation constant for the breakdown of IE complex to E+I; k_p: the rate constant for the breakdown of ES complex to E+P; αK_i: the dissociation constant for the breakdown of IES complex to ES+I and αK_s: the dissociation constant for the breakdown of IES complex to IE+S.

Equations I and III were derivated from simplified rapid equilibrium model for linear mixed-type inhibition (Scheme I; β=0). If $[E]_{Total}$ is written in terms of [ES], equation I can be obtained: (v: initial velocity; k_p: the rate constant for the breakdown of ES to E+P; V_{max}: maximum velocity; $[E]_{Total}$: total concentration of enzyme; [E]: concentration of free enzyme, [ES]: concentration of enzyme-substrate complex; [EI]: concentration of enzyme-inhibitor complex; [EIS]: concentration of enzyme-inhibitor-substrate complex; K_s: the dissociation constant for ES complex; K_i: the dissociation constant for the breakdown of IE complex to E+I; αK_i: the dissociation constant for the breakdown of IES complex to ES+I; αK_s: the dissociation constant for the breakdown of IES complex to IE+S).

$$V = k_p[ES]$$

$$[E]_{Total} = [E] + [ES] + [EI] + [EIS]$$

$$K_s = \frac{[E][S]}{[ES]}; [E] = \frac{[ES]K_s}{[S]}$$

$$K_i = \frac{[E][I]}{[EI]}; [EI] = \frac{[E][I]}{K_i} = \frac{[ES]K_s[I]}{[S]K_i}$$

$$\alpha K_i = \frac{[ES][I]}{[EIS]}; [EIS] = \frac{[ES][I]}{\alpha K_i}$$

$$[E]_{Total} = \frac{[ES][K_s]}{[S]} + [ES] + \frac{[ES]K_s[I]}{[S]K_i} + \frac{[ES][I]}{\alpha K_i}$$

$$[E]_{Total} = [ES]\left[\frac{K_s}{[S]} + 1 + \frac{K_s[I]}{[S]K_i} + \frac{[I]}{\alpha K_i}\right]; [ES] = \frac{[E]_{Total}}{\frac{K_s}{[S]} + 1 + \frac{K_s[I]}{[S]K_i} + \frac{[I]}{\alpha K_i}}$$

▪

$$V = k_p[ES]$$

$$V = \frac{k_p[E]_{Total}[S]}{K_s\left[1+\frac{[I]}{K_i}\right]+[S]\left[\frac{[I]}{\alpha K_i}\right]}$$

$$V_{max} = k_p[E]_{Total}$$

$$V = \frac{V_{max}[S]}{K_s\left(1+\frac{[I]}{K_i}\right)+[S]\left(1+\frac{[I]}{\alpha K_i}\right)} \quad \text{(I)}$$

The rate equation (Eq.1) for linear mixed type may be converted a linear form in which the varied ligand is [I] (Eq.2).

$$V^{-1} = \frac{[S]+\alpha K_s}{\alpha K_i V_{max}[S]}[I]+\frac{[S]+K_s}{V_{max}[S]} \quad \text{(II)}$$

(Dixon Equation)

$$Dixon\ \ slope = \frac{K_s}{K_i V_{max}[S]}+\frac{1}{\alpha K_i V_{max}} \quad \text{(III)}$$

RESULTS

Inhibitory Potency of DMMB

The human BChE activity was found to be inhibited in a dose-dependent manner, with an estimated IC_{50} value of 116 nM (Figure 2). The percent remaining activity showed a residual activity of 18% at the highest dose of DMMB.

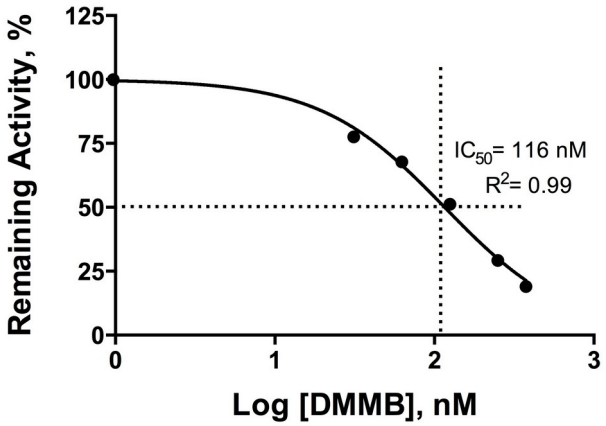


Figure 2. Inhibitory activity of DMMB against human plasma BChE. Dose response curve for the quantification of the correlation between DMMB concentration and the inhibitory effect. 100% equals the BChE activity in the absence of DMMB. IC_{50}: Half-maximal inhibitory concentration.

Inhibition of Human Butyrylcholinesterase by DMMB

Kinetic analysis results showed that DMMB acts as a linear, reversible inhibitor of human plasma BChE. The Dixon plots were found to be linear ($\beta=0$) at different substrate concentrations. Dixon plots and the secondary slope replot of the inhibition of human BChE by DMMB are shown in Figure 3A and Figure 3B. The slope replot of DMMB pointed to a linear mixed-type inhibition ($1<\alpha<\infty$) of human plasma BChE. K_i value was found to be 23 (±0.004) nM and α value was 3.6 (±1.6) derived from Dixon slope replot (based on K_s=32.2 (±2.03) μM and V_{max}=10 (±0.58) μM/min using Lineweaver-Burk plot

without DMMB) (Table I). Since the value of α is greater than 1 and smaller than ∞, this situation also supports that DMMB acts as a linear mixed-type inhibitior of BChE.

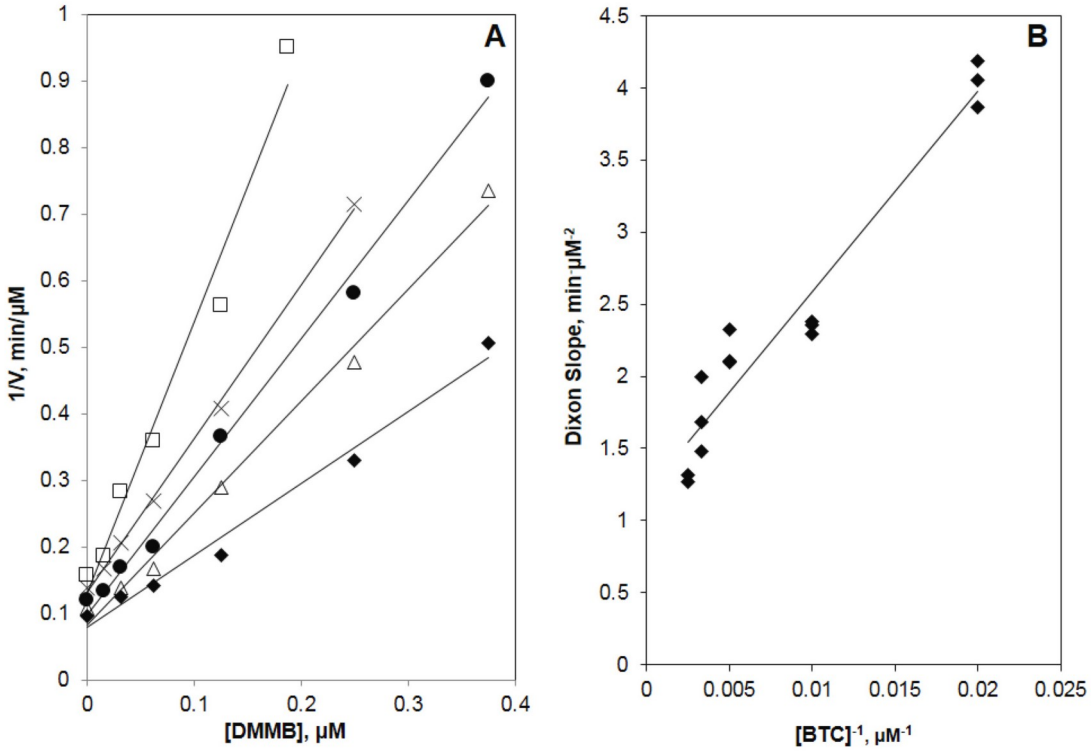


Figure 3. The inhibition of human BChE by 1,9-dimethyl-methylene blue. (A) Dixon plots of the inhibition at 400 (◆), 300 (△), 200 (●), 100 (×) and 50 (□) μM butyrylthiocholine. Each point is the average of three independent experiments (B) Slope replot of pooled data from three independent experiments.

Table 1: Kinetic parameters for the inhibition of human BChE by DMMB.

Enzyme	Inhibitor	Inhibition type	K_i, nM	α[b]	β[c]
Human Butyrylcholinesterase	1,9-dimethyl-methylene blue	Linear mixed	23±0.004[a]	3.6±1.6	0

[a] All data are shown as mean ± SD. [b] α is the factor by which Ks changes when inhibitor occupies the enzyme. [c] reduction in the catalytic kinetic constant is captured by a factor, β.

DISCUSSION

BChE can hydrolyze the neurotransmitter acetylcholine like AChE and it is one of the important targets in the treatment of AD (30). Recent evidences have suggested that BChE shows an important effect on modulation of motor control, cognition and behavior due to possible regulatory function of BChE on acetylcholine levels. Besides, BChE inhibitors have been shown to improve learning performance in rats and mitigate neurotoxic β-amyloid peptide levels (31). It was reported that there is a positive correlation between increased levels of BChE and development of amyloid-rich neuritic plaques in AD patient brain tissues (32). Darvesh et al. has suggested that knock-out of BChE gene in a mouse model, including five human familial AD genes (5XFAD) showed reduced fibrillar Aβ plaque deposition due to lack of BChE (33). Also, according to an AChE knockout mouse study, BChE was found to be more abundant than AChE activity in most tissues of mice (34). In AD, AChE activity decreases progressively in specific brain regions while BChE activity dramatically increases (35, 36). Therefore, it is noteworthy to explore new BChE inhibitors due to their possible inhibitory effects on cholinesterases or amyloid precursor protein (APP) metabolism for the treatment of AD. Cationic phenothiazine-derived compounds have been used since long time ago because of their pharmacological or biological activities (37) and most of them are in clinical use (38). Among these compounds, MethB has recently gained prominence as a potential therapeutic for the treatment of neurodegenerative disorders like AD. MethB can significantly inhibit both AChE and BChE (25, 39). This inhibition may show, at least in part, beneficial contribution to treatment of AD (40). Despite having a cationic structure, MethB can cross through the blood-brain barrier (BBB) after

conversion to its reduced form (uncharged leuco-form) (40). The fact that nerve tissues have a high affinity for the leuco form of MethB was confirmed by exposing tissue sections to air (or by treating them with iron chloride). This resulted in the conversion of the reduced form of MethB to its oxidized colored form (41).

DMMB, also known as Taylor's blue, is a structurally related derivative of MethB and contains two additional methyl groups (42). DMMB is widely used in the tissue staining applications due to its metachromatic property (43). In terms of its photodynamic action, DMMB has been shown to be more efficacious on tumor cells, viruses, and parasites (43, 44) than MethB. DMMB has lipophilic nature. Although there is no evidence, DMMB is expected to cross BBB similar to MethB. The results of Taylor et al. indicate that DMMB not only stains erythrocyte membranes but also stains the entire erytrocyte blue-green (42). It has shown that pharmacological actions of DMMB are superior to those of MethB. For instance, DMMB exhibits higher potency in inhibiting monoamine oxidase A, compared to MethB (45). The photodynamic action of DMMB has been found to be more efficacious in treatment of microbial infections due to its high lipophilic character, compared to other photosensitizers, including MethB (46). An earlier study has demonstrated that MethB yielded a complex pattern of human BChE inhibition (Intrinsic K'=420±0.04 nM) and indicates cooperative binding at more than one site (47) whereas our results show that DMMB is a linear mixed-type inhibitior of human BChE (23±0.004 nM) and it is ~18-fold more potent than MethB. Although DMMB shows the same kinetic pattern as ethopropazine (K_i=37±0.07; α=8.4±2), a phenothiazine-derived compound (47), DMMB inhibits human plasma BChE more strongly. A recent study also demonstrated that DMMB has a 55-fold higher potency than MethB for inhibition of tau-tau binding in vitro (48). Therefore, DMMB deserves more detailed studies as a potential candidate for the treatment of AD.

In our previous studies, various phenothiazine-structured compounds have been searched for possible inhibition of human cholinesterases (15). The findings showed that methylene violet (MV) caused a linear mixed type-inhibition and K_i value was 0.66±0.06 μM. Toluidine blue O (TBO; K_i=0.008±0.003 μM) and thionine (TH; K_i=2.1±0.42 μM) acted as nonlinear inhibitor of human BChE. Besides, TBO also caused linear mixed-type inhibition of human erythrocyte AChE with K_i=0.041±0.05 μM (15). In addition to its strong inhibitory effects on both AChE and BChE, it was shown that both TBO and TH mitigate the levels of secreted Aβ peptides in cell model of AD (PS70 cells) while only TBO affects hippocampal amyloid pathology by decreasing the levels of insoluble Aβ species in a transgenic mouse model of AD (16, 17). In our recent study, azure B, the major metabolite of MethB, has been shown to decrease the levels of secreted APPα and Aβ peptides significantly. A significant decrease has also been observed in the levels of intracellular total APP (49). Considering our previous results, a phenothiazine-structured compound, DMMB which is a highly effective BChE inhibitor (23±0.004 nM) can be also a potent inhibitor of AChE. Further testing would be also useful for designing and development of new cholinesterase inhibitors. This study represents the first report of inhibition of human BChE by DMMB. AD is a multifactorial disease and due to direct relationship between cholinergic system and APP metabolism, it is also worth determining the effects of DMMB on APP metabolism in future investigations.

CONCLUSION

These kinetic findings indicate that DMMB is a potent inhibitor of human plasma BChE with K_i value in nM range. In conclusion, DMMB may be useful in designing new cholinesterase inhibitors for the treatment of AD.

CONFLICT OF INTEREST

The author declares that there are no conflicts of interest.

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