Research Article

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New insights into the interaction between mammalian butyrylcholinesterase and amitriptyline: a combined experimental and computational approach

Memeli bütünilerikolinesterazı ile amitriptilin arasındaki etkileşime yönelik yeni anlayışlar: Birleşik deneysel ve hesapsal bir yaklaşım

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Abstract

Background: Today, there is a growing recognition in the scientific community of the many roles of butyrylcholinesterase (BChE) in both physiological and pathological contexts.

Objective: Here, we aim at providing an accurate and comprehensive understanding of the mechanistic and structural aspects of mammalian BChE inhibition by the tricyclic antidepressant amitriptyline (AMI).

Materials and methods: The present work involves enzyme kinetic studies as well as protein–ligand docking and interaction profiling studies.

Results: We verify that AMI acts as an effective, mixed-type inhibitor of mammalian BChE, with an IC$_{50}$ value of 10 μM and a K$_i$ value of 2.25 μM. We also provide evidence showing that AMI penetrates deep into the active-site gorge of BChE where it interacts noncovalently with both the choline-binding and catalytic residues.

Conclusion: These findings could facilitate the prevention of the adverse metabolic sequelae of acquired BChE deficiency and also the design of new reversible anticholinesterase drugs.

Keywords: Butyrylcholinesterase; Plasma cholinesterase; Amitriptyline; Tricyclic antidepressant; Enzyme inhibition; Molecular docking.

Öz

Genel bilgiler: Günümüzde bütünilerikolinesterazın (BChE) hem fizyolojik hem de patolojik bağlamlardaki sayısız rolü, bilim camiasında giderek artan bir şekilde kabul görmekteirdir.

Amaç: Bu çalışmada memeli BChE’nin trisiklik antidepresan amitriptilin (AMI) tarafından inhibisyonunun mekanistik ve yapısal yönlerinin doğru ve kapsamlı bir biçimde anlaşılmasını hedefledik.

Gereç ve yöntemler: Mevcut araştırmada, enzim kинettiği çalışmalarının yanı sıra protein–ligand yerleştirmeye ve etkileşim profilendirme çalışmalarını içinde barındırmaktadır.

Bulgular: AMI’nin 10 μM’lık IC$_{50}$ ve 2.25 μM’lık K$_i$ değerleri ile etkin, karşı tıpte bir memeli BChE inhibitörü olarak faaliyet gösterdiğini doğruladık. Aynı zamanda AMI’nin aktif bölge oluşuna derinlemesine nüfuz ettiği ve burada hem kolin bağlayıcı hem de katalitik amino asitlerle kovalent olmayan şekilde etkileşimde bulunduğu gösteren kanıtlar sunduk.

Sonuç: Bu bulgular, kazandığımız BChE eksikliğinin olumsuz metabolik neticelerinin önlenmesinde ve ayrıca yeni, tersinir antikolinesteraz ilaçların tasarımıda kolyak yayılabilecektir.

Anahtar kelimeler: Bütirilikolinesteraz; Plazma kolinesterazi; Amitriptilin; Trisiklik antidepresan; Enzim inhibisyonu; Moleküler yerleştirmeye.

Introduction

Butyrylcholinesterase (BChE; EC 3.1.1.8) belongs to the carboxylic ester hydrolase family of enzymes and, in spite of its almost ubiquitous distribution among tissues and organs, is best known for its abundant presence in plasma (hence its alternative names of ‘plasma cholinesterase’ and ‘pseudocholinesterase’). For many years, the physiological function of BChE remained unknown, since individuals with effectively no BChE activity appear to be...
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in good health. This, however, is not in accord with the continued existence of the enzyme through many millions of years in a relatively large number of vertebrate species. It is now established that BChE promotes detoxification by neutralizing drugs, pesticides, and other reactive chemicals including alkaloids both in plasma and in the brain and facilitates neurotransmission by hydrolyzing acetylcholine both in the central nervous system and at the neuromuscular junction of the somatic nervous system [reviewed in 1]. BChE also hydrolyzes ghrelin, possibly modulating levels of this so-called ‘hunger hormone’ in the peripheral circulation [2]. Perhaps paralleling the aforementioned emerging roles of the enzyme, there is a growing body of evidence demonstrating that reduced BChE activity is related with a number of diseases such as liver cirrhosis [3] and chronic obstructive pulmonary disease [4]. In addition, BChE deficiency, which usually arises from natural variations in the gene encoding BChE, leads to prolonged apnea and paralysis following the administration of local anesthetics (e.g. cocaine) and muscle relaxants (e.g. succinylcholine and mivacurium) [reviewed in 5]. On the other hand, elevated BChE activity is associated with Alzheimer’s disease [6] and type 2 diabetes [7]. Putting together these data and the results of numerous studies on this curious enzyme, BChE inhibition is expected to have wide-ranging effects in health and disease.

Amitriptyline (AMI; also known by the trade name Elavil® among others) is pharmacologically classed as a tricyclic antidepressant (TCA) and works by boosting the neurotransmitters serotonin and norepinephrine/noradrenaline. Although AMI is at least as efficacious as other TCAs or newer drugs, it is linked to some notable side effects (e.g. sedative effects, dry mouth, constipation, and blurred vision) that may be explained by its anticholinergic activity [8]. Çokuğraş and Tezcan [9] were the first to report that AMI inhibited human serum BChE in a reversible, partially competitive mode. That is, using the substrate analog acetylthiocholine (ATCh), they provided evidence showing that the Michaelis constant ($K_m$) of BChE for its substrate increased and that the maximum velocity ($V_{max}$) of the BChE-catalyzed reaction remained unchanged. A plausible explanation for their findings could lie in the ability of AMI to bind at a subsite other than the catalytic subsite (which was postulated by the same authors to be the choline-binding pocket or the acyl-binding pocket) so as to lower the affinity of the enzyme towards its substrate. Intriguingly, Müller et al. [10] found out that AMI inhibited human serum cholinesterase (which was used interchangeably with BChE in the same report) in a reversible, linear mixed-type mode, where it interfered with ATCh binding (thus increasing the $K_m$) as well as hampered catalysis (thus decreasing the $V_{max}$) in a dose-dependent manner. The presence of two distinct AMI-binding sites, among which one was the catalytic subsite, could possibly provide a rational explanation for the occurrence of the aforementioned mixture of competitive and noncompetitive inhibitory effects. Here, using enzyme kinetic measurements as well as protein–ligand docking and interaction profiling, we attempted to resolve the discrepancy between those two experiments and obtain further information on the structural basis of BChE–AMI interaction.

Materials and methods

Reagents

A highly-purified formulation of butyrylcholinesterase from equine serum (BChE; catalog no.: CI057), the substrate analog S-butyrylthiocholine iodide (BTCh; catalog no.: B3253), the chromogenic reagent 5,5′-dithio-2-bis-nitrobenzoate (DTNB; catalog no.: D8130), and amitriptyline hydrochloride (AMI; catalog no.: A8404) were all purchased from Sigma-Aldrich, Inc., St. Louis, MO, USA.

Protein preparation and quantification

BChE was reconstituted from a lyophilized powder with 20 mM MOPS–KOH buffer at pH 7.5. Protein concentration was determined by using a UVS-99 Micro-volume UV/Vis Spectrophotometer (ACTGene, Inc., Piscataway, NJ, USA) based on the spectrophotometric protein assay of Kalb and Bernlohr [11].

Enzyme activity assay

BChE activity was assayed in a 1-cm-pathlength quartz cuvette blackened on sides on a LAMBDA 25 UV/Vis Spectrophotometer (PerkinElmer, Inc., Waltham, MA, USA) according to the colorimetric method of Ellman et al. [12] with slight modifications. The final reaction volume was 500 μL, consisting of 160 μL of double-distilled water, 250 μL of 200 mM MOPS–KOH buffer at pH 7.5, 10 μL of BTCh (dissolved in 20 mM MOPS–KOH buffer, pH 7.5), 50 μL of DTNB (dissolved in 200 mM MOPS–KOH buffer, pH 7.5), 25 μL of equine serum BChE (dissolved in 20 mM MOPS–KOH buffer, pH 7.5), and 5 μL of AMI (dissolved
in methanol or solvent (methanol). The presence of 1% (v/v) methanol in the reaction mixture did not affect BChE activity. The reaction was initiated by the addition of BChE and subsequent rapid mixing of the contents. The resulting increase in absorbance at 412 nm due to the development of yellow color was monitored for 20 s at 37 °C. Reactions were independently carried out three times in series, and mean values were calculated from triplicate measurements. One unit of BChE activity was defined as the amount of enzyme producing 1 μmol of the chromophore thionitrobenzoate per minute. Volume activities were converted into specific activities (in U mg⁻¹ protein) by using the extinction coefficient for thionitrobenzoate, 13.6 mM⁻¹ cm⁻¹.

**Dose–response relationship assessment**

The BChE inhibitory potency of AMI was measured at varying inhibitor concentrations (0.24 μM, 0.49 μM, 0.98 μM, 1.95 μM, 3.91 μM, 7.81 μM, 15.63 μM, 31.25 μM, 62.5 μM, 125 μM) in the presence of 1 mM BTCh, 0.25 mM DTNB and 1.95 μg mL⁻¹ equine serum BChE. The resulting data were fitted with a dose–response curve shaped like an inverted, or upside-down, hyperbola [i.e. a percent remaining activity versus (AMI) graph], from which the half-maximal inhibitory concentration (IC₅₀) of AMI was derived. BChE activity in the absence of AMI was regarded as 100%.

**Kinetics of enzyme inhibition**

The determination of the overall BChE activity pattern was achieved by varying the concentration of BTCh (gradually from 0.05 mM to 2 mM) at one of the six different fixed AMI concentrations (0.25 μM, 0.5 μM, 1 μM, 2 μM, 4 μM, 8 μM) that spanned the steepest, or near-linear, region of the dose–response curve. The resulting data were fitted with a Lineweaver–Burk plot [i.e. a 1/specific activity versus 1/(BTCh) graph] as well as with slope and y-intercept replots, from which preliminary information regarding the mode of inhibition was obtained [13].

**Statistical analysis**

Curve-fitting of the enzyme kinetic data with all known mathematical models for inhibitor binding was performed by using the Nonlinear Estimation module of STATISTICA '99 Edition (StatSoft, Inc., Tulsa, OK, USA). The parameter estimates, namely $V_m$ (maximum velocity), $K_s$ (dissociation constant for the enzyme–substrate complex), $K_i$ (dissociation constant for the enzyme–inhibitor complex) and $\alpha$ (factor by which inhibitor binding alters the affinity of the enzyme for its substrate), and their standard errors were calculated with the help of the Hooke–Jeeves pattern moves procedure.

**Receptor/Ligand selection and preparation**

The amino acid sequence of equine BChE was downloaded from the UniProt Knowledgebase (entry: P81908) and used as input to the protein homology modeling Web server CPHmodels Version 3.2 [14]. A comparative model of equine BChE was built based on the high-resolution crystal structure of full-length, recombinant human BChE (entry: 3O9M). The quality of the resulting protein model was assessed by using the three-dimensional profile calculator Verify3D [15]. Equine BChE was subjected to receptor preparation for docking with the aid of the Dock Prep tool of UCSF Chimera, Version 1.11.2 [16]; accordingly, hydrogen atoms were added in a process where the hydrogen-bonding network was optimized and the protonation states of residues at physiological pH were determined. Also, atoms in standard residues were assigned correct formal charges based on the AMBER ff14SB force field. The ready-to-dock, three-dimensional structure of amitriptyline (entry: 00968257) was downloaded from the ZINC Database [17].

**Receptor–ligand docking and interaction profiling**

The prepared receptor and its annotated ligand were collectively used as input to Blind Docking Server [18], which employs a customized version of AutoDock Vina, also known as Vina_vision, to perform an exhaustive search of the whole protein surface. The top clusters ranked according to their computed binding energies (in kcal mol⁻¹) were downloaded from the associated Web site, and the docking pose with the highest affinity in each cluster was visually inspected in the PyMOL Molecular Graphics System, Version 1.8 (Schrödinger, LLC, Portland, OR, USA). The complete enumeration of noncovalent bonds between equine BChE and AMI in the most relevant docking pose was achieved by PLIP [19], a Web server for detecting and displaying intermolecular interactions based on atom types and normal geometric constraints.
Results and discussion

Equine BChE shares 90% sequence identity spread over its entire length (i.e. 574 amino acids) with human BChE, despite the current lack of X-ray crystallographic (or nuclear magnetic resonance spectroscopic) data on its three-dimensional structure. Furthermore, all fourteen critical residues constituting the enzyme’s active-site gorge (D70 and Y332 at the peripheral anionic site; W82, Y128, and F329 in the choline-binding pocket; G116, G117, and A199 in the oxyanion hole; S198, E325, and H438 at the catalytic subsite; and W231, L286, and V288 in the acyl-binding pocket) are absolutely conserved between equine BChE and human BChE. When combined with the relatively low cost and biochemically and biophysically well-characterized nature of the commercially available enzyme, the aforementioned structural features make equine serum BChE a valuable mammalian model enzyme providing a reliable basis for human toxicologic and pharmacologic projections. In this study, our primary aim was to gain an accurate and deep understanding of the mechanistic and structural aspects of mammalian BChE inhibition by AMI in an in vitro setting.

When the percent remaining activity of equine serum BChE as a function of AMI concentration was graphed, AMI was found to inhibit the enzyme in a dose-dependent fashion, with an estimated IC50 value of 10 µM (Figure 1). Within the concentration range tested here, percent remaining activity did not reach zero, even though a gradual decrease in BChE activity was noticeable to the human eye. Given that the therapeutic plasma concentration of AMI in major depression is between 80 and 200 ng mL⁻¹ [20], AMI emerges as a potent inhibitor of mammalian BChE at near-therapeutic doses. And considering that the normal plasma concentration of BChE is around 4.64 µg mL⁻¹ [21], the present enzymatic setting maximizes the clinical relevance of our findings.

Graphing the kinetics of BChE inhibition by AMI, the Lineweaver–Burk plot of 1/specific activity versus 1/[AMI] yielded a family of straight lines intersecting in the second quadrant (Figure 2A). This overall pattern reflects the unique characteristics of mixed-type inhibition, where the effective Kᵢ increases and the effective Vₘₐₓ decreases upon inhibitor binding. As a means to produce helpful insights into the detailed mechanism of BChE–AMI interaction, the replots of slope versus [AMI] and y-intercept versus [AMI] were graphed on the same set of axes (Figure 2B). The resulting pattern illustrated that α is different from and greater than 1. Statistical analysis of the kinetics of BChE inhibition by AMI revealed that the best fit to the experimental data was obtained with a linear mixed-type inhibition model. The corresponding parameter estimates Vₘₐₓ, Kᵢ, Kᵢ', and α were found to be 1070 ± 28 U mg⁻¹ protein, 0.17 ± 0.02 mM, 2.25 ± 0.66 µM, and 3.26 ± 1.52, respectively. These results are in good agreement with the graphical solutions above, further supporting the correct assignment of the mode of inhibition. Because the value of α is small but greater than 1, the mixed-type model becomes a composite of competitive and noncompetitive inhibitory effects. Consequently, AMI binding in the enzyme’s active-site gorge decreases the affinity of mammalian BChE towards BTCh and also decreases the rate of transformation of bound BTCh. Our findings corroborate the study by Müller et al. [10], in which the researchers reported a Kᵢ value of 1.22 µM and an α value of 2.6 for AMI, as derived from their mixed-type model of BChE inhibition. It should be noted, however, that the same research group directly used human serum in their enzyme assays, unlike the highly-purified formulation of equine serum BChE we used. Human plasma is known to also contain paraoxonase, albumin esterase, and acetylcholinesterase, the former two being at high enough levels to play an important part in ester hydrolysis in this body fluid [22]. In their study, Çokuğraş and Tezcan [9] reported a very large α value — that is to say, 1425. It is a known fact that when the α is very large, inhibitor binding prevents the substrate transformation process and the mixed-type model becomes practically identical to competitive inhibition. Although they used a purified formulation of human

![figure1](image)  
**Figure 1:** Inhibitory activity of AMI against horse serum BChE. Dose–response curve for the quantification of the relationship between AMI concentration and the inhibitory effect it produces. IC₅₀: half-maximal inhibitory concentration.
serum BChE in their enzyme assays, there is serious conflict between their findings and our findings.

In an attempt to predict the AMI-binding site(s) on mammalian BChE, we performed a blind docking simulation. The highest-ranked docking solution (calculated binding energy $= -8.50$ kcal mol$^{-1}$) showed that AMI penetrated deep into the active-site gorge of BChE, with its three-ring structure facing the choline-binding subsite and its propylidene side chain, which extends from the central carbocyclic ring, terminating close to the catalytic subsite (Figure 3A). The position of AMI in the active-site gorge of BChE was found to be stabilized through a combination of several noncovalent interactions: (i) one of the two outer benzene rings of the three-ring structure established $\pi$-$\pi$ stacking interactions with W82 in the choline-binding pocket; (ii) the other outer benzene ring and the central carbocyclic (imino) ring formed hydrophobic interactions with W82 and A328, respectively; and the tertiary amino group of the propylidene side chain was involved in a hydrogen-bonding interaction with S198 of the catalytic triad (Figure 3B). The engagement of AMI in various interactions with the choline-binding tryptophan may be the main reason causing the lowered affinity of BChE towards BTCh, while its engagement in a hydrogen-bonding interaction with the catalytic serine may explain the lowered rate of BTCh transformation. Although the unique position of AMI relative to the critical residues involved in the binding/hydrolysis of choline esters is likely to add sufficient structural evidence to the linear mixed-type mode of BChE inhibition, we do not exclude the existence of a second (allosteric) AMI-binding site on the enzyme. This bound AMI, however, could only be visualized in an electron density map of a crystal of the BChE–AMI complex.

In view of these results, it is tempting to speculate that with long-term use of AMI in major depressive disorder, acquired BChE deficiency and subsequent peri- and postoperative complications may develop. This phenomenon has previously been illustrated in a case where sertraline-associated acquired BChE deficiency resulted in prolonged apnea after general anesthesia with succinylcholine [23]. On the other hand, the observed BChE inhibitory potency of AMI can possibly be exploited in the rational design of new reversible anticholinesterases. Reversible anticholinesterases, such as donepezil, rivastigmine, and galantamine, have been approved by the U.S. Food and Drug Administration (FDA) for the symptomatic treatment of mild-to-moderate Alzheimer’s disease. They are believed to stimulate cholinergic transmission indirectly by inhibiting the activity of acetylcholinesterase (AChE) and thus delaying the hydrolysis of acetylcholine at receptor sites. Given the increasingly prominent role of BChE in the pathophysiology of Alzheimer’s disease [24], AMI may guide researchers through the structure-based design of more potent and selective BChE (and perhaps AChE) inhibitors that can be tested as anti-Alzheimer’s drugs.

Intriguingly, our findings suggest a cholinergic role for AMI in the body, which is justified by BChE inhibition in vitro. This seems to be in conflict with the general view that TCAs result in anticholinergic side effects, the central ones being sedation (especially with AMI), memory impairment, and confusion. These effects most probably...
arise from the affinities of TCAs for the muscarinic acetylcholine receptors of the human brain [25]. A tolerance to TCAs normally develops after a few days, and their undesirable side effects then gradually subside in severity. Therefore, it is reasonable to assume that the cholinesterase inhibitory activity of AMI may be partly responsible for the increasing tolerance to AMI over time.

Overall, with its IC$_{50}$ and $K_i$ values in the low-micromolar range AMI is a relatively potent inhibitor of mammalian BChE. Perhaps more importantly, BChE inhibition appears to be clinically relevant at near-therapeutic doses of AMI. The mechanism of the interaction between BChE and AMI is best described by a linear mixed-type model where both competitive and noncompetitive inhibitory regimes are observed. This alters the affinity of the enzyme for its substrate and also impedes the hydrolysis of the bound substrate. We propose that the resulting mechanistic paradigm arises from the concurrent interaction of AMI with both the choline-binding and catalytic subsites of the enzyme’s active-site gorge. Repeated intoxication and/or long-term use of AMI may pose serious health risks by blocking BChE in the circulatory and nervous systems. But considering that TCAs are well studied, widely prescribed and well tolerated (usually at low doses), AMI may serve as a safe template for the design of new anti-Alzheimer’s drugs with cholinesterase-inhibiting activity.

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References


