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Research paper

Mechanism of stereoselective interaction between butyrylcholinesterase and ethopropazine enantiomers

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ABSTRACT

Stereoselectivity of reversible inhibition of butyrylcholinesterase (BChE; EC 3.1.1.8) by optically pure ethopropazine [10-(2-diethylaminopropyl)phenothiazine hydrochloride] enantiomers and racemate was studied with acetylthiocholine (0.002–250 mM) as substrate. Molecular modelling resulted in the reaction between BChE and ethopropazine starting with the binding of ethopropazine to the enzyme peripheral anionic site. In the next step ethopropazine 'slides down' the enzyme gorge, resulting in interaction of the three rings of ethopropazine through π–π interactions with W82 in BChE. Inhibition mechanism was interpreted according to three kinetic models: A, B and C. The models differ in the type and number of enzyme–substrate, enzyme–inhibitor and enzyme–substrate–inhibitor complexes, i.e., presence of the Michaelis complex and/or acetylated BChE. Although, all three models reproduced well the BChE activity in absence of ethopropazine, model A was poor in describing inhibition with ethopropazine, while models B and C were better, especially for substrate concentrations above 0.2 mM. However model C was singled out because it approaches fulfilment of the one step-one event criteria, and confirms the inhibition mechanism derived from molecular modelling. Model C resulted in dissociation constants for the complex between BChE and ethopropazine: 61, 140 and 88 nM for *R*-enantiomer, *S*-enantiomer and racemate, respectively. The respective dissociation constants for the complexes between acetylated BChE and ethopropazine were 268, 730 and 365 nM. Butyrylcholinesterase had higher affinity for *R*-ethopropazine.

1. Introduction

Ethopropazine [10-(2-diethylaminopropyl)phenothiazine hydrochloride] is a chiral molecule with a stereogenic centre on the aminoalkyl side chain (Fig. 1). Racemic ethopropazine is a potent reversible inhibitor of butyrylcholinesterase (BChE; EC 3.1.1.8), with enzyme–inhibitor dissociation constants in the nanomolar range [1–3]. There are no published reports on the activities of the separated enantiomers in the reaction with BChE. Namely, ethopropazine is a member of anticholinergic drugs, known as Parsidol in US or Parsidan in Canada. Ethopropazine and to it related benztropine, biperiden, diphenhydramine, orphenadrine, procyclidine and trihexyphenidyl, are used in the treatment of Parkinson's disease [4]. Some of their *R*-enantiomers have been shown to have marked stereoselectivity with regard to their muscarinic receptor-binding affinities [5].

BChE is present in vertebrate plasma, and has important pharmacological and toxicological roles due to its hydrolysis of various xenobiotics (drugs) [6,7]. The three-dimensional structure of human BChE has been solved and it has revealed an active site at the bottom of an approximately 20-Å-deep 'gorge' [8]. Kinetic and structural studies have also defined four sub-domains within this active site, one at the gorge entrance, known as the peripheral anionic site (PAS). At the bottom of the active site is the catalytic anionic site (CAS), composed of the acylation site accompanying the oxyanion hole, a choline-binding pocket and acyl-binding pocket [9–11]. Due to the relatively large active-site gorge, BChE can accommodate a broader variety of substrates and inhibitors compared to the related acetylcholinesterase (AChE; EC 3.1.1.7) with which it shares more than 50% identical amino acids [12] and almost the same backbone structure [6]. It appears that differences in the amino acid residues lining the active site gorge contribute substantially to the exceptional and versatile kinetic behaviour of the cholinesterases [13]. Active site gorge residues define the stereoselectivity of cholinesterases discriminating enantiomers, i.e., chiral organophosphorus compounds or carbamates [14–16].

In the present study, we have analyzed the properties of separated ethopropazine enantiomers relative to BChE inhibition to determine the nature of ethopropazine binding to BChE. Previous studies of cholinesterase inhibition by racemic ethopropazine have

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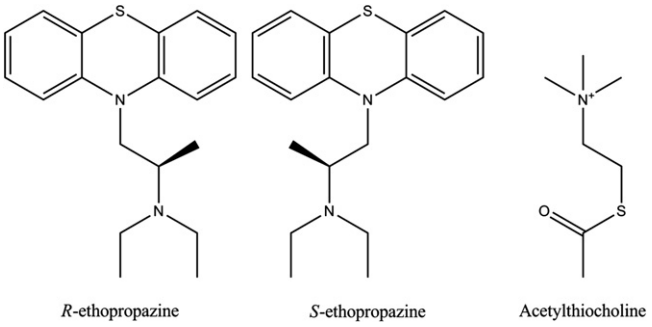

Fig. 1. Chemical structures of the ethopropazine enantiomers and the enzyme–substrate acetylthiocholine.

reported one, two or even three enzyme–inhibitor dissociation constants (inhibition constants), and these constants have been attributed to the binding of ethopropazine to CAS or PAS, and to simultaneous binding of two ethopropazine molecules on BChE (Table 1). This information was obtained using different experimental conditions, BChE preparations and different kinetic models, all of which might have significantly contributed to inconsistencies in the results obtained [3,10,17–21]. Therefore, our aim was to clarify the kinetics of BChE inhibition by the ethopropazine racemate and its separated enantiomers, to determine the likely positions, the extent of stereoselectivity, and the sequence of events during these interactions.

For the experimental analysis, we used latest kinetic model of substrate hydrolysis for cholinesterases [22] and two kinetic models for cholinesterases described earlier in the literature [23,24]. The models differ in prediction of events during substrate turnover, and in the number and type of enzyme–substrate complexes. Our aim was also to propose the most suitable model for the complex reaction between BChE and ethopropazine. For that purpose each of the models was expanded with inhibition reactions by ethopropazine. Along with kinetic and *in silico* investigation of ethopropazine–BChE interactions, we also examined the conformity of the models for acetylthiocholine (ACTh) hydrolysis by BChE, which included inhibition by ATCh at concentrations above 75 mM.

2. Materials and methods

2.1. Chemicals

Purified horse serum BChE (min. 80%), acetylthiocholine (ATCh), 5,5′-dithio-bis(2-nitrobenzoic acid) (DTNB) and ethopropazine hydrochloride [10-(2-diethylamino-propyl)phenothiazine hydrochloride] were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Human BChE was isolated from venous blood after centrifugation. Recombinant mouse BChE and AChE were provided by Professor Palmer Taylor (Skaggs School of Pharmacy and Pharmaceutical Sciences, UCSD, La Jolla, USA). Ethopropazine enantiomers were separated using fractional crystallization with chiral agents [25], and by chiral HPLC using a polysaccharide column Chiralcel-OJ [25]. Enantiomeric purities of ethopropazine enantiomers were at least 97%, as confirmed by chiral HPLC [25]. All other reagents were of analytical grade. Experiments were carried out at 25 °C in 100 mM sodium phosphate buffer, pH 7.4.

Table 1
Dissociation constants for racemic ethopropazine and BChEs from various species. Constants K_1 and K_2 represent complexes between single ethopropazine and BChE at two different binding sites, while constant K_3 represents a BChE complex with two ethopropazine molecules.

BChE source	Dissociation constant			Reference
	K_1 (nM)	K_2 (nM)	K_3 (nM)	
Mouse	61	130	–	[10]
Mouse	82	–	–	[19]
Human	20	26	–	[17]
Human	160	–	–	[3]
Horse	160	450	4400	[18]
Horse	200	–	–	[21]
Horse	77[a]	–	–	[21]

[a] Dissociation constant for ethopropazine and BChE measured in the absence of substrate by intrinsic fluorescence quenching of the active site tryptophans.

2.2. Substrate hydrolysis assays

Hydrolysis of ATCh catalyzed by the horse-serum BChE in the absence or presence of ethopropazine was recorded on a stopped-flow apparatus (HI-Tech Scientific SFA20-MX [UK] with Cary 300 Varian Inc. [Australia]). Aliquots of two solutions, one containing only the enzyme and the other the substrate, ethopropazine and the DTNB reagent, were mixed together in the mixing chamber of the apparatus. Absorbance of the reaction mixture was recorded spectrophotometrically (according to Ellman et al. [26,27]) at different substrate concentrations (0.002–250 mM) with a minimum of 10 repetitions per concentration. At each substrate concentration, four different inhibitor concentrations (0.5–20.0 μM) were tested, with a minimum of two repetitions per concentration. The final DTNB concentration was 0.5 mM. At low substrate concentrations, the reaction was followed until its completion, while at higher concentrations, only the initial portions were measured. To avoid possible product modulation, we stopped the measurement when the concentration of the product formed was between 60 μM and 80 μM. Concentration of BChE active sites were determined by stoichiometric titration with various concentrations of a high-affinity phosphorylating agent DEPQ [7-(O,O′-diethylphosphinyloxy)-1-methyl-quinolinium methylsulfate] [28]. Concentration of the BChE active sites during the assays was 2.3 nM.

2.3. Simultaneous fitting of the initial rate curves

Initial rates were calculated as a slope of the line in case of a linear absorbance increase over time or as a tangent of activity curve at time zero in case of a progressive decline in rate due to substrate depletion. The parameters (i.e., kinetic constants) of the initial rate equations derived from models A, B and C were fitted to the same sets of experimental data (i.e., initial rates). Evaluation of each model was carried out by simultaneous analysis of the data for substrate hydrolysis and for inhibition of substrate hydrolysis in the presence of ethopropazine racemate and separated enantiomers. Four sets of parameters for the racemate, *R*-enantiomer, *S*-enantiomer and the substrate were fitted using an appropriate computer program [22]. To obtain convergence in the fit with the lowest possible residual sum of squares and standard deviations of the constants, we decreased the number of unknown parameters in two ways, either unstable parameters were omitted or some of the parameters in the equations were linked. According to the principles of kinetic theory, unstable parameters suggest that the fluxes through the corresponding steps are unimportant and can be omitted [13,29,30]. Parameters expected to be similar for enantiomers or unaffected by the ligand were set as being equal (for model C $a = c$, $b = d$). Parameter d in model B was set as zero due to its infinitesimal value.

2.4. Docking of ethopropazine into BChE

The structural model of horse BChE was prepared by revised homology building using human BChE as a template (PDB code