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Inhibition of Human Blood Acetylcholinesterase and Butyrylcholinesterase by Ethopropazine

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Inhibition of human erythrocyte acetylcholinesterase (AChE; EC 3.1.1.7) and serum butyrylcholinesterase (BChE; EC 3.1.1.8) by ethopropazine, 10-(2-diethylaminopropyl)phenothiazine hydrochloride, was measured with acetylthiocholine (ATCh) as substrate. Dissociation constants for the enzyme-inhibitor complexes were calculated from the effect of ATCh concentration on the apparent dissociation constants by applying non-linear regression to fit the model to experimental data. Inhibition of AChE revealed a competitive inhibition for two binding sites (K_a = 161 and K_i = 393 μmol dm^{-3}), inhibition of the atypical BChE was non-competitive (K_i = 7.5 μmol dm^{-3}) while that of the usual BChE was competitive ($K_{(I)}$ = 0.16 μmol dm^{-3}). At the ethopropazine concentration of 20 μmol dm^{-3} and the acetylthiocholine concentration of 1.0 mmol dm^{-3} (conditions used for differentiation between AChE and BChE activities), the erythrocyte AChE was 8% inhibited and the BChE phenotypes UU, UA, FF/FS, AF, AJ/AK and AA/AS between 98% and 74%.

Key words: acetylcholinesterase, butyrylcholinesterase, ethopropazine, selective inhibition, inhibition mechanism, enzyme-inhibitor and enzyme-substrate dissociation constants.

INTRODUCTION

Ethopropazine is one of the phenothiazine derivatives that are potent inhibitors of butyrylcholinesterase (BChE, EC 3.1.1.8), but poor inhibitors of acetylcholinesterase (AChE, EC 3.1.1.7). As the most potent and selective

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inhibitor among phenothiazine derivatives, ethopropazine is used to inhibit selectively BChE in samples where both BChE and AChE are present.[1–4] Ethopropazine has a quaternary nitrogen in the structure (Figure 1), and one may expect that it inhibits the usual human serum BChE to a higher degree than the atypical enzyme or its heterozygotes. We confirmed this assumption in a preliminary study.[5]

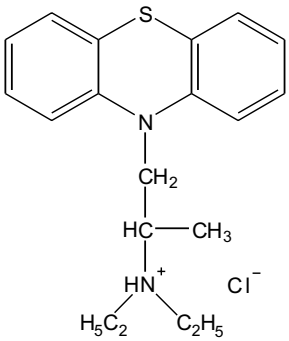

Figure 1. Chemical structure of ethopropazine.

This paper deals with the inhibition of human serum BChE variants and of human erythrocyte AChE by racemic ethopropazine. The selectivity of ethopropazine for different human BChE phenotypes and for human erythrocyte AChE was determined under the experimental conditions used for assessment of AChE and BChE activities in human blood. To get more information on the inhibition mechanism of the two enzymes, the dissociation constants of the enzyme-inhibitor complexes were determined from the effect of the substrate upon the degree of inhibition.

EXPERIMENTAL

Enzyme Source

The source of AChE was human erythrocytes washed with buffer to remove the plasma. Sera were taken from individuals whose BChE phenotypes had previously been determined by measuring the activity with propionylthiocholine as substrate and the inhibition with benzoylcholine as substrate using dibucain, sodium fluoride and Ro 02-0683 as differentiating inhibitors.[6–8] The phenotypes were homozygotes or heterozygotes of the usual (U), atypical (A), fluoride-resistant (F), Kalow (K), J, or silent (S) BChE variants.

Reagents

Ethopropazine (Sigma, St. Louis, USA) was a gift from Professor P. Eyer. Acetylthiocholine iodide (ATCh) was from Sigma St. Louis, USA.

Activity Measurement

Activity of the enzymes was measured with ATCh in 0.1 mol dm^{-3} phosphate buffer, pH = 7.4 at 37 °C with the thiol reagent DTNB.[9] In the final reaction mixture, erythrocytes and serum were diluted 600 and 150 times, respectively. Activities measured at ATCh concentrations of 0.5 mmol dm^{-3} or higher were corrected for spontaneous substrate hydrolysis. More details of the procedure were described earlier.[10,11]

Enzyme Inhibition

The selectivity of ethopropazine for AChE and BChE was tested with ethopropazine concentration of 20 μmol dm^{-3} and ATCh concentration of 1.0 mmol dm^{-3}. To study the competition between ethopropazine and the substrate, the activity of AChE and BChE was measured in the absence and presence of ethopropazine with ATCh concentrations ranging between 0.1 and 10 mmol dm^{-3}. From experimental data thus obtained, enzyme-inhibitor and enzyme-substrate dissociation constants were calculated from the equations, based on the assumption that the enzyme has a peripheral (allosteric) site where substrates or inhibitors can bind.[13]

If both, substrate and inhibitor, bind to both sites on the enzyme (catalytic and peripheral), the equation which relates substrate and inhibitor concentrations to enzyme activities is:[13]

$$K_{\mathrm{app}} = \frac{v \cdot i}{v_0 - v} = \frac{(1 + K_{\mathrm{s}} / s)(1 + s / K_{\mathrm{ss}})}{(K_{\mathrm{s}} / s)(1 / K_{\mathrm{a}} + 1 / K_{\mathrm{i}} + i / (K_{\mathrm{a}} K_{\mathrm{i}})) + 1 / K_{\mathrm{i}} + K_{\mathrm{s}} / (K_{\mathrm{a}} K_{\mathrm{ss}})} \quad (1)$$

where v_0 and v are the enzyme activities at a given substrate concentration (s) in the absence and presence of the inhibitor (concentration: i). In this equation, the apparent dissociation constant K_{app} is a function of both inhibitor and substrate concentrations. The K_{s}, K_{ss}, K_{a} and K_{i} are dissociation constants of enzyme-substrate and enzyme-inhibitor complexes at the catalytic and peripheral sites, respectively.

If the inhibitor binds only to the catalytic site or only to the peripheral site, and the substrate binds to both sites, the effect of substrate concentration upon K_{app} is expressed by the following equation:[13]

$$K_{\mathrm{app}} = \frac{v \cdot i}{v_0 - v} = K_{(\mathrm{I})} + \frac{K_{(\mathrm{I})}}{K_{(\mathrm{S})}} s \quad (2)$$

$K_{(\mathrm{I})}$ is either the enzyme-inhibitor dissociation constant at the catalytic site (K_{a}) or the dissociation constant at the peripheral site (K_{i}), and $K_{(\mathrm{S})}$ is the enzyme-substrate dissociation constant at the respective sites (K_{s} or K_{ss}). It follows from Eq. (2) that K_{app} is only a function of the substrate concentration.

Finally, if K_{app} does not change with the change of substrate concentration, a non-competitive inhibition occurs, and consequently

$$K_{app} = \frac{v \cdot i}{v_0 - v} = K_i \quad (3)$$

The enzyme-inhibitor and enzyme-substrate dissociation constants were calculated from the above equations by the MacCurvefit Programme applying the Quasi-Newton minimisation algorithm.

RESULTS

Selectivity of Ethopropazine for Acetylcholinesterase and Butyrylcholinesterase

Absorption of cholinesterase inhibitors is commonly assessed by measuring the decrease of AChE and/or BChE activities in human blood. If activities are measured in whole blood, *i.e.* without separating the erythrocytes from plasma, one should apply either a specific substrate or a specific inhibitor of AChE or BChE in order to assess the activity of only one enzyme. Ethopropazine has been suggested as a specific BChE inhibitor, which at the concentration of 20 μmol dm^{-3} fully inhibits the BChE activity without inhibiting AChE, and procedures have been worked out for the differentiation of the two enzymes in whole blood.[2,3]

In this paper we have tested ethopropazine as an inhibitor of BChE variants and of erythrocyte AChE. Inhibition was measured with the ethopropazine concentration of 20 μmol dm^{-3} using ATCh (1.0 mmol dm^{-3}) as substrate (Table I).

The mean activities (μmol min^{-1} mL^{-1}) of the studied BChE variants ranged from 2.86 for the usual BChE (UU) to 0.41 for the atypical enzyme (AA/AS). All six variants were inhibited by the ethopropazine concentration of 20 μmol dm^{-3}. The degree of inhibition decreased in almost the same order as the rates of ATCh hydrolysis: the UU BChE, which is most active, was 98% inhibited while the AA/AS BChE was only 74% inhibited. Under the same experimental conditions, ethopropazine (20 μmol dm^{-3}) inhibited 8% of erythrocyte AChE, which is consistent with 5% inhibition reported by Worek *et al.*[3]

Consequently, ethopropazine is a very potent inhibitor of BChE. However, when ethopropazine is applied as inhibitor of BChE in order to measure the AChE activity in human whole blood, AChE will also be slightly inhibited. On the other hand, when blood contains a homozygous or heterozygous A variant of BChE, inhibition of the serum BChE by ethopropazine

TABLE I

Activities of AChE and BChE, and inhibition of cholinesterases by ethopropazine (20 μmol dm^{-3}), measured with ATCh (1.0 mmol dm^{-3})

Enzyme	*N*	Activity[a] μmol min^{-1} mL^{-1}	Inhibition[a] %
Erythrocyte AChE	6	4.66 ± 1.08	8 ± 4
Serum BChE phenotype			
UU	4	2.86 ± 0.57	98 ± 2
UA	4	1.92 ± 0.33	94 ± 1
FF/FS	5	0.66 ± 0.11	92 ± 1
AF	3	0.85 ± 0.26	87 ± 1
AJ/AK	6	0.51 ± 0.14	85 ± 3
AA/AS	5	0.41 ± 0.04	74 ± 4

[a] Mean ± SD. Mean values were calculated from measurements done on erythrocytes or sera from *N* individuals.

(20 μmol dm^{-3}) will be less than 98%. Both of these facts should be taken into account when validating procedures for the AChE activity measurements in whole blood, based on BChE inhibition by ethopropazine.[12]

Reversible Binding of Ethopropazine to Acetylcholinesterase and Butyrylcholinesterase

To determine the dissociation constants of the ethopropazine complexes with AChE or BChE, inhibition was measured with a broad range of ethopropazine concentrations in order to reach a degree of inhibition between 20 to 80%. Experimental data were interpreted according to the models described by Eqs. (1–3).

The apparent dissociation constants K_{app} for inhibition of AChE and BChE as a function of the ATCh concentration (Hunter-Downs plots) are shown in Figure 2.

The Hunter-Downs plot for the inhibition of erythrocyte AChE displayed a curve. Applying Eq. (1), a theoretical curve and the best fitting dissociation constants K_s, K_{ss}, K_a and K_i were calculated (Table II). The curve fitting was done for the ethopropazine concentration of 200 μmol dm^{-3} because that concentration was used in the experiments. The theoretical curves calculated for ethopropazine concentrations of 200 and 400 μmol dm^{-3} fell within one standard deviation of the experimental points. The calculated K_s and K_{ss} values (Table II) are in good agreement with the K_m and K_{ss} constants we

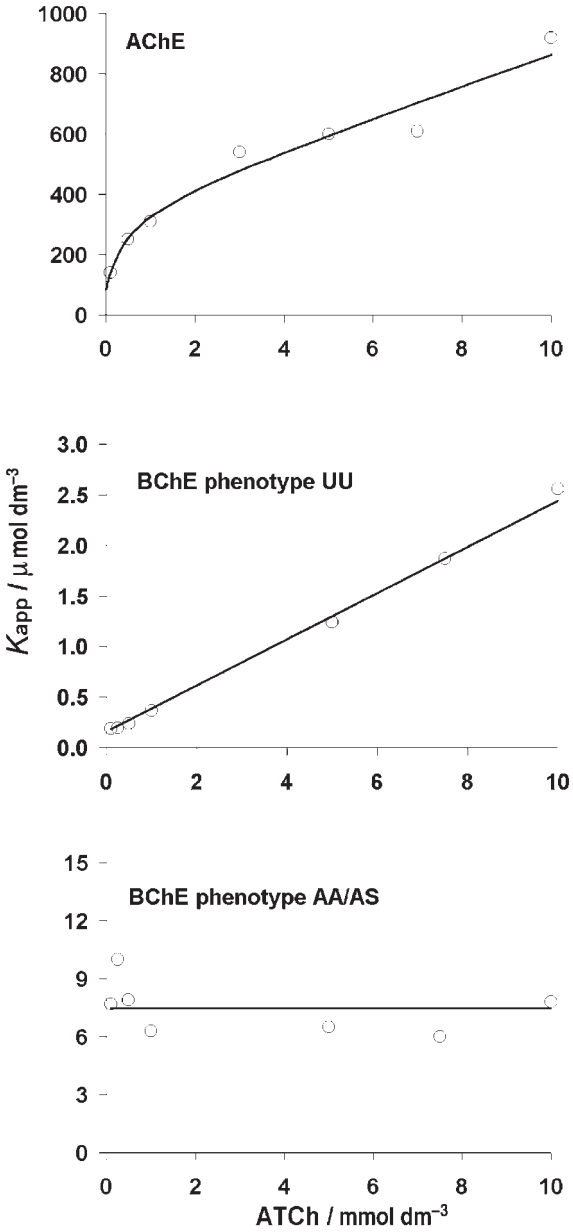

Figure 2. Inhibition of erythrocyte acetylcholinesterase (AChE) and serum butyrylcholinesterase (BChE) variants (UU and AA/AS) by ethopropazine, measured with acetylthiocholine (ATCh) as substrate. K_{app} is defined by Eqs. (1), (2) or (3). Each point is the mean value of 4–7 experiments. The lines were calculated as described under Experimental.

TABLE II

Dissociation constants for enzyme-inhibitor and enzyme-substrate complexes calculated by Eqs. (1), (2) or (3) from K_{app} values shown in Figure 2

Enzyme	ATCh	Ethopropazine	Inhibitor constants	Substrate constants
	mmol dm^{-3}	μmol dm^{-3}	μmol dm^{-3}	mmol dm^{-3}
Erythrocyte AChE	0.1–10	200	K_a = 161 K_i = 393	K_s = 0.10 K_{ss} = 7.3
UU phenotype BChE	0.1–10	0.25–2	$K_{(I)}$ = 0.16 ± 0.03	$K_{(S)}$ = 0.69 ± 0.14
AA phenotype BChE	0.1–10	2–10	K_i = 7.5 ± 1.4	–

calculated from the data for erythrocyte AChE and ATCh in the absence of ethopropazine (0.1 and 5.7 mmol dm^{-3}, respectively).

The Hunter-Downs plot for the usual BChE displayed competitive inhibition and Eq. (2) was therefore applied (Table II). However, the K_{app} values measured at ATCh concentrations of 0.10 and 0.25 mmol dm^{-3} were almost the same, 0.19 and 0.20 μmol dm^{-3} respectively, indicating a non-competitive component of inhibition at low substrate concentrations. As the $K_{(I)}$ constant derived from Eq. (2) (Table II) was not significantly different from the above two K_{app} values, we find it justified to attribute only one enzyme-inhibitor dissociation constant to the inhibition of the usual BChE by ethopropazine. The value of the $K_{(S)}$ constant derived from Eq. (2) (Table II) was between two enzyme-substrate dissociation constants calculated in the absence of ethopropazine either from data in this paper (K_1 = 0.04 mmol dm^{-3} and K_2 = 1.2 mmol dm^{-3}) or in a previous study (K_1 = 0.04 mmol dm^{-3} and K_2 = 1.8 mmol dm^{-3}).[10] These enzyme-substrate dissociation constants were calculated by applying an equation which assumes two binding sites for the substrate on the enzyme.[14]

Inhibition of the atypical BChE was non-competitive over the total ATCh concentration range studied. This follows from Figure 2 and also from the analysis of K_{app} values at ATCh concentrations of 0.10 and 10 mmol dm^{-3}, which were not significantly different (t = 1.99, at the 0.094 significance level). The non-competitive inhibition constant K_i was therefore calculated as the mean of all the measured K_{app} values according to Eq. (3) (Table II). According to the model underlying Eqs. (1–3), the non-competitive feature of inhibition points to the involvement of the peripheral site in the inhibition mechanism.

DISCUSSION AND CONCLUSION

Differentiation between AChE and BChE activities is routinely done with ethopropazine (20 μmol dm^{-3}) as a potent BChE inhibitor, and the activities are measured with ATCh (1 mmol dm^{-3}) as substrate. It follows from the evaluated enzyme-inhibitor dissociation constants given in Table II that under these conditions the erythrocyte AChE and the usual BChE variant would be inhibited 6% and 98%, respectively. The atypical BChE variant would be inhibited 73% at any ATCh concentration between 0.1 and 10 mmol dm^{-3}. The measured percents of inhibition given in Table I agree well with these values, calculated from the enzyme-inhibitor dissociation constants. This in turn means that the equations applied to the calculation of the dissociation constants are valid over a broad range of ethopropazine concentrations, including the concentration of 20 μmol dm^{-3} which was not used in the experiments wherefrom the respective dissociation constants were calculated (Table II).

The competition between ethopropazine and acetylthiocholine clearly shows a difference between AChE and BChE. AChE displays two binding sites, and ATCh competes with ethopropazine at both sites. The atypical BChE displays a non-competitive inhibition, which indicates a peripheral binding site for ethopropazine. The usual BChE reveals primarily a competitive inhibition, which however has a non-competitive component at low substrate concentrations. Attribution of only one enzyme-inhibitor dissociation constant might therefore be an oversimplification of the inhibition mechanism.

The affinity of ethopropazine for the usual BChE is three-orders of magnitude higher than for the AChE. Similar differences between the inhibition of AChE and BChE were found for enzymes from other sources. The specificity of ethopropazine in interaction with cholinesterases was demonstrated through molecular modelling and confirmed by site specific mutagenesis. The selectivity was related to the presence of a bulky aromatic residue, tyrosine or phenylalanine, in AChE, in place of an alanine found at the corresponding position in BChE. For this reason, the binding site is more spacious in BChE and is capable of admitting the bulky ethopropazine.[4,15,16]

Positively charged compounds are better inhibitors of the usual BChE variant than of the atypical variant,[6,17] and this was also confirmed for ethopropazine (Tables I and II) which has a quaternary nitrogen in the molecule (Figure 1). The affinity of ethopropazine for the usual phenotype was about 50-fold higher than for the atypical phenotype, which results from the one-point mutation in the atypical enzyme (Asp 70 Gly) as compared to the usual enzyme. The AJ/AK sera were inhibited less than the usual enzyme due to the presence of the atypical variant in heterozygotes; mutations on J

and K variants are outside the active site and they are not critical for the binding of substrates or inhibitors.[18,19]

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SAŽETAK

Inhibicija acetilkolinesteraze i butirilkolinesteraze iz ljudske krvi etopropazinom

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Inhibicija ljudske eritrocitne acetilkolinesteraze (AChE, EC 3.1.1.7) i serumske butirilkolinesteraze (BChE, EC 3.1.1.8) etopropazinom, 10-(2-dietilaminopropil)fenotiazin-hidrokloridom, mjerena je pri raznim koncentracijama supstrata acetiltiokolina. Disocijacijske konstante kompleksa enzim-inhibitor izračunane su iz utjecaja supstrata na prividne disocijacijske konstante nelinearnom regresijom, prema modelima definiranim teorijskim jednadžbama. Inhibicija AChE bila je kompetitivna u cijelom području koncentracije supstrata i uočena su dva vezna mjesta (K_a = 161 i K_i = 393 μmol dm^{-3}). Inhibicija atipične BChE bila je nekompetitivna (K_i = 7,5 μmol dm^{-3}), a obične BChE kompetitivna ($K_{(I)}$ = 0,16 μmol dm^{-3}). Pri koncentracijama etopropazina od 20 μmol dm^{-3} i acetiltiokolina od 1,0 mmol dm^{-3} (uvjeti korišteni za razlikovanje aktivnosti BChE i AChE) eritrocitna AChE bila je inhibirana 8%, a BChE (fenotipovi UU, UA, FF/FS, AF, AJ/AK i AA/AS) između 98% i 74%.