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Exploring the Active Sites of Cholinesterases by Inhibition with Bambuterol and Haloxon

Zrinka Kovarik,* Anita Bosak, Goran Šinko, and Tatjana Latas

Institute for Medical Research and Occupational Health, Ksaverska c. 2, POB 291, HR-10001 Zagreb, Croatia

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Key words
• mouse acetylcholinesterase
• mouse, human and horse butyrylcholinesterase
• inhibition
• carbamate
• organophosphate
• haloxon
• bambuterol

The paper describes the inhibition of mouse acetylcholinesterase (AChE; EC 3.1.1.7) and mouse, human, and horse butyrylcholinesterase (BChE; EC 3.1.1.8) by 5-[2-(*tert*-butylamino)-1-hydroxyethyl]-*m*-phenylene-bis(dimethylcarbamate) hydrochloride (bambuterol) and by *O,O*-bis-(2-chloroethyl)-*O*-(3-chloro-4-methylcoumarin-7-yl) phosphate (haloxon). The haloxon inhibition rate constant (k_i) for mouse BChE was 3.7 x 10^7 min^{-1} mol^{-1} dm^3, which was 40-fold higher than the rate constant for mouse AChE. Bambuterol inhibition of horse BChE ($k_i = 2.1 \times 10^5$ min^{-1} mol^{-1} dm^3) was about 25-fold slower than that of human or mouse BChE, whereas the respective haloxon inhibition of horse BChE (k_i = 1.2 x 10^7 min^{-1} mol^{-1} dm^3) was about 2-3-fold slower. Sequence alignments and the computational model of the three-dimensional structure of horse BChE suggest that residues inside the active site at positions 69, 277 and 285 are important for the differences in the inhibition of these three BChE species.

INTRODUCTION

Acetylcholinesterase (AChE; EC 3.1.1.7) and butyrylcholinesterase (BChE; EC 3.1.1.8) are serine hydrolases, that share about 55 % of amino acid sequence identity, and have similar, yet distinct catalytic properties.[1] The different specificity for substrates, irreversible inhibitors and reversible ligands is dictated by the difference in amino acid residues of the active sites of AChE and BChE.[2–5] The AChE active centre is located at the bottom of a deep and narrow gorge, with the surface predominantly lined with aromatic residues.[6,7] Three-dimensional modelling of BChE revealed an active site gorge with fewer hydrophobic residues.[8] However, the BChE primary structures from various species also differ, and not all residues inside the active site are conserved.[9]

Carbamates and organophosphates are potent inhibitors of both BChE and AChE.[10] We studied the inhibition of mouse AChE and mouse, human, and horse BChE by the carbamate 5-[2-(*tert*-butylamino)-1-hydroxyethyl]-*m*-phenylene-bis(dimethylcarbamate) hydrochloride (bambuterol), and by the organophosphate *O,O*-bis(2-chloroethyl)-*O*-(3-chloro-4-methylcoumarin-7-yl) phosphate (haloxon) (Figure 1). The choice of the compounds was based upon previous findings that there was a large difference between AChE and BChE in the rate of inhibition by bambuterol or haloxon.[11–13] We compared the structures of inhibitors and discussed the relationship between the size of those inhibitors and the size of AChE and BChE active sites with inhibition rate constants. The kinetic data in conjunction with the model of horse BChE and the alignment of sequences of horse, human

* Author to whom correspondence should be addressed. (E-mail: zrinka.kovarik@imi.hr)

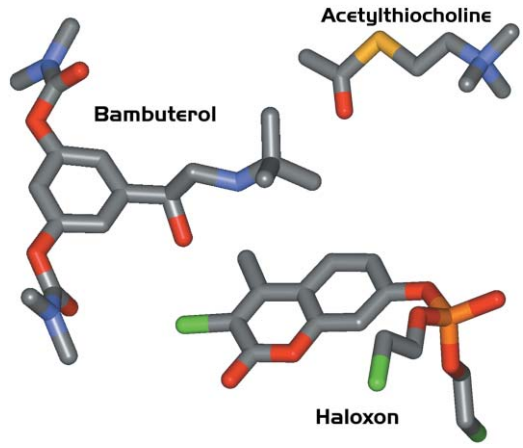


Figure 1. Minimized three-dimensional structure of the substrate, acetylthiocholine, and the inhibitors, bambuterol and haloxon. Hydrogen atoms were subtracted for better visibility. Minimization was performed using the MM2 molecular mechanics module in ChemOffice Software, ChambridgeSoft Corporation, USA.

and mouse BChE pointed to the residues inside the active site gorge of BChE as the probable reason for the differences in inhibition between these species. This is why we identified the residues, that differ in the active sites of the three BChE species.

EXPERIMENTAL

Chemicals

Acetylthiocholine iodide (ATCh), 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB) and bovine serum albumin (BSA) were purchased from Sigma Chemical Co., USA. Bambuterol, 5-[2-(*tert*-butylamino)-1-hydroxyethyl]-*m*-phenylene-bis(dimethylcarbamate) hydrochloride, was a gift from Astra Draco AB, Sweden. Haloxon, *O,O*-bis(2-chloroethyl)-*O*-(3-chloro-4-methylcoumarin-7-yl) phosphate, was from Aldrich, USA. Stock solutions (10 mmol dm^{-3} bambuterol in water, 10 mmol dm^{-3} haloxon in ethanol) were kept at 4 °C.

Enzymes

Horse serum BChE was purchased from Sigma Chemical Co., USA (partially purified; declared activity for butyrylthiocholine is 22 μmol per min, per 1 mg of protein, pH = 8.0, 37 °C). Recombinant mouse BChE and mouse AChE were gifts from Professor Palmer Taylor, University of California at San Diego, USA (purified by affinity chromatography). Native human serum, as the source of BChE, was collected at the Institute for Medical Research and Occupational Health, Zagreb. Enzyme preparations were kept at –20 °C.

Enzyme Activity Measurements

Enzyme activities were determined at 25 °C using the Ellman method and acetylthiocholine (1 mmol dm^{-3}) as the substrate.[14] The reaction was started by adding the substrate to 100 mmol dm^{-3} phosphate buffer, pH = 7.4, containing the enzyme, 0.33 mmol dm^{-3} DTNB and 0.01% BSA. All spectrophotometric measurements were performed on a Cary 300 spectrophotometer (Varian, Inc.).

Progressive Inhibition

In inhibition experiments enzyme samples were incubated for up to 30 minutes with inhibitors in the absence of substrate; typically, 3–5 inhibitor concentrations were used (ranging from 2 nmol dm^{-3} to 2 μmol dm^{-3} of haloxon, and from 1 μmol dm^{-3} to 1 mmol dm^{-3} of bambuterol). In all experiments, the inhibitor concentration exceeded the enzyme concentration. The inhibition reaction was stopped by the addition of ATCh, and the extent of inhibition was determined by measuring the residual activity. To obtain the enzyme activity at zero time inhibition, the enzyme was added to a mixture of inhibitor and ATCh.

The first-order rate constants (k_{obs}) were calculated by linear regression for any given inhibitor concentration using the formula:

$$\ln (v_0 / v_i) = k_{obs}\, t \qquad (1)$$

where v_0 and v_i stand for the enzyme activity in the absence and in the presence of the inhibitor (I) at time t.[11] The second-order inhibition rate constant (k_i) was calculated from the linear dependency of k_{obs} from the inhibitor concentration:

$$k_i = k_{obs} / [I] \qquad (2)$$

RESULTS AND DISCUSSION

In all experiments a linear relationship between the logarithm of activity (percents) *vs.* time was obtained for any given inhibitor concentration, and the first-order rate inhibition constants, k_{obs}, were calculated by Eq. 1 (Figure 2). Table I displays the resulting bimolecular inhibition rate constants k_i for the inhibition by haloxon and bambuterol calculated by Eq. 2. The obtained constants are similar to the values published earlier.[11–13,15]

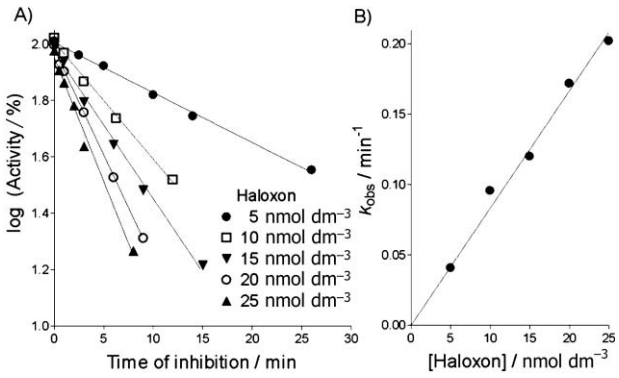
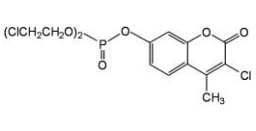
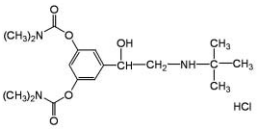


Figure 2. Inhibition of horse BChE by haloxon. Points on (A) indicate the measured values. The slopes of the lines (k_{obs}) were plotted as a function of inhibitor concentrations on plot (B). The overall inhibition rate constant is the slope of the line on plot B.

TABLE I. Rate constants (k_i) for inhibition of butyrylcholinesterase and acetylcholinesterase by haloxon and bambuterol, determined in at least 3 experiments[a]

	$10^{-6}\ k_i$ / min^{-1} mol^{-1} dm^3	
		
	Haloxon	Bambuterol
BChE		
Horse	12 ± 0.8	0.21 ± 0.01
Human	19 ± 0.4	4.7 ± 0.9
Mouse	37 ± 0.6	5.7[b]
AChE		
Mouse	0.87 ± 0.05	0.00036[b]

[a] Published constants for haloxon inhibition were: for horse BChE[12] $k_i = 15 \times 10^6$ min^{-1} mol^{-1} dm^3, for human BChE[12] $k_i = 25 \times 10^6$ min^{-1} mol^{-1} dm^3, for mouse AChE[15] $k_i = 0.25 \times 10^6$ min^{-1} mol^{-1} dm^3 and for bovine AChE[11] $k_i = 0.21 \times 10^6$ min^{-1} mol^{-1} dm^3. [b] Ref. 13.

All three BChEs were inhibited more rapidly than AChE by any of the two inhibitors. Mouse BChE was 16000-fold more rapidly inhibited by bambuterol and 40-fold by haloxon than mouse AChE (Table I). Six aliphatic amino acids in the active site gorge of BChE, corresponding to six aromatic amino acids in AChE, make the BChE active site gorge wider (Figure 3). Consequently, bambuterol[13] and haloxon have easier access to the catalytic centre, resulting in a more efficient inhibition of BChE than of AChE. The high specificity of bambuterol for mouse BChE, compared to AChE, depends primarily on the difference in the structure of the choline binding site where AChE has the tyrosine side chain 337 but BChE has alanine 328.[13] On the basis of the enhanced hydrolysis of butyrylthiocholine (a specific substrate of BChE) by AChE acyl pocket mutants[5] and of the size similarity between the haloxon chloroethoxy group and the butyryl group, we presume a possible restriction of phosphorylation by haloxon, imposed by the acyl pocket of AChE.

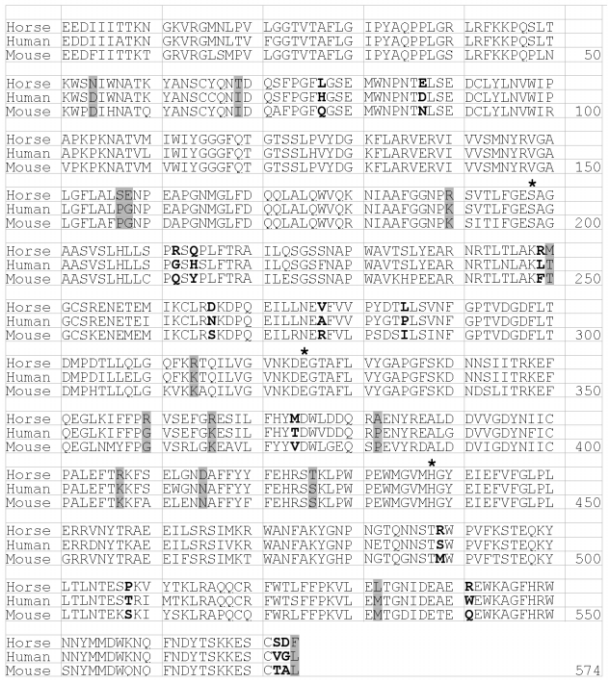


Figure 4. Amino acid sequence alignment of horse, human and mouse BChE.[1,9,18] Letters in grey represent residues in which horse BChE differs from human or mouse BChE. Letters in bold are non-conserved residues inside mouse, human or horse BChE active sites. Asterisks denote the catalytic triad S198-E325-H438.

The inhibition of all cholinesterases by haloxon was more potent than that by bambuterol. As shown in Figure 1, bambuterol is a bulkier molecule than haloxon, and the difference between haloxon inhibition of mouse AChE and mouse BChE is not very pronounced. However, the size is not the only criterion, because also relatively big molecules (MW > 400) have a high affinity for the enzyme.[16] Besides the molecule size, the number of single bonds with freedom of rotation is also important. Molecular mechanics calculations resulted in minimized

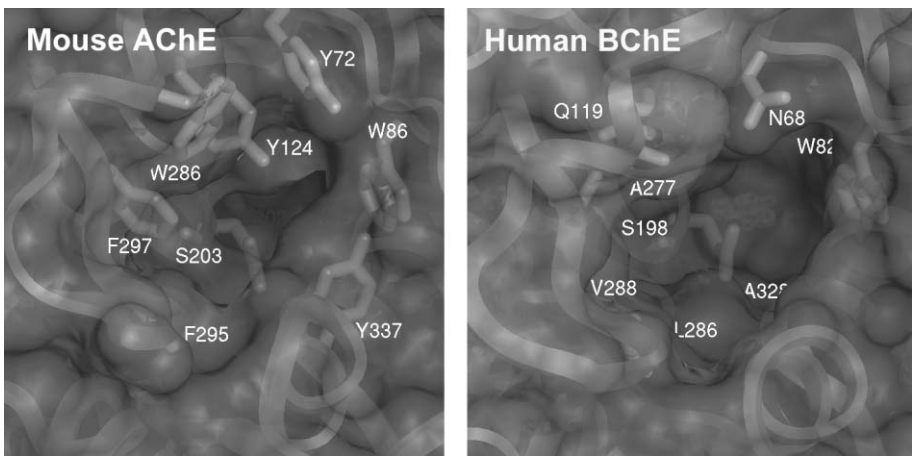


Figure 3. View of the residues of the mouse AChE and human BChE active site gorge critical for the difference in AChE and BChE specificities. Aromatic residues Y72, Y124, W286, F295, F297 and Y337 in mouse AChE correspond to aliphatic N68, Q119, A277, L286, V288 and A328 in human BChE, respectively (modified from Ref. 4).

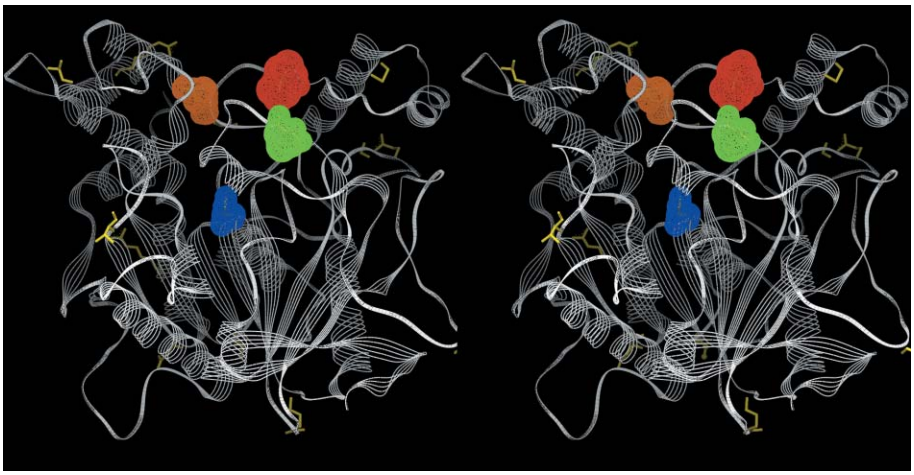


Figure 5. Stereo view of horse BChE (catalytic S198 in blue). Residues in yellow represent amino acids which differentiate horse BChE from human and mouse BChE. T69 (green) is substituted by isoleucine in both human and mouse BChE. V277 (red) and L285 (orange) are not conserved in BChE of the other two species.

three-dimensional structures of haloxon, bambuterol and the substrate acetylthiocholine (Figure 1). Bambuterol, the bulkiest molecule in our case, has nine single bonds with freedom of rotation that can cause a significant change in the three-dimensional structure. The smaller haloxon has eight single bonds with freedom of rotation, and acetylthiocholine has four such bonds. Bambuterol with the elongated 2-(*tert*-butylamino)-1-hydroxyethyl group and a relatively large phenyl-bis(dimethylcarbamate) group has less chance for proper orientation inside the active site and for successful carbamoylation of cholinesterases. Haloxon has a planar 3-chloro-4-methylcoumarin group, which makes it a less bulky compound with an easier access to the catalytic centre. This could be one of the reasons why haloxon phosphorylates both BChE and AChE at faster rates than bambuterol.

Bambuterol significantly differentiates horse from mouse and human BChE; it inhibits horse BChE about 25-fold slower than mouse or human BChE (Table I). Haloxon inhibited horse BChE only 3-fold faster than mouse BChE and only 1.5-fold faster than human BChE. The percentage of identity of horse, mouse and human BChE was calculated from the amino acid sequence alignments shown in Figure 4. Horse BChE shares 90.4 % of the amino acid sequence with human BChE and 82.2 % with mouse BChE. Sequence alignment of three BChE, aligned with respect to the catalytic triad: Ser198-Glu325-His438, pointed to 15 amino acid residues distinguishing horse from mouse and human BChE. However, in the three-dimensional model of horse BChE, only one of the 15 amino acid residues, threonine 69, was close to the peripheral binding site of BChE.[17] We believe that this may influence the enzyme interactions with haloxon or bambuterol (Figure 5). From 14 amino acid residues, which are not conserved in any of the three BChE species, only two amino acid residues line the active site gorge of BChE.[9] Residues at position 277, valine, alanine, and arginine, and at position 285, leucine, proline and isoleucine, are found in horse, human and mouse butyrylcholinesterases, respectively.

In conclusion, we assume that residues at positions 69, 277 and 285 are responsible for the inhibition differences of horse, human and mouse BChE. The inhibition difference between BChE and AChE is probably dictated by the choline binding site for bambuterol, and by the acyl pocket for haloxon.

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SAŽETAK

Proučavanje aktivnih mjesta kolinesteraza inhibicijom bambuterolom i haloksonom

Zrinka Kovarik, Anita Bosak, Goran Šinko i Tatjana Latas

Acetilkolinesteraza (AChE; EC 3.1.1.7) miša i butirilkolinesteraze (BChE; EC 3.1.1.8) miša, čovjeka i konja inhibirane su 5-[2-(*tert*-butilamino)-1-hidroksietil]-*m*-fenilen-bis(dimetilkarbamat)-hidrokloridom (bambuterol) i *O*,*O*-bis(2-kloroetil)-*O*-(3-klor-4-metilkumarin-7-il)-fosfatom (haloksonom). Konstanta brzine inhibicije (k_i) BChE miša haloksonom je $3{,}7 \times 10^7$ min^{-1} mol^{-1} dm^3 i 40 puta veća od konstante brzine inhibicije AChE miša. Inhibicija BChE konja bambuterolom ($k_i = 2{,}1 \times 10^5$ min^{-1} mol^{-1} dm^3) je oko 25 puta sporija nego inhibicija BChE čovjeka ili miša. Inhibicija BChE konja haloksonom ($k_i = 1{,}2 \times 10^7$ min^{-1} mol^{-1} dm^3) je 2–3 puta sporija nego inhibicija BChE čovjeka ili miša. Razlike primarnih struktura BChE konja, čovjeka i miša, zajedno s modelom trodimenzijske strukture BChE konja, upućuju na zaključak da su aminokiseline unutar aktivnih mjesta na pozicijama 69, 277 i 285 važne za razlike u inhibiciji tih triju BChE.