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Interactions of chiral quinuclidin-3-yl benzoates with butyrylcholinesterase: kinetic study and docking simulations†

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epoc ABSTRACT: Both enantiomers of quinuclidin-3-yl benzoate (RQBz and SQBz) were synthesized in order to examine the stereoselectivity of the hydrolysis of these esters catalyzed by horse serum butyrylcholinesterase (BChE). The hydrolysis of benzoylcholine (BzCh) was also studied in order to determine the influence of the alcohol part of the esters upon the kinetics. The k_{cat} value for the substrates decreased in order BzCh > RQBz (4-fold slower) ≫ SQBz (76-fold slower reaction). K_M values determined for quinuclidinium substrates revealed that the binding affinity of RQBz (0.28 mM) is approximately 2-fold lower than that of SQBz (0.13 mM) towards BChE. From the ratio of the enantiomeric k_{cat}/K_M values, an enantiomeric excess of 78% was calculated, indicating that the resolution of racemic quinuclidin-3-yl benzoate can be achieved by hydrolysis with BChE. The orientations of all the studied benzoate esters and butyrylcholine (BuCh) in the active site of human BChE were proposed by flexible ligand docking with AutoDock 3.0. Analyses of the Michaelis complexes obtained revealed that there are numerous similar close contacts in the active site. The main difference in binding of quinuclidinium and choline esters was found in the ammonium electrostatic region which includes cation–π interaction of the ammonium moiety of substrates with the indole ring of Trp^{84}. The important cation–π interaction with Trp^{84} was lowest in the case of the *S*-enantiomer of QBz, which might be the main explanation for the slowest rate of hydrolysis of that compound. Copyright © 2002 John Wiley & Sons, Ltd.
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KEYWORDS: (*R*)- and (*S*)-quinuclidin-3-yl benzoates; butyrylcholinesterase; hydrolysis kinetics; benzoylcholine; butyrylcholine; docking

INTRODUCTION

Quiniclidin-3-ol and its esters may be looked upon as bicyclic analogues of acetylcholine, and therefore their interaction with various receptors in a cholinergic system is not surprising. Many esters of quinuclidin-3-ol have been tested and recognized as pharmacological agents, and as such they are commercially available as therapeutics.[1] Compounds containing the quinuiclidine moiety have also been found to be potential antidotes against poisoning by organophosphorus (anticholinesterase) compounds, including pesticides, insecticides and warfare agents.[2] Since 3-quiniclidinol contains an asymmetric carbon atom, many investigations have concentrated on the resolution of racemic compounds using chemical[3] and biocatalytic[4] methods in order to provide a simple, efficient and inexpensive procedure.

One of the enzymes tested as a biocatalyst was butyrylcholinesterase (BChE, EC 3.1.1.8). BChE is a serine hydrolase that has a structure similar to that of acetylcholinesterase.[5] The active site of this enzyme consists of several major domains: (i) esteratic site, containing active serine as a part of the catalytic triad (Ser–His–Glu); (ii) acyl pocket, a hydrophobic region which accommodates the acyl group of an ester; (iii) choline subsite, consisting of tryptophan for molecular recognition of the substrate's quaternary ammonium group; and (iv) oxyanion hole, formed by the main chain N—H dipoles interacting with the carbonyl oxygen of the substrate. The catalytic steps in ester hydrolyses involve initial formation of an enzyme–substrate complex, followed by an acylation step, and finally water causes deacylation of the enzyme (Scheme 1).

BChE has been used previously for the resolution of

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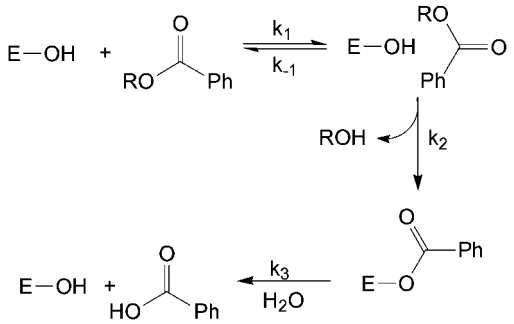


Scheme 1

racemic 3-quinuclidinyl butyrate. The stereoselectivity of that hydrolysis was in favour of the *R*-enantiomer.[4a] However, the difference in hydrolysis rates was not sufficient to achieve complete resolution of enantiomers. In the course of our work with quinuclidinium esters, a problem of resolution of racemic mixtures arose.[2] Therefore, we chose to determine the kinetics of BChE-catalysed hydrolysis of benzoate esters: (*R*)-quinuclidin-3-yl benzoate (RQBz) and (*S*)-quinuclidin-3-yl benzoate (SQBz) in comparison with benzoylcholine (BzCh) (Fig. 1).

Furthermore, in order to investigate the nature of the interaction of benzoates with the enzyme and to predict possible structural changes for obtaining better resolution, docking simulations were also performed on butyrylcholine (BuCh) (Fig. 2).

EXPERIMENTAL

Materials and instrumentation

BChE (EC 3.1.1.8), type IV-S lyophilized powder from horse serum (Sigma Chemical), benzoylcholine chloride (BDH) and (±)-3-quinuclidinol (Aldrich Chemical) were used as received. Optical rotations were measured on an Optical Activity AA-10 automatic polarimeter at ambient temperature in ethanol. ^{1}H and ^{13}C, 1D and 2D NMR spectra were recorded on a Varian XL-GEM 300 spectrometer. Chemical shifts are given in ppm downfield from TMS as internal standard. UV spectra were recorded

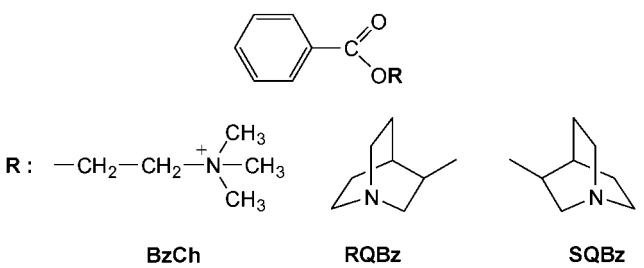


Figure 1. Substrates for enzymatic hydrolysis

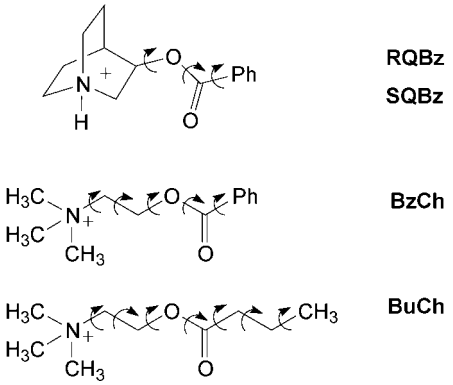


Figure 2. The ligands chosen for docking, with rotatable bonds highlighted with arrows

on a Varian Cary 3 UV–Visible spectrophotometer. HPLC analyses (Thermo Separation Prod-ucts, Spectra-SYSTEM 2000) were performed on an RP-18 column (Waters, SymmetryShield, 5 µm, 150 × 3.9 mm i.d.) at 40 °C. The mobile phase used was water–methanol–acetonitrile–acetic e.g. acid–triethylamine (60:25:15:0.33:0.2) at a flow-rate of 0.7 ml min^{-1}. The reactions were carried out in a Heidolph UNIMAX 1100 shaker.

Preparation of substrates

(*R*)- and (*S*)-3-quinuclidinol were resolved according to the published procedure using L- and D-tartaric acid.[3a]

(*R*)-Quinuclidin-3-yl benzoate (RQBz) was prepared by dissolving (*R*)-3-quinuclidinol (500 mg, 3.9 mmol) in benzoic anhydride[6] (3.7 ml, 20 mmol) and stirring the mixture for 1 h at 130 °C. The reaction mixture was then cooled, made alkaline with saturated aqueous K_2CO_3 and extracted with chloroform. The extract was dried over Na_2SO_4 and evaporated under reduced pressure. The residue was purified by column chromatography [aluminium oxide 90 active neutral (70–230 mesh ASTM); Merck, Darmstadt, Germany] with dichloromethane–methanol–ammonia solution (8:2:0.35) as the eluent and distilled under reduced pressure to give the title compound as a colourless oil, yield 860 mg (95%), $[\alpha]_D^{26}$ + 12.3°(*c* = 2.0, EtOH). ESMS: *m/z* calculated for $C_{14}H_{18}NO_2^+$, 232.1; found, 232.0. ^{1}H NMR (300 MHz, DMSO-d_6), δ (ppm): 1.33–1.77 (m, 4H, H_5 and H_8); 1.99–2.07 (m, 1H, H_4); 2.47–2.88 (m, 5H, H_6, H_7 and H_2); 3.20 (dd, 1H, ^2J = 14.7 and ^3J = 8.2 Hz, H_2); 4.88–4.96 (m, 1H; H_3); 7.46–7.54 (m, 2H, H_3 and H_5 *Ph*); 7.60–7.69 (m, 1H, H_4 *Ph*); 7.97 (d, 2H, *J* = 7.93 Hz, H_2 and H_6 *Ph*). ^{13}C NMR (300 MHz, DMSO-d_6), δ (ppm): 19.44 (C_5); 24.16 (C_8); 25.19 (C_4); 46.01 (C_6); 46.98 (C_7); 55.24 (C_2); 71.94 (C_3); 129.03 (C_2 and C_6 *Ph*); 129.39 (C_3 and C_5 *Ph*); 130.26 (C_1 *Ph*); 133.57 (C_4 *Ph*); 165.79 (C═O). UV (EtOH), λ_{max} (nm): 238.4 (ε = 4766 dm^3 mol^{-1} cm^{-1}).

(*S*)-Quinuclidin-3-yl benzoate (SQBz) was prepared by the same method, $[\alpha]_D^{26} - 11.8°$ ($c = 2.0$, EtOH).

Kinetic procedure

The hydrolysis of benzoate esters catalysed by BChE was monitored by following the production of benzoic acid at 238 nm by a UV detector (HPLC). The reactions were conducted at 30 °C in 0.1 M sodium phosphate buffer (pH 7.4). All experiments were performed in a total volume of 1.0 ml and an enzyme concentration of 1.5×10^{-9} M (0.016 mg ml^{-1}). The enzymatic reaction was stopped by addition of aliquots (20 µl) of the reaction mixture to the HPLC mobile phase (200 µl). Two to four measurements were made with each substrate concentration (0.08–0.5 mM). Rates were corrected to account for non-enzymatic hydrolysis. The concentration of active sites of BChE was determined by titration with a high-affinity phosphorylating agent 7-(diethoxyphosphoryl)oxy-1-methylquinolinium iodide (DEPQ), and ascertaining the minimal concentration that produced complete inhibition.[7] K_M and k_{cat} values were obtained by non-linear regression of the experimental data to the Michaelis–Menten equation.

Structures of BChE and substrates

A theoretical 3D structure of human BChE was derived by homology modelling on the basis of the known x-ray structure of *Torpedo* acetylcholinesterase.[5] Using the AutoDock 3.0 suite of programs,[11] we added polar hydrogen atoms to amino acid residues. Gasteiger–Marsili[8] atomic partial charges were assigned to all atoms of the enzyme. The charge of the non-polar hydrogens was added to the atom to which that hydrogen was bonded. Then, atomic solvation parameters and fragmental volumes were assigned to the protein atoms using AutoDock's AddSol utility.

Geometry optimizations of all substrates were carried out using the Gaussian 94 program.[9] Structures were optimized by the density functional theory (DFT) method using the B3LYP functional and 6–31G* basis set.[10] The structure of SQBz was simply prepared by the mirroring of the coordinates obtained for RQBz. Further preparation of substrates included addition of Mulliken's atomic charges, removal of hydrogen atoms and addition of their atomic charges to skeleton atoms, and finally, assignment of proper atomic types. AutoTors was then used to define the rotatable bonds in the ligands (Fig. 2).

Docking simulations

All docking studies were performed by using the AutoDock 3.0 suite of programs.[11] This program requires a precalculated electrostatic grid map for each atom type present in the substrate molecule. This was achieved by the AutoGrid part of the suite. All electrostatic maps were calculated with 0.2 Å spacing between grid points and in all cases the centre of the grid was attached to the γ-oxygen atom of catalytic Ser^{200}. The dimensions of the active site box were set at $10 \times 20 \times 10$ Å, which ensured an appropriate size of the ligand-accessible space. The consistencies of the maps were ascertained by checking maximum and minimum van der Waals energies and electrostatic potentials for each calculated grid map.

Flexible ligand docking was performed for choline (BuCh and BzCh) and two enantiomeric quinuclidinium (RQBz and SQBz) esters. Docking calculations were carried out using the Lamarckian genetic algorithm (LGA) and all parameters were the same for each docking. We used initially a population of random individuals (population size: 400), a maximum number of 2×10^6 energy evaluations, a maximum number of generations of 200 000, an elitism value of 1 and a mutation rate of 0.08. For the local search, the pseudo-Solis and Wets method was used: maximum of 300 iterations per local search, the probability of performing a local search on an individual in the same population was 0.06, the maximum number of consecutive successes or failures before doubling or halving the local search step size was 4 in both cases and the termination criterion for the local search was 0.01. At the end of a docking procedure (50 docking runs), the resulting positions were clustered according to an r.m.s. criterion of 0.5 Å and the orientations obtained were analysed visually. To ensure the validity of the results, the docking procedure was repeated 10 times for each substrate.

RESULTS AND DISCUSSION

Reactivity of BChE towards substrates

According to Scheme 1, the Michaelis constant (K_M) corresponds to $(k_{-1}/k_1 + k_2/k_1)[k_3/(k_2 + k_3)]$. The apparent catalytic first-order rate constant (k_{cat}) contains the first-order rate constants for acylation (k_2) and deacylation (k_3) and is equal to $k_2k_3/(k_2 + k_3)$. The ratio k_{cat}/K_M is often used as a measure of catalytic efficiency and it reflects the initial steps leading to the formation of the acylated enzyme, $k_1k_2/(k_{-1} + k_2)$.[12] Using non-linear

Table 1. Michaelis–Menten parameters for BChE-catalyzed hydrolysis of choline esters

Substrate	K_M (mM)	k_{cat} (10^3 min^{-1})	(k_{cat}/K_M) (10^7 M^{-1} min^{-1})
BzCh	0.17 ± 0.01	19 ± 1	11
RQBz	0.28 ± 0.02	4.4 ± 0.1	1.6
SQBz	0.13 ± 0.02	0.25 ± 0.01	0.2

regression, K_M, k_{cat} and the apparent bimolecular rate constants (k_{cat}/K_M) were calculated for the hydrolysis of benzoate esters by BChE. Kinetic constants and standard errors are displayed in Table 1.

Despite the marked difference in experimentally observed k_{cat}, the difference in K_M values between substrates was relatively small. Because of the relatively small k_2, K_M can be used as a measure of the binding affinity of SQBz and RQBz. Actual K_M values determined for the quinuclidinium substrates indicate that the binding affinity RQBz toward BChE is approximately 2-fold lower than that of SQBz. The greater affinity of the *S*-enantiomer towards the active site indicates that the binding of that substrate does not always lead to the tetrahedral intermediate and therefore hydrolysis proceeds at a much slower rate than that of the *R*-enantiomer or BzCh.

A change of the alcohol part from the charged quaternary ammonium choline moiety in BzCh to quinuclidinium resulted in a decreased catalytic efficiency of BChE for both enantiomers. The activity trend for both k_{cat} and k_{cat}/K_M was BzCh > RQBz ≫ SQBz. The combined effects on K_M and k_{cat} resulted in a 7-fold reduction for RQBz and a 53-fold reduction in the apparent bimolecular rate constant for SQBz compared with BzCh. A comparison of the substrate acylation rates for the quinuclidinium derivatives showed that despite the lower affinity towards the enzyme, the *R*-enantiomer acylates the serine in the active site 8-fold faster. The catalytic first-order rate constant (k_{cat}) for quinuclidinium derivatives reveals an 18-fold slower reaction for the *S*-enantiomer. Since k_3 is the same for all substrates, the difference in the k_{cat} values indicates that the major reason for the 4-fold slower reaction of RQBz and the 76-fold slower reaction of SQBz compared with BzCh lies in the acylation step (Scheme 1).

The tested quinuclidinium benzoate esters are poorer substrates for BChE than butyrylcholine, which is a good substrate.[13] Hence it can be assumed that catalysis is limited by substrate orientation or a possible unimolecular isomerization step, as proposed for some other substrates.[14] Possible alternative binding modes in the active gorge can compete with that which leads to the tetrahedral intermediate and hydrolysis.

From the difference in free energy, $\Delta\Delta G‡$, one can determine the selectivity of the reaction and the optical purity of the product when performing the resolution of enantiomers.[15] $\Delta\Delta G‡$ was calculated from the ratio of 'specificity constants' (k_{cat}/K_M) obtained for RQBz and SQBz. The value of 1.25 kcal mol^{-1} corresponds to an enantiomeric excess of 78% (50% conversion) in favor of the *R*-enantiomer.

Substrate-BChE complexes derived from docking studies

Three different benzoate esters and butyrylcholine were docked into the active site of BChE by utilizing the AutoDock program. We chose to explore the binding at the active site of the enzyme to see whether differences in interaction with those residues can explain the rates of hydrolyses. Furthermore, observed differences in binding in the ground states are probably also important for the stability of the tetrahedral intermediates.

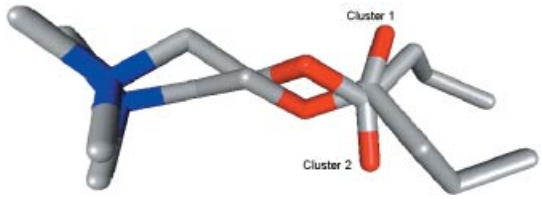


Figure 3. Two orientations of BuCh obtained by flexible ligand docking with AutoDock 3.0 in the active site of BChE: cluster 1 (−7.21 kcal mol^{-1}), the most energetically favourable orientation with the carbonyl group pointed towards catalytic triad; cluster 2 (−7.13 kcal mol^{-1}), carbonyl group oriented towards oxyanion hole. Two clusters obtained for each benzoate ester (BzCh, SQBz and RQBz) are related in a similar way to those of BuCh. This figure is available in colour at www.interscience.wiley.com/epoc

According to the energy values and substrate orientations in the BChE–ester complexes, AutoDock generated two clusters of binding orientations for all substrates in the active site. Based on the knowledge of the mechanism of hydrolysis of AChE and BChE,[12] we chose the most probable orientation obtained for BuCh which can result in the most stable tetrahedral intermediate during hydrolysis (Fig. 3).

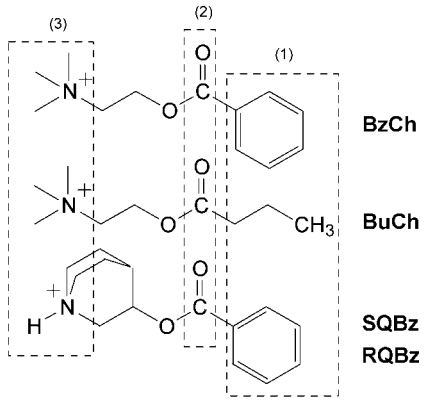
That orientation was then compared with all other similar orientations obtained for the benzoate esters (Table 2). However, AutoDock is not able to deal with conformational flexibility of the enzyme, so that should be taken into account when evaluating these results.

The results obtained from flexible ligand docking revealed that all esters have similar interactions in the active site. There are three energetically favourable regions for the interaction of the tested esters with the active site of BChE: (1) the hydrophobic region, which

Table 2. Final intermolecular energy (E_{FIE}), interaction energy contributed by van der Waals interactions (E_{vdW}) and interaction energy contributed by electrostatic interactions (E_{Elect}) obtained by AutoDock for the second, energetically favourable orientation (cluster 2) for all substrates[a]

Substrate	E_{FIE} (kcal mol^{-1})	E_{vdW} (kcal mol^{-1})	E_{Elect} (kcal mol^{-1})
BuCh	−7.13	−6.62	−0.51
BzCh	−8.81	−8.31	−0.50
RQBz	−8.72	−8.77	0.05
SQBz	−8.71	−8.87	0.16

[a] 1 kcal = 4.184 kJ.



Scheme 2

includes the propyl group and the benzene ring interacting with the amino acids of the acyl pocket, (Leu[285], Val[287], Trp[233], Phe[400], Phe[331]); (2) the carbonyl electrostatic region, which includes partial hydrogen bonds formed between the carbonyl oxygen of substrates and the amide backbone hydrogen of Gly[119] or hydroxyl hydrogen of Ser[200], and the electrostatic interaction between the electrophylic carbonyl carbon of substrate and γ-oxygen of catalytic Ser[200]; and (3) the ammonium electrostatic region, which includes strong cation–π interaction of the ammonium moiety of substrate with the indole ring of Trp[84] and close contacts with carboxylate of Glu[199] and aromatic ring of Tyr[130] (Scheme 2).

BuCh was docked to test whether AutoDock can reproduce the binding of the 'natural' substrate of BChE. The docking results obtained for this ligand showed two main ways of binding which differed only in the position of the carbonyl group of the substrate (Fig. 3). The orientation with slightly lower energy was the one with a direct H-bond between the carbonyl group and the hydroxyl group of Ser[200], which showed the strongest tendency to form a hydrogen bond. The second complex obtained differed only slightly in energy (0.08 kcal mol^{-1}) but described nicely the expected mode of binding (Fig. 4). The distances of the carbonyl oxygen atom from the amide nitrogen atom of Gly[118] and Gly[119] were 4.0 and 2.9 Å, respectively, and the distance of the carbonyl carbon atom from the γ-oxygen atom of the catalytic Ser[200] was 3.2 Å. Hydrophobic interactions in the acyl pocket are between the propyl chain of the substrate and Leu[288], Val[290], Phe[400], Phe[331] and Trp[233]. The cation–π interactions between the quaternary ammonium group and Trp[84] are very important in binding of the charged choline moiety.[16] The quaternary ammonium group of BuCh is oriented in such a way that

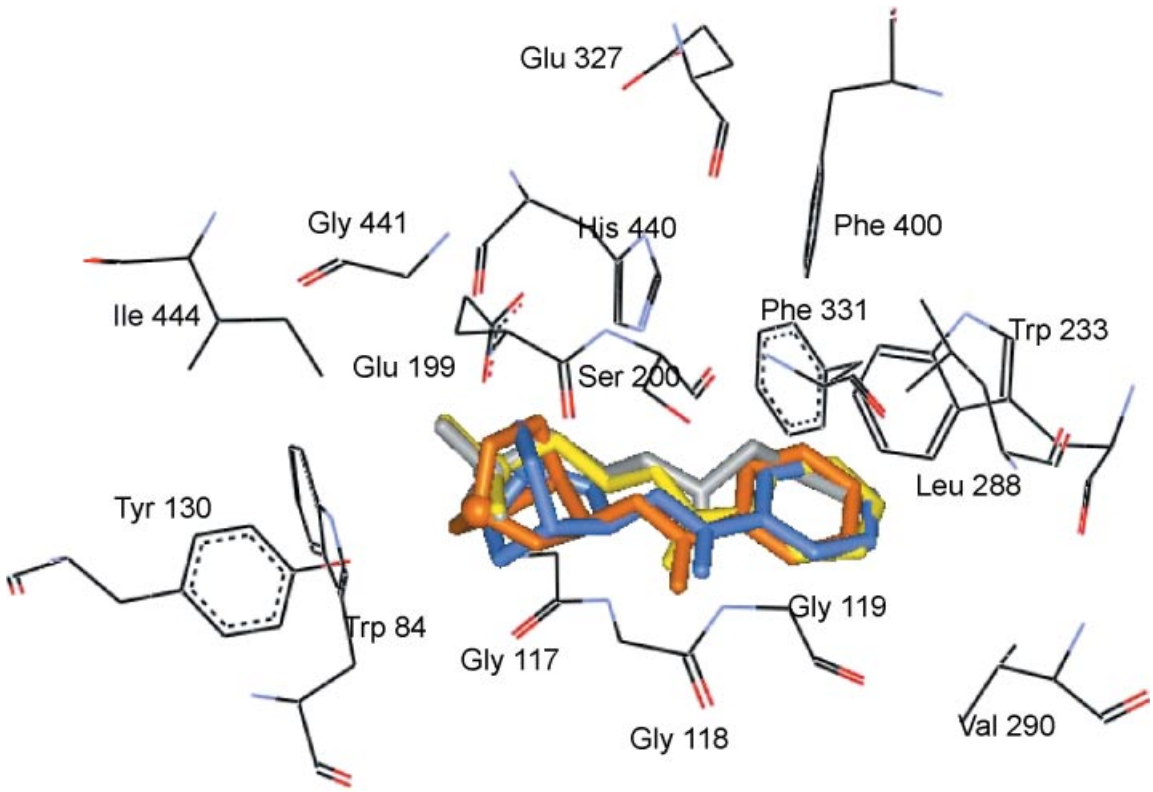


Figure 4. Proposed orientations for BuCh (grey), BzCh (yellow), RQBz (orange) and SQBz (blue) in the active site of human BChE (cluster 2) obtained by flexible ligand docking with AutoDock 3.0, showing the residues involved in the interactions. Hydrogens atoms are omitted for clarity. The nitrogen atom of the substrate is represented with a ball model. This figure is available in colour at www.interscience.wiley.com/epoc

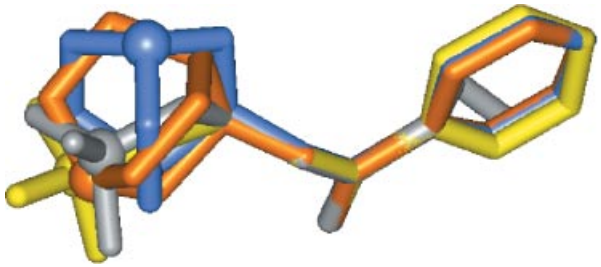


Figure 5. Overlay of carbonyl groups of structures obtained by flexible ligand docking with AutoDock 3.0 in the active site of human BChE (cluster 2) showing the similarity of obtained orientations for BuCh (grey), BzCh (yellow) and RQBz (orange) and difference in the position of the quaternary ammonium nitrogen of SQBz (blue). This figure is available in colour at www.interscience.wiley.com/epoc

the cation–π interactions with the indole ring of Trp^{84} are optimal, the distance to the centre of the indole ring being 3.7–4.5 Å. This group also interacts with Glu^{199} ($\sim$3.0 Å from the N^+ atom and one methyl group to the OE2 oxygen of glutamic acid), but it was proved by mutation studies that Trp^{84} is more important for efficient catalysis.[17]

Benzoate esters. In many respects the Michaelis complexes obtained with flexible ligand docking display interactions with the active site residues that are very similar to those obtained for BuCh. For all benzoates, the most energetically favourable orientation was that with the H-bond with the catalytic Ser^{200}, while the orientation in which the carbonyl group is pointed toward the oxyanion hole was slightly higher in energy as obtained for BuCh (Fig. 3). The modelling of the benzoate esters in the acyl pocket indicated that the acyl pocket provides a tight fit for the phenyl ring. The van der Waals energy is $\sim$2 kcal mol^{-1} higher in the case of the phenyl ring compared with the propyl group of BuCh. It is worth noting that the phenyl ring of all substrates has almost exactly the same location in the acyl pocket making a T-shaped aromatic complex with Phe^{331}, Phe^{400} and Trp^{233} and having close contacts with Leu^{288} and Val^{290}. This strong interactions and lower flexibility of the phenyl ring resulted in a longer distance of the carbonyl carbon atom from the γ-oxygen of the catalytic Ser^{200}, 4.0 Å for BzCh, 4.5 Å for RQBz and 4.7 Å for SQBz. Furthermore, the distances of the carbonyl oxygen atom and the amide nitrogen atom of Gly^{119} were extended to 3.8, 4.1 and 4.7 Å for BzCh, RQBz and SQBz, respectively (Fig. 4).

The quaternary ammonium group of BzCh is located at the same position as that of BuCh, while the positions of the quinuclidiniums are in a less favourable position. The nitrogen and adjacent methylenic groups of (*R*)-quinuclidinium are 3.8–5.0 Å from the centre of the indole ring of Trp^{84}. The position of the same atoms in (*S*)-quinuclidinium is even more distant from Trp^{84} (4.9–5.8 Å), but is closer to Glu^{199} (2.7–3.5 Å). Therefore, cation–π stabilization of the alcohol part of RQBz and especially of SQBz is lower than in the case of choline esters, and that is probably the main contributor to the lower reaction rates.

Overlay of carbonyl groups of all obtained structures (cluster 2) by flexible ligand docking showed that all substrates ended in a similar orientation regarding the position of the quaternary nitrogen atom and the carbonyl group, except for SQBz (Fig. 5).

The quinuclidinium part of SQBz is properly positioned in the complex with the active site when its carbonyl oxygen is turned toward His^{440} and makes a hydrogen bond with Ser^{200}. Such an orientation of SQBz, which is unsuitable for hydrolysis, may contribute significantly to its binding to the enzyme, explaining why the *S*-enantiomer of QBz is a poor substrate for BChE.

This knowledge should facilitate the search for and identification of other structural analogues likely to exhibit improved stereoselectivity towards BChE.

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