Identification of the human enzymes responsible for the enzymatic hydrolysis of aclidinium bromide

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Abbreviations

AChE, acetylcholinesterase
AEBSF, 4-(2-aminoethyl)benzenesulfonyl fluoride
ATCI, acetylthiocholine iodide
BPNP, bis(p-nitrophenyl)phosphate
BChE, butyrylcholinesterase
BTCI, butyrylthiocholine iodide
BW 284c51, 1,5-Bis(4-allyldimethylammoniumphenyl)pentan-3-one
CbE, carboxylesterase
ChE, cholinesterase
COPD, chronic obstructive pulmonary disease
CYP450, cytochrome P450
DFP, diisopropyl fluorophosphates
DMSO, dimethyl sulphoxide
DTNB, 5,5'-dithio-bis(2-nitrobenzoic acid) or Ellman’s reagent
DNPP, paraoxon
ESE, serine
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ESI, electrospray ionization

Iso-OMPA, tetraisopropyl pyrophosphoramide

k_h, hydrolysis rate constant

LC, liquid chromatography

MS, mass spectrometry

PCMB, p-chloromercuribenzoate

PMSF, phenylmethanesulfonfyl fluoride

SDS, sodium dodecyl sulfate
Abstract

Aclidinium bromide, a novel, long-acting, inhaled muscarinic antagonist for the treatment of chronic obstructive pulmonary disease, has demonstrated rapid hydrolysis in human and animal plasma. This process occurred both non-enzymatically ($k_h 0.0075 \text{ min}^{-1}$) and enzymatically. The purpose of the current study was to investigate the in vitro enzymatic hydrolysis of aclidinium in humans. Human butyrylcholinesterase was identified as the most important esterase responsible for the enzymatic hydrolysis of aclidinium from inhibition studies in human plasma with selective paraoxonase, arylesterase, carboxylesterase, acetylcholinesterase, and butyrylcholinesterase chemical inhibitors, as well as from incubations with pure human cholinesterases. Furthermore, neither human CYP450 nor human serum albumin participated in the enzymatic ester cleavage of aclidinium. Butyrylcholinesterase activity in the human lung was lower than that observed in human plasma, suggesting a limited first-pass effect after administration by inhalation of the drug. Aclidinium was shown to inhibit competitively both human butyrylcholinesterase ($K_i, 2.7 \mu M$) and acetylcholinesterase ($6.3 \mu M$), but did not have any effect on the activity of other human esterases, as well as its hydrolysis metabolites. These results suggest that the potential for clinical interactions involving human cholinesterases is remote at clinically relevant plasma concentrations, which are below 10 nM.
Introduction

Aclidinium bromide [3R-(2-hydroxy-2,2-dithiophen-2-yl-acetoxy)-1-(3-phenoxy-propyl)1-azonia bicyclo[2.2.2] octane bromide] is a novel, long-acting, inhaled muscarinic antagonist currently in Phase III clinical trials (Gavaldà et al., 2009). It is intended for the maintenance treatment of chronic obstructive pulmonary disease (COPD). Aclidinium demonstrated non-enzymatic hydrolysis of its ester bond at neutral and basic pH and was rapidly hydrolyzed in plasma of different animal species and humans to yield an alcohol (LAS34823) and carboxylic acid metabolite (LAS34850) (Prat et al., 2009). The marked difference in the rate of aclidinium hydrolysis observed in human plasma (t1/2 2.4 minutes) and in phosphate buffer at pH 7.4 (t1/2 1.2 hours) suggested that enzymatic hydrolysis plays a major role in the overall hydrolysis of aclidinium (Sentellas et al., manuscript submitted for publication). It has been demonstrated in clinical studies that, upon reaching systemic circulation, the ester bond of aclidinium undergoes rapid hydrolytic cleavage, and both hydrolysis metabolites are the major circulating compounds following administration of aclidinium using a multidose dry-powder inhaler (Jansat et al., 2009). The alcohol and carboxylic acid metabolites are devoid of any significant affinity to the muscarinic receptors and did not show any relevant bronchodilatory activity in vivo (Sentellas et al., manuscript submitted for publication).

Different human proteins such as esterases, CYP450 and albumin may be involved in the hydrolysis of ester compounds. Esterase classification is difficult because esterases exhibit overlapping substrate specificities and a single substrate is often hydrolyzed by more than one enzyme. There is classification of esterases, based on the interaction of these enzymes with organophosphorous esters (Aldridge 1953). According to this classification, esterases can be divided into three different classes: A-esterases, which hydrolyze paraoxon and other organophosphorous esters; B-esterases, which are inhibited by organophosphates such as paraoxon; and C-esterases, which do not interact with organophosphates. A-esterases include paraoxonase (aryldialkylphosphatase; EC 3.1.8.1) and arylesterase activity (EC 3.1.1.2). Three paraoxonases – PON1, PON2, and PON3 – have been identified. Human paraoxonase (PON1) may be present in two structural isoforms, where paraoxon is the discriminatory substrate and phenylacetate the non-discriminatory substrate (Mackness et al., 1998). B-esterases belong to the α,β-hydrolase-fold family of proteins, and include cholinesterases and carboxylesterases (Satoh et al., 2002). In mammals, cholinesterases are divided into acetylcholinesterase (AChE; EC 3.1.1.7) and butyrylcholinesterase (BChE; EC 3.1.1.8). Acetylcholinesterase, also called red blood cell cholinesterase, is more abundant in the central nervous system, skeletal muscle, and erythrocyte...
membranes, whereas butyrylcholinesterase, also called pseudocholinesterase or plasma cholinesterase, is synthesized in the liver and secreted into plasma (Rhynaen 1983). Human plasma contains BChE almost exclusively, whilst plasma in other animals contains variable proportions of BChE and AChE (Schwarz et al., 1995). The exact physiological function of BChE remains elusive, although it could act as an alternative to the homologous AChE as a scavenger for anticholinesterase compounds. Carboxylesterases (CbE, EC 3.1.1.1) belong to a family of proteins encoded by multiple genes that are localized in the endoplasmatic reticulum of many tissues. CbEs have been classified into four main groups, namely CES-1, CES-2, CES-3, and CES-4. There are two major human liver isoenzymes (hCE-1 and hCE-2) found predominantly in the microsomal fraction of the human liver (Satoh et al., 1998).

In addition to esterases, CYP450-catalyzed oxidative ester cleavage may also occur, as has been demonstrated for different ester and amide compounds (Guengerich 1987, Peng et al., 1995). Furthermore, it has been postulated that human serum albumin may also present esterase-like activity (Yang et al., 2007). However, it has also been proposed that the esterase-like activity displayed by purified human serum albumin could be a result of contamination by other soluble hydrolases (Chapuis et al., 2001).

The rapid hydrolysis of aclidinium bromide is an important part of its safety profile since low circulating drug levels can reduce the potential for unwanted effects at sites other than the lung, which is the intended target. The purpose of this study was to identify the human enzymes involved in the enzymatic hydrolysis of aclidinium.
Materials and methods

Chemicals

Aclidinium bromide (Almirall code no. LAS34273, >99% purity), its alcohol metabolite (LAS34823 [3(R)-hydroxy-1-(3-phenoxy-propyl)-1-azonia-bicyclo[2.2.2]octane, bromide]), and its carboxylic acid metabolite (LAS34850 [dithienyl-glycolic acid, sodium salt]) were synthesized at Ranke Química S.L. (Barcelona, Spain). Chemical structures of aclidinium bromide and its hydrolysis metabolites LAS34823 and LAS34850 are depicted in Fig.1. Gradient grade LC methanol and acetonitrile, dimethylsulphoxide (DMSO), and sodium-1-octanesulphonic acid were obtained from Scharlab S.L. (Barcelona, Spain). All other chemicals were purchased from Merck (Darmstadt, Germany) or Sigma-Aldrich (Steinheim, Germany). Stock solutions of aclidinium bromide (50 mM) were prepared by dissolution of the drug in a 0.1 N hydrochloric acid:acetonitrile (10:90, v/v) mixture or in 0.1 N hydrochloric acid:DMSO (10:90, v/v). Stock solutions (5 mM) of LAS34823 and LAS34850 were prepared in 0.1 N hydrochloric acid:acetonitrile (10:90, v/v) mixture. Stock solutions were stored at -20°C. Stock solutions of esterase substrates and inhibitors were prepared in water or TRIS buffer at pH 7.4 unless indicated otherwise. Stock solution of 0.1% fast red ITR (5-Diethylaminosulfonyl-2-methoxybenzenediazonium chloride hemi-[zinc chloride] salt) was prepared in 2.5% Triton X-100 aqueous solution. Stock solutions of p-chloromercuribenzoate (PCMB), 4-(2-aminoethyl)benzenesulfonyl fluoride (AEBSF), and phenylacetate were prepared in 0.01N sodium hydroxide, 50 mM phosphate buffer (pH 5.0), and ethanol, respectively. Stock solutions of diisopropyl fluorophosphate (DFP) and 1-naphthyl acetate were prepared daily before use.

Biological materials

Individual plasma and erythrocyte samples from six human volunteers (three male and three female) were obtained by centrifuging blood samples at 2000 g (4°C) using lithium heparin as anticoagulant. After centrifugation, individual plasma and erythrocyte samples (0.5 ml aliquots) were immediately frozen at -80°C. Pooled human plasma samples (n=6) were prepared daily before use from frozen individual plasma samples. The mean content of total protein in plasma samples was 77.0 ± 5.0 mg/ml (n=6). Protein concentration in human plasma samples was determined by the method of Lowry et al., (1951) using human serum albumin as standard. Pooled human liver microsomes and S9 fraction were obtained from Xenotech (Kansas, USA). Human pulmonary microsomes and S9 fraction (smoker and non-smoker) were obtained from Human Biologics International (Phoenix, USA) and from Xenotech. Recombinant human CYP1A1, 1A2, 2C8, 2C9*1, 2C19*1,
2A6, 2B6, 2D6*1, 2E1, 3A4, 3A5, 4A11, 4F2, 4F3A, and 4F3B expressed in microsomes of baculovirus-infected cells (Supersomes™) were obtained from BD Gentest (Woburn, USA). Human recombinant acetylcholinesterase (2230 units/mg protein) and purified human butyrylcholinesterase from human serum (12.7 units/mg protein) were purchased from Sigma-Aldrich. One unit of AChE or BChE hydrolyzes 1.0 µmole of acetylcholine or butyrylcholine to choline and acetate/butyrate per minute at pH 8.0 and 37°C.

**Hydrolysis in human plasma**

In preparation for kinetic and inhibition assays, the linearity of the formation of the hydrolysis metabolites was investigated by incubating aclidinium bromide (2-250 µM) in diluted pooled human plasma (1/100, 2/100, and 5/100, v/v) with 50 mM potassium phosphate buffer (pH 7.4) at 37°C in a final reaction volume of 2 ml. After 3 minutes of pre-incubation at 37°C, reactions were initiated by adding 0.1 ml of the corresponding aclidinium solution. The final concentration of DMSO in the incubation mixtures was 2.5%. At pre-defined times of 15, 30, and 60 minutes, aliquots of 200 µl of the incubation mixtures were separated and the reactions stopped by adding 100 µl of 0.2N HCl:acetonitrile (80:20, v/v). The precipitated protein was removed by centrifuging at 2000xg and 4°C for 10 minutes. Sample analysis was conducted using a LC-UV system (**Method A**). Control incubations in the absence of human plasma were also conducted and an equivalent amount of human plasma was added at the end of the incubation period. All the incubations were carried out in duplicate. The kinetic study of the hydrolysis of aclidinium bromide (1-500 µM) was conducted as described above by incubating individual human plasma samples (2/100, v/v) at 37°C for 30 minutes in a final volume of 0.2 ml. Reactions were stopped by the addition of 0.1 ml of 0.2N HCl:acetonitrile (80:20, v/v). All the samples were maintained at 4°C once obtained and during analysis in order to prevent aclidinium hydrolysis. Plasma hydrolysis assays of aclidinium at the nanomolar range (from 2 nM to 50 µM) were conducted as described above but in the absence of DMSO as solvent. The final analysis of aclidinium and LAS34823 in incubation samples was conducted using the LC-MS/MS method.

Incubations of aclidinium bromide in human erythrocytes were conducted as described above in a final volume of 1 ml. At pre-defined times of incubation (0, 5, 15, 30, and 60 minutes), 150 µl aliquots of the incubation mixtures were transferred to another vessel containing 0.9 ml of 0.2N HCl:acetonitrile (80:20, v/v) and processed as described above. Sample analysis was conducted using the LC-UV system (**Method A**).
**Hydrolysis in human pulmonary and hepatic subcellular fractions**

Aclidinium bromide (5 µM) was incubated in the presence of pooled human liver and pulmonary microsomes and S9 fraction (0.5 mg/ml) at 37°C in 0.5 ml incubation mixtures containing potassium phosphate buffer (50 mM, pH 7.4), MgCl₂ (3 mM), EDTA (1 mM), NADP⁺ (1 mM), glucose-6-phosphate (5 mM) and glucose-6-phosphate dehydrogenase (2 unit/ml). After 3 minutes of incubation, aclidinium bromide was added to the 0.5 ml incubation mixtures in 10 µl of 0.01N HCl:acetonitrile (80:20, v/v). The final concentration of acetonitrile in the incubation mixtures was 0.5%. At different pre-defined times (10, 20, 30, and 60 minutes), 100 µl aliquots of the incubation mixtures were separated and the reactions stopped as described in previous sections. Control incubations without the NADPH-generating system and in 50 mM phosphate buffer (pH 7.4) were also prepared. All the incubations were carried out in duplicate. The amounts of the acid and alcohol hydrolysis products (LAS34850 and LAS34823, respectively) in the clear supernatant fraction were determined by LC-UV (Method B). All the samples were kept at 4°C once obtained and during LC analysis in order to prevent LAS34273 ester cleavage.

**Inhibition of aclidinium hydrolysis**

The effect of selective chemical esterase inhibitors on aclidinium hydrolysis was evaluated using PCMB (paraoxonase/arylesterase), DFP and AEBSF (general B-esterase), eserine (cholinesterases), BW284c51 (AChE), iso-OMPA (BChE), and BPNP and NaF (CbE). Selective esterase inhibitors at final concentrations of 1, 10, and 100 µM were pre-incubated with diluted pooled human plasma (3.85 mg/ml, 5/100, v/v) for 15 minutes at 37°C in a final volume of 0.2 ml. The final concentration of DMSO in the incubation mixtures was 2.5%. The reactions were initiated by the addition of aclidinium bromide at a final concentration of 5 µM and were stopped after 30 minutes of incubation as indicated above. Sample analysis was conducted using the LC-UV system (Method A). All the incubations were carried out in duplicate and control incubations in the absence of human plasma were also conducted, with the addition of an equivalent amount of plasma at the end of the incubation period.

Additional assays at a low concentration of aclidinium bromide (10 nM) were conducted as described above but in the absence of DMSO as solvent. Final analysis of aclidinium and LAS34823 in incubation samples was conducted using the LC-MS/MS method.
Incubations with human esterases

Aclidinium bromide (5 μM) was incubated in the presence of human recombinant acetylcholinesterase and purified human butyrylcholinesterase (0, 0.001, 0.01, 0.1, and 1 U/ml, relative units) in 50 mM phosphate buffer (pH 7.4) for 30 minutes at 37°C (final volume of 0.2 ml). The final protein concentration in the incubation mixtures was adjusted to 0.25 mg/ml with human serum albumin. The reactions were initiated by the addition of aclidinium bromide solution and after 30 minutes of incubation were stopped and treated as indicated above. Sample analysis was conducted using the LC-UV system (Method A).

Kinetic study of aclidinium hydrolysis in purified human butyrylcholinesterase was conducted at a fixed concentration of 0.2 units/ml (relative units). Samples were incubated at 37°C in 0.2 ml (final volume) containing potassium phosphate buffer (50 mM, pH 7.4) and aclidinium bromide (1-500 μM). The final concentration of DMSO in the incubation mixtures was 2.5%. Final protein concentration in the incubation mixtures was adjusted to 0.125 mg/ml by adding human serum albumin. After 5 minutes of pre-incubation, reactions were started by the addition of aclidinium bromide solutions. After 30 minutes of incubation, the reactions were stopped and treated as indicated above. Sample analysis was conducted using the LC-UV system (Method A). Control incubations in 50 mM phosphate buffer (pH 7.4) were also prepared. All the incubations were carried out in duplicate.

Human recombinant CYP450 isoforms

cDNA-expressed human isoforms (25 pmol CYP450/ml) were incubated at 37°C in 0.2 ml (final volume) incubation mixtures containing potassium phosphate buffer 50 mM, pH 7.4 (CYP1A1, 1A2, 2B6, 2C8, 2C19*1, 2D6*1, 2E1, 3A4, 3A5, 4F2, 4F3A, and 4F3B) or TRIS buffer 100 mM, pH 7.4 (CYP2A6, 2C9*1, and 4A11), MgCl₂ (3 mM), EDTA (1 mM), NADP (1 mM), glucose-6-phosphate (5 mM), glucose-6-phosphate dehydrogenase (2 unit/ml) and aclidinium (20 μM) at the final concentrations indicated. Aclidinium bromide was added to the 0.2 ml incubation mixtures in 10 μl of HCl 0.01N:acetonitrile (90:10, v/v). The final concentration of acetonitrile in the incubation mixtures was 0.5%. Protein content was fixed at 0.25 mg/ml in all incubation mixtures by adding appropriate volumes of insect cell control microsomes (0.5 mg protein/ml). After 5 minutes of pre-incubation, reactions were started by the addition of the NADPH-generating system. After 60 minutes of incubation, reactions were stopped and treated as indicated above. All the incubations were carried out in duplicate. Sample analysis was carried out using the LC-UV system (Method B).
Esterase activities

Paraoxonase activity was determined spectrophotometrically using a substrate paraoxon as previously described (Rodrigo et al., 2001). Briefly, diluted human plasma (1/150, v/v, approximately 0.5 mg protein/ml) was incubated at 37°C with 5 mM paraoxon in 100 mM HEPES containing 2 mM CaCl₂ (pH 7.4). The generation of p-nitrophenol was monitored at 405 nm for 20 minutes at one-minute intervals. The extinction coefficient of p-nitrophenol at the assay conditions (9.65 mM⁻¹.cm⁻¹) was calculated from a regression curve (10-100 μM).

Arylesterase activity was determined spectrophotometrically using phenylacetate as substrate as described by Rodrigo et al., 2001. Briefly, diluted human plasma (0.01 mg protein/ml) was incubated at 37°C with 2 mM phenylacetate in 100 mM HEPES containing 2 mM CaCl₂ (pH 7.4) in a final volume of 3 ml. Reactions were stopped by adding 2 ml of 0.1N HCl, and UV absorbances at 270 nm were measured. Quantification was conducted using a calibration curve obtained from standard samples of phenol (0.1-0.75 mM) prepared in the assay conditions. Carboxylesterase activity was determined using α-naphthyl acetate as a substrate based on its hydrolysis to yield naphthol, which reacts with the dye Fast Red ITR to yield a colored product that was measured spectrophotometrically (Thompson, 1999). Diluted human plasma (0.25 mg protein/ml) was incubated at 37°C with 35 μg/ml of α-naphthyl acetate in 100 mM TRIS buffer (pH 7.4) in a final volume of 3 ml for 5 minutes. Reactions were stopped by the addition of 0.25 ml of 2.5% sodium dodecyl sulfate (SDS) solution and 0.25 ml of 0.1% fast red ITR. The mixtures were maintained at room temperature in the dark for 10 minutes and the absorbance of the napthol fast red ITR complex was measured at 530 nm. AChE and BChE activities were assayed using acetylthiocholine iodide (ATCI) and butyrylthiocholine iodide (BTCI) as substrate, respectively. In both cases, the thiocholine generated reacts with DTNB (5,5-dithiobis[2-nitrobenzoic acid]) to produce the yellow-colored 5-thio-2-nitrobenzoate ion (Ellman et al., 1961). The selective inhibitor iso-OMPA (0.1 mM final concentration) was used to suppress butyrylcholinesterase activity for acetylcholinesterase determinations. In brief, diluted human plasma (AChE: 0.25 mg protein /ml or BChE: 0.125 mg protein/ml) or tissue subcellular fractions (0.08 mg protein/ml) were incubated for 15 minutes with 2 mM DTNB in 100 mM TRIS buffer (pH 7.4) at 37°C. Then the corresponding substrate ATCI or BTCI (0.5 mM) was added and the formation of 5-thio-2-nitrobenzoate was monitored at 415 nm for 20 minutes at one-minute intervals. Enzyme activities were calculated using the extinction coefficient of 5-thio-2-nitrobenzoate (13.6 mM⁻¹.cm⁻¹).
Esterase inhibition assays

The inhibition of human paraoxonase, BChE, AChE, and CbE by aclidinium bromide and its hydrolysis metabolites was conducted in human plasma as described above. However, the conversion of phenylacetate to phenol (arylesterase activity) was quantified by using LC-UV (254 nm) to prevent UV interferences from aclidinium and its metabolites. In these assays, reactions were stopped by adding 2 ml of ice-cold acetonitrile. Phenylacetate and phenol were separated on a Waters Novapack C18 column (150x3.9 mm, 4 µm) protected by Tracer Spherisorb ODS-2CN (10x4.6 mm) and eluted using a gradient with (solvent A) 10 mM ammonium formiate (pH 4.5) and (solvent B) acetonitrile. The initial mobile phase composition was 25%B for 4 minutes and progressed linearly to 90%B in 5 minutes, maintaining this percentage for 4.5 minutes. The mobile phase was returned to the starting solvent mixture in 0.5 minutes and the system equilibrated for 5 minutes between runs. A constant flow rate of 1 ml/min was employed. The injection volume was 10 µl and the retention times were 3.5 minutes and 7.4 minutes for phenol and phenylacetate, respectively. Neither aclidinium nor its two hydrolysis metabolites interfered in the phenol determination.

Instrumentation and LC-UV analysis

Incubation assays were carried out using a programmable Packard Multiprobe II automated liquid handling system (Perkin Elmer, Illinois, USA) and 1-ml Nunc 96-deepwell (round bottom) plates (Kamstrup, Denmark) or Eppendorf conical bottom plastic tubes (incubation volume >1 ml). All spectrophotometric determinations (esterase activities) were conducted using a double-beam Hitachi U2001 spectrophotometer (San Jose, USA) assisted with an on-line Huber Polysat K6 water-bath (Hothenburg, Germany) set at 37°C. Aclidinium and its acid and alcohol hydrolysis products (LAS34850 and LAS34823) in the clear supernatant fraction were determined using a Waters Alliance 2790 LC system (Waters, Milford, MA, USA) with UV detection (Waters 2996PDA) at 219 nm (LAS34823) and 238 nm (aclidinium and LAS34850). Aclidinium and its metabolites were separated on a Waters Symmetry C18 column (250x4.6 mm, 5 µm) protected by Tracer Spherisorb ODS-2CN (10x4.6 mm) and eluted using a gradient with (solvent A) 10 mM sodium phosphate containing 5 mM 1-octanesulphonic acid (pH 2.8) and (solvent B) acetonitrile. Both methods used a constant flow rate of 1 ml/min and the injection volume was 90 µl. Method A: The initial mobile phase composition was 35%B and progressed linearly to 50%B in 12 minutes. The mobile phase was returned to the starting solvent mixture in 1 minute and the system equilibrated for 8 minutes between runs. The retention times were 4.5 minutes, 6.5 minutes, and 11.6 minutes for LAS34823, LAS34850, and aclidinium, respectively. Method B: The initial mobile phase
composition was 10%B and progressed linearly to 55%B in 30 minutes. The mobile phase was returned to the starting solvent mixture in 1 minute and the system equilibrated for 7 minutes between runs. The retention times were 17.8 minutes, 18.8 minutes, and 26.8 minutes for LAS34850, LAS34823, and aclidinium, respectively.

All the samples generated in incubations were always kept at 4°C in order to prevent aclidinium ester cleavage. Quantification of LAS34823, LAS34850, and aclidinium was carried out using a calibration curve of standard samples containing the three compounds in 50 mM phosphate buffer (pH 4.0):acetonitrile (90:10, v/v). These standard samples were diluted and treated as incubation mixtures. The chromatographic gradient was slightly modified for the analysis of incubation samples containing PCMB (arylesterase/paraoxonase inhibitor) in order to avoid the interference of the inhibitor chromatographic peak. The LAS34850 (carboxylic acid derivative) and LAS34823 (alcohol derivative) concentrations in the aclidinium hydrolysis studies were similar, so only the results for the carboxylic acid derivative (LAS34850) are presented unless indicated otherwise.

**LC-MS/MS analysis**

The determinations of aclidinium and LAS34823 in incubation samples from plasma stability studies (nanomolar range) were conducted by using LC with mass spectrometry detection. LC-MS analyses were performed using an Agilent 1100 Series liquid chromatograph (Waldbronn, Germany) coupled to an Applied Biosystems API 4000 QTrap hybrid triple quadrupole linear ion trap mass spectrometer (Applied Biosystems, Foster City, CA). Sample separation was carried out on a Spherisorb ODS-2 Waters (4.6x50 mm, 5 µm) column at a flow-rate of 0.5 ml/min using acetonitrile: 50 mM ammonium formiate (pH 4.0) (80:20, v/v) as mobile phase in isocratic mode. The TurboIonSpray ion source conditions were optimized and set at the following values: curtain gas = 20, collision gas = medium, ion spray voltage = 5500, temperature = 600°C, ion source gas 1 = 40, ion source gas 2 = 60. Nitrogen was used as the nebulizer and auxiliary gas. Quantification was performed using multiple reaction monitoring (MRM) for the transitions 484→262 (aclidinium; declustering potential 126; collision energy, 47), 262→140 (LAS34823; declustering potential 56; collision energy, 43), and 332→166 (ipratropium as internal standard; declustering potential 396; collision energy, 37).

**Data analysis**

Substrate inhibition was determined on the basis of visual examination of the Eadie-Hofstee plots, according to the following equation for a one-site binding model (Houston and Kenworthy, 2000):
where \([S]\) is the substrate concentration, \(K_s\) is the constant describing the substrate inhibition interaction and \(V_{\text{max}}\) and \(K_m\) are the kinetic constants, respectively.

The IC\(_{50}\) value was determined by plotting the percentage of inhibition of each test article versus log concentration and determining the concentration of test article that results in 50% inhibition of the probe substrate reaction. The mode of inhibition was determined on the basis of visual examination of the Dixon and Eadie-Hofstee plots. The goodness of fit was assessed by visual examination of the data and residual plots and by the precision of the parameter estimates. Calculation of competitive inhibition constant (\(K_i\)) values from Dixon plots was performed by fitting the data to the following equation:

\[
v = \frac{V_{\text{max}} [S]}{K_m [S] + K_i [I] + [S]}
\]

where \(V_{\text{max}}\) and \(K_m\) are the kinetic constants, \([S]\) is the substrate concentration, \([I]\) is the inhibitor concentration, and \(K_i\) is the inhibition constant. The experimental data were fitted by nonlinear regression using Grafit version 5.0 software (Erithacus Software Limited, Staines, UK).
Results

Aclidinium hydrolysis in human plasma and subcellular fractions

The *in vitro* hydrolysis of aclidinium bromide (5 μM) in human plasma and lung and liver subcellular fractions was studied in order to select the most appropriate human material for further studies. In all cases, the net enzymatic hydrolysis was calculated by subtracting the non-enzymatic hydrolysis obtained in control incubations in the presence of the corresponding incubation buffer (pH 7.4) from incubations with biological material. The net enzymatic hydrolysis of aclidinium bromide was higher in human plasma (93.2 pmol alcohol/min/mg protein) than in lung and liver subcellular fractions (Table 1).

Esterase activities in human plasma

The activities of the different human esterases were determined in the six human plasma samples used in this study using selective substrates. The mean activities (± SD, n=6) were 50.9 ± 16 nmol/min.mg protein for BChE (Table 1), 3.8 ± 1.4 nmol/min.mg for AChE, 12.6 ± 3.0 nmol 1-naphthol/min/mg for CbE, 2083 ± 295 nmol phenol/min/mg for arylesterase, and 2.8 ± 1.8 nmol p-nitrophenol/min/mg for paraoxonase. The results obtained demonstrate the presence of the activities of the reported esterases in human plasma and the suitability of human plasma to conduct further *in vitro* studies.

Human plasma hydrolysis assays

Initial experiments with aclidinium bromide (2, 10, 50, and 250 μM) in diluted pooled human plasma (1/100, 2/100, and 5/100, v/v) were conducted to establish reaction conditions that would ensure linearity of product formation with incubation time at 37°C. The time course of aclidinium hydrolysis was linear up to 30 minutes in all ranges of protein concentrations. Total substrate depletion was around 50% at an aclidinium concentration of 2 μM after 30 minutes of incubation in diluted pooled human plasma (2/100, 1.5 mg/ml). Although parent compound disappearance was elevated under these conditions, they were selected for further kinetic studies in order to ensure proper differentiation between enzymatic and non-enzymatic hydrolysis.

The hydrolysis of aclidinium in phosphate buffer at pH 7.4 was linear in the range of concentrations assayed (1-500 μM). The *k*ₐ in the incubation buffer was calculated from the slope of the regression line (*r*²=0.9969) and presented a value of 0.0074 min⁻¹ (results not shown). Following incubation of aclidinium bromide (1-500 μM)
in diluted human plasma from different donors, no differences were observed in the hydrolysis rates between phosphate buffer and human plasma at substrate concentrations higher than 50 μM. The percentage of non-enzymatic hydrolysis at aclidinium bromide concentrations between 5 and 500 nM was 17.4 ± 2.8%, suggesting a major contribution of the enzymatic hydrolysis. Following visual inspection of Eadie-Hofstee plots, two different phases can be clearly differentiated in the aclidinium hydrolysis profile in diluted human plasma (Fig. 2A). Accordingly, a new model is proposed to take into account independently both enzymatic and non-enzymatic processes. Thus, the determination of the apparent Michaelis-Menten parameters (K_m and V_max) and non-enzymatic hydrolysis rate constant (k_h) was performed using the following equation:

$$v = \frac{V_{max}[S]}{K_m + [S]} + k_h[S]$$

where v is the reaction rate, [S] is the substrate concentration, K_m is the apparent Michaelis-Menten constant, V_max is the apparent maximum velocity and k_h is the non-enzymatic hydrolysis rate constant. The apparent K_m values ranged between 1.9 and 4.8 μM (mean: 3.3 μM) and the apparent V_max ranged from 42.6 to 95.4 pmol acid/min/mg protein (mean: 61.6 pmol acid/min/mg protein). The mean calculated k_h was 0.0079 ± 0.0006 min⁻¹, which is in agreement with the hydrolysis constant obtained in phosphate buffer at pH 7.4.

For a better characterization of the kinetic profile of aclidinium metabolism, further incubations at lower concentrations (from 5 nM to 50 μM) were also conducted with final analysis by LC-MS/MS (positive ESI) of the alcohol metabolite (LAS34823) and parent compound. In this case, the net enzymatic formation of the alcohol derivative was calculated by subtracting the LAS34823 obtained in the incubation buffer from that measured in diluted human plasma. The net enzymatic formation rates of alcohol derivative reached maximum levels and then declined thereafter, suggesting substrate inhibition kinetics for the hydrolysis process. Accordingly, the net enzymatic LAS34823 formation rate was analyzed according to a substrate inhibition model and showed a good fit (Fig. 2B). The apparent K_m value was 8.0 μM and the apparent V_max was calculated as 179.1 pmol alcohol/min/mg protein. The autoinhibition constant was calculated to be 14.6 μM.

**Inhibition of aclidinium hydrolysis**

All the inhibition experiments on aclidinium hydrolysis were carried out at a substrate concentration of 5 μM, final plasma dilution of 5/100 (v/v) and in 30 minutes in order to maximize the differences between enzymatic
and non-enzymatic hydrolysis. The selective arylesterase/paraoxonase inhibitor (PCMB) and the selective CbE inhibitors (BPNP and NaF) had little effect on the enzymatic hydrolysis of aclidinium at any of the concentrations assayed (Fig. 3). Notable inhibition of aclidinium enzymatic hydrolysis was observed in the presence of B-esterase inhibitors. Hence, the non-specific type-B inhibitor AEBSF decreased aclidinium enzymatic hydrolysis to about 50% at 100 µM. In contrast, another general inhibitor of B-esterases (DFP) completely inhibited the enzymatic hydrolysis of aclidinium at the lowest concentration assayed of 1 µM. Eserine, specific inhibitor of cholinesterases, completely inhibited the enzymatic hydrolysis at all concentrations assayed. The selective BChE inhibitor iso-OMPA completely inhibited the enzymatic hydrolysis of aclidinium at 100 µM only, whereas selective AChE inhibitor BW284c51 at the same concentration inhibited aclidinium enzymatic hydrolysis by some 50%.

Additional incubations in the presence of 1, 10, and 100 µM inhibitors were conducted at a concentration of 10 nM aclidinium, which is closer to C_max plasma values in a clinical situation. The inhibition results were practically identical to those observed at an aclidinium concentration of 5 µM for all inhibitors assayed, with complete inhibition by eserine and DFP at all concentrations assayed. Inhibitors BW284c51 and iso-OMPA (100 µM) caused a 66% and 85% inhibition of aclidinium enzymatic hydrolysis, respectively.

**Incubations with human esterases**

In the presence of recombinant human AChE or human serum albumin (0.25 mg/ml), the rate of hydrolysis of aclidinium was similar to the rate observed in phosphate buffer (pH 7.4) at all the enzyme concentrations assayed. On the other hand, purified BChE was active in the hydrolysis of aclidinium and enzymatic hydrolysis increased with the enzyme concentration (Table 2).

The Eadie-Hofstee plot of LAS34850 formation in purified human BChE was nearly identical to that obtained in human plasma. The kinetic analysis using the same enzymatic model previously described resulted in apparent K_m and V_max values of 4.98 µM and 1.03 nmol/min/unit BChE, respectively. The hydrolysis rate constant was 0.0085 min⁻¹ (Fig. 2C). The hydrolysis rate constant (k_h) of aclidinium in phosphate buffer at pH 7.4 (BChE assay) was 0.0085 min⁻¹ (r² 0.9972), agreeing completely with previous results.
Esterase inhibition assays

The effect of different concentrations of aclidinium (1, 10, and 100 µM) on esterase activities was evaluated in pooled human plasma. No relevant inhibition of paraoxonase and arylesterase activities was observed. Carboxylesterase activity was slightly inhibited by aclidinium. The calculated IC₅₀ value was 43.9 µM. Aclidinium appeared to inhibit acetylcholinesterase and butyrylcholinesterase activities. According to the Dixon plots, the experimental values appeared to fit a pure competitive type of inhibition for both enzymes. The Kᵢ values for aclidinium were calculated to be 6.3 µM and 2.7 µM for acetylcholinesterase and butyrylcholinesterase, respectively (Fig. 4). The carboxylic acid metabolite (LAS34850) did not show relevant inhibition of any of the human esterases at the highest concentration of 100 µM. Similarly, the alcohol metabolite (LAS 34823) did not have any effect on paraoxonase, carboxylesterase, and arylesterase activities at 100 µM. However, the activities of acetylcholinesterase and butyrylcholinesterase were inhibited approximately 50% by 100 µM LAS34823.

Human recombinant CYP450 isoforms

Incubations of aclidinium in the presence of human recombinant CYP450 isoforms and NADPH were carried out at a substrate concentration of 20 µM. The rate of formation for the hydrolysis metabolites LAS34850 and LAS34823 was similar in all human CYP450 isoforms assayed (Fig. 5). However, the rate of disappearance of aclidinium was higher in the presence of CYP2D6, which may be explained by the formation of different oxidative and NADPH-dependent metabolites (results not shown).
Discussion

Aclidinium is rapidly hydrolyzed in vitro and in vivo by human plasma esterases to its two main alcohol and acid metabolites (Jansat et al., 2009, Sentellas et al., 2009). Aclidinium was confirmed as an unstable compound that undergoes non-enzymatic hydrolysis at the physiological pH of 7.4 (t1/2 70 min). However, its hydrolysis occurred very rapidly in human plasma (t1/2< 5 min), suggesting that enzymatic hydrolysis may be a key factor in the metabolism of this compound (Sentellas et al., 2009). The current study examined the role of human enzymes such as esterases, CYP450 isoenzymes, and albumin in the in vitro enzymatic hydrolysis of aclidinium in humans. Although these enzymes are known to be mainly expressed in human plasma and liver, the human lung was also considered, since this is the target organ after administration of aclidinium by inhalation.

Following incubation of aclidinium in different human liver, lung, and blood subcellular fractions, a higher net enzymatic hydrolysis was found in human plasma (Table 1). These results suggest that human plasma may be the most appropriate in vitro system for further hydrolysis studies. Nevertheless, the activities of different human esterases were determined in human plasma to demonstrate that all of them were actively expressed. Although comparisons among esterase activities are constrained by differences in substrates and analytical determinations, BChE activity was much higher than AChE activity in human plasma, which is consistent with what has been described in the literature (Brimijoin et al., 1988). In our hands, the CbE activity found in human plasma using α-naphthyl acetate as a substrate was important. However, it has been demonstrated that CbE activity found in human plasma should be considered as a residual activity from albumin and PON1 (Li et al., 2005), which suggests that the substrate used was not selective for CbE. In any case, the enzymatic hydrolysis of aclidinium in subcellular liver fractions was lower than that found in plasma despite the fact that different human CbE isoforms are highly expressed in human liver (Jewell et al., 2007), suggesting that the potential involvement of CbE in aclidinium hydrolysis may be of comparatively low relevance.

The kinetic study in human plasma was carried out under linear conditions with respect to time and plasma protein concentration. The hydrolysis of aclidinium (1-500 μM) in diluted human plasma showed a clear biphasic Eadie-Hofstee profile, consistent with two independent processes described by (a) enzymatic hydrolysis at low substrate concentrations, and (b) non-enzymatic hydrolysis, which becomes predominant at higher substrate concentrations, leading to a constant value for the v/[S] relationship (Fig. 2A). The range of aclidinium concentrations assayed was limited by the sensitivity of the LC-UV method. The lack of
experimental points where enzymatic hydrolysis was predominant obviated any attempt to conduct additional analyses using net enzymatic hydrolysis. Therefore, further incubations were conducted using a more sensitive analytical method, namely LC-MS/MS, which made possible incubations of aclidinium concentrations in the nanomolar range. The results of this experiment showed that enzymatic hydrolysis was predominant at the lowest aclidinium concentrations assayed (5-500 nM), whereas the enzymatic hydrolysis became less important at high aclidinium concentrations. This effect can be explained through a process of autoinhibition, which is consistent with the inhibition caused by aclidinium on the enzyme responsible for its own hydrolysis (see below). However, the Michaelis-Menten constants ($K_m$) calculated using both fitting models were similar (3.5 µM and 8 µM), confirming the validity of the proposed model.

The inhibition experiments in the presence of selective chemical inhibitors were carried out at a substrate concentration of 5 µM (approximately apparent $K_m$) and at a lower concentration down to 10 nM, closer to the plasma $C_{\text{max}}$ observed in humans (approximately 0.05 nM). Cholinesterase inhibitors (DFP and eserine) caused the maximal inhibition of the enzymatic hydrolysis of aclidinium. The selective BChE inhibitor iso-OMPA provided greater inhibition than that observed in the presence of AChE inhibitor BW248c51. However, these results did not enable us to determine which of these two enzymes was the most important catalyst in the enzymatic hydrolysis of aclidinium. Further incubations with pure human enzymes confirmed that enzymatic hydrolysis only occurred with BChE (Table 2). Unfortunately, other purified or recombinant human esterases were not commercially available for testing. In addition, the kinetic profile of aclidinium hydrolysis (1-500 µM) in human BChE was nearly identical to the results obtained previously in human plasma, with a similar Michaelis-Menten constant (Fig. 2C). These results are consistent with the fact that BChE is predominantly expressed in human plasma, in contrast to AChE, which is mainly located in human red blood cells (Chantonet et al., 1989, Von Bernhardi et al., 2005).

BChE activities in the different tissues were also determined in order to establish a potential relationship between enzymatic aclidinium hydrolysis and BChE activities (Table 1). The correlation coefficient ($r$) was 0.866, with a clear tendency to increasing aclidinium hydrolysis with increased BChE activity. The results for BChE activity in the human lung agree with a study carried out by Jbilo et al. (1994), where similar activity distribution was found in S9 and microsomal fractions. The differences in BChE content found in human liver microsomal and S9 fraction could be explained by the different commercial origin and donors of the biological
material. This observation reinforces the argument that aclidinium hydrolysis is not mediated by human AChE, the main esterase found in human red blood cells. To better understand the extent of aclidinium hydrolysis in vivo, BChE activities and the rates of aclidinium hydrolysis were normalized to protein concentration. The protein content in human liver, lung microsomal fraction and human plasma are reported to be 90 mg/g, 2.9 mg/g, and 77 mg/g, respectively (Sohlenius-Stenberck, 2006 and Sipal et al., 1979). Therefore, taking into account BChE activities and the enzymatic aclidinium hydrolysis rates measured in these subcellular fractions, the in vivo hydrolysis of aclidinium would occur mainly in the systemic circulation and to a lower extent in the human lung and liver. However, an important enzymatic hydrolysis was also observed in human lung subcellular fractions, which is consistent with the low absolute bioavailability (< 5%) of aclidinium bromide following a single inhaled 200 μg dose (Almirall S.A., data on file).

Regarding other human enzymes that could potentially be involved in aclidinium hydrolysis, human albumin did not show any effect. Furthermore, oxidative hydrolysis of aclidinium by CYP450 is very unlikely since the hydrolysis metabolites, the alcohol and the acid metabolites were formed to a similar extent by all the CYP450 isoforms. However, the oxidative metabolism observed during incubations with different CYP450 isoforms could be further understood with additional investigation.

The results of the inhibition study on esterase activities in human plasma showed that aclidinium was a competitive inhibitor of both BChE and AChE enzymes. The inhibition potential of the two hydrolysis metabolites was comparatively much lower or had no influence on the activities of esterases. Taking into consideration that in clinical settings, the C\text{max} plasma values of unchanged drug and alcohol metabolite are below 1 nM (Jansat et al., 2009), drug-drug interactions involving aclidinium and human esterases are unlikely. Furthermore, potent marketed BChE and AChE oral inhibitors for Alzheimer’s disease, such as tacrine, donezepil, galantamine, and rivastigmine or other anticholinergic and antidepressant drugs, have been shown to be BChE inhibitors in vitro at the micromolar range with no related clinical side effects (Bodur et al., 2001, Müller et al., 2002).

Butyrylcholinesterase is widely distributed in the human body and is known to be produced in the liver and secreted into the blood stream. In human plasma, BChE represents approximately 0.1% of total serum proteins (Chantonet et al., 1989). The estimated prevalence of the wild-type homozygous BChE genotype in Caucasian
populations is around 95%. To date, approximately 65 genetic variants of human BChE have been identified and the most predominant variants are the K, A (atypical), and J variants. Individuals with this genotype show reduced enzyme activity caused by a depletion of the circulating BChE molecules. There are also around 20 silent genotypes characterized by the near complete absence of BChE activity. In addition, other rare variants (e.g., C5+, Cynthiana and Johannesburg) with increased BChE activity have been also identified (Table 3). Although the influence of the BChE genotype on aclidinium disposition has not been studied, one could speculate that in those patients with genetic BChE variants with decreased activity, the rate of aclidinium ester hydrolysis would be reduced. However, it is not expected to be clinically significant due to the important non-enzymatic hydrolysis ($t_{1/2}$ 1.2 hours) at physiological pH.

The results obtained in this study demonstrate that human butyrylcholinesterase is the most important enzyme involved in the enzymatic hydrolysis of aclidinium and that this process takes place mainly in plasma.
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References


Footnotes

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Legends for figures

FIG. 1. Hydrolysis of aclidinium bromide hydrolysis into its alcohol (LAS34823) and carboxylic acid (LAS34850) derivatives

FIG. 2. Kinetic analyses of aclidinium hydrolysis. (A) Eadie-Hofstee plot of total hydrolysis in human plasma (1-500 µM), (B) Net enzymatic hydrolysis in human plasma (5-50,000 nM), and (C) Eadie-Hofstee plot of total hydrolysis in human butyrylcholinesterase (1-500 µM).

Aclidinium bromide was incubated in diluted human plasma (1.5 mg/ml protein) and 0.2 units BChE/ml (relative units) for 30 minutes. Concentrations were expressed as nmol aclidinium/mg protein and nmol aclidinium/unit BChE for kinetic analyses and graph representation in (A) and (C), respectively. Results are expressed as (A) the mean ± standard deviation of six different plasma samples (represented by different symbols) or (B, C) mean value of duplicate incubations. One unit of BChE activity corresponds to the increase of absorbance/min at the assay conditions.

FIG. 3. Effect of different selective chemical esterase inhibitors on aclidinium enzymatic hydrolysis in human plasma.

Incubations of aclidinium bromide (5 µM) were conducted in pooled diluted human plasma (3.85 mg/ml) for 30 minutes in the presence of esterase inhibitors DFP and AEBSF (general type-B esterase), eserine (ChE), BW248c51 (AChE), iso-OMPA (BChE), PCMB (arylesterase/paraoxonase inhibitor), BPNP, and NaF (CbE) at three different concentrations (1, 10, and 100 µM) after 15 minutes of pre-incubation. Each bar represents the mean of net enzymatic inhibition (against control) from duplicate measurements (<15% variance).

FIG. 4. Dixon plots for the inhibition of thiocholine formation by aclidinium in the (A) butyrylcholinesterase and (B) acetylcholinesterase activity assays.

S: substrate concentrations (BChE:butyrylthiocholine, AChE: acetylthiocholine). Rates expressed as unit of BChE or AChE activity/mg protein. Each point represents the mean of duplicate measurements. One unit BChE or AChE activity corresponds to the increase of absorbance/min at the assay conditions. K_i expressed as mean value ± SE of the model fit.

FIG. 5. Hydrolysis of aclidinium in the presence of human recombinant CYP450 isoforms.

Aclidinium (20 µM) was incubated in recombinant human CYP450 isoforms (25 pmol/ml) for 60 minutes. Incubations were conducted with phosphate buffer at pH 7.4 (Ctrl A) or TRIS buffer at pH 7.4 (Ctrl B). Rates are expressed as nmol compound formed or nmol of aclidinium disappearance per min and nmol CYP at the end of the incubations. Each bar represents the mean from duplicate measurements (<20% variance).
**TABLE 1.** Aclidinium hydrolysis and BChE activities in different human subcellular fractions.

The net enzymatic hydrolysis results are expressed as the mean of duplicate measurements. BChE activities were determined as reported in the experimental section and expressed as the mean of three measurements ± SD, unless indicated otherwise.

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Fraction</th>
<th>Source/Batch</th>
<th>Net enzymatic aclidinium hydrolysis (pmol alcohol/min/mg protein)</th>
<th>BChE activity (nmol thiocholine/min/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blood</td>
<td>plasma</td>
<td>--</td>
<td>93.2</td>
<td>50.9 ± 16.0</td>
</tr>
<tr>
<td></td>
<td>red blood cells</td>
<td>--</td>
<td>1.1</td>
<td>1.9 ± 0.1</td>
</tr>
<tr>
<td>Liver</td>
<td>microsomal</td>
<td>Xenotech/0210002</td>
<td>19.0</td>
<td>7.0 ± 0.1</td>
</tr>
<tr>
<td></td>
<td>S9</td>
<td>HBI/1.0</td>
<td>42.0</td>
<td>4.2 ± 0.1</td>
</tr>
<tr>
<td></td>
<td>microsomal</td>
<td>Xenotech/0210354</td>
<td>38.0</td>
<td>15.5 ± 0.2</td>
</tr>
<tr>
<td>Lung</td>
<td>S9</td>
<td>Xenotech/0210352</td>
<td>54.0</td>
<td>13.3 ± 0.2</td>
</tr>
</tbody>
</table>

* Mean value ± SD (n=6).
TABLE 2. Aclidinium hydrolysis in recombinant human acetylcholinesterase, purified human butyrylcholinesterase, and human albumin.

Incubations of aclidinium (5 μM) were conducted with pure enzymes containing human serum albumin (0.25 mg/ml) for 30 minutes. Mean values from duplicate measurements (<10% variance).

<table>
<thead>
<tr>
<th>Enzyme concentration</th>
<th>Rate</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>mU/ml</td>
</tr>
<tr>
<td>Phosphate buffer (pH 7.4)</td>
<td>--</td>
</tr>
<tr>
<td>Human serum albumin</td>
<td>0.25 mg/ml</td>
</tr>
<tr>
<td></td>
<td>1</td>
</tr>
<tr>
<td>AChE</td>
<td>10</td>
</tr>
<tr>
<td></td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>1000</td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td>BChE</td>
<td>10</td>
</tr>
<tr>
<td></td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>1000</td>
</tr>
</tbody>
</table>

a relative units. One unit of AChE or BChE hydrolyzes 1.0 μmole of acetylcholine or butyrylcholine to choline and acetate/butyrate per minute at pH 8.0 and 37°C.

<table>
<thead>
<tr>
<th>Variant</th>
<th>Aminoacid substitution</th>
<th>Homozygote frequency</th>
<th>Activity</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild Type, Usual (WT)</td>
<td>Wild-Type</td>
<td>95% population</td>
<td>Normal activity</td>
<td>Lockridge, 1990</td>
</tr>
<tr>
<td>K variant</td>
<td>539Ala → Thr</td>
<td>1:100</td>
<td>Same activity as WT BChE. 33% reduction in circulation molecules. (KK) 33% reduction in serum BChE activity</td>
<td>Whittaker and Britten, 1988</td>
</tr>
<tr>
<td>Atypical (A) Dibucaine-resistant</td>
<td>70Asp → Gly</td>
<td>1:2,500</td>
<td>(AA) 40% reduction in serum BChE activity</td>
<td>Bartels et al., 1992</td>
</tr>
<tr>
<td>J variant</td>
<td>497Glu → Val</td>
<td>1:150,000</td>
<td>Same activity as WT BChE 66% reduction in circulation molecules</td>
<td>Bartels et al., 1992</td>
</tr>
<tr>
<td>Fluoride resistant (F-1)</td>
<td>243Thr → Met</td>
<td>1:150,000</td>
<td>(FF) 50% reduction in serum BChE activity</td>
<td>Harris et al., 1961</td>
</tr>
<tr>
<td>Fluoride resistant (F-2)</td>
<td>390Gly → Val</td>
<td>1:160,000</td>
<td>F2 more frequent than F1. Different substrate kinetics</td>
<td>Nogueira et al., 1992</td>
</tr>
<tr>
<td>Fluoride resistant (Jap)</td>
<td>330Leu → Ile</td>
<td>Jap: 0.29% freq</td>
<td>Low serum BChE activity</td>
<td>Sudo et al., 1997</td>
</tr>
<tr>
<td>H variant</td>
<td>142Val → Met</td>
<td>2 families</td>
<td>90% reduction in BChE activity</td>
<td>Whittaker and Britten, 1987</td>
</tr>
<tr>
<td>Silent variants</td>
<td>≈ 30 variants</td>
<td>S-1 variant (more frequent): 1:110,000</td>
<td>&gt; 90% reduction in BChE activity</td>
<td>Nogueira et al., 1990</td>
</tr>
<tr>
<td>C5+, Cynthiana, Johannesburg</td>
<td>No genetic (phenotype)</td>
<td>Very rare</td>
<td>Increased protein → increased BChE activity</td>
<td></td>
</tr>
<tr>
<td>-----------------------------</td>
<td>------------------------</td>
<td>-----------</td>
<td>---------------------------------------------</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>(C5+: 30-54% increased activity, association of BChE subunits with a non-cholinesterase protein)</td>
<td></td>
</tr>
</tbody>
</table>

Krause et al., 1988
Akizuki et al., 2004
Figure 2a

$K_r: 3.5 \pm 1.0 \mu M$

$V_{max}: 0.056 \pm 0.020 \text{ nmol/min/mg}$

$k_r: 0.0079 \pm 0.0006 \text{ min}^{-1}$
Figure 2b
Figure 2c

$K_m = 4.98 \mu M$

$V_{max} = 1.03 \text{ nmol/min/unit BChE}$

$k_i = 0.0085 \text{ min}^{-1}$
Figure 4

A

\[ \frac{1}{\text{Rate}} = \frac{S}{K_i + S} \]

- \( S = 0.25 \text{ mM} \)
- \( S = 0.5 \text{ mM} \)
- \( S = 1 \text{ mM} \)
- \( S = 2 \text{ mM} \)

\( K_i = 2.7 \pm 0.8 \text{ µM} \)

B

\[ \frac{1}{\text{Rate}} = \frac{S}{K_i + S} \]

- \( S = 0.25 \text{ mM} \)
- \( S = 0.5 \text{ mM} \)
- \( S = 1 \text{ mM} \)
- \( S = 2 \text{ mM} \)

\( K_i = 6.3 \pm 0.8 \text{ µM} \)