Identification of the Human Enzymes Responsible for the Enzymatic Hydrolysis of Aclidinium Bromide

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ABSTRACT:

Aclidinium bromide [LAS34273, 3R-(2-hydroxy-2,2-dithiophen-2-yl-acetoxy)-1-(3-phenoxy-propyl)-1-azonia bicycle-[2.2.2]-octane bromide] is a novel, long-acting, inhaled muscarinic antagonist for the treatment of chronic obstructive pulmonary disease. Aclidinium showed rapid hydrolysis in human and animal plasma. This process occurred both nonenzymatically 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 cytochrome P450 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. Aclidinium was shown to inhibit competitively both human butyrylcholinesterase (Kᵢ, 2.7 μM) and acetylcholinesterase (6.3 μM) 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, which are less than 1 nM.

Aclidinium bromide [LAS34273, 3R-(2-hydroxy-2,2-dithiophen-2-yl-acetoxy)-1-(3-phenoxy-propyl)-1-azonia bicycle-[2.2.2]-octane bromide] is a novel, long-acting, inhaled muscarinic antagonist for the treatment of chronic obstructive pulmonary disease, has shown rapid hydrolysis in human and animal plasma. This process occurred both nonenzymatically 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 cytochrome P450 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. Aclidinium was shown to inhibit competitively both human butyrylcholinesterase (Kᵢ, 2.7 μM) and acetylcholinesterase (6.3 μM) 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, which are less than 1 nM.

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ABBREVIATIONS: aclidinium bromide/LAS34273, 3R-(2-hydroxy-2,2-dithiophen-2-yl-acetoxy)-1-(3-phenoxy-propyl)-1-azonia bicycle-[2.2.2]-octane bromide; LAS34273, [3R]-hydroxy-1-(3-phenoxy-propyl)-1-azonia bicycle[2.2.2]octane, bromide; LAS34850, [dithienyl-glycolic acid, sodium salt]; P450, cytochrome P450; CbE, carboxylesterase; AChE, acetylcholinesterase; BChE, butyrylcholinesterase; LC, liquid chromatography; DMSO, dimethyl sulfoxide; ITR, 5-diethylaminosulfonyl-2-methoxy-benzenediazonium chloride hemi-[zinc chloride]-salt; PCMB, p-chloromercuribenzoate; AEBSF, 4-(2-aminoethyl)benzenesulfonyl fluoride; DFP, diisopropyl fluorophosphate; MS/MS, tandem mass spectrometry; BW284c51, 1,5-bis[4-allyldimethylammoniumphenyl]pentan-3-one; iso-OMPA, tetraisopropyl pyrophosphoramide; BPNP, bis[p-nitrophenyl]phosphate; kᵢ, hydrolysis rate constant.
PON2, and PON3—have been identified. Human paraoxonase (PON1) may be present in two structural isoforms, where paraoxon is the discriminatory substrate and phenylacetate is the nondiscriminatory substrate (Mackness et al., 1998). The B-esterases belong to the α,β-hydrolase-fold family of proteins and include cholinesterases and carboxylesterases (ChE) (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). AChE, also called red blood cell cholinesterase, is more abundant in the central nervous system, skeletal muscle, and erythrocyte membranes, whereas BChE, also called pseudocholinesterase or plasma cholinesterase, is synthesized in the liver and secreted into plasma (Ryhänen, 1983). Human plasma contains BChE almost exclusively, whereas 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 (EC 3.1.1.1) belong to a family of proteins encoded by multiple genes that are localized in the endoplasmic reticulum of many tissues. ChEs 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., 2002).

In addition to esterases, P450-catalyzed oxidative ester cleavage may also occur, as has been shown for different ester and amide compounds (Guengerich, 1987; Peng et al., 1995). Furthermore, it has been postulated 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 because 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 (LAS3427, >99% purity), its alcohol metabolite (LAS34823), and its carboxylic acid metabolite (LAS34850) were synthesized at Ranké Química S.L. (Barcelona, Spain). Chemical structures of aclidinium bromide and its hydrolysis metabolites LAS34823 and LAS34850 are depicted in Fig. 1. Gradient-grade liquid chromatography (LC) method and acetonitrile, dimethyl sulfoxide (DMSO), and sodium-1-octanesulfonic acid were obtained from Scharlab S.L. (Barcelona, Spain). All the 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) or 0.1 N hydrochloric acid/DMSO (10:90, v/v) mixture. Stock solutions (5 mM) of LAS34823 and LAS34850 were prepared in a 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 5-diethylaminosulfonyl-2-methoxy-benzenediazonium chloride hemi-[zinc chloride]-salt (ITR) was prepared in 2.5% Triton X-100 aqueous solution. Stock solutions of p-chloromercuribenzoate (PCMB), 4-(2-aminoethyl)benzenesulfonfluryl fluoride (AEBSF), and phenylacetate were prepared in 0.01 N sodium hydroxide, 50 mM phosphate buffer (pH 5.0), and ethanol, respectively. Stock solutions of diisopropyl fluorophosphates (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 2000g (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, LLC (Lenexa, KS). Human pulmonary microsomes and S9 fraction (smoker and non-smoker) were obtained from Human Biologics International (Phoenix, AZ) and from Xenotech, LLC. Recombinant human CYP1A1, CYP1A2, CYP2C8, CYP2C9*, CYP2C19*, CYP2D6*, CYP2E1, CYP3A4, CYP3A5, CYP4A11, CYP4F2, CYP4F3A, and CYP4F3B expressed in microsomes of baculovirus-infected cells (Supersomes) were obtained from BD Gentest (Woburn, MA). Human recombinant AChE (2230 units/mg protein) and purified human BChE from human serum (12.7 units/mg protein) were purchased from Sigma-Aldrich. One unit of AChE or BChE hydrolyzes 1.0 μmol 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 min of preincubation 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 predefined times of 15, 30, and 60 min, aliquots of 200 μL of the incubation mixtures were separated, and the reactions were stopped by adding 100 μL of 0.2 N HCl/acetonitrile (80:20, v/v). The precipitated protein was removed by centrifuging at 2000g and 4°C for 10 min. Sample analysis was conducted using an 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 previously by incubating individual human plasma samples (2:100, v/v) at 37°C for 30 min in a final volume of 0.2 mL. Reactions were stopped by the addition of 0.1 mL of 0.2 N HCl/acetonitrile (80:20, v/v). All the samples were maintained at 4°C once obtained and during analysis to prevent aclidinium hydrolysis. Plasma hydrolysis assay of aclidinium at the nanomolar range (from 2 nM to 50 μM) was conducted as described previously but in the absence of DMSO as solvent. The final analysis of aclidinium and LAS34823 in incubation samples was conducted using the LC/UV system (Method A).

**Incubations of aclidinium bromide in human erythrocytes were conducted as described previously in a final volume of 1 mL. At predefined times of incubation (0, 5, 15, 30, and 60 min), 150-μL aliquots of the incubation mixtures were transferred to another vessel containing 0.9 mL of 0.2 N HCl/acetonitrile (80:20, v/v) and processed as described previously. Sample analysis was conducted using the LC/UV system (Method A).**
Hydrolysis in Human Pulmonary and Hepatic Subcellular Fractions. Acidinium 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 50 mM potassium phosphate buffer (pH 7.4), 3 mM MgCl₂, 1 mM EDTA, 1 mM NADP⁺, 5 mM glucose-6-phosphate, and 2 units/ml glucose-6-phosphate dehydrogenase. After 3 min of incubation, acidinium bromide was added to the 0.5-ml incubation mixtures in 10 μl of 0.01 N HCl/acetonitrile (80:20, v/v). The final concentration of acetonitrile in the incubation mixtures was 0.5%. At different predefined times (10, 20, 30, and 60 min), 100-μl aliquots of the incubation mixtures were separated, and the reactions were 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 (System B). All of the samples were kept at 4°C once obtained and during LC analysis to prevent LAS34273 ester cleavage.

Inhibition of Acidinium Hydrolysis. The effect of selective chemical esterase inhibitors on acidinium hydrolysis was evaluated using PCMB (paraoxonase/arylesterase), DFP and ABEFF (general B-esterase), eserine (cholinesterases), 1,5-bis(4-allyldimethylammoniumphenyl)pentan-3-one (BW284c51; AChE), tetrapropyl pyrophosphoramide (iso-OMPA; BChE), and bis[p-nitrophenyl]phosphate (BPNP) and NaF (CbE). Selective esterase inhibitors at final concentrations of 1, 10, and 100 μM were preincubated with diluted pooled human plasma (3.85 mg/ml, 5:100, v/v) for 15 min 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 acidinium bromide to a final concentration of 5 μM and were stopped after 30 min of incubation as indicated previously. 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 acidinium bromide (10 nM) were conducted as described previously but in the absence of DMSO as solvent. Final analysis of acidinium and LAS34823 in incubation samples was conducted using the LC/MS/MS method.

Incubations with Human Esterases. Acidinium bromide (5 μM) was incubated in the presence of human recombinant AChE and purified human BChE (0, 0.001, 0.01, 0.1, and 1 unit/ml, relative units) in 50 mM phosphate buffer, pH 7.4, for 30 min 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 acidinium bromide solution, and after 30 min of incubation they were stopped and treated as indicated previously. Sample analysis was conducted using the LC/UV system (Method A).

Kinetic study of acidinium hydrolysis in purified human BChE 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 acidinium bromide (1–500 μM). The final concentration of DMSO in the incubation mixtures was 2.5%. The final protein concentration in the incubation mixtures was adjusted to 0.125 mg/ml by adding human serum albumin. After 5 min of preincubation, reactions were started by the addition of acidinium bromide solution. After 30 min of incubation, the reactions were stopped and treated as indicated previously. 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 P450 Isomers. cDNA-expressed human isozymes (25 pmol of P450/50 ml) were incubated at 37°C in 0.2-ml (final volume) incubation mixtures containing 50 mM potassium phosphate buffer, pH 7.4 (CYP1A1, CYP1A2, CYP2B6, CYP2C8, CYP2C9*1, CYP2D6*1, CYP2E1, CYP3A4, CYP3A5, CYP4F2, CYP4F3A, and CYP4F3B) or 100 mM Tris buffer, pH 7.4 (CYP2A6, CYP2C9*1, and CYP4A11), 3 mM MgCl₂, 1 mM EDTA, 1 mM NADP⁺, 5 mM glucose-6-phosphate, 2 units/ml glucose-6-phosphate dehydrogenase, and 20 μM acidinium at the final concentrations indicated. Acidinium bromide was added to the 0.2-ml incubation mixtures in 10 μl of 0.01 N HCl/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 the incubation mixtures by adding appropriate volumes of insect cell control microsomes (0.5 mg of protein/ml). After 5 min of preincubation, reactions were started by the addition of the NADPH-generating system. After 60 min of incubation, reactions were stopped and treated as indicated previously. 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 paraaxon as described previously (Rodrigo et al., 2001). In brief, diluted human plasma (1:150, v/v, approximately 0.5 mg of protein/ml) was incubated at 37°C with 5 mM paraaxon in 100 mM HEPES containing 2 mM CaCl₂, pH 7.4. The generation of p-nitrophenol was monitored at 405 nm for 20 min at 1-min intervals. The extinction coefficient of p-nitrophenol at the assay conditions (9.65 mM/cm) 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). In brief, diluted human plasma (0.01 mg of 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 0.1 N HCl, and UV absorbance at 270 nm was measured. Quantification was conducted using a calibration curve obtained from standard samples of phenol (0.1–0.75 mM) prepared in the assay conditions. CbE activity was determined using α-naphthyl acetate as a substrate and 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 of protein/ml) was incubated at 37°C with 35 μg/ml α-naphthyl acetate in 100 mM Tris buffer, pH 7.4, in a final volume of 3 ml for 5 min. Reactions were stopped by the addition of 0.25 ml of 2.5% SDS solution and 0.25 ml of 0.1% Fast Red ITR. The mixtures were maintained at room temperature in the dark for 10 min, and the absorbance of the naphthol Fast Red ITR complex was measured at 530 nm. AChE and BChE activities were assayed using acetylthiocholine iodide and butyrylthiocholine iodide as substrate, respectively. In both cases, the thiocarbamoyl generated reacts with 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 BChE activity for AChE determinations. In brief, diluted human plasma (AChE, 0.25 mg of protein/ml; or BChE, 0.125 mg of protein/ml) or tissue subcellular fractions (0.08 mg of protein/ml) were incubated for 15 min with 2 mM 5,5'-dithiobis[2-nitrobenzoic acid] in 100 mM Tris buffer, pH 7.4, at 37°C. Then the corresponding substrate acetylthiocholine iodide or butyrylthiocholine iodide (0.5 mM) was added, and the formation of 5-thio-2-nitrobenzoate was monitored at 415 nm for 20 min at 1-min 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 acidinium bromide and its hydrolysis metabolites was conducted in human plasma as described previously. However, the conversion of acetylcholine to phenylacetate (arylesterase activity) was quantified by using LC/UV (254 nm) to prevent UV interferences from acidinium 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 (Milford, MA) NovaPack C18 column (150 × 3.9 mm, 4 μm) protected by Tracer Spherisorb ODS-2CN (10 × 4.6 mm; Teknokroma SCCL, Barcelona, Spain) and eluted using a gradient with 10 mM ammonium formate, pH 4.5 (solvent A) and acetonitrile (solvent B). The initial mobile phase composition was 25% B for 4 min and progressed linearly to 90% B in 5 min, maintaining this percentage for 4.5 min. The mobile phase was returned to the starting solvent mixture in 0.5 min, and the system was equilibrated for 5 min between runs. A constant flow rate of 1 ml/min was used. The injection volume was 10 μl, and the retention times were 3.5 and 7.4 min for phenol and phenylacetate, respectively. Neither acidinium nor its two hydrolysis metabolites interfered in the phenol determination.

Instrumentation and LC/UV Analysis. Incubation assays were carried out using a programmable Packard Multiplate II automated liquid handling system (PerkinElmer Life and Analytical Sciences, Waltham, MA) and 1-ml 96-deepwell (round-bottom) plates (NUNC A/S, Kamstrup, Denmark) or Eppendorf AG (Hamburg, Germany) conical-bottom plastic tubes (incubation volume >1 ml). All of the spectrophotometric determinations (esterase activities) were conducted using a double-beam Hitachi (San Jose, CA) U2001
spectrophotometer assisted with an on-line Huber (Hoftenburg, Germany) Polysat K6 water-bath set at 37°C. Aclidinium and its acid and alcohol products were separated on a Waters Alliance 2790 LC system with UV detection (Waters 2996PDA) at 219 nm (LAS34823) and 238 nm (acridinium and LAS34850). Aclidinium and its metabolites were separated on a Waters Symmetry C18 column (250 × 4.6 mm, 5 μm) protected by Trace Spherisorb ODS-2CN (10 × 4.6 mm; Teknokroma) and eluted using a gradient with 10 mM sodium phosphate containing 5 mM 1-octanesulfonic acid, pH 2.8 (solvent A) and acetonitrile (solvent B). 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 min. The mobile phase was returned to the starting solvent mixture in 1 min, and the system was equilibrated for 8 min between runs. The retention times were 4.5, 6.5, and 11.6 min for LAS34823, LAS34850, and acridinium, respectively. Method B: the initial mobile phase composition was 10% B and progressed linearly to 55% B in 30 min. The mobile phase was returned to the starting solvent mixture in 1 min, and the system was equilibrated for 7 min between runs. The retention times were 17.8, 18.8, and 26.8 min for LAS34850, LAS34823, and acridinium.

All of the samples generated in incubations were always kept at 4°C to prevent acridinium ester cleavage. Quantification of LAS34823, LAS34850, and acridinium 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) to avoid the interference of the inhibitor chromatographic peak. The LAS34850 (carboxylic acid derivative) and LAS34823 (alcohol derivative) concentrations in the acridinium hydrolysis studies were similar; thus, only the results for the carboxylic acid derivative (LAS34850) are presented unless indicated otherwise.

LC/MS/MS Analysis. The determination of acridinium and LAS34823 in incubation samples from plasma stability studies (nanomolar range) was conducted by using LC with MS detection. LC/MS analyses were performed using an Agilent (Waldbronn, Germany) 1100 series liquid chromatograph coupled to an Applied Biosystems (Foster City, CA) API 4000 QTrap hybrid triple quadrupole linear ion trap mass spectrometer. Sample separation was carried out on a Spherisorb ODS-2 Waters (4.6 × 50 mm, 5 μm) column at a flow rate of 0.5 mL/min using acetonitrile/50 mM ammonium formate, 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; and ion source gas 2, 60. Nitrogen was used as the nebulizer and auxiliary gas. Quantification was performed using multiple reaction monitoring for the transitions 484→262 (acridinium; declustering potential, 126; collision energy, 47), 262→140 (LAS34823; declustering potential, 56; collision energy, 43), and 332→166 (paratropium as internal standard; declustering potential, 396; collision energy, 37).

Data Analysis. Substrate inhibition was determined based on visual examination of the early Hodfleeve plots according to the following equation for a one-site binding model (Houston and Kenworthy, 2000):

$$v = \frac{v_{max} \cdot [S]}{K_m + \left[\frac{[I]}{K_i}\right] + [S]}$$

where [S] is the substrate concentration, $K_m$ is the constant describing the substrate inhibition interaction, and $v_{max}$ and $K_m$ are the kinetic constants, respectively.

The IC50 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 based on 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:

$$v = \frac{v_{max}[S]}{K_i\left(1 + \left[\frac{[I]}{K_i}\right]\right) + [S]}$$

where $v_{max}$ and $K_i$ 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 Graftit version 5.0 software (Erithacus Software, Surrey, UK).

Results

Aclidinium Hydrolysis in Human Plasma and Subcellular Fractions. The in vitro hydrolysis of acridinium bromide (5 μM) in human plasma and lung and liver subcellular fractions was studied 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 acridinium bromide was higher in human plasma (93.2 pmol of 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 (± S.D.; n = 6) were 50.9 ± 16 nmol/min/mg protein for BChE (Table 1), 3.8 ± 1.4 nmol/min/mg protein for AChE, 12.6 ± 3.0 nmol of 1-naphthol/min/mg protein for CbE, 2083 ± 295 nmol of phenol/min/mg protein, and 2.8 ± 1.8 nmol of p-nitrophenol/min/mg protein for paraoxonase. The results obtained show 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 acridinium 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

### TABLE 1

Aclidinium hydrolysis and BChE activities in different human subcellular fractions

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Fraction</th>
<th>Source/Batch</th>
<th>Net Enzymatic Aclidinium Hydrolysis</th>
<th>BChE Activity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>pmol alcohol/min/mg protein</td>
<td>nmol thiocholine/min/mg protein</td>
</tr>
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<td>Blood</td>
<td>Plasma</td>
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<td>Red blood cells</td>
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<td>1.9 ± 0.1</td>
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<tr>
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<td>XenoTech,0210002</td>
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<td>7.0 ± 0.1</td>
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<tr>
<td></td>
<td>S9</td>
<td>Human Biologics International/1.0</td>
<td>42.0</td>
<td>4.2 ± 0.1</td>
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<tr>
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<td>XenoTech,0210355</td>
<td>54.0</td>
<td>13.5 ± 0.2</td>
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</table>

NA, not applicable.

*Mean value ± S.D. (n = 6).
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 min in all the ranges of the protein concentrations. Total substrate depletion was around 50% at an aclidinium concentration of 2 µM after 30 min of incubation in diluted pooled human plasma (2:100, v/v, 1.5 mg/ml). Although parent compound disappearance was elevated under these conditions, they were selected for further kinetic studies to ensure proper differentiation between enzymatic and nonenzymatic hydrolysis.

The hydrolysis of aclidinium in phosphate buffer at pH 7.4 was linear in the range of concentrations assayed (1–50 µM). The $k_h$ 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). After 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. After 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 nonenzymatic processes. Thus, the determination of the apparent Michaelis-Menten parameters ($K_m$ and $V_{max}$) and nonenzymatic 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 nonenzymatic 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 of acid/min/mg protein (mean, 61.6 to 56 pmol of 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 electrospray ionization) 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 percentage of nonenzymatic hydrolysis at aclidinium bromide concentrations between 5 and 500 nM was 17.4 ± 2.8%, suggesting a major contribution of the enzymatic hydrolysis. 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 of 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 min to maximize the differences between enzymatic and nonenzymatic 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 nonspecific type-B inhibitor AEBSF decreased aclidinium enzymatic hydrolysis to approximately 50% at 100 μM. In contrast, another general inhibitor of B-estrases (DFP) completely inhibited the enzymatic hydrolysis of aclidinium at the lowest concentration assayed of 1 μM. Eserine, a specific inhibitor of cholinesterases, completely inhibited the enzymatic hydrolysis at all the concentrations assayed. The selective BChE inhibitor iso-OMPA completely inhibited the enzymatic hydrolysis of aclidinium at 100 μM only, whereas selective AChE inhibitor BW248c51 at the same concentration inhibited aclidinium enzymatic hydrolysis by approximately 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 \( c_{\text{max}} \) plasma values in a clinical situation. The inhibition results were practically identical to those observed at an aclidinium concentration of 5 μM for all the inhibitors assayed, with complete inhibition by eserine and DFP at all the concentrations assayed. Inhibitors BW248c51 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 \( V_{\text{max}} \) 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\(^{-1}\) (\( r^2 = 0.9972 \)), 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. ChE activity was slightly inhibited by aclidinium. The calculated IC50 value was 43.9 μM. Aclidinium appeared to inhibit ACHE and BChE activities. According to the Dixon plots, the experimental values appeared to fit a pure competitive type of inhibition for both enzymes. The \( K_i \) values for aclidinium were calculated to be 6.3 and 2.7 μM for ACHE and BChE, respectively (Fig. 4). The carboxylic acid metabolite (LAS34823) did not show relevant inhibition of any of the human esterases at the highest concentration of 100 μM. Likewise, the alcohol metabolite (LAS34823) did not have any effect on paraoxonase, ChE, and arylesterase activities at 100 μM. However, the activities of ACHE and BChE were inhibited approximately 50% by 100 μM LAS34823.

**Human Recombinant P450 Isoforms.** Incubations of aclidinium in the presence of human recombinant P450 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 the human P450s 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).

**TABLE 2**

<table>
<thead>
<tr>
<th>Enzyme Concentration</th>
<th>Rate (pmol acid/min/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phosphate buffer, pH 7.4</td>
<td>NA 29.7</td>
</tr>
<tr>
<td>Human serum albumin</td>
<td>0.25 mg/ml 30.2</td>
</tr>
<tr>
<td>AChE</td>
<td>1 19.0</td>
</tr>
<tr>
<td>10 22.7</td>
<td></td>
</tr>
<tr>
<td>100 26.3</td>
<td></td>
</tr>
<tr>
<td>1000 30.7</td>
<td></td>
</tr>
<tr>
<td>BChE</td>
<td>1 31.0</td>
</tr>
<tr>
<td>10 33.0</td>
<td></td>
</tr>
<tr>
<td>100 53.8</td>
<td></td>
</tr>
<tr>
<td>1000 180.8</td>
<td></td>
</tr>
</tbody>
</table>

NA, not applicable.

Relative units. One unit of ACHE or BChE hydrolyzes 1.0 nmol of acetylcholine or butyrylcholine to choline and acetate/butyrate per minute at pH 8.0 and 37°C.
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., 2010). Aclidinium was confirmed as an unstable compound that undergoes nonenzymatic hydrolysis at the physiological pH of 7.4 ($t_{1/2} < 5$ min), suggesting that enzymatic hydrolysis may be a key factor in the metabolism of this compound (Sentellas et al., 2010). The current study examined the role of human enzymes such as esterases, $P_{450}$ 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 because this is the target organ after administration of aclidinium by inhalation.

After 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 show 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 and Hammond, 1988). In our hands, the ChE activity found in human plasma using $\alpha$-naphthyl acetate as a substrate was important. However, it has been shown that ChE 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 ChE. 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 ChE isoforms are highly expressed in human liver (Jewell et al., 2007), suggesting that the potential involvement of ChE 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 $\mu$M) in diluted human plasma showed a clear biphasic Eadie-Hofstee profile, consistent with two independent processes described by 1) enzymatic hydrolysis at low substrate concentrations, and 2) nonenzymatic hydrolysis, which becomes predominant at higher substrate concentrations, leading to a constant value for the $v/f[S]$ 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 and 8 $\mu$M), 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 $\mu$M (approximately apparent $K_{m}$) and at a lower concentration down to 10 nM, closer to the plasma $C_{max}$ 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). It is unfortunate that other purified or recombinant human esterases were not commercially available for testing. In addition, the kinetic profile of aclidinium hydrolysis (1–500 $\mu$M) 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 (Chatonnet and Lockridge, 1989; von Bernhardi et al., 2005).
BChE activities in the different tissues were also determined 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, 2.9, and 77 mg/g, respectively (Sípal et al., 1979; Sohlenius-Sternbeck, 2006). 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 after a single inhaled 200-$\mu$g 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 P450 is very unlikely because the hydrolysis metabolites were formed to a similar extent by all the P450 isoforms. However, the oxidative metabolism observed during incubations with different P450 isoforms could be further understood with additional investigation.

**TABLE 3**

*Summary of known mutations of human BChE gene in white and Japanese populations*

<table>
<thead>
<tr>
<th>Variant</th>
<th>Amino Acid Substitution</th>
<th>Homozygote Frequency</th>
<th>Activity Description</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>A539T</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></td>
<td></td>
<td>1:69</td>
<td></td>
<td>Bartels et al., 1992</td>
</tr>
<tr>
<td>Atypical (A)</td>
<td>D70G</td>
<td>1:2500</td>
<td>(AA) 40% reduction in serum BChE activity</td>
<td>Altmirano et al., 2000</td>
</tr>
<tr>
<td>Dibucaine-resistant (J) variant</td>
<td>E497V</td>
<td>1:1945 (Japanese)</td>
<td>Same activity as WT BChE 66% reduction in circulation molecules</td>
<td>McGuire et al., 1989</td>
</tr>
<tr>
<td>Fluoride-resistant (F-1)</td>
<td>T243M</td>
<td>1:150,000</td>
<td>(FF) 50% reduction in serum BChE activity</td>
<td>Bartels et al., 1992</td>
</tr>
<tr>
<td>Fluoride-resistant (F-2)</td>
<td>G390V</td>
<td>1:160,000</td>
<td>F2 more frequent than F1; different substrate kinetics</td>
<td>Harris and Whittaker, 1961</td>
</tr>
<tr>
<td>Fluoride-resistant (Japanese)</td>
<td>L330I</td>
<td>Japanese: 0.29% frequency</td>
<td>Low serum BChE activity</td>
<td>Sudo et al., 1997</td>
</tr>
<tr>
<td>H variant</td>
<td>V142M</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-I variant (more frequent): 1:110,000</td>
<td>Increased protein–increased BChE activity (C5 +: 30–54% increased activity, association of BChE subunits with a noncholinesterase protein)</td>
<td>Nogueira et al., 1990</td>
</tr>
<tr>
<td>C5+, Cynthiana, Johannesburg</td>
<td>No genetic (phenotype)</td>
<td>Very rare</td>
<td></td>
<td>Krause et al., 1988</td>
</tr>
</tbody>
</table>

KK, AA, and FF indicate BChE genotype.
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 $C_{max}$ plasma values of unchanged drug and alcohol metabolite are less than 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, donepezil, 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).

BChE is widely distributed in the human body and is known to be produced in the liver and secreted into the bloodstream. In human plasma, BChE represents approximately 0.1% of total serum proteins (Chatonnet and Lockridge, 1989). The estimated prevalence of the wild-type homozygous BChE genotype in white populations is approximately 95%. To date, approximately 65 genetic variants of human BChE have been identified, and the most prevalent 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 approximately 20 silent genotypes characterized by the near complete absence of BChE activity. In addition, other rare variants (e.g., C5+, Cynthia 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 as a result of the important nonenzymatic hydrolysis ($t_{1/2}$, 1.2 h) at physiological pH. The results obtained in this study show that BChE 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


