

Stereoselective Inhibition of Human Butyrylcholinesterase by the Enantiomers of Bambuterol and Their Intermediates^[S]

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ABSTRACT

This work describes the sequential hydrolysis of bambuterol enantiomers and their monocarbamate metabolites (MONO) catalyzed by human butyrylcholinesterase (BChE) as well as the enzyme inhibition resulting from this process. Particular emphasis is given to the contribution given by MONO to the enzyme inhibition because it was not fully characterized in previous works. Bambuterol and MONO enantiomers displayed the same time- and concentration-dependent mechanism of interaction with the enzyme. The hydrolysis kinetics of both bambuterol and MONO was enantioselective, and the (R)-enantiomer of each compound was hydrolyzed fourfold faster than the respective (S)-enantiomer. Even though the enzyme inhibition rates of (R)- and (S)-MONO were much slower than those of their respective bambuterol enantiomers (~15-fold), both MONO enantiomers showed a significant BChE inhibition when physiologically

relevant concentrations of enzyme and inhibitors were used (~50% of their respective bambuterol enantiomers). The kinetic constants obtained by testing each single compound were used to model the contribution given by MONO to the enzyme inhibition observed for bambuterol. The hydrolysis of MONO enantiomers enhanced the inhibitory power of bambuterol enantiomers of about 27.5% (R) and 12.5% (S) and extended more than 1 hour the duration of inhibition. The data indicate that MONO contribute significantly to the inhibition of BChE occurring in humans upon administration of normal doses of bambuterol. In addition, the hydrolysis of MONO resulted in the rate-limiting step in the conversion of bambuterol in its pharmacologically active metabolite terbutaline; therefore, MONO concentrations should always be monitored during pharmacokinetic studies of bambuterol.

Introduction

Bambuterol (BMB) is a bis-dimethylcarbamate prodrug of terbutaline (TBT) classified as a long-acting β_2 -adrenoceptor agonist. BMB is given orally for the treatment of the symptoms of asthma and other affections characterized by bronchospasm (Olsson and Svensson, 1984; Petrie et al., 1993). In humans, the oral administration of BMB results in a slow production of TBT, making a single daily dose of BMB sufficient to maintain therapeutic levels of TBT (Nyberg et al., 1998). BMB itself has no bronchodilating effect, but it is metabolized

in vivo by plasma butyrylcholinesterase (BChE; EC 3.1.1.8) into bambuterol monocarbamate (MONO; inactive), which is further metabolized by the same enzyme into the pharmacologically active compound, TBT (Tunek and Svensson, 1988). The two-step hydrolysis is described by the second-order kinetic constants k_{11} and k_{12} (Fig. 1). The hydrolysis mechanism in each step consists of a first stage in which one carbamate group of BMB or MONO is transferred to the active site of the enzyme, which therefore results in it being temporarily inhibited. In a successive stage, the enzyme activity is restored by water that removes the carbamyl group from the active site with a rate constant k_3 (O'Brien et al., 1966; Main, 1979). The inhibition of plasma BChE occurring during the hydrolysis of BMB into TBT is traditionally considered the main reason accounting for the slow production of TBT observed in vivo (Sitar, 1996). An oxidative pathway for the biotransformation of BMB into TBT, most likely driven by cytochrome P450-dependent oxidases, has been observed in rat liver microsomes (Svensson and Tunek, 1988; Lindberg et al., 1989). Some evidence suggests that this metabolism might be significant also in humans (Bang et al., 1998), although its real impact on the pharmacokinetics of BMB is still not clear and requires further investigation.

The inhibition of BChE by BMB has significant effects on the pharmacokinetics of other coadministered drugs that are substrates of

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ABBREVIATIONS: AUC, area under time-concentration curve; BChE, human butyrylcholinesterase (EC 3.1.1.8); BMB, bambuterol; BTCh, butyrylthiocholine; DTNB, 5-5'-dithiobis(2-nitrobenzoic acid); HPLC, high-performance liquid chromatography; MONO, bambuterol monocarbamate; RSS, residual sum of squares; SPE, solid-phase extraction; TBT, terbutaline; UPLC-MS/MS, ultra-performance liquid chromatography-tandem mass spectrometry.

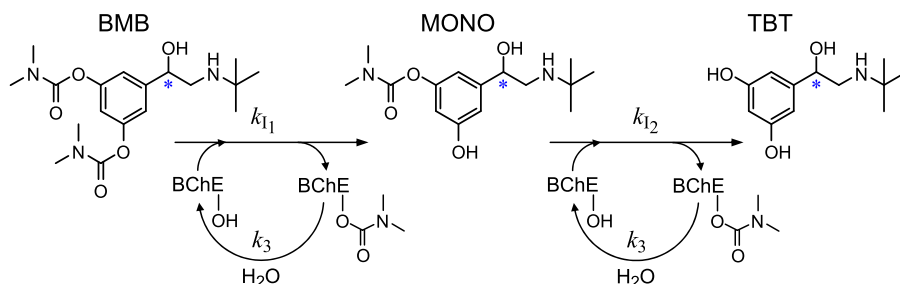


Fig. 1. Hydrolysis of BMB by BChE. *, Chiral center.

BChE. Well-known examples of these effects include the accumulation and the prolonged duration of action of the muscle-relaxant drugs succinylcholine (Fisher et al., 1988; Staun et al., 1990), suxamethonium (Bang et al., 1990), and mivacurium (Ostergaard et al., 2000) observed in patients under BMB medication. BMB, which contains a chiral center, is currently synthesized and administered as a racemate, but there is some evidence that the (R)-enantiomer is more potent and less toxic than its mirror image (Morley, 2002; Tan and Cheng, 2009). On this basis, (R)-BMB hydrochloride has been recently approved by the Chinese State Food and Drug Administration for phase I clinical trials, and a preliminary pharmacokinetic study has been published (Zhou et al., 2014a). In light of the recent indications reported by the European Medicines Agency (EMA, 2012), the Food and Drug Administration (U.S. FDA, 2012), and successive suggestions (Yu and Tweedie, 2013), metabolites that are present at 25% of the parent area under time-concentration curve (AUC) or 100% of the parent AUC (Yu and Tweedie, 2013) should be subject of further in vitro characterization of the metabolite as a possible contributor to drug-drug interactions. This is clearly the case of BMB and MONO, because the AUC of MONO (in molar units) was found to be about 300% of the AUC of BMB in a recent pharmacokinetic study (Zhou et al., 2014b). The renewed interest over BMB and the indications of the regulatory bodies emphasized the gaps in the current knowledge of BMB pharmacokinetics, and in particular on the interaction of its primary metabolite, MONO with BChE. BMB enantiomers have been characterized for their BChE inhibitory effects, and (R)-BMB resulted in inhibiting the enzyme about fivefold faster than (S)-BMB in vitro (Bosak et al., 2008). The racemic mixture of MONO was found about 10-fold less potent as an inhibitor of human BChE than racemic BMB (Tunek and Svensson, 1988), but its hydrolysis kinetics has not been fully characterized. In addition, the inhibitory effect of MONO enantiomers on BChE has not yet been studied.

This study presents the stereoselective hydrolysis kinetics of BMB and MONO enantiomers and their BChE inhibition properties. The kinetic parameters obtained were used to model the BChE inhibition caused by BMB without the contribution of MONO and to evaluate the effect of the enzyme inhibition on the production rate of TBT. The contribution of TBT to the inhibition of BChE was not investigated because its role at therapeutic plasma concentrations can be considered insignificant (Kovarik and Simeon-Rudolf, 2004).

Materials and Methods

Chemicals

(RS)-[3-[2-(tert-butylamino)-1-hydroxyethyl]-5-(dimethylcarbamoyloxy)phenyl] N,N-dimethylcarbamate hydrochloride (racemic BMB hydrochloride, mol. wt. 403.9), its single enantiomers, and racemic terbutaline hemisulfate (mol. wt. 274.3) were supplied by Dongguan Keyphar (Dongguan, PRC). The chemical purity of all compounds exceeded 99.5%, and the enantiomeric excess of the single enantiomers was $\geq 99.5\%$. Butyrylcholinesterase derived from human plasma (EC 3.1.1.8), S-butyrylthiocholine iodide (BTCh), 5-5'-dithiobis

(2-nitrobenzoic) acid (DTNB), and neostigmine methyl sulfate (mol. wt. 223.3) were purchased from Sigma-Aldrich (Shanghai, PRC). Salbutamol hemisulfate (mol. wt. 288.4) was obtained from the Chinese National Institute for Food and Drug Control. Buffer salts (ammonium acetate, NaH_2PO_4 , Na_2HPO_4 , NaHCO_3), NaCl, sodium EDTA, acetic acid, sodium hydroxide, ammonium hydroxide, ethyl acetate, and glycerol were of analytical grade and purchased from Sigma-Aldrich. Solvents and additives used for chromatography (methanol, acetonitrile, reagent alcohol, hexanes, formic acid) were high-performance liquid chromatography (HPLC) grade and purchased from Fisher Scientific (Guangzhou, PRC). BChE, supplied as a lyophilized powder, was dissolved in a buffer containing sodium phosphate (10 mM, pH 7.2), NaCl (50 mM), EDTA (1 mM), and glycerol (10% v/v) at a nominal concentration (according to the analysis certificate provided by the supplier) of 38.5 U butyrylcholine/mL and stored at -80°C . All the enzyme activity data reported in this work refer to the nominal activity provided by the supplier.

Synthesis of Rac-Bambuterol Monocarbamate Hydrochloride and Its Single Enantiomers

General Synthetic Approach. Racemic MONO [(RS)-[3-[2-(tert-butylamino)-1-hydroxyethyl]-5-hydroxyphenyl] N,N-dimethylcarbamate hydrochloride, MONO] and its (S)- and (R)-enantiomers were synthesized by using racemic, (S)-, and (R)-BMB as starting materials, respectively. Five aliquots of 10 mg BMB hydrochloride (*rac*-S- or R-, 0.124 mmol) were solubilized in aqueous NaOH (0.25 M, 0.5 mL/aliquot) and left at room temperature (~ 26 – 28°C) for 12 hours. The reaction was stopped by adding aqueous acetic acid (20% v/v, 100 μL /aliquot). The fractions containing MONO (*rac*-S- or R-) were then isolated by solid-phase extraction (SPE).

Isolation of MONO by SPE. A C-18 SPE cartridge (1 g bed; Autoscience, Tianjin, PRC) was sequentially washed with 10 mL water, 10 mL methanol, and equilibrated with 10 mL NH_4OH 0.2% in water (SOL 1). One aliquot of reaction mixture (600 μL) was loaded onto the cartridge, and terbutaline was eluted with 10 mL SOL 1/methanol 90:10. MONO was eluted with 10 mL SOL 1/methanol 60:40. The first mL eluate was collected separately and discarded. BMB was eluted with 5 mL buffer ammonium acetate (50 mM, pH 4.6)/methanol 10:90. The cartridge was washed with 5 mL water/methanol 10:90, and the procedure was repeated for the other aliquots of reaction mixture. The fractions of MONO obtained by SPE were pooled, and the methanol content was reduced by a vacuum concentrator; aqueous HCl (0.05 M, 5 mL) was added, and the fraction was lyophilized.

Chemical Characterization. The identity of MONO was confirmed by mass (Xevo TQ-S, Waters, UK) and ^1H NMR (500 MHz, Bruker) spectrometries. The HPLC methods used to characterize chemical purity and enantiomeric excess of the synthesized compounds are described in Supplemental Material 1 and 2).

In Vitro Measurements of BChE Activity

The activity of BChE was measured in 96-well plates by an EnSpire Multimode 2300 plate reader (Perkin Elmer, Waltham, MA) using Ellman's spectrophotometric assay (Ellman et al., 1961). Stock solutions of substrate (BTCh) were freshly prepared in water. Stock solutions of DTNB (Ellman's reagent) were freshly prepared in sodium phosphate buffer (100 mM, pH 7, 0.15% NaHCO_3 w/v). Stock solutions of the tested compounds [*rac*-BMB, (R)-BMB, (S)-BMB, *rac*-MONO, (R)-MONO, (S)-MONO] were prepared in

water. Ellman's assay was used to determine the second-order carbamylation rate constants and IC_{50} at low enzyme concentrations, the carbamylation kinetics at physiologically relevant concentrations, and the decarbamylation rate constant of N,N'-dimethylcarbamy-BChE.

Inhibition Kinetics at Low Concentration of Enzyme: Determination of k_1 and IC_{50} . Solutions of purified BChE (200 μ L, 0.03 U/mL) in assay buffer (sodium phosphate buffer 100 mM, pH 8) were preincubated at 37°C with solutions of each inhibitor (25 μ L, in water) at different concentrations. At set times, the preincubation was stopped by adding a solution (25 μ L) containing the substrate (BTCh, 2.5 mM) and DTNB (3.4 mM) in assay buffer, and the enzyme activity (v_i) was determined by monitoring the increase of concentration of yellow product at 412 nm over 5–6 minutes at 37°C. A sample incubated with water in place of the inhibitor was analyzed in parallel to measure the maximum enzyme activity (v_0) at each incubation time. A blank sample containing assay buffer in place of the enzyme was measured for each inhibitor concentration to account for the spontaneous conversion of the substrate into product, and the values obtained were subtracted from each measured activity.

Enzyme Inhibition at Physiologically Relevant Concentrations of BChE and Inhibitors. The physiologically relevant concentration of BMB and MONO was set at 40 nM to equal the highest sum of BMB, MONO, and TBT concentrations measured in the plasma of healthy volunteers during a pharmacokinetic study of BMB (Zhou et al., 2014b). This value represents a hypothetical minimum concentration of BMB reaching the circulation (i.e., considering plasma protein binding, other metabolisms, distribution to tissues, and elimination of the three species negligible). Because the plasma protein binding of BMB is low [40–50% (Rosberg et al., 1993)] and that of TBT is even lower [20% (Ryrfeldt and Ramsay, 1984)], this factor is unlikely to modify significantly the hydrolysis kinetics of BMB. The physiologically relevant concentration of BChE was set at 6.42 U/mL. This value was chosen on the basis of the lowest activity found among the previously cited healthy volunteers (Zhou et al., 2014b). An aliquot of the stock solution of BChE was diluted fourfold in sodium phosphate buffer (50 mM, pH 7.4) and divided in fractions of 12 μ L. A solution of each inhibitor (6 μ L, 120 nM) in phosphate buffer (50 mM, pH 7.4) was added to the enzyme fraction and preincubated at 30°C (both solutions were prewarmed 10 minutes at 30°C before mixing), giving a solution of 6.42 U/mL BChE and 40 nM inhibitor. At set times, the preincubation was stopped by diluting an aliquot of the inhibitor-enzyme mixture (2 μ L) into a solution prewarmed at 37°C (498 μ L) containing the substrate (250 μ M BTCh) and DTNB (340 μ M) in assay buffer (100 mM sodium phosphate buffer, pH 8). A total of 250 μ L of this solution was quickly transferred to a well of a 96-well plate, and the enzyme activity (v_i) was determined by monitoring the increase of concentration of yellow product at 412 nm over 3 minutes at 37°C. A sample preincubated with water in place of the inhibitor was also analyzed to measure the maximum enzyme activity (v_0) at each incubation time. A blank sample containing assay buffer in place of the enzyme was measured to account for the spontaneous conversion of the substrate into product, and the obtained values were subtracted from each measured activity.

Determination of the Decarbamylation Rate Constant of N,N'-Dimethylcarbamy-BChE, k_3 . The decarbamylation rate constant was determined by using a concentration of inhibitor able to almost completely inhibit the enzyme activity (Perola et al., 1997). This solution was then diluted down to a concentration at which the inhibitor does not show any significant inhibition, and the recovery of the activity was followed over time.

An aliquot of BChE stock solution (3 μ L) was mixed with a solution of 400 nM *rac*-BMB (3 μ L) in water and preincubated 30 minutes at room temperature (final condition: 19.25 U/mL BChE, 200 nM *rac*-BMB). After the preincubation, 5 μ L of this solution was diluted 300-fold into phosphate buffer (50 mM, pH 7.4) at 37°C or 30°C. At set times, an aliquot of this solution (225 μ L) was added to a well of a 96-well plate containing a solution (25 μ L) of substrate (BTCh, 2.5 mM) and DTNB (3.4 mM). The enzyme activity (v_i) was determined by monitoring the increase of concentration of yellow product at 412 nm over 3–5 minutes at 37°C or 30°C, respectively. A sample preincubated with water in place of the inhibitor was also analyzed to measure the maximum enzyme activity (v_0) at each incubation time. A blank sample containing assay buffer in place of the enzyme was measured to account for the

spontaneous conversion of the substrate into product, and the obtained values were subtracted from each measured activity. The determination of k_3 was performed in duplicate (30°C) or in triplicate (37°C).

BMB and MONO Used as Substrates: Simultaneous Quantitation of BMB, MONO, and TBT upon Incubation of Physiologically Relevant Concentrations of BChE and Substrates

Sample Preparation. Solutions of *rac*-BMB, *rac*-MONO, and their single enantiomers (50 μ L, 48 nM) in sodium phosphate buffer (60 mM, pH 7.4) were added to aliquots of BChE stock solution (10 μ L) and incubated at 30°C (final condition: 6.42 U/mL BChE, 40 nM drug). At set times (1, 2, 4, 8, 15, 30, 60, 120, and 180 minutes), the hydrolysis was stopped by mixing an aliquot (5 μ L) of the enzyme-inhibitor mixture with 5 μ L neostigmine methyl sulfate (60 μ g/mL in water). An aliquot (8 μ L) of this solution was then mixed with 120 μ L internal standard (125 pg/mL salbutamol in mobile phase B) and analyzed by ultra-performance liquid chromatography-tandem mass spectrometry (UPLC-MS/MS; Xevo-TQS; Waters, Manchester, UK). Each experiment was performed in triplicate. The incubation was performed at 30°C, because the hydrolysis of BMB at 37°C [in particular, *rac*-BMB and (R)-BMB] was too fast to be monitored by a manual sampling; moreover, 30°C resulted in the highest temperature at which the evaporation of water did not influence significantly the measured concentrations within the whole duration of the experiment (3 hours).

UPLC-MS/MS Analysis. After the addition of the internal standard, the samples were centrifuged at 10,000g at 4°C for 10 minutes. The supernatant (2 μ L) was injected into the UPLC-MS/MS system. The chromatographic separation and the detection of BMB, MONO, and TBT were performed by using a method validated for plasma samples (Zhou et al., 2014b). The details of the analysis are reported as Supplemental Material 3.

Data Analysis

Inhibition Kinetics at Low Concentration of Enzyme: Determination of k_1 and IC_{50} . The pseudo-first-order rate constant (k_{obs}) values were calculated by plotting the residual fractional activity obtained from each inhibitor concentration (R_t , eq. 1) as a function of time and fitted to a one-phase exponential decay equation (eq. 2) (Feaster and Quinn, 1997).

$$R_t = v_i/v_0 \quad (1)$$

$$R_t = R_0 \cdot \exp(-k_{obs} \cdot t) + R_\infty \quad (2)$$

where v_i is the measured activity of the enzyme treated with the inhibitor after a given time of preincubation; v_0 is the activity of the enzyme treated with water at the same given time of preincubation; and R_t , R_0 , and R_∞ represent the residual fractional activity at time t , 0, and ∞ , respectively. The k_{obs} values were then plotted versus the concentration of inhibitor $[I]$. Since the relationship was linear for all the molecules under investigation, the data were analyzed as for a simple irreversible inhibition, and the second-order inhibition (carbamylation) rate constant (k_I) was calculated from eq. 3 (Marangoni, 2003; Gazić et al., 2006):

$$k_{obs} = k_I \cdot [I] \quad (3)$$

The second-order carbamylation rate constants were defined as k_{I1} when referring to the hydrolysis of BMB to MONO and k_{I2} when referring to the hydrolysis of MONO to TBT. The residual fractional activities obtained after 1 hour of incubation were plotted against the logarithm of inhibitor concentrations, and the IC_{50} values were obtained by fitting the data with a standard four-parameter logistic nonlinear regression analysis (Brooks et al., 2012). The values of k_{I1} and IC_{50} measured for *rac*-BMB, R-BMB, and S-BMB were not corrected for the potential influence of the MONO metabolite generated during the incubation, which was considered negligible. In fact, the concentration of MONO generated during the experiment is expected to be of the same order of magnitude as the concentration of enzyme (about 0.4 nM), and, in that range of concentrations, MONO did not show significant inhibition.

Determination of the Decarbamylation Rate Constants of N,N'-dimethyl-BChE, k_3 . The decarbamylation rate constant (k_3) was calculated by plotting

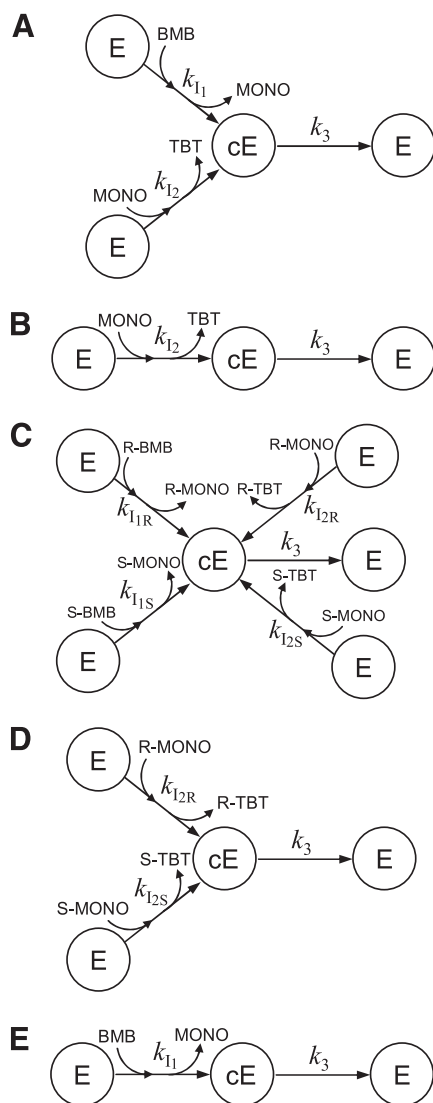


Fig. 2. Hydrolysis models used to calculate the second-order carbamylation rate constants (k_i) of BMB racemate and its single enantiomers (A), and MONO and its single enantiomers (B) by the web tool ENZO. (C and D) Models for *rac*-BMB and *rac*-MONO (respectively), in which the enantiomers compete kinetically for the hydrolysis. (E) Model used to evaluate the contribution of MONO enantiomers to the inhibition of BChE by BMB enantiomers showed in Fig. 4, C and D. Legend: E = BChE; cE = N,N'-dimethylcarbamyl-BChE. The ordinary differential equations describing each model are reported as Supplemental Methods 4.

the natural logarithm of the measured inhibited fraction against time according to eq. 4 (Perola et al., 1997):

$$\ln[1 - (v_i/v_0)] = -k_3 \cdot t \quad (4)$$

where v_i is the measured activity of the enzyme preincubated with the inhibitor at a given time (t) after dilution and v_0 is the activity of the enzyme preincubated with water at the same time after dilution. The slope of the linear data regression gives $-k_3$.

Determination of k_i Values from UPLC-MS/MS Data Combined with Enzymatic Inhibition Data Obtained at the Same Physiologically Relevant Concentration of BChE and Inhibitors. The averaged time-course concentration profiles of BMB, MONO, and TBT as measured by UPLC-MS/MS upon incubation of BMB and MONO with BChE were combined to the enzymatic inhibition data obtained in the same experimental conditions and analyzed by the WEB server ENZO [http://enzo.cmm.ki.si (Bevc et al., 2011)] to obtain the second-order carbamylation rate constants (k_i). The hydrolysis model used to analyze BMB enantiomers is reported in Fig. 2A: BMB is hydrolyzed in

a single step (i.e., the BMB-enzyme complex is considered irreversible), yielding MONO and the inactive carbamyl-BChE with a kinetic constant k_{i1} . MONO is then hydrolyzed in a single step with a kinetic constant k_{i2} to give TBT and carbamyl-BChE. At the same time, the carbamyl-enzyme originating from the hydrolysis of BMB and MONO is hydrolyzed by water to the active form with a kinetic constant k_3 . Because the two carbamate groups of BMB are identical, both BMB and MONO generate the same carbamyl-enzyme. A similar model, which considers only the step involving MONO, was used to analyze the hydrolysis of MONO enantiomers (Fig. 2B). In the case of racemic compounds (*rac*-BMB and *rac*-MONO), the above-mentioned models, considering the racemate as a single compound, were used to obtain a numerical value of k_{i1} and k_{i2} ; in addition, more complex models were evaluated, for which the two enantiomers (at half concentration of the respective racemate) act as different compounds and compete kinetically for the hydrolysis (Fig. 2, C and D). The concentration of BChE (necessary to obtain the k_i values) was set at 80 nM, as this value best fit both UPLC-MS/MS and enzymatic inhibition experimental data for all the compounds. The value of k_3 was fixed for all the computational analyses at the experimental value obtained at 30°C (0.006424 minute⁻¹; Table 1). Residual sum of squares (RSS) values were used to evaluate the goodness of fit of the different models to the experimental mass spectrometry data. The ordinary differential equations describing each model are reported as supplemental data (Supplemental Material 4).

Calculation of the Contribution of MONO to the Inhibition of BChE Displayed by BMB. To estimate the contribution given by MONO to the enzyme inhibition observed for BMB, the k_i values obtained at 30°C and 37°C were used to calculate the theoretical BChE inhibition profiles generated by the hydrolysis of BMB enantiomers without the contribution of the MONO metabolites, according to the model reported in Fig. 2E. For *rac*-BMB, the model reported in Fig. 2D was used, with BMB in place of MONO, MONO in place of TBT, and k_{i1R} and k_{i1S} in place of k_{i2R} and k_{i2S} , respectively. The isoactivity point was defined as the time at which BMB enantiomers display the same enzyme inhibition and was used to evaluate the contribution of MONO to the enzyme inhibition in terms of enzyme recovery speed.

Calculation of the Effect of the Inhibition of BChE by BMB on the Rate of TBT Production. The k_i values obtained at 37°C were used to evaluate the effect of BChE inhibition on the production of TBT at different concentrations of R-BMB and *rac*-BMB, using the models reported in Fig. 2, A and C, respectively.

Results

Synthesis of Rac-Bambuterol Monocarbamate Hydrochloride and Its Single Enantiomers

The products were obtained as a white powder (yield, 79%). Electrospray Ionization Mass Spectrometry (m/z): 297.6 [MH]⁺; 241.4; 223.4. ¹H NMR, *rac*-MONO (500 MHz, CD₃OD) δ 6.77 (t, J = 1.8 Hz, 1H), 6.66 (t, J = 1.7 Hz, 1H), 6.49 (t, J = 2.2 Hz, 1H), 4.86 – 4.80 (m, 1H), 3.17 (dd, J = 12.5, 3.2 Hz, 1H), 3.10 (s, 3H), 3.02 (dd, J = 12.5, 9.8 Hz, 1H), 2.99 (s, 3H), 1.38 (s, 9H). Chemical purity $\geq$ 99.3% (HPLC-UV at 260 nm). Enantiomeric excess: 99.5% (R-MONO), 99.4% (S-MONO).

Inhibition Kinetics at Low Concentration of Enzyme: Determination of k_i and IC₅₀

Both BMB enantiomers showed a time- and concentration-dependent inhibition of purified BChE (Supplemental Fig. 1). For both the enantiomers, the relationship between concentration and k_{obs} was linear (Fig. 3B), and the line passed through the origin indicating that the enzyme-inhibitor Michaelis complex is virtually irreversible (i.e., all the inhibitor that binds the enzyme is hydrolyzed) (Main, 1969). The inhibition kinetics was highly enantioselective, as (R)-BMB inhibited the enzyme about fourfold faster than (S)-BMB (Table 1). The measured second-order carbamylation rate constants (k_i) and the enantioselectivity of BMB enantiomers are in agreement with data obtained with the homozygous usual variant (UU) of BChE in

TABLE 1

Summary of the kinetic parameters measured for the hydrolysis of bambuterol and bambuterol monocarbamate enantiomers by human BChE

Data are expressed as mean (S.E.).

Tested Compound	Experimental Approach (Temperature)					
	As Inhibitors (37°C)			As Substrates (30°C)		
	k_i^a ($\times 10^6 \text{ M}^{-1}\text{min}^{-1}$)	IC_{50}^b (nM)	k_3 (min^{-1})	k_{f1} ($\times 10^6 \text{ M}^{-1}\text{min}^{-1}$)	k_{f2} ($\times 10^6 \text{ M}^{-1}\text{min}^{-1}$)	k_3 (min^{-1})
<i>rac</i> -BMB	8.30 (0.17)	3.9 (0.6)	0.01175 (0.0003)	3.88 (0.90) ^c	0.255 (0.071) ^c	0.0064 (0.0004)
R-BMB	13.20 (0.74)	2.7 (0.6)		10.3 (6.9)	0.482 (0.305)	
S-BMB	3.23 (0.12)	8.7 (0.9)		1.56 (0.26)	0.091 (0.030)	
<i>rac</i> -MONO	0.341 (0.019)	44.7 (4.4)		—	0.298 (0.183) ^c	
R-MONO	0.839 (0.015)	23.4 (2.8)		—	0.544 (0.258)	
S-MONO	0.215 (0.021)	65.1 (9.6)		—	0.127 (0.008)	

^aThese k_i values are referred to as k_{f1} when the tested compound is bambuterol (BMB) and k_{f2} when it is bambuterol monocarbamate (MONO).^bCalculated from data measured after 60 min of incubation.^cData obtained by using the simple hydrolysis model, which does not consider the kinetic competition between the two enantiomers (see Fig. 2A).

plasma, measured with the same method at 25°C (Gazić et al., 2006). The relatively small difference between those data and the values reported in this work (about twofold) is consistent with the 12°C higher incubation temperature used in this work. As observed for BMB, MONO enantiomers inhibited the enzyme in a time- and concentration-dependent manner (Fig. 3A; Supplemental Fig. 1), with the same inhibition mechanism and the same enantioselectivity displayed by BMB (Fig. 3B; Table 1). The inhibition rate of MONO enantiomers was about 15-fold slower compared with the respective BMB enantiomers, whereas the fastest MONO enantiomer, (R)-MONO, was only fourfold slower than the slowest BMB enantiomer, (S)-BMB (Table 1).

All the compounds showed IC_{50} values in the nanomolar range (Table 1). The IC_{50} of *rac*-BMB, (R)-BMB, and (S)-BMB were about 11-fold, ninefold, and sevenfold lower than their respective MONO counterparts. The IC_{50} values of the (R)-enantiomers of both BMB and MONO were about threefold lower than their respective (S)-enantiomers. The measured IC_{50} for *rac*-BMB and *rac*-MONO are in good agreement with previously reported data obtained with whole blood (2.7 nM and 50 nM for *rac*-BMB and *rac*-MONO, respectively) (Tunek and Svensson, 1988).

Determination of the Decarbamylation Rate Constants, k_3

The inhibition of BChE by BMB and MONO enantiomers was completely reversible, as the enzyme was able to recover 95% of its activity in 4 hours at 37°C (Fig. 3C). The recovery speed at 30°C was about half of that at 37°C (Fig. 3C; Table 1). The decarbamylation rate constant (k_3) measured at 37°C (Table 1) is in very good agreement with the value of 0.011 minute^{-1} obtained with whole blood at the same temperature (value obtained reploting data published in Tunek and Svensson, 1988, according to eq. 4).

Enzyme Inhibition at a Physiologically Relevant Concentration of BChE and Inhibitors

The increase of concentration of BChE up to a physiologically relevant range (6.42 U/mL) did not modify the inhibition pattern observed for the different inhibitors at lower concentrations of enzyme. (R)-BMB was the fastest inhibitor, followed by (S)-BMB, (R)-MONO, and (S)-MONO (Fig. 4A). BMB and MONO racemates inhibited the enzyme with an intermediate kinetics within those of their respective single enantiomers (Fig. 4, A and B). Moreover, the inhibitory power followed the same pattern observed with lower enzyme concentrations: after 1 hour of incubation, (R)-BMB inhibited about 64% of the enzyme, followed by *rac*-BMB, 50%; (S)-BMB, 43%; (R)-MONO, 38%; *rac*-MONO, 28%; and (S)-MONO, 15%.

BMB and MONO as Substrates of BChE at Physiologically Relevant Concentrations

The concentration profiles of BMB, MONO, and TBT obtained upon incubation of BMB and MONO enantiomers with physiologic concentrations of BChE at 30°C are reported in Fig. 5. The profiles obtained for the racemic mixtures are shown in Fig. 6.

(R)-BMB was hydrolyzed very fast when incubated with a physiologic concentration of BChE. After 1 minute of incubation, ~70% of (R)-BMB was hydrolyzed to MONO, and a small amount of TBT was already present [6% of the initial (R)-BMB concentration]. The highest concentration of MONO was reached after 4 minutes of incubation [85% of the initial (R)-BMB concentration]. (R)-BMB was completely hydrolyzed within 8 minutes, and the MONO produced from its hydrolysis was almost completely hydrolyzed (96%) to TBT in 3 hours. Half of the initial concentration of (R)-BMB was transformed to TBT ($t_{1/2 \text{ TBT}}$) in about 45 minutes (Fig. 5).

(S)-BMB showed a much slower reactivity compared with (R)-BMB. However, most of the drug (93%) was hydrolyzed within 30 minutes, and the highest concentration of MONO [85% of the initial (S)-BMB concentration] was reached after 30 minutes. After 3 hours, the hydrolysis was still incomplete, and the concentration of TBT was only 57% of the initial (S)-BMB concentration ($t_{1/2 \text{ TBT}} \sim 160$ minutes; Fig. 5).

Initially, the hydrolysis of *rac*-BMB was only slightly slower than that of (R)-BMB. About 50% of *rac*-BMB was already hydrolyzed to MONO within 1 minute, and traces of TBT were detectable. About 95% of the initial concentration of *rac*-BMB was hydrolyzed within 30 minutes. The highest concentration of MONO was reached after 8 minutes (72% of the initial BMB concentration). After 3 hours of incubation, about 78% of the initial *rac*-BMB was hydrolyzed to TBT ($t_{1/2 \text{ TBT}} \sim 60$ minutes; Fig. 6).

The incubation of (R)-MONO with the enzyme resulted in a faster hydrolysis than that displayed by the (R)-MONO originating from the hydrolysis of (R)-BMB. The hydrolysis was completed in 3 hours, but 96% of the initial concentration of (R)-MONO was hydrolyzed to TBT within 2 hours ($t_{1/2 \text{ TBT}} \sim 20$ minutes; Fig. 5). As observed for (R)-MONO, the hydrolysis of (S)-MONO was faster than that displayed by the (S)-MONO originating from the hydrolysis of (S)-BMB. After 3 hours, about 78% of the initial concentration of (S)-MONO was hydrolyzed to TBT ($t_{1/2 \text{ TBT}} \sim 70$ minutes; Fig. 5). The hydrolysis of *rac*-MONO proceeded with an intermediate speed within those of its single enantiomers. After 3 hours of incubation, about 94% of the initial concentration was hydrolyzed to TBT ($t_{1/2 \text{ TBT}} \sim 38$ minutes; Fig. 6).

Data Fitting. To obtain reliable k_i values, the concentration profiles of BMB, MONO, and TBT obtained upon incubation of BMB

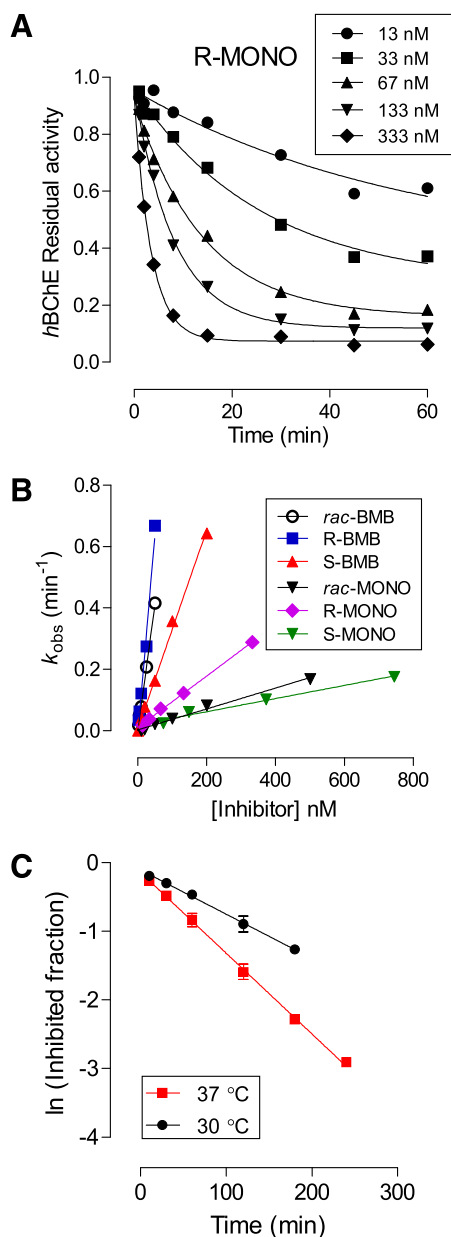


Fig. 3. (A) Time- and concentration-dependent inhibition of BChE after preincubation with various concentrations of (R)-MONO. The fitted one-phase exponential decay equations used to calculate the k_{obs} values for each concentration are represented as solid lines. (B) Linear correlations of the k_{obs} values obtained for BMB and MONO racemates and single enantiomers versus their respective concentrations. The slope of each line corresponds to k_i . Coefficients of determination (r^2): 0.9987 (*rac*-BMB), 0.9907 (R-BMB), 0.9913 (S-BMB), 0.9883 (*rac*-MONO), 0.9991 (R-MONO), 0.9818 (S-MONO). (C) Recovery of the activity of BChE after preincubation with *rac*-BMB, followed by dilution, reflecting the decarbamylation kinetics of N,N'-dimethylcarbamyl-BChE, plotted according to eq. 4 (mean $\pm$ SD, $n = 2-3$). The slope of the linear fitting corresponds to $-k_3$. The recovery was monitored up to 70% and 95% at 30°C and 37°C, respectively.

and MONO with a physiologically relevant concentration of BChE at 30°C were analyzed together with the inhibition profiles of BChE obtained in the same experimental conditions (Fig. 4). In fact, because a quite large range of enzyme concentrations gave a reasonable fitting of the concentration profiles (as far as they were higher than the concentration of inhibitor), the exact concentration of enzyme was required to calculate reliable k_i . The molar concentration of active enzyme in the purified preparation used for the experiments was not known. Anyway, only a very small range of enzyme concentrations,

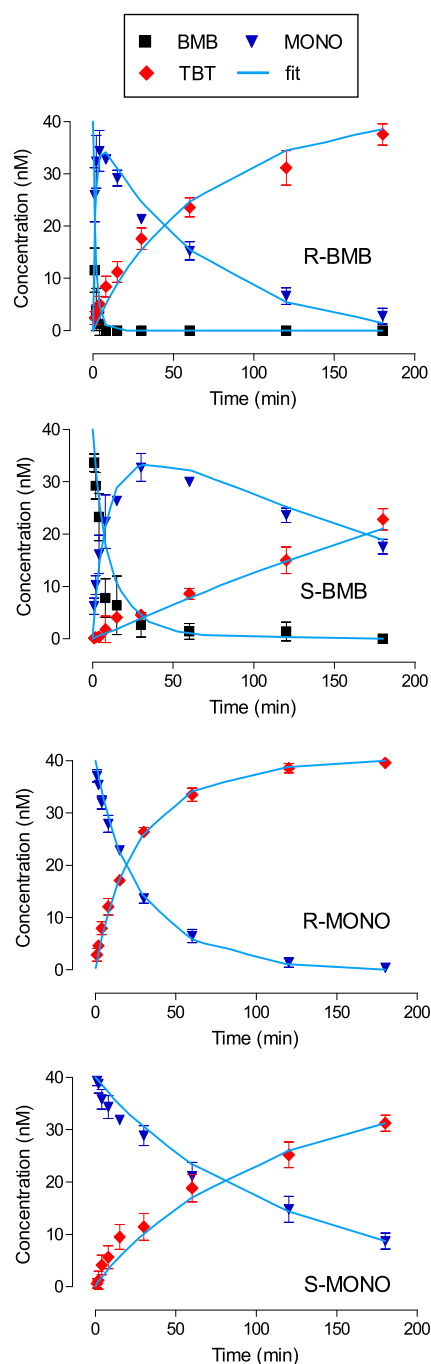


Fig. 4. Hydrolysis of BMB and MONO enantiomers incubated with purified BChE at physiologic concentrations of drugs and enzyme. The drugs were preincubated with the enzyme (drug = 40 nM; BChE = 6.4 U/mL) in phosphate buffer (60 mM, pH 7.4) at 30°C. At set times, the concentration of BMB, MONO, and TBT was measured by UPLC-MS/MS. The picture shows the average of three different experiments ($\pm$ S.E.M.) performed for each enantiomer. The concentration profiles of each molecule were combined together with their respective enzyme inhibition data displayed in Fig. 4A to compute the second-order carbamylation rate constants (k_i) reported in Table 1. The hydrolysis models employed for BMB and MONO enantiomers are depicted in Fig. 2, A and B, respectively. The fitted curves obtained by ENZO are shown for visual comparison with the experimental data (light blue solid line).

centered at about 80 nM, could fit both the concentration profiles and the inhibition profiles of all the tested compounds. This concentration of BChE is in good agreement with the value (79 nM) that was previously estimated in plasma (Myers, 1952), confirming that the concentration of enzyme used for the experiments was representative

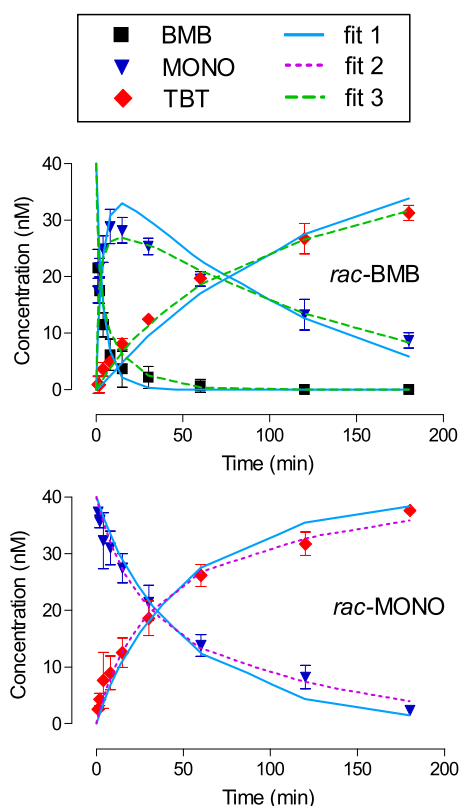


Fig. 5. Hydrolysis of BMB and MONO racemates incubated and analyzed, as described in Fig. 5. Each plot shows the average of three different experiments ($\pm$ S.E.M.). The concentration profiles obtained were combined together with their respective enzyme inhibition data displayed in Fig. 4B to compute the second-order carbamylation rate constants (k_i) reported in Table 1. The best fit curves obtained using different models are displayed as solid lines: fit 1, light blue, simple model (*rac*-BMB, $\text{RSS} = 3.61 \times 10^{-16}$; *rac*-MONO, $\text{RSS} = 1.05 \times 10^{-16}$); fit 2, violet, competitive model (*rac*-MONO, $\text{RSS} = 4.08 \times 10^{-17}$); fit 3, green, competitive model with k_i values fixed to those obtained for the single enantiomers (*rac*-BMB, $\text{RSS} = 2.08 \times 10^{-16}$). The k_{i2} values for (R)- and (S)-MONO calculated from the application of the competitive model on *rac*-MONO data were $0.622 \times 10^6 \text{ M}^{-1} \text{ min}^{-1}$ and $0.142 \times 10^6 \text{ M}^{-1} \text{ min}^{-1}$, respectively.

of physiologic conditions. The second-order carbamylation rate constants (k_i) obtained by this analysis are reported in Table 1. The computed best-fit curves of the concentration profiles are reported

together with the experimental data in Figs. 5 and 6, whereas the computed best-fit curves for the BChE inhibition profiles are reported together with the experimental data in Fig. 4, A and B. The analysis of the concentration profiles of BMB and MONO racemates gave a reasonable good fit when the simple models, in which racemic mixtures are considered as a single species, were used (Fig. 6, $\text{RSS}_{\text{rac-BMB}} = 3.61 \times 10^{-16}$; $\text{RSS}_{\text{rac-MONO}} = 1.05 \times 10^{-16}$), and the k_i values obtained were consistent with those obtained in the inhibition experiments (Table 1). However, the fit was definitely better when a competitive model was used (Fig. 6, $\text{RSS}_{\text{rac-BMB}} = 2.08 \times 10^{-16}$; $\text{RSS}_{\text{rac-MONO}} = 4.08 \times 10^{-17}$). Interestingly, the k_{i2} values for R- and S-MONO hydrolysis calculated from *rac*-MONO data were very close to those obtained for the single enantiomers separately (see caption to Fig. 6 and Table 1). In contrast, the software failed in calculating reliable k_{i1} and k_{i2} values for BMB and MONO enantiomers from *rac*-BMB data, although a very good fit to the experimental data was obtained when k_{i1} and k_{i2} values were fixed to those obtained for the single enantiomers (Fig. 6).

Calculation of the Contribution of MONO to the Enzyme Inhibition Shown by BMB. The k_i and k_3 values measured at 30°C and 37°C were used to calculate the theoretical enzyme inhibition profiles of BMB with and without the contribution of MONO. The calculated inhibition profiles at 30°C and 37°C are depicted in Fig. 4, C and D, respectively, and the results are summarized in Table 2.

Calculation of the Effect of the Enzyme Inhibition on the Production of TBT. The calculated inhibition profiles of a physiologic concentration of BChE (80 nM) caused by *rac*-BMB and (R)-BMB concentrations obtainable in human plasma with a therapeutic dose of BMB (20–120 nM) are depicted in Fig. 7A, whereas the corresponding formation of TBT is depicted in Fig. 7B. In the case of *rac*-BMB, the initial production rate of TBT increases with the concentration up to 40 nM, it remains almost constant in the range 40–80 nM, and decreases temporarily at higher concentrations (Fig. 7B). (R)-BMB shows a similar behavior with a slightly faster kinetics, and at 80 nM a temporary reduction of the initial production rate of TBT is already observable. At the highest concentration tested (120 nM), both *rac*-BMB and (R)-BMB show initially a low rate of production of TBT, and after about 1 hour the rate increases to values similar to those calculated at lower concentrations (Fig. 7B). The range of concentrations for which the initial rate of production of TBT is almost constant (40–80 nM)

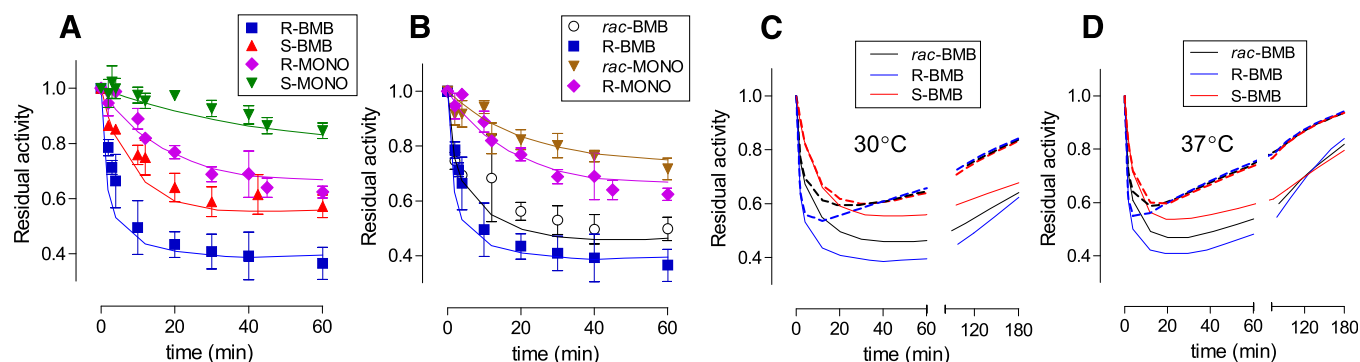


Fig. 6. (A) Time course of the inhibitory activity of (R)- and (S)-BMB, and their MONO metabolites (R)-MONO and (S)-MONO on purified BChE at physiologic concentrations. (B) Time course of the inhibitory activity of *rac*-BMB, (R)-BMB, and their MONO metabolites *rac*-MONO and (R)-MONO on purified BChE at physiologic concentrations. The drugs were preincubated with the enzyme (drug = 40 nM; BChE = 6.4 U/mL) in phosphate buffer (60 mM, pH 7.4) at 30°C. At set times, an aliquot of each mixture was diluted, and the residual enzymatic activity was measured. The experimental results (mean $\pm$ S.E.M., $n = 3-6$) were compared with the theoretical residual activity (dashed lines) calculated by ENZO (as in A and B) for racemic BMB and its single enantiomers (solid lines) depicted in Fig. 2 (the theoretical curves displayed were obtained with a concentration of 80 nM BChE). The theoretical curves for *rac*-BMB and *rac*-MONO correspond to the competitive models reported in Fig. 2, C and D, respectively. (C and D) Contribution of MONO to the inhibition of BChE displayed by BMB at 30°C and 37°C. The theoretical time-course residual activity calculated by ENZO (as in A and B) for racemic BMB and its single enantiomers (solid lines) are compared with the theoretical residual activity for the three species calculated considering only the first step of hydrolysis (from BMB to MONO, dashed lines) according to the model depicted in Fig. 2E for (R)-BMB and (S)-BMB and the respective competitive version for *rac*-BMB.

TABLE 2
Theoretical human BChE inhibition by BMB with and without the contribution of MONO

BChE maximal inhibition % (time).				
Compound	30°C		37°C	
	With MONO Contribution	Without MONO Contribution	With MONO Contribution	Without MONO Contribution
rac-BMB	54% (40 min)	40% (19 min)	53% (20 min)	41% (12 min)
R-BMB	61% (40 min)	46% (12 min)	59% (19 min)	45% (4 min)
S-BMB	45% (40 min)	40% (29 min)	46% (19 min)	40% (12 min)
Isoactivity point	>180 min	38 min	~120 min	19 min

corresponds to an inhibition of the enzyme activity in the range of about 50–80%, whereas a lower and higher inhibition correspond to a positive or to a temporary negative dependence of the production rate of TBT versus the concentration of BMB, respectively (Fig. 7A).

Discussion

It has been known for nearly three decades that BMB is a substrate of BChE, and that their interaction results in a temporary inhibition of the enzyme. In the early reports, the contribution given by the MONO intermediate to the inhibition of BChE observed for BMB was not fully investigated and the effect of MONO has not been taken into account in the following studies related to BMB. The present study demonstrated that at physiologically relevant concentrations of enzyme, the MONO originating from the hydrolysis of BMB plays a significant and long lasting inhibitory effect on BChE.

The interaction of BMB and MONO enantiomers with BChE was studied from two different perspectives. First, the compounds were characterized as inhibitors of the enzyme, to obtain an indirect picture of the hydrolytic process. Then the hydrolysis model was confirmed by studying BMB and MONO as substrates of the enzyme by measuring the concentrations of reagents and products over the time. The enantioselective time- and concentration-dependent inhibition mechanism obtained when BMB enantiomers were used as inhibitors is in agreement with previously reported findings (Tunek and Svensson, 1988) and confirms the hydrolysis model (Fig. 1) previously proposed (Kovarík and Simeon-Rudolf, 2004; Gazić et al., 2006). In fact, according to this model, the interaction between BMB and BChE results in a virtually irreversible complex formation, and this is consistent with the linear correlation between the pseudo-first-order rate constant (k_{obs}) and BMB concentration (Fig. 2B). In addition, the IC_{50} , k_I , and k_3 values obtained for BMB within this work are in good agreement with those obtained with plasma and whole blood, confirming that the purified enzyme is a reliable model to study the hydrolysis of BMB catalyzed by BChE and that their interaction is not significantly affected by other blood constituents. In this study, we demonstrated that the interaction between MONO enantiomers and the enzyme follows the same mechanism observed for BMB enantiomers (Fig. 2, A and B), but their hydrolysis is about 15-fold slower than their respective BMB counterparts, as indicated by the k_I values reported in Table 1.

As a further confirmation of the hydrolysis model (Fig. 1), when BMB and MONO enantiomers were studied as substrates of the enzyme, the experimental concentration profiles of substrates and products provided a very good fit with those computed according to the model (Fig. 5), and the computed k_I values were consistent with those obtained by studying the drugs as enzyme inhibitors (Table 1).

Second-order carbamylation rate constants (k_I) and IC_{50} are often considered indicative of the potency of inhibitors, and they are used to compare different compounds. However, in the case of irreversible inhibitors, their interpretation in this sense might be misleading

because the concentration of enzyme used to test the potency greatly influences the final result. This issue was taken into account for BMB (Tunek and Svensson, 1988), but not for MONO. For instance, the ratio between the IC_{50} values of (R)-BMB and (R)-MONO is about 10 (Table 1), and the ratio between their k_I values is about 15 (Table 1). However, when physiologically relevant concentrations of (R)-BMB and (R)-MONO were incubated with a physiologically relevant concentration of enzyme, (R)-BMB showed an inhibition only double as much as (R)-MONO (Fig. 4A). Therefore, IC_{50} ratios and k_I ratios clearly overestimate the inhibitory power of BMB with respect to MONO, or more in general, they overestimate the faster inhibitor with respect to the slower. For this reason, the contribution of the MONO intermediate to the inhibition of the enzyme by BMB enantiomers was previously underestimated (Tunek and Svensson, 1988). To emphasize this aspect, the experimental k_I values were used to calculate the theoretical BChE inhibition profiles of BMB enantiomers without the contribution of the MONO generated during their hydrolysis (Fig. 4, C

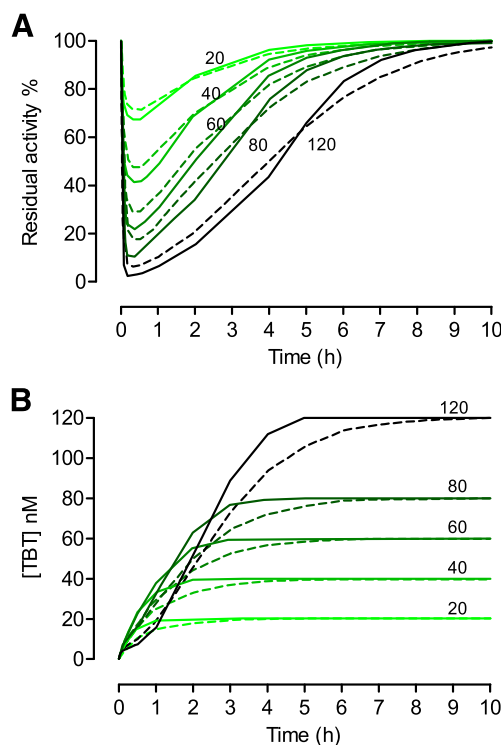


Fig. 7. (A) Theoretical time-course residual activity of purified BChE calculated by ENZO at different concentrations of (R)-BMB (solid lines) and *rac*-BMB (dashed lines), and (B) the corresponding theoretical time course of the production of TBT. The theoretical curves displayed were obtained by applying the models depicted in Fig. 2A for (R)-BMB and Fig. 2C for *rac*-BMB with a concentration of 80 nM BChE. The concentrations of (R)-BMB and *rac*-BMB are indicated in nM unit.

and D; Table 2). As an example, the calculation of the contribution of (R)-BMB to the total enzyme inhibition revealed that the hydrolysis of (R)-MONO enhanced the inhibitory effect of (R)-BMB by 25% and, at 37°C, it increased the recovery time of the enzyme of about 40% (Table 2). In addition, the contribution of MONO greatly enhances the effect of BMB chirality on BChE inhibition in terms of both maximum inhibition and duration of effect (Fig. 4, C and D; Table 2). In other words, without the contribution of the respective MONO enantiomers, the maximum inhibition and the duration of inhibition of (R)-BMB and (S)-BMB are quite similar.

The analysis of the impact of BChE inhibition by *rac*-BMB and (R)-BMB over the initial production rate of TBT (Fig. 7) showed that the increase of production rate expected upon the increase of substrate concentration is almost perfectly balanced by the decrease of rate due to the reduction of active enzyme. This is especially noticeable in the range of BMB concentrations responsible for a maximum inhibition within 50% and 80%, which is the normal range of inhibition measured upon oral administration of *rac*-BMB in pharmacokinetic studies (Sitar et al., 1992; Nyberg et al., 1998; Ahlström et al., 1999). Thus, the inhibition of the enzyme affects the initial production rate of TBT by keeping it constant over a relatively wide range of concentrations of administered BMB, rather than lowering it (Fig. 7). However, the calculations showed that a dose of BMB able to inhibit the enzyme up to 95% can slow down the rate of production of TBT, even though only for a very short time (less than 90 minutes; Fig. 7). This effect can be reasonably ascribed mainly to a reduction of the hydrolysis rate of MONO, rather than a reduction of the hydrolysis rate of BMB. In fact, due to its lower k_1 value, the hydrolysis rate of MONO is much more sensitive to the concentration of active enzyme than BMB.

Taken together, these data demonstrate that the inhibition of BChE by MONO accounts for a significant part of the inhibition observed for BMB in physiologic conditions, particularly for the (R)-enantiomer. Furthermore, the hydrolysis of MONO by BChE has a larger impact on the pharmacokinetics of BMB than previously considered; therefore, the concentration profiles of MONO should be always monitored during pharmacokinetic studies involving BMB.

The whole hydrolytic process involving BMB and BChE is described in this work, giving a complete picture of the hydrolysis kinetics and the concurrent enzyme inhibition. For the first time, the role of MONO is elucidated, and the effect of chirality is demonstrated. These findings offer a valuable support to the interpretation of pharmacokinetic data obtained in humans and will help to fully unveil the properties of the two enantiomers of BMB that underlie their different therapeutical activity and toxicity, thus facilitating a better clinical use of BMB.

Authorship Contributions

Participated in research design: Pistolozzi, Tan.

Conducted experiments: Pistolozzi, Du, Wei.

Contributed new reagents or analytic tools: Pistolozzi, Du, Wei.

Performed data analysis: Pistolozzi, Du, Wei.

Wrote or contributed to the writing of the manuscript: Pistolozzi, Tan.

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