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Immobilized butyrylcholinesterase in the characterization of new inhibitors that could ease Alzheimer's disease

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Abstract

Focus of this work was the development and characterization of a new immobilized enzyme reactor (IMER) containing human recombinant butyrylcholinesterase (rBChE) for the on-line kinetic characterization of specific, pseudo-irreversible and brain-targeted BChE inhibitors as potential drug candidates for Alzheimer's disease (AD). Specifically, a rBChE-IMER containing 0.99U of covalently bound target enzyme was purposely developed and inserted into a HPLC system connected to a UV-vis detector. Selected reversible cholinesterase inhibitors, (-)-pheneserine and (-)-cymserine analogues, were then kinetically characterized by rBChE-IMER, and by classical in solution assays and their carbamoylation and decarbamoylation constants were determined. The results support the elucidation of the potency, inhibition duration, mode of action and specific structure/activity relations of these agents and allow cross-validation of the two assay techniques.

Keywords

Human butyrylcholinesterase; Covalent immobilization; Monolithic disks; Pseudo-irreversible inhibitors; Kinetic constants; On-line studies; Pheneserine; Cymserine

1. Introduction

Alzheimer's disease (AD), the most prevalent form of dementia, is thought to affect between 2 and 10% of North Americans and Europeans over the age of 65 years. In the search for new AD therapeutics, investigation of the mechanism of action of new cholinesterase inhibitors (ChEIs) represents a key strategy to aid new lead selection and development, as this drug class has demonstrated consistent efficacy in AD and current clinically approved agents can be improved upon. The ex vivo characterization of drug candidates on isolated target enzymes, unfortunately, is often associated with long, labor-intensive assays and a large amount of disposable expensive material. However, with recent developments and applications of stable immobilized enzyme reactors (IMERS) [1-5], several in solution methods may prove of value for the development of automated procedures to support rapid generation of useful data and the more efficient use of both target proteins and synthesized drug candidates.

In this context, the focus of this work was the development and characterization of a new IMER containing immobilized human recombinant butyrylcholinesterase (rBChE, EC 3.1.1.8) for the online kinetic characterization of specific, pseudo-irreversible and brain-targeted ChEIs. The

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drug candidates, (-)-phenserine and (-)-cymserine derivatives, were studied as representatives of this drug class as they are in current development for mild to moderate forms of AD.

BChE together with its better known sister enzyme, acetylcholinesterase (AChE, EC 3.1.1.7), are serine hydroxylases that belong to a large family of proteins that collectively share a common α/β fold, which includes enzymes that are esterases, lipases proteases, in addition to non-enzymatic proteins that function as adhesion molecules and pro-hormones [6,7]. BChE, like AChE, is classically associated with catalyzing the hydrolysis of the neurotransmitter acetylcholine (ACh) to yield choline and acetic acid, but is more promiscuous in the array of substrates it cleaves than AChE [6,7]. Whereas its function remains to be fully elucidated, within the brain BChE has a clear role in neural functions [6,7]; in the co-regulation of cholinergic and non-cholinergic neurotransmission [6-9].

In healthy human brain, BChE plays prevalently a secondary role in the hydrolysis of ACh, whereas AChE activity predominates [9,10]. In AD brain, however, as AChE levels rapidly decline with disease progression, BChE levels rise [11,12] and its role becomes more important [6,7,9,10]. Clearly, this imbalance in the cholinesterase activity in AD brain modifies the normally supportive role of BChE and represents the rationale behind the recent synthesis of new BChE selective and brain targeted inhibitors for mild to moderate forms of AD [13].

BChE and AChE share 52% amino acid sequence homology [14]. However, differences in the internalized binding pockets of the two enzymes into which ACh diffuses and is hydrolyzed allow for rational design of selective BChE inhibitors [6,13,15]. In particular, for carbamate derivatives structurally related to the natural compound (-)-physostigmine, activity and selectivity has been proven to be modulated by substituents at the N¹ and N⁸ positions of the eseroline fragment as well as by residues at the carbamoyl moiety (see Fig. 1 for general structure) [6,15].

A basis underpinning the notion that inhibiting BChE would be of value in AD was recently provided by experimental agents with either enhanced or complete selectivity for BChE versus AChE. Specifically, *in vivo* studies [15], demonstrated that pseudo-irreversible inhibitors structurally related to the natural compound (-)-physostigmine readily enter the central nervous system following systemic administration, provide an extended selective of brain BChE and elevate synaptic ACh levels in rodent. Moreover, selective BChE inhibition augmented long-term potentiation (LTP) and improved the cognitive performance of elderly rats without the classic adverse actions associated with AChE inhibitors. In the same animals, brain levels of the putative AD toxic peptide, amyloid- β (A β), were dramatically lowered [15,16]. This latter action, however, was likely mediated by non-cholinergic mechanisms, as cholinergically inactive (+) enantiomeric forms of the pseudo-irreversible inhibitors dose- and time-dependently lowered amyloid- β precursor protein (APP) and A β levels by reducing the APP synthesis rate [17].

For carbamate derivatives, inhibition is time-dependent and, hence, the assessment of their kinetic parameters is of utmost importance because they describe the duration of enzyme inhibition that will, in turn, influence the duration of the inhibitors' *in vivo* pharmacological actions. Determination of kinetics constants may therefore not only help to elucidate the mechanism of action and rationalize *in vivo* studies, but also aid in the optimization of clinical studies.

Available kinetic data on isolated human BChE are very limited, primarily due to the relatively recent interest on this enzyme as a specific target in drug discovery for AD. Nevertheless, on the basis of its emerging role, the elucidation of this aspect is of key importance both for better understanding of *in vivo* studies as well as for future drug design. 'In solution' kinetic studies are the most used technique, but such studies are often tedious, highly material consuming and

require experienced investigators. To overcome these potential drawbacks and obtain a reliable and automated method for kinetics investigation, on the basis of previous studies on an AChE-IMER [3,18] a human recombinant BChE-IMER was purposely developed and characterized in terms of retained active units, kinetic constants and optimal chromatographic conditions.

For kinetic investigation, several (-)-physostigmine-based pseudo-irreversible inhibitors were characterized alongside (-)-physostigmine. These compounds differed in the substituent in the carbamic moiety (R) and at the N¹ and N⁸ position (R₁ and R₂) in their common eseroline structure. In particular, (-)-tolserine, (-)-pheneserine and (-)-cymserine analogues were kinetically characterized by a reliable on-line method [19] and results were compared with those obtained with in solution assay.

2. Materials and methods

2.1. Materials

Ethylendiamino (EDA) CIM Disks (12 × 3 mm I.D.) were obtained from BIA Separations (Ljubljana, Slovenia). Human recombinant BChE (E.C. 3.1.1.8) was kindly supplied by Dr. A. Saxena (Division of Biochemistry, Walter Reed Army Institute of Research, Silver Spring, MD, USA). Butyrylthiocholine iodide, 5,5'-dithio-bis(2-nitrobenzoic acid) (DTNB; Ellman's reagent), glutaraldehyde 70% aqueous solution, HPLC grade methanol, ethanol, 2-propanol and dimethyl sulfoxide (DMSO) were purchased from Sigma Chemical (Milan, Italy). Monoethanolamine was obtained from Aldrich Italia (Milan, Italy). Potassium chlorate and sodium cyanoborohydride were purchased from Fluka (Milan, Italy). Sodium hydrogencarbonate and gelatin were from Merck (Darmstadt, Germany).

(-)-Phenserine, (+)-posiphen, (-)-bisnorcymserine and (-)-isobutylbisnorcymserine were synthesized and chemically characterized as previously reported [15,20-22]. All were >99.9% chemically and chirally pure. Physostigmine was purchased from Sigma (Milan, Italy).

Purified water from a TKA ROS 300 system (Millipore, Ireland) was used to prepare buffers and standard solutions. To prepare the buffer solutions, potassium dihydrogenphosphate, dipotassium hydrogenphosphate trihydrate of analysis quality and tris(hydroxymethyl) aminomethane (Carlo Erba Reagenti, Milan, Italy) were used.

The buffer solutions were filtered through a 0.22-μm membrane filter (Millipore, Ireland) and degassed before their use for HPLC.

2.2. Apparatus

Spectrophotometric determinations were performed using a Jasco double beam V-530 UV-Vis spectrophotometer, with a slit width of 2 nm and 0.5 s data pitch.

For HPLC-based analyses, the solvent delivery system was a Jasco BIP-I HPLC pump equipped with a Rheodyne Model 7125 injector with a 20 μl sample loop. The eluates were monitored by a Jasco 875-UV Intelligent UV-Vis detector. Data were processed using Borwin chromatography software (version 1.21) from Jasco Europe (Cremella, Italy). For routine analyses the detector wavelength was set at 480 nm.

The chromatographic analyses on rBChE-IMER were performed at 25 °C unless otherwise stated. An Haake DC30 open heating bath (Haake, Rezzato, Italy) was used for temperature-controlled studies.

2.3. rBChE immobilization

The EDA CIM disk was connected to an HPLC system and conditioned for 30 min with a mobile phase consisting of phosphate buffer (20 mM, pH 7.0) at 0.5 mL/min. Then, the CIM disk was removed, placed in a glass beaker, covered with 6mL of a 12.5% glutaraldehyde solution in phosphate buffer (20mM, pH 6.0) and kept under stirring for 6 h, in the dark. The reacted matrix was then washed with phosphate buffer (20mM, pH 6.0). An aliquot of 709 μ L of rBChE solution (16.93 U/mL) in phosphate buffer (20mM, pH 8.0) was added to the matrix and left to react overnight under gentle stirring. After immobilization, the enzyme solution was analyzed by Ellman's assay [23] to determine the unreacted enzyme units.

Schiff bases were reduced by stirring the rBChE-CIM disk in a 10 mL solution of cyanoborohydride (0.1M) in phosphate buffer (20mM, pH 6.0) for 3 h at 25 °C.

The matrix was then washed with phosphate buffer (20mM, pH 6.0) to remove the unreacted reagent and stirred for 3 h with monoethanolamine (0.2M) in phosphate buffer (20mM, pH 8.0) at room temperature.

The rBChE-IMER was then inserted in the appropriate holder, connected to the HPLC system and washed with phosphate buffer (20mM, pH 7.4) for 1 h at a flow rate of 0.8 mL/min.

When not in use, the BChE-IMER was stored at 4 °C in phosphate buffer (20mM, pH 7.4) containing 0.1% sodium azide.

2.4. Determination of immobilized rBChE activity

The activity of BChE was determined by measuring the formation of the yellow anion obtained from the reaction between Ellman's reagent and the thiocholine generated by enzymatic hydrolysis of the substrate, butyrylthiocholine (BTCh) [23]. One unit of enzymatic activity is defined as the amount of enzyme catalysing the hydrolysis of 1 μ mol of BTCh/min at pH 8.0 and 37 °C, i.e. the μ mol formation of the stoichiometrically correspondent yellow anion.

Therefore, the evaluation of the rBChE active units retained after immobilization was performed at 37 °C. rBChE-IMER was conditioned with the optimized mobile phase consisting of Tris HCl buffer (0.1M, pH 8.0) containing Ellman's reagent (0.126mM) and KClO₃ (100mM) (buffer A). Flow rate was set at 1.0 mL/min and UV detection at 480 nm. Aliquots of 20 μ L BTCh aqueous solution at increasing concentrations (range comprised between 3.10 and 300mM) were injected. In order to account for μ moles of BTCh hydrolyzed, eluates for each substrate injection were collected in 5mL volumetric flasks during 5min of chromatographic elution. The absorbance at 412 nm of each eluate was spectrophotometrically acquired, using the mobile phase as a blank. Micromoles of hydrolyzed substrate were calculated by applying Lambert-Beer law. As previously reported [3] the catalysis rates (μ mol/min) were then derived by dividing the μ moles of substrate hydrolyzed by the contact time (0.34min).

A linear correlation between absorbance values, relative chromatographic peak areas and μ moles of product formed per min was obtained and used in further analysis.

A Michaelis-Menten plot was obtained by plotting the catalysis rates versus normalized substrate concentration, and k_m^{app} and V_{max}^{app} derived (GraphPad). From the V_{max} value the immobilized active units were then determined. Normalized substrate concentration was calculated by the following formula:

$$[\text{BTCh}]_{\text{normalized}} = \frac{C_{\text{inj}} \times V_{\text{inj}}}{\text{BV}}$$

where C_{inj} is the injected substrate concentration, V_{inj} is the injected volume and BV is the bed volume of the rBChE-IMER.

2.5. Optimization of the chromatographic conditions

2.5.1. Mobile phase composition—Potassium chlorate (0-100mM) as a selective anion exchanger competitor for the protonated amine groups on the matrix, magnesium sulfate (0-40mM) as enzyme activator and Ellman's reagent concentration (0.079×10^{-4} - 5.04×10^{-4} M) were evaluated. The peak symmetry and peak area obtained by the injection of a fixed saturating BTCh concentration (200mM) were determined for each buffer type and additive concentration, by using a flow rate of 1mL/min and setting UV detection at 480 nm.

2.5.2. Mobile phase pH—The enzyme column was equilibrated for 30 min with 0.1M Tris-HCl containing 0.1M KClO_3 , 0.126 mM Ellman's reagent in a pH range comprised between 6.0 and 8.5.

A fixed BTCh concentration (200mM) was injected in triplicate onto the HPLC. Flow rate was 1.0 mL min^{-1} and UV detection was set at 480 nm. The product peak area was integrated and plotted against the pH value of the mobile phase.

2.5.3. Temperature—rBChE-IMER activity in the presence of saturating concentrations of substrate (200mM) was assayed at 25, 30, 34, and 37 °C, after appropriate conditioning for at least 30min. The product peak area was integrated after injection of saturating concentration of substrate (200mM).

2.5.4. Organic modifier—Methanol, ethanol, *n*-propanol, 2-propanol and DMSO were individually added to the mobile phase at a percentage ranging between 1 and 5%. The product peak area was integrated after injection of saturating concentration of substrate (200mM).

2.6. Apparent kinetic constants variation upon flow rate

Apparent k_m^{app} and $V_{\text{max}}^{\text{app}}$ for rBChE-IMER were determined at increasing flow rates (0.6-0.8-1.0-1.2-1.4 mL/min) by injecting in duplicate 20 μL aliquots of BTCh aqueous solution (concentrations comprised in the range 3.1-300mM) with UV detection at 480 nm at constant temperature (25.0 ± 0.8 °C) and using buffer A as mobile phase. By plotting the rates of catalysis ($\mu\text{moles of product formed per min}$) versus the normalized substrate concentrations (range 0.09-15mM), Michaelis-Menten plots [24] were obtained and k_m^{app} and $V_{\text{max}}^{\text{app}}$ derived at each flow rate.

2.7. rBChE-IMER stability

rBChE-IMER stability was determined by using buffer A as mobile phase and injecting every day saturating aqueous solution (200mM) of substrate under optimized flow and detection conditions (1.0 mL min^{-1} and $\lambda = 480\text{nm}$).

2.8. Determination of rate constants for pseudo-irreversible inhibitors

Kinetic studies were carried out at 30 °C, with a flow rate of 1.0 mL min^{-1} and setting the UV detection at 480 nm. rBChE-IMER was first equilibrated with buffer A or buffer A at pH 7.4 and reference enzyme activity was assessed by injecting saturating substrate concentration

(BChE, 200mM) in duplicate and determining the average product peak area (A_0). Then the pseudo-irreversible inhibitor, previously dissolved in methanol, was added at the selected concentration to the mobile phase. The mobile phase containing the inhibitor was run through the BChE-IMER ($t = 0$, carbamoylation phase). Every 5 min 20 μ L aliquots of saturating concentration of substrate were injected and the time-dependent decrease of the product peak area (A_i) was monitored down to a constant plateau value. At least five different concentrations of each inhibitor were tested. In particular, selected ranges of concentrations were: 0.02-1.0 μ M for (-)-physostigmine, 3.0-50 μ M (-)-tolserine, 2.0-5.0 μ M (-)-phenserine, 1.0-50 nM (-)-bisnorcymserine and 2.5-50 nM (-)-isobutylbisnorcymserine. Residual percent enzyme activity $[(A_i/A_0) \times 100]$ was plotted versus time. Data were fitted to the Eq. (1), and pseudo first order rate constant k_{obs} values were calculated accordingly [25].

$$R = R_0 \exp(-k_{\text{obs}}t) + R_{\infty} \quad (1)$$

where R , R_0 and R_{∞} are ratios of the inhibited enzyme activity (A_i) to the control activity (A_0) at times t , 0 and ∞ , respectively.

Double reciprocal plots of k_{obs} versus inhibitor concentration ($[I]$) were then used to compute k_2 from the intercept, and from the ratio of the slope to the intercept, respectively, according to the equation:

$$\frac{1}{k_{\text{obs}}} = \frac{K_c}{k_2} \frac{1}{[I]} + \frac{1}{k_2} \quad (2)$$

2.8.1. Determination of decarbamoylation rate constants for pseudo-irreversible inhibitors—Once a stable inhibition plateau was reached, clear mobile phase was flushed through rBChE-IMER and the recovery of enzymatic activity was followed over time by injecting aliquots of saturating substrate at fixed time intervals up to the initial activity was recovered.

Decarbamoylation rate (k_3) was determined according to Perola et al. [26] by using the inhibitor's concentration able to give an almost complete inhibition of the enzyme activity. Specifically, 50 μ M for (-)-physostigmine and (-)-tolserine and 5 μ M for (-)-phenserine and 0.05 μ M for (-)-bisnorcymserine and (-)-isobutylbisnorcymserine were used, respectively. k_3 values were obtained by plotting $\ln(A_t/A_p)$ versus time, where A_p is the percent inhibition at plateau product peak area, that is $t = 0$ in the decarbamoylation phase ($A_p = \{100 - [100 \times (A_i/A_0)]\}_{t=\text{zero}}$) and A_t is the percent inhibition determined over time, during the enzyme activity recovery phase ($A_t = \{100 - [100 \times (A_i/A_0)]\}_{t=t}$).

Decarbamoylation rate constants (k_3) were derived as the slope values.

2.9. In solution assays

2.9.1. Activity and inhibition assay—The method of Ellman et al. [23] was followed. BTCh iodide solution (0.037M) was prepared in water. 5,5'-Dithiobis(2-nitrobenzoic acid) (DTNB, Ellman's reagent) (0.01 M) was dissolved in pH 7.0 phosphate buffer, and 0.15% (w/v) NaHCO_3 was added. Human recombinant BChE solution was prepared by dilution of rBChE stock solution in gelatine (0.2% aqueous solution) to obtain a final enzyme activity comprised between 0.150 and 0.100 $\Delta\text{AU}/\text{min}$. Stock solutions of the test compounds (0.5-1mM) were prepared in methanol as well as the (-)-physostigmine reference stock solution. The assay solutions were prepared by diluting the stock solutions in water. Five different concentrations of each compound were used in order to obtain inhibition of BChE activity comprised between

20 and 80%. The assay solution consisted of a 0.1M phosphate buffer pH 8.0, with the addition of 340 μ M DTNB, 0.02 unit/mL rBChE, and 550 μ M BTCh iodide. The final assay volume was 1 mL. Test compounds were added to the assay solution and preincubated with the enzyme for 20 min, with the addition of substrate following. Initial rate assays were performed at 37 °C: the rate of absorbance increase at 412 nm wavelength was followed for 5 min. Assays were performed with a blank containing all components except rBChE, to account for the non-enzymatic reaction. The reaction rates were compared, and the percent inhibition due to the presence of test compounds was calculated. Inhibition curves were obtained for each compound by plotting the percent inhibition versus the logarithm of inhibitor concentration in the assay solution. The linear regression parameters were determined for each curve and the IC₅₀ extrapolated.

2.9.2. Determination of carbamylation constants—To characterize the carbamylation step, a traditional stopped time assay was performed, in which rBChE and inhibitor were mixed in the assay buffer, and aliquots were transferred to the spectrophotometer cell at various times for the determination of the residual BChE activity. The data were fitted to the Feaster and Quinn equation [25] and carbamylation constants were derived.

3. Results and discussion

3.1. BChE-IMER preparation and characterization

Previous attempts to develop BChE-based IMERs have been carried out with the serum equine isoform. Specifically, BChE-IMERs were developed by Luckarift et al. on silica support [27, 28] by two different silica-entrapment methods with high immobilization efficiency. Some preliminary tests could be performed with reversible inhibitors. However, some non-specific interactions were observed even with relatively small molecules and the IMER stability was monitored during a very limited period of time (50 h) [28].

On the basis of very good results obtained in previous work with monolithic-based IMERs [3,5,18,29], we chose to perform a covalent immobilization of the target enzyme on a small disk-shaped monolithic column (3mm×12mm I.D., 0.34mL internal volume) which previously has been demonstrated to be a suitable chromatographic support for enzyme immobilization and for the rapid bioconversion of substrates, due to complete loss of diffusion resistance during mass transfer, reduced time analysis and high enzyme efficiency [3,5,18,29]. In fact, silica-based beads support may have some drawbacks, such as a longer conditioning time together with a lower stability at high or low pH values and a limitation in mass transfer, as previously reported [18].

Therefore, rBChE has been covalently immobilized on a monolithic EDA-CIM disks endowed with EDA (ethylenediamine) functions. Moreover, in accord with our application for drug discovery for AD, human recombinant BChE was used in this study.

By following our previous studies on recombinant human AChE-IMERs [3,18], rBChE was cross-linked with a suitable bifunctional reagent, glutaric dialdehyde to the primary amino groups of the monolithic disk by performing an in situ immobilization. Recombinant human BChE was anchored to the modified disk by reaction between the free aldehydic groups resulting from the previous reaction and the primary amino groups of the lysine residues. Final end-capping of free remaining aldehyde functions was carried out by ethanolamine.

The amount of rBChE active immobilized units was assessed by inserting the resulting IMER into an HPLC system connected with a conventional UV-vis detector. Under the described conditions, the immobilization yield was found to be 8.3% and the retained active units resulted to be 0.99±0.01. This amount is compatible with the concentration of BChE (Units/g of tissue)

in healthy brains. However, it has to be taken into account that this value significantly varies upon brain area, age, and in pathological conditions.

Similar to AChE, BChE is able to catalyze the hydrolysis of acetylcholine, even if less efficiently [7]. However, since butyrylcholine is its most preferred substrate, butyrylthiocholine was employed as a substrate for on-line studies.

Optimal chromatographic conditions were evaluated to access the unmodified behavior of the enzyme after immobilization.

In particular, mobile phase composition was optimized in terms of the type of buffer, saline concentration and Ellman's reagent content. Data obtained showed that optimal chromatographic conditions for immobilized rBChE overlapped almost perfectly with the ones selected for the parent AChE-IMER, with the exception of magnesium chloride.

For the rBChE-IMER, the presence of magnesium salt proved not so detrimental for activity as is the case for AChE. Magnesium chloride was therefore not added to the mobile phase.

In the optimized conditions, IMER conditioning time was within 10min (Fig. 2A) and the chromatographic run lasted less than 5min. A rapid IMER conditioning time is a suitable feature when developing tools for application in HTS (high throughput) analyses.

The influence of the temperature and of the mobile phase pH was also evaluated and data obtained are shown in Fig. 2B and C. As expected, temperature strongly influenced enzyme activity. Consequently, studies were performed in controlled temperature conditions using an open heating bath. To define the influence of the pH on rBChE activity, a comparison between data obtained with the enzyme in the free and immobilized formats showed that immobilization did not alter the enzyme behavior: enzyme activity increased with increasing pH values up to an optimal value at 8.0.

Finally, the effect of the addition of a small percentage of organic modifiers to the mobile phase was investigated with the aim of limiting non-specific interactions between tested inhibitors and the used chromatographic support. The introduction of an organic modifier may be required to suppress some non-specific interactions that may occur with more hydrophobic compounds. On the other hand, an organic modifier may influence the enzyme activity. Therefore, as general rule, the type and the optimal amount of an organic modifier is a compromise between the best eluting agent (chromatographic requirements) and the less activity influencing one (enzymatic requirements).

Fig. 2D shows rBChE-IMER activity with and without 1% (v/v) of the most common organic modifiers. Among the selected solvents, 2-propanol gave the best elution profile and provided an increase in product peak area, as well. In particular, the addition of 1% 2-propanol increased activity by 34%, while concentrations ranging from 3 to 5% provided an increase of 41% of activity ($\mu\text{mol}/\text{min}$ of product formed). 2-Propanol was hence used in all following experiments when non-specific interactions were presumed.

3.2. Kinetic studies

The insertion of an immobilized enzyme into a flow-through system as an HPLC-IMER may affect the overall substrate conversion. To assess optimal chromatographic conditions, apparent kinetic constants (k_m^{app} and $V_{\text{max}}^{\text{app}}$) were studied as a function of flow rate (range 0.6-1.4 mL min^{-1}). Aliquots of 20 μL of increasing BTCh concentration (3.1-200mM) were injected into the HPLC system under the optimized chromatographic conditions reported in Section 2.6. Michaelis-Menten curves were obtained by plotting velocity (μmoles of product formed

per minute) versus normalized substrate concentration (0.09–5.88 mM), which is the concentration of substrate in the BChE-IMER (0.34 mL). As shown in Fig. 3A, k_m^{app} values were found to be unaffected by the flow rate (average k_m^{app} value of 1.0 ± 0.10 mM), while maximal velocities (V_{max}^{app}) slightly decreased. Since only the product formation is affected, it might be speculated that the increasing friction due to higher flow rate could negatively influence the enzyme catalytic efficiency by reversibly modifying the three-dimensional structure of the immobilized enzyme. However, by extrapolating at zero flow rate, the influence of flow rate was zeroed. The $V_{max(0)}^{app}$ value was found to be 0.721 ± 0.021 $\mu\text{mol min}^{-1}$ (Fig. 3B).

3.3. Inhibition studies

Carbamates are cholinesterase inhibitors characterized by the presence of a carbamoyl moiety which is responsible for time-dependent inhibition. The inhibition of BChE by carbamates involves a reversible complex formation, followed by carbamoylation of the enzyme, and production of a covalent adduct (the carbamoylation constant k_2 describes this phase) [25,30, 31]. The carbamoylated enzyme is then hydrolyzed to regenerate the free enzyme (the decarbamoylation constant k_3 kinetically describes this phase). The kinetic constants k_2 and k_3 describe the mode and duration of enzyme inhibition and will, in turn, influence the duration of the inhibitors' in vivo pharmacological action. Due to the formation of a reversible covalent adduct this class of inhibitors is also known as pseudo-irreversible inhibitors.

In this study, the well-known AChE-selective ((-)-phenserine, (-)-tolserine), non-selective ((-)-physostigmine) and recently published BChE highly selective ((-)-bisnorcymserine and (-)-isobutylbisnorcymserine) pseudo-irreversible inhibitors were chosen for characterization.

Utilizing a previously designed method developed for the online determination of the kinetic constants of pseudo-irreversible AChE inhibitors [19], the k_2 and k_3 values of the selected carbamates were determined by rBChE-IMER. This system may offer potential advantages over conventional methods. Specifically, due to the covalent and stable linkage of the target enzyme to a chromatographic support, the enzyme activity can be monitored over time during both the inactivation and re-activation phases.

In particular, after assessing the initial enzyme activity (A_0), the inhibitor was dissolved in the mobile phase and the progressive enzyme inhibition, due to the time-dependent carbamoylation of the enzyme active site, was monitored by injecting fixed concentrations of substrate at regular intervals of time. Fig. 4A shows the time-dependent inactivation of immobilized BChE by (-)-phenserine at five different concentrations ranging from 2.0 to 50.0 μM . Once the inhibition plateau was reached, by switching the mobile phase back to buffer A, it was possible to regenerate the inactivated enzyme (decarbamoylation phase). For these compounds, the carbamoylation phase of the reaction is considerably more rapid than the decarbamoylation phase (i.e. $k_2 > k_3$, minutes vs. hours), and the two phases can be characterized separately even when within a single experiment. In accord with the Feaster and Quinn method [25] and as described in Section 2.8, the values of the observed pseudo-first order inhibition rate constants (k_{obs}) were obtained for each tested concentration and replotted versus inhibitor concentration ([I]) (see Fig. 4B). K_2 values were then calculated from the double reciprocal plot of the latter graph.

The obtained values are reported in Table 1, which also shows the carbamoylation constants (k_2) obtained with rBChE in solution, by classical Ellman's assay. These results demonstrate that all the tested inhibitors generated a rapid BChE inhibition, the fastest inhibitor being (-)-phenserine in both in solution (3.094 ± 0.536 min^{-1}) and IMER-based (0.486 ± 0.037 min^{-1}) assays. Moreover, values of the carbamoylation constants for phenylcarbamates were inversely dependent on the lipophilicity of the R moiety, as reported for other pseudo-irreversible

inhibitors [32-35]. In particular, derivatives with bulkier carbamate nitrogen substituents showed a slower carbamoylation and decarbamoylation (see the decarbamoylation section). This phenomenon might be ascribed to constrained access to active site during the inactivation phase and consequently longer time for inhibition or, as speculated in previous work, to torsion of the substituted phenyl ring that may change the mode of interaction and the kinetic behavior of the inhibitor within the enzyme binding domain [36].

In comparison with data obtained in solution, the k_2 values determined with the immobilized enzyme were found slightly lower, however a similar trend could be noticed in both assays. The slower inactivation of the immobilized BChE (lower k_2) might be partially ascribed to some partitioning of the inhibitors on the surface of the stationary monolithic phase and to a concomitant withdrawal of the inhibitors from the enzyme active site caused by the flow rate.

Nevertheless, it should be remarked that, due to the very fast inactivation kinetics, the time required for reaching the maximum inhibition in the two systems differs only by few minutes.

As previously stated, once the inhibition plateau was reached, by changing mobile phase, it was possible to hydrolyze carbamoyl-BChE adducts, regenerate the active enzyme and evaluate decarbamoylation rates. Calculation for k_3 was performed by applying Perola's mathematical equation [26] that assumes that, by starting from an almost complete inhibition, k_3 can be calculated from the slope of the linear plot obtained by monitoring the inhibition degree over time. As an example, Fig. 4C shows the linear plots obtained by applying Perola's equation to (-)-phenserine. Less than 2 h flushing was required to achieve a complete recovery of rBChE-IMER activity after complete inhibition by the fastest inhibitor (-)-phenserine, whereas a 16 h flushing was required after inhibition by (-)-isobutylbisnorcymserine, the slower tested carbamate. As for carbamoylation, decarbamoylation constants were inversely dependent on the lipophilicity of the R moiety (Table 1). Limited access of hydrolytic water molecules to the carbamoylated active site, in consequence of the filling of the BChE gorge by the larger carbamic residue could be the explanation for longer reactivation time. It is worth noting that even with stronger covalent inhibitors, the initial activity could be fully recovered and the same IMER could be reused for numerous further studies. In Fig. 5, the overlaid full profiles obtained for (-)-phenserine kinetic characterization are shown. Carbamoylation and decarbamoylation studies were carried out in temperature-controlled conditions. In particular, the temperature of 30 °C represents a good compromise between high enzymatic activity and inhibitor stability. Physostigmine derivatives may in fact suffer from light and temperature sensitivity.

The inhibitory potencies of selected (-)-phenserine and (-)-cymserine analogues (see Fig. 1 for structures), as well as of (-)-physostigmine as a reference compound were determined on-line and compared with the IC_{50} values generated classically, with the enzyme in solution (Table 2). Since inhibition is time-dependent, % inhibition was calculated at the inhibition plateau, when the maximum inhibition was reached.

The on-line IC_{50} values were in agreement with values obtained with the same enzyme in solution (within the same order of magnitude), as well as with published in depth enzyme kinetic analyses [37,38]. The slightly higher value obtained for (-)-isobutylbisnorcymserine, likely, reflects some minor non-specific interactions between this inhibitor and the chromatographic support that may reduce the amount of inhibitor available for enzyme inhibition. Another parameter which can be determined by Eq. (2) (Section 2.7) is K_c (thermodynamic constant of the inhibitor-enzyme reversible complex). Similarly to IC_{50} , K_c values were found slightly higher than those determined in solution, but in the same order of magnitude.

Covalent immobilization may alter the stereoselective properties of the target enzyme. In the case of BChE, it was demonstrated that the enzyme discriminates between R and S enantiomers at the 3a position of the natural compound (-)-physostigmine and derivatives, with the natural (-)-(S)-enantiomer being far more potent than the unnatural (+)-(R)-chiral form.

(-)-Phenserine (see Fig. 1), the phenyl derivative of (-)-physostigmine, was first described as a potent and selective inhibitor of AChE and was shown to improve cognition in rodents and dogs [16,20,39]. It was clinically tested for AD with moderate success in initial phase-II studies [16,39,40]. As for the parent compound physostigmine, phenserine (-)- and (+)-enantiomers are endowed with different biological profiles [16,20]. Whereas, (-)-phenserine, the cholinergically active enantiomer [3aS-(-)-phenylcarbamoyleseroline], is a potent cholinesterase inhibitor, the (3aR)-(+)-enantiomer, posiphen, has weak activity as an AChE inhibitor and hence can be dosed much higher to animals and humans. By contrast, both enantiomers are equipotent in down-regulating APP expression. On the basis of this interesting profile, (+)-posiphen has entered phase I clinical trials where it may prove to be a promising drug, either alone or in combination with (-)-phenserine, to attenuate disease progression in AD.

In a classical in solution assay, (-)-enantiomer phenserine is one order of magnitude more active than (+)-enantiomer posiphen (Table 2). The same stereoselectivity was found when the two enantiomers were tested by the rBChE-IMER. This result confirms that the immobilization of BChE did not alter the stereoselective properties of BChE active site.

Fig. 6 shows the overlaid carbamoylation profiles of both (-)-phenserine and (+)-posiphen at their IC₅₀ value. The overlaid profiles indicate that the chiral center on the tricyclic eseroline residue while influencing the inhibitory potency (see Table 2 for IC₅₀ values), does not cause any difference in the kinetics of their binding to the active site of BChE. These findings are in agreement with the fact that carbamoylation/decarbamoylation kinetics mainly depend on the R substituent on the carbamic nitrogen, while affinity for the BChE active site depends on the overall inhibitor structure (both eseroline and R residues) and reflects the BChE stereoselectivity. Moreover these results indirectly confirmed that immobilization did not alter the stereoselective properties of BChE, i.e., the stereoselective recognition of (-)- versus (+)-eseroline derivatives.

3.4. Stability

Long life is a suitable feature for IMERs that is not always achieved. Nevertheless, it has been demonstrated that in most cases immobilization and inclusion in a closed system, such as a chromatographic column, usually increase enzyme stability. Stability over time of the developed rBChE-IMER was, therefore, evaluated using optimized conditions and a saturating concentration of substrate. Both half-life and number of injections were considered since the latter is a parameter that better describes the stress of use. As a general conclusion of the results obtained, immobilization greatly increased enzyme stability: more than 2300 injections have been made, and the rBChE-IMER has been in use for more than a year. Moreover, no inactivation was observed when the IMER was stored for an extended time at 4 °C. This finding suggests that any observed inactivation is not related to an intrinsic instability of the rBChE-IMER but, more likely, to the stress caused by the inactivation-regeneration cycles. Notwithstanding the progressive loss of activity, after one year the IMER residual activity was still suitable for inhibitor screening, by a simple normalization of the reference activity. Noteworthy, the stability of rBChE-IMER resulted further higher than previously published silica-based ones [27,28].

4. Conclusions

The availability of the here presented automated, accurate, precise HPLC method, in which the amount of enzyme is maintained constant for months, guaranteed fast and reliable assessment of the mechanism of action of the selected inhibitors endowed with a carbamic function. When compared with traditional 'stopped time' assays, the proposed rBChE-IMER system shows advantages in terms of simplicity of use, cost and automation. In fact, the carbamates' inactivation and regeneration of the immobilized enzyme activity was performed in one single experiment, with increased accuracy and precision. Even if with the upcoming HTS multi-well plates-based assays, fast data output might not represent a limitation anymore, the use of HPLC-coupled IMER offers advantages in terms of simplicity of use (no purposely designed or expensive instrument is required) and almost full automation, which still represent limitations of multi-well plates-based kinetic studies.

The amount of required enzyme was in the order of few nanograms and it could be reused throughout this study. Conversely, traditional assays require replenishment of new enzyme aliquots for each single determination. Therefore, IMER development may be of key importance when the target protein is in short supply, is not commercially available or expensive, or when its production and isolation is difficult. In conclusion, the rBChE-IMER may represent a suitable new screening tool for the fast and automated evaluation of the inhibitory potency and the mechanism of action of new inhibitors during the drug discovery process which may be valuable for the future treatment of AD, myasthenia gravis and nerve gas poisoning.

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References

- [1]. Markoglou, N.; Wainer, IW. Bioanalytical Separations. In: Wilson, ID., editor. Handbook of Analytical Separations. Elsevier; Amsterdam: 2003. p. 215
- [2]. Bertucci C. J. Chromatogr 2003;B 797:111.
- [3]. Andrisano V, Bartolini M, Gotti R, Cavrini V, Felix G. J. Chromatogr 2001;B 753:375. Bartolini M, Cavrini V, Andrisano V. J. Chromatogr 2004;A 1031:27.
- [4]. Markoglou N, Wainer IW. J. Chromatogr 2001;B 766:145.
- [5]. Nicoli R, Bartolini M, Rudaz S, Andrisano V, Veuthey J-L. J. Chromatogr 2008;A 1206:2.
- [6]. Greig, NH.; Yu, Q-S.; Brossi, A.; Martin, E.; Lahiri, DK.; Darvesh, S. Medicinal Chemistry of Alzheimer's Disease. Martinez-Gil, A., editor. Transworld Research Network; Kerala, India: 2008. p. 79 ISBN 978-81-7895-342-7
- [7]. Darvesh S, Hopkins DA, Geula C. Nat. Rev. Neurosci 2003;4:131. [PubMed: 12563284]
- [8]. Mesulam MM, Guillozet A, Shaw P, Levey A, Duysen E, Lockridge O. Neuroscience 2002;110:627. [PubMed: 11934471]
- [9]. Greig NH, Lahiri DK, Sambamurti K. Int. Psychogeriatrics 2002;14:77.
- [10]. Giacobini E. Int. J. Geriatr. Psychiatry 2003;18:S1. [PubMed: 12973744]
- [11]. Perry E, Perry R, Blessed G, Tomlinson B. Neuropathol. Appl. Neurobiol 1978;4:273. [PubMed: 703927]
- [12]. Arendt T, Bruckner M, Lange M, Bigl V. J. Neurochem 1992;21:381.
- [13]. Greig NH, Utsuki T, Ingram DK, Wang Y, Pepeu G, Scali C, Yu QS, Mamczarz I, Holloway HW, Giordano T, Chen D, Furukawa K, Sambamurti K, Brossi A, Lahiri DK. Proc. Natl. Acad. Sci. U.S.A 2005;102:17213. [PubMed: 16275899]

- [14]. Soreq H, Ben-Aziz R, Prody CA, Seidman S, Gnatt A, Neville L, Lieman-Hurwitz J, Lev-Lehman E, Ginzberg D, Lapidot-Lifson Y, Zakut H. *Proc. Natl. Acad. Sci. U.S.A* 1990;87:9688. [PubMed: 2263619]
- [15]. Yu Q-S, Holloway HW, Utsuki T, Brossi A, Greig NH. *J. Med. Chem* 1999;42:1855. [PubMed: 10346939]
- [16]. Greig NH, Sambamurti K, Yu Q-S, Brossi A, Bruinsma RG, Lahiri DK. *Curr. Alzheimer Res* 2005;2:281. [PubMed: 15974893]
- [17]. Lahiri DK, Chen D-M, Maloney B, Holloway HW, Yu Q-S, Utsuki T, Giordano T, Sambamurti K, Greig NH. *J. Pharmacol. Exp. Ther* 2007;320:386. [PubMed: 17003227]
- [18]. Bartolini M, Cavrini V, Andrisano V. *J. Chromatogr* 2005;A 1065:135.
- [19]. Bartolini M, Cavrini V, Andrisano V. *J. Chromatogr* 2007;A 1144:102.
- [20]. Pei X-F, Yu Q-S, Lu B-Y, Greig NH, Brossi A. *Heterocycles* 1996;42:229.
- [21]. Greig NH, Pei X-F, Soncrant TT, Ingram DK, Brossi A. *Med. Res. Rev* 1995;15:3. [PubMed: 7898167]
- [22]. Zhu X, Greig NH, Holloway HW, Whittaker NF, Brossi A, Yu Q-S. *Tetrahedron Lett* 2000;41:4861.
- [23]. Ellman GL, Courtney KD, Andres V Jr. Featherstone M. *Biochem. Pharmacol* 1961;7:88. [PubMed: 13726518]
- [24]. Dixon, M.; Webb, EC. *Enzymes*. Vol. 2nd ed.. Dixon, M.; Webb, EC., editors. Longmans; London, UK: 1964. p. 63
- [25]. Feaster SR, Quinn DM. *Methods Enzymol* 1997;286:231. [PubMed: 9309653]
- [26]. Perola E, Cellai L, Lamba D, Filocamo L, Brufani M. *Biochim. Biophys. Acta* 1997;1343:41. [PubMed: 9428657]
- [27]. Luckarift HR, Spain JC, Naik RR, Stone MO. *Nat. Biotechnol* 2004;22:211. [PubMed: 14716316]
- [28]. Luckarift HR, Johnson GR, Spain JC. *J. Chromatogr* 2006;B 843:310.
- [29]. Mancini F, Naldi M, Cavrini V, Andrisano V. *J. Chromatogr* 2007;A 1175:217.
- [30]. Main AR, Hastings FL. *Science* 1966;154:400. [PubMed: 5917090]
- [31]. Ariel N, Ordentlich A, Barak D, Bino T, Velan B, Shafferman A. *Biochem. J* 1998;335:95. [PubMed: 9742217]
- [32]. Rampa A, Bisi A, Valenti P, Recanatini M, Cavalli A, Andrisano V, Cavrini V, Fin L, Buriani A, Giusti P. *J. Med. Chem* 1998;41:3976. [PubMed: 9767635]
- [33]. Rampa A, Piazzi L, Belluti F, Gobbi S, Bisi A, Bartolini M, Andrisano V, Cavrini V, Cavalli A, Recanatini M, Valenti P. *J. Med. Chem* 2001;44:3810. [PubMed: 11689067]
- [34]. Bolognesi ML, Bartolini M, Cavalli A, Andrisano V, Rosini M, Minarini A, Melchiorre C. *J. Med. Chem* 2004;47:5945. [PubMed: 15537349]
- [35]. Belluti F, Rampa A, Piazzi L, Bisi A, Gobbi S, Bartolini M, Andrisano V, Cavalli A, Recanatini M, Valenti P. *J. Med. Chem* 2005;48:4444. [PubMed: 15974596]
- [36]. Yu Q-S, Holloway HW, Flippen-Anderson JL, Hoffman B, Brossi A, Greig NH. *J. Med. Chem* 2001;44:4062. [PubMed: 11708910]
- [37]. Kamal MA, Klein P, Yu Q-S, Tweedie D, Li Y, Holloway HW, Greig NH. *J. Alzheimer Dis* 2006;10:43.
- [38]. Kamal MA, Al-Jafari AA, Yu Q-S, Greig NH. *Biochim. Biophys. Acta* 2006;1760:200. [PubMed: 16309845]
- [39]. Klein J. *Expert Opin. Investig. Drugs* 2007;16:1087.
- [40]. Kadir A, Andreasen N, Almkvist O, Wall A, Frosberg A, Engler H, Hagman G, Lärksäter M, Winblad B, Zetterberg H, Blennow K, Långström B, Nordberg A. *Ann. Neurol* 2008;63:621. [PubMed: 18300284]
- [41]. Pei X-F, Greig NH, Bi S, Brossi A, Toome V. *Med. Chem. Res* 1995;5:265.
- [42]. Brzostowska M, He XS, Greig NH, Brossi A. *Med. Chem. Res* 1992;2:238.
- [43]. Yu Q-S, Holloway HW, Utsuki T, Brossi A, Greig NH. *J. Med. Chem* 1999;42:1855. [PubMed: 10346939]

	R	R ₁	R ₂
Physostigmine (3aS)-(-)	—CH ₃	—CH ₃	—CH ₃
Phenserine (3aS)-(-)		—CH ₃	—CH ₃
Posiphen (3aR)-(+)		—CH ₃	—CH ₃
Tolserine (3aS)-(-)		—CH ₃	—CH ₃
Bisnorcymserine (3aS)-(-)		—H	—H
Isobutylbisnorcymserine (3aS)-(-)		—H	—H

Fig. 1.
Chemical structures of (-)-physostigmine and its derivatives.

Fig. 2. rBChE-IMER characterization. (A) BChE-IMER conditioning time. Flow rate: 1.0 mL min^{-1} ; Mobile phase: buffer A; [BTCh] 200mM. (B) Influence of the temperature on the IMER activity. Flow rate, 1.0 mL/min ; mobile phase, buffer A; [BTCh] 200mM. (C) Influence of the pH on BChE-IMER activity. (■) rBChE in solution; (○) rBChE-IMER; flow rate, 1.0 mL min^{-1} ; mobile phase, Tris-HCl at appropriate pH containing KClO_3 (100mM) and Ellman's Reagent (0.126mM); [BTCh] 200mM. (D) Influence of several organic modifiers (1%) on the BChE-IMER activity.

Fig. 3.

Trend of (A) k_m^{app} and (B) $V_{\text{max}}^{\text{app}}$ as a function of flow rate.

Fig. 4.

(A) Time course of BChE-IMER carbamoylation by increasing (-)-phenserine concentration in the mobile phase. (B) Trend of k_{obs} dependence on (-)-phenserine concentration. Double reciprocal plots of k_{obs} versus (-)-physostigmine concentration ($[I]$) were then used to compute k_2 (Eq. (2)). (C) Linear plots obtained by applying Perola's equations [26] to rBChE decarbamoylation. Slope of the linear plot was used to compute decarbamoylation kinetic constant (k_3).

Fig. 5.

Representative carbamoylation and decarbamoylation profiles obtained at five different concentrations of (-)-phenserine. %RA stands for percentage of residual activity and was calculated by the following formula: $\%RA = 100A_t/A_0$ where A_t is the activity at the time t and A_0 is the initial enzyme activity.

Fig. 6. Overlaid carbamylation profiles obtained for the two enantiomers of the phenylcarbamoyleseroline at their IC_{50} concentration. In grey (+)-posiphen profile (50 μM) and in black (-)-phenserine profile (5.0 μM) are shown, respectively. v_i and v_0 stand for the enzymatic activity (v) in the presence and in absence of the tested inhibitor, respectively.

Table 1

Carbamoylation (k_2) and decarbamoylation (k_3) constants of the selected pseudo-irreversible cholinesterase inhibitors by rBChE-IMER and carbamoylation constants (k_2) determined by in solution assay.

	rBChE-IMER		rBChE in solution k_2 (min^{-1})
	k_2 (min^{-1})	k_3 (h^{-1})	
(-)-Physostigmine	0.161 ± 0.025	0.681 ± 0.010	0.479 ± 0.070
(-)-Phenserine	0.486 ± 0.037	2.593 ± 0.042	3.094 ± 0.536
(-)-Tolserine	0.174 ± 0.012	1.030 ± 0.014	0.609 ± 0.082
(-)-Bisnorcymserine	0.081 ± 0.004	0.501 ± 0.006	0.573 ± 0.068
(-)-Isobutylbisorcymserine	0.049 ± 0.002	0.163 ± 0.003	0.634 ± 0.022

Table 2

Inhibitory potencies expressed as IC₅₀ values of the selected pseudo-irreversible compounds. All the inhibitors were tested as L-tartrate salts.

	IC ₅₀ (nM) ± SEM	
	rBChE-IMER	rBChE in solution
(-)-Physostigmine	87.3 ± 5.0	16.0 ± 2.9 ^a
(-)-Phenserine	4610 ± 140	1300 ± 400 ^a
(+)-Posiphen	49300 ± 1500	23500 ± 300 ^a
(-)-Tolserine	8120 ± 244	1950 ± 240 ^b
(-)-Bisnorcymserine	2.66 ± 0.09	1.00 ± 0.10 ^c
(-)-Isobutylbisnorcymserine	8.01 ± 0.24	0.46 ± 0.03

SEM = standard error of the mean.

^aData from Ref. [41].

^bData from Ref [42].

^cData from Ref. [43].