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CNS-selective noncompetitive cholinesterase inhibitors derived from the natural piperidine alkaloid (−)-spectaline

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Abstract

LASSBio-767 [(−)-3-*O*-acetyl-spectaline] and LASSBio-822 [(−)-3-*O*-*tert*-Boc-spectaline] were recently described as cholinesterase inhibitors derived from the natural piperidine alkaloid (−)-spectaline, obtained from the flowers of *Senna spectabilis* (Fabaceae). We investigated their mechanism of inhibition of acetylcholinesterase and their efficacy in reversing scopolamine-induced amnesia. Competition assays with the substrate acetylthiocholine showed a concentration-dependent reduction in rat brain cholinesterase V_{max} without changes in apparent K_m. The kinetic data for LASSBio-767 and LASSBio-822 were best fit by a model of simple linear noncompetitive inhibition with K_i of 6.1 μM and 7.5 μM, respectively. A dilution assay showed a fast and complete reversal of inhibition, independent of incubation time. Simulated docking of the compounds into the catalytic gorge of *Torpedo* acetylcholinesterase showed interactions with the peripheral anionic site, but not with the catalytic triad. Anti-amnestic effects in mice were assessed in a step-down passive avoidance test and in the Morris water maze 30 min after injection of scopolamine (1 mg/kg i.p.). Saline, LASSBio-767, or LASSBio-822 was administered 15 min before scopolamine. Both compounds reversed the scopolamine-induced reduction in step-down latency at 0.1 mg/kg i.p. LASSBio-767 reversed scopolamine-induced changes in water maze escape latency at 1 mg/kg i.p. or p.o., while its cholinergic side effects were absent or mild up to 30 mg/kg i.p. (LD_{50} above 100 mg/kg i.p.). Thus, the (−)-spectaline derivatives are potent cholinergic agents in vivo, with a unique profile combining noncompetitive cholinesterase inhibition and CNS selectivity, with few peripheral side effects.

Keywords: Acetylcholinesterase; Alzheimer; Water maze; Passive avoidance; (−)-spectaline; Noncompetitive inhibitor

1. Introduction

Alzheimer's disease is a highly prevalent neurodegenerative disease, characterized initially by selective loss of cholinergic neurons in the basal forebrain (Whitehouse et al., 1982), followed by cognitive and behavioral impairments that progressively disrupt activities of daily living, leading to institutionalization and eventually death (Blennow et al., 2006). The prevalence of Alzheimer's disease is almost exponentially associated with age; consequently, the disease burden is already high in the industrialized world but is increasing faster in developing nations, where the rate of change in life expectancy is higher. Between 2001 and 2040 the

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prevalence of dementia (including non-Alzheimer's disease) is predicted to increase 2.3 times worldwide and to quadruple in Latin America (Ferri et al., 2005).

In spite of the identification of novel potential targets for disease-modifying drugs (Jacobsen et al., 2005), the most effective approach to treatment so far is based on the long-standing evidence that the cognitive impairments observed in Alzheimer's disease are associated with cholinergic hypofunction (Bartus et al., 1982; Terry and Buccafusco, 2003). Thus, cholinesterase inhibitors are standard therapy for Alzheimer's disease, three of which are presently in clinical use in many countries: rivastigmine, donepezil and galantamine (Lleo et al., 2006). Their use is no longer restricted to mild and moderate disease stages, at least in the case of donepezil, whose effectiveness in severe disease has been shown (Black et al., 2007; Winblad et al., 2006). Clinical studies have ascertained the significant efficacy of all three inhibitors (Birks, 2006; Burns et al., 2006; Fillit et al., 2006). Although these drugs generally appear to have a palliative effect on existing cognitive disturbances without proven effects on disease progression, there is preclinical evidence that they also may be neuroprotective (Nordberg, 2006).

Acetylcholinesterase (EC 3.1.1.7) is a globular protein containing a 20-Å deep groove (gorge) at the bottom of which sits the catalytic serine hydrolase triad. Because acetylcholine has to dive into and interact with the narrow gorge on its way to the active site (Bourne et al., 2006), different mechanisms of inhibition are possible, with distinct structural requirements. Novel compounds having a unique mechanism of inhibition combined with other favourable properties regarding selectivity, pharmacokinetics, tolerability, and cost may prove to be useful alternatives to the available anticholinesterase drugs. A marked variety of structural motifs occur in natural cholinesterase inhibitors isolated from plants and microorganisms and these are a valuable source of novel Alzheimer's disease drug candidates (Houghton et al., 2006). The piperidine alkaloid (−)-3-*O*-acetyl-spectaline can be isolated from flowers of the tree *Senna spectabilis* (DC.) Irwin and Barneby (sin. *Cassia spectabilis*) together with large amounts of (−)-spectaline (Viegas et al., 2004). Structural homologies between acetylcholine and (−)-3-*O*-acetyl-spectaline led us to propose its synthesis from the abundant natural precursor (−)-spectaline and to design derivatives aiming at an anticholinesterase activity (Viegas et al., 2005).

Here we describe the biochemical and behavioral pharmacological profiles of (−)-3-*O*-acetyl-spectaline hydrochloride (LASSBio-767) and its *tert*-Boc analogue (LASSBio-822), which are chemically novel, centrally acting cholinesterase inhibitors under development as candidate drugs for Alzheimer's disease therapy (Fig. 1).

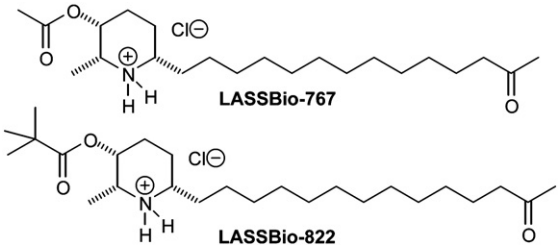

Fig. 1. Chemical structure of the new piperidine derivatives from natural *S. spectabilis* alkaloids, compounds (2R, 3R, 6S)-2-methyl-6-(13-oxotetradecyl) piperidin-3-yl acetate hydrochloride (LASSBio-767) and *tert*-butyl (2R, 3R, 6S)-2-methyl-6-(13-oxotetradecyl) piperidin-3-yl carbonate hydrochloride (LASSBio-822).

2. Materials and methods

2.1. Animals

Male Wistar rats (250–300 g) were sacrificed under deep ether anesthesia and their brains were removed for biochemical assays. Male Swiss albino mice weighing 25–35 g were used for behavioral tests. Mice were kept in the experimental room for one week prior to testing, and had free access to food and water, in acclimatized conditions (22±2 °C) on a 12/12 h light/dark cycle with lights on at 6:00 am. The behavioral tests were performed in the same room and all experiments were conducted during the daylight period. Animals were maintained and used in accordance with the Guide for the Care and Use of Laboratory Animals (USA National Research Council).

2.2. Drugs

Compounds LASSBio-767 [(2R,3R,6S)-2-methyl-6-(13-oxotetradecyl)piperidin-3-yl acetate hydrochloride] and LASSBio-822 [*tert*-butyl-(2R,3R,6S)-2-methyl-6-(13-oxotetradecyl)-piperidin-3-yl carbonate hydrochloride] were synthesized in house from natural (−)-spectaline, obtained from the flowers of *S. spectabilis* as previously described (Viegas et al., 2005). Sample quality was monitored by HPLC chromatography coupled to a light scattering detector and the results indicated that all derivatives were present at high purity. However, recent accurate mass measurements performed by electrospray ionization tandem mass spectrometry (ESI-MS/MS) of the (−)-spectaline preparation evidenced the co-occurrence of a C12 inferior homologue, known as (−)-cassine. Like (−)-spectaline, (−)-cassine may have undergone conversion to the 3-*O*-acetyl and *tert*-Boc derivatives during the synthetic steps. Acetylthiocholine iodide, 5, 5′-dithiobis-(2-nitrobenzoic) acid (DTNB), scopolamine hydrobromide, and tacrine (9-amino-1,2,3,4-tetrahydroacridine hydrochloride) were purchased from Sigma-Aldrich (St. Louis, MO, USA). For in vivo experiments, tacrine and scopolamine were dissolved in 0.9% saline and were administered i.p. at a volume of 10 ml/kg. Vehicle, NaCl 0.9%, or DMSO 1% in saline was injected i.p. in control animals. Compounds LASSBio-767 and LASSBio-822 were dissolved in DMSO (maximum 1% for injection).

2.3. Cholinesterase activity assays

Cholinesterase activity was evaluated in rat brain homogenates. Brain tissue from adult Wistar rats was homogenized at 8% w/v (or 40%, for the reversal assay) in 0.1 M sodium phosphate buffer, pH 7.4, with added Triton X-100 0.05% v/v.

Ellman's colorimetric method (Ellman et al., 1961) was adapted for reading in a microplate spectrophotometer (SpectraMax 250, Molecular Devices, Sunnyvale, CA, USA). Aliquots of homogenate (10 μL) were incubated with anticholinesterase compounds and with DTNB for 10 min in phosphate buffer pH 7.4, before addition of acetylthiocholine iodide. The reaction was run at room temperature (22–25 °C) in a final volume of 200 μL in 96-well microplates and was followed at 412 nm for 5 min. In every experiment three replicates were made per condition.

2.4. Substrate competition assay and analysis

Different concentrations of inhibitors – LASSBio-767 and LASSBio-822 – (2, 5 and 10 μM) were incubated with brain homogenates for 10 min before addition of the substrate, acetylthiocholine (0.02 to 0.5 mM). Cholinesterase-independent (nonspecific) substrate hydrolysis was determined by including one experimental group treated with tacrine 20 μM. Initial reaction velocities (v) were determined by regression from the progress curves; the average velocity of nonspecific hydrolysis was subtracted from all other v values obtained in the same experiment. The nonspecific (tacrine-resistant) reaction rate was 1–2% of the control rate observed with 0.5 mM acetylthiocholine and no inhibitors.

Data were first plotted as v versus acetylthiocholine concentration and fitted by unweighted nonlinear regression with the Michaelis–Menten equation. To normalize for differences in enzyme activity among brain homogenates, the v values in each experiment were expressed as percent of maximum velocity (V_{max}) obtained from the Michaelis–Menten fit of the control curve (without inhibitors). For a preliminary visual analysis, Lineweaver–Burk transforms of the original data were then plotted with superimposed lines corresponding to the Michaelis–Menten best fits, extrapolated beyond the axis limits. Selected models of enzyme inhibition were then tested by global fitting of the entire data set of each experiment. Models were compared based on the difference of Akaike information criterion (AIC) associated with the global fitting of the same data set. Graphical and numerical analyses were made with Microsoft Excel and GraphPad Prism software.

2.5. Reversal of cholinesterase inhibition

The dilution method of Aldridge (Aldridge, 1950) was used with minor modifications to study the potential recovery of cholinesterase activity and time-dependent inhibition by LASSBio-767 and LASSBio-822. The enzymatic activity was evaluated by Ellman's method as described above, except that a more concentrated rat brain homogenate was used (40% w/v). The protocol was validated using tacrine as enzyme-inhibitor.

Four conditions were compared in each experiment and assayed in triplicates, after different incubation times. A control group was without inhibitor and a "concentrated" group contained the inhibitor at 400 μM. In two "diluted" groups the inhibitor was assayed at 10 μM, which is close to their IC_{50} (Viegas et al., 2005). In one of these groups, the inhibitor was at 10 μM throughout the incubation ("pre-diluted"). In the other group, the inhibitor was incubated at 400 μM with the tissue homogenate and a 40-times dilution was made immediately before the readings, when the substrate (0.5 mM final) was added in a larger volume of phosphate buffer ("post-diluted"). The concentrations of brain tissue, DTNB and substrate were the same in all groups during the readings.

2.6. Molecular modeling

All docking runs were performed on an Intel Pentium 4 computer with FlexE (Claussen et al., 2001) embedded within SYBYL Molecular Modeling Software version 7.2 (Tripos Associates, St. Louis, MO, USA). Formal charges were assigned and the FlexX scoring function was chosen to evaluate the docking poses (Kramer et al., 1999). The three-dimensional structures of *Torpedo californica* acetylcholinesterase complexed with huperzine A (Raves et al., 1997), tacrine (Harel et al., 1993) and donepezil (Kryger et al., 1999) were retrieved from the Protein Data Bank (PDB; entry codes 1VOT, 1ACJ and 1EVE, respectively). Only one chain was kept in all crystal structures for docking purposes. After removal of water molecules and cofactors, protein geometries were checked and corrected for wrong atom types and bumps with the Biopolymer module, using the "Protein Preparation" tool, followed by addition of hydrogen atoms and Amber99 charges (Weiner et al., 1984).

The active site was defined as the collection of residues within 15.0 Å of the bound inhibitor, present in the reference structure (1ACJ). The active site of the ensemble was defined by the union of all ligands of the ensemble. All atoms located less than 15.0 Å from any ligand atom were considered in the ensembles. The bound inhibitors were not included in the docking runs. 1ACJ was used as a reference structure for the united protein preparation.

The assignment of hydrogen positions was made on the basis of default rules, except for the definition of the torsion angles at the hydroxyl groups of the amino acid residues serine, threonine, tyrosine, and the hydrogen position inside the histidine side-chain. The side-chains of lysine, arginine and the carboxylate groups of aspartic and glutamic acid have been modeled in their ionized states. Water molecules contained in the PDB file have been removed.

Ligands were prepared for docking by first generating their coordinates using the Sketcher module in SYBYL. Next, the correct atom types (including hybridization states) and correct bond types were defined, the hydrogen atoms were added, Gasteiger–Hückel charges were assigned to each atom, and finally the structures were energy-minimized. All carboxylic-acid groups were modeled in their anionic form while all amino groups were protonated.

2.7. Passive avoidance test

The tests were performed as previously described (Cumin et al., 1982) with some modifications (Camacho et al., 1996). Briefly, in the first day of testing, the animals acclimated to the

laboratory, experimenter and experimental chamber, a steel box (20 × 20 cm) with electrified grid floor made of bars spaced 1 cm apart, and a wooden platform (4 × 4 × 4 cm). Each mouse placed in the passive avoidance chamber was allowed to explore during 10 s, then was immediately removed to the home cage (acclimation session). After 24 h (day 2), animals were injected i.p. with vehicle, tacrine (1, 3, 5.6 or 10 mg/kg), compound LASSBio-767 (0.01, 0.1 or 1 mg/kg) or LASSBio-822 (0.1, 1 or 3 mg/kg), and after 15 min, with saline or scopolamine (1 mg/kg i.p.). Injection volume was always 0.01 ml/g of body weight. Thirty minutes after the last injection, animals were gently placed on the wooden platform inside the chamber and their latencies to step-down were recorded. When all four paws touched the grid, a low level constant current foot-shock (0.6 mA) was delivered for 3 s (training session). When the shock ceased, the mice were immediately removed from the grid and placed again on the platform. If they stepped down before 60 s, a second foot-shock was delivered. The mice returned to their home cage if they stayed on the platform for more than 60 s or immediately after the second shock. Animals that showed a step-down latency longer than 15 s in the training session were excluded from the experiment. After 24 h (day 3), the same mice were again placed on the wooden platform for the retention test. Their step-down latencies were measured (maximum 120 s) and no shock was applied. Latencies from the 24-h retention session for each drug group were then compared with the latencies of the vehicle control group.

2.8. Spatial learning test

The effect of cholinesterase inhibitors in scopolamine-induced spatial memory deficits was evaluated in a modified Morris water maze (Morris, 1984). The maze was a large white circular pool (180 cm diameter, 50 cm height) filled with water to a depth of approximately 25 cm and divided into four equal quadrants. In addition to distinct extra-maze visual cues, the maze was enriched with eight cue cards of different colors and shapes mounted onto the inner wall of the pool. A clear platform (8 cm in diameter), not visible to the mice, was submerged 0.5 cm below the opaque water surface in the center of the fourth quadrant, and kept on the same position during all experiments.

Mice were challenged to locate the hidden platform during 5 consecutive days (5 trials per day). Animals were individually released from 5 different start points with an inter-trial interval of 10 min. Each animal received one i.p. injection of vehicle, tacrine (1 or 10 mg/kg), or LASSBio-767 (0.1 or 1 mg/kg) and, after 15 min, saline or scopolamine i.p. (1 mg/kg). Thirty minutes after the second i.p. injection, mice were allowed 120 s to locate the hidden platform in the water maze, and the escape latency was measured. If the animal didn't find the escape platform after 120 s, it was gently placed on the platform for 15 s, in order to correlate the visual cues of the room with the position of the platform, and returned to the home cage. The test for effects after oral administration were similar, except that each mouse received one p.o. dose of vehicle, tacrine (1 or 10 mg/kg), or LASSBio-767 (1 or 15 mg/kg) and, after 15 min, saline or scopolamine i.p. (1 mg/kg). Thirty minutes after the second i.p. injection the mice were challenged in the water maze.

2.9. Toxicity and cholinergic side effects

Groups of 6–7 male mice per dose were used for evaluation of acute toxicity. Observations were made just before drug administration (zero time), at 15, 30, 60, 120, 180, 240, 300, and 360 min. Scores were assigned to salivation (0–4), lacrimation (0–3), and tremor (0–4) (e.g., Liston et al., 2004); body temperature was measured with a rectal probe. The mean values of the parameters were calculated at each time point in groups with at least 4 survivors. The maximum effect observed during the 6-hour period was used for plotting the dose–response curves.

2.10. Statistical analysis

Results were expressed as mean ± S.E.M. Differences between groups in the kinetic reversibility experiments were analyzed by 2-way ANOVA and Tukey post-hoc test. The behavioral tests were analyzed first with ANOVA on ranks, then with the Mann–Whitney rank sum test for particular inter-group comparisons.

3. Results

3.1. Mechanism of cholinesterase inhibition

In order to elucidate the mechanism of cholinesterase inhibition by LASSBio-767 and LASSBio-822, kinetic studies of enzyme activity were made in rat brain homogenates. The relationship between substrate concentration and reaction velocity was in good agreement with Michaelis–Menten kinetics (Fig. 2). Three complete data sets were obtained for LASSBio-767 and 4 sets were obtained for LASSBio-822, using brain tissue from different rats. In the absence of inhibitors, the average K_m for acetylthiocholine was 83.8 ± 9.3 μM ($n = 7$ independent experiments). In all data sets, when the Michaelis–Menten relationship was fitted separately to each curve, with or without inhibitor, the apparent Michaelis constants (K_m') were not very different and showed no particular trend related to inhibitor concentration. In contrast, both compounds reduced the maximum velocity V_{max}' in a concentration-dependent way, when compared with control (Fig. 2). Even in the highest substrate concentration (0.5 mM), the compounds effectively inhibited the reaction. This pattern of interaction with substrate pointed to a noncompetitive inhibitory mechanism, for both compounds. In our previous study, full concentration-inhibition curves were obtained for determination of IC_{50} and both compounds were found to fully inhibit the reaction (Viegas et al., 2005). Therefore, only linear (not partial) mechanisms of inhibition were considered.

Two models of linear inhibition were selected based on preliminary Lineweaver–Burk (double reciprocal) plots of the data: a simple noncompetitive inhibition and a general mixed

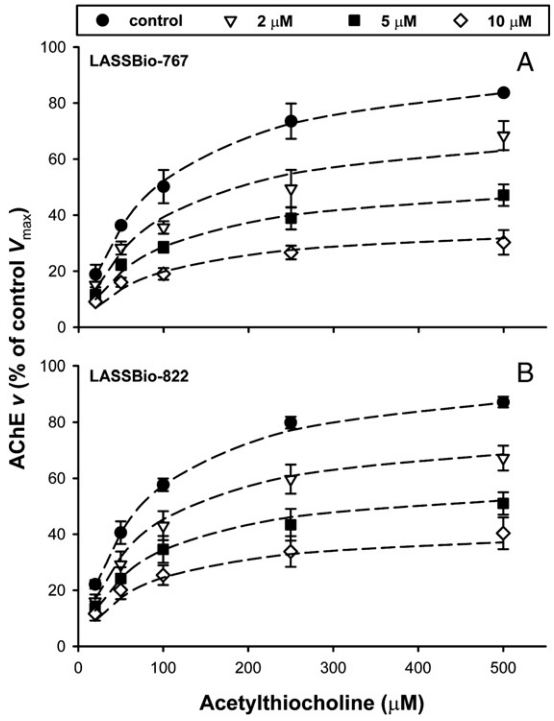

Fig. 2. Substrate competition assays of LASSBio-767 (A) and LASSBio-822 (B). The initial velocities of reaction (v) were obtained from progression curves in different concentrations of substrate and inhibitor. The solid lines are derived from the best-fitting noncompetitive inhibition model. Data points are means±S.E.M. from 3 (A) and 4 experiments (B), made in triplicate.

inhibition, that includes one additional parameter (alpha) (Segel, 1993). For model comparison, the data sets were averaged across experiments (different rats). Both models yielded good fits of the data, with small differences in total sum of squares. Based on the AIC, the preferred model was that of simple or "classical" noncompetitive inhibition, where the addition of inhibitor alters V_{max}' values, but not K_m', according to the expressions:

$$V_{max}' = V_{max} \cdot \left(1 + [I] \cdot K_i^{-1}\right)^{-1}$$

$$v = V_{max}' \cdot [S] \cdot (K_m + [S])^{-1} \quad (1)$$

where $[I]$ and $[S]$ are the concentrations of inhibitor and substrate, respectively, and K_i is the noncompetitive inhibition constant. For LASSBio-767, this model was 3.6 times more likely than mixed inhibition, and the best-fitting K_i was 6.1 μM. For LASSBio-822, the model was 4.5 times more likely and K_i was 7.5 μM.

A dilution assay was used to investigate the time-dependence and reversibility of binding of the compounds to acetylcholinesterase (Aldridge, 1950). LASSBio-767 pre-diluted to 10 μM led to 26% of inhibition after 5 min of incubation, and 45% after 10 min, without further change at 60 min (Fig. 3A). Incubation at 400 μM (concentrated group) led to 89–93% inhibition at all incubation times, but rapid post-dilution to 10 μM led to immediate relief of acetylcholinesterase inhibition to 23–38%. There was no significant difference in V_{max} between pre- and post-diluted samples (P=0.876, 0.065, and 0.508, at 5, 10, and 60 min, respectively). LASSBio-822 10 μM inhibited the reaction to the same extent (47–51%) after 5, 10, or 60 min of incubation (Fig. 3B). Inhibition was nearly complete at 400 μM (96–99%), but after dilution to 10 μM it quickly re-equilibrated at 37–42% inhibition. There was no significant difference in V_{max} between pre- and post-diluted samples (P=0.805, 0.141, and 0.776, at 5, 10, and 60 min, respectively). Therefore, it seems that the inhibition of acetylcholinesterase by LASSBio-767 and LASSBio-822 was fully reversible. The delay between dilution with substrate-containing buffer and spectrophotometric reading was less than 1 min, showing that dissociation of both (−)-spectaline derivatives was very fast, irrespective of the incubation time.

3.2. Proposed molecular docking mode in acetylcholinesterase

In order to obtain preliminary information *in silico* about the recognition pattern of LASSBio-767 and LASSBio-822 by the active site of the enzyme, flexible docking experiments with FlexE were performed, using the default FlexX scoring function and the structure of *Torpedo* acetylcholinesterase in complex with three marketed drugs.

The top docking positions showed that neither of the derivatives was able to penetrate deeply in the active site gorge, possibly due to the volume of their long aliphatic side-chain (Fig. 4A–B). In the simulation, the compounds did not interact with any of the residues that make up the catalytic triad,

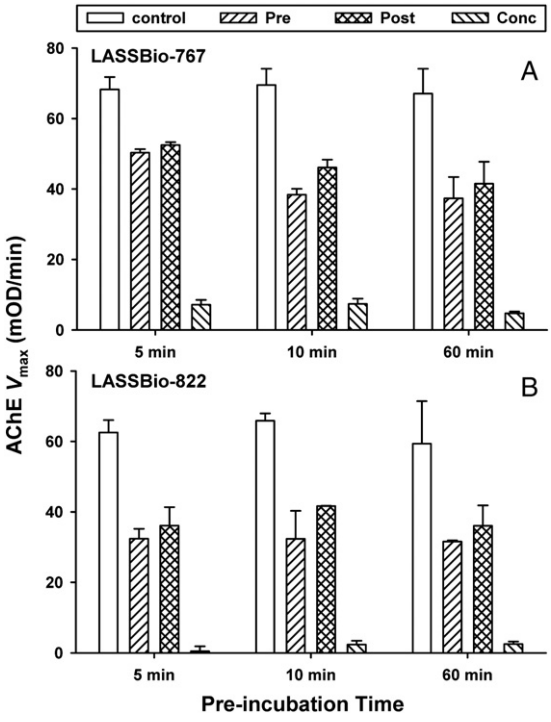

Fig. 3. Reversibility of ChE inhibition by LASSBio-767 (A) and LASSBio-822 (B). Maximum enzyme velocity without inhibitor (control), or pre-diluted (Pre) and post-diluted (Post) to 10 μM, and 40×concentrated (Conc, 400 μM), compared after different periods of incubation. Data points are means±S.D. from a representative experiment made in triplicate. The differences between pre- and post-diluted samples were not significant.

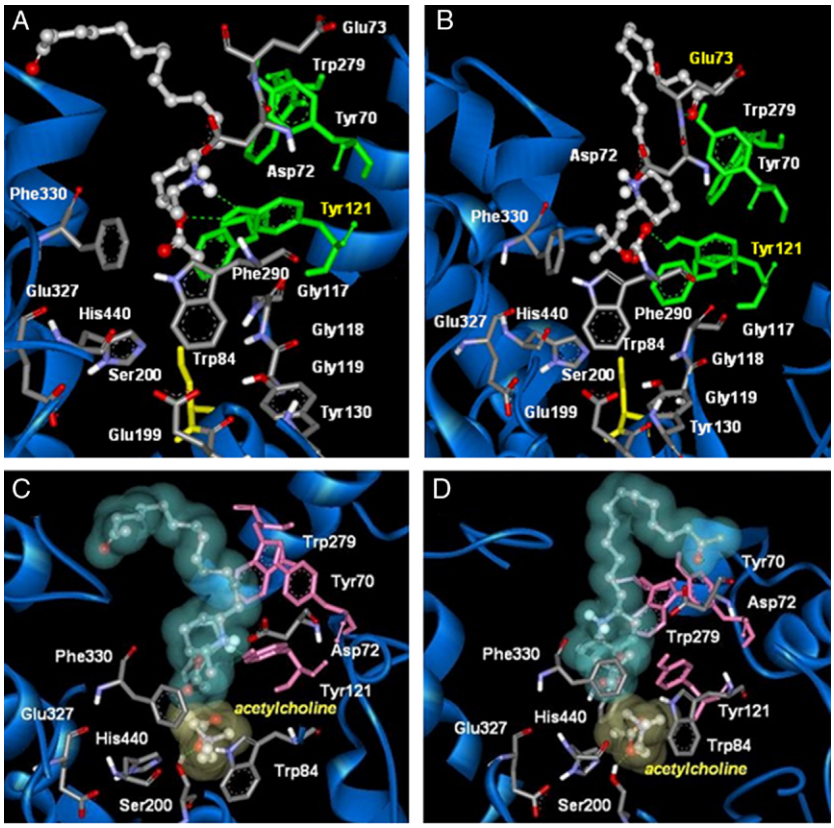

Fig. 4. Molecular modeling. The best docking solutions obtained with FlexE for LASSBio-767 (A, C) and LASSBio-822 (B, D) are shown with relevant residues of *Torpedo* acetylcholinesterase. In A and B, interacting residues are labeled in yellow and the catalytic Ser200 is shown in yellow sticks. Panels C and D show the compounds with vdW surfaces (green) in their best poses together with acetylcholine (yellow vdW) bound in the active site. Some amino acid residues from the peripheral anionic site are depicted in green (A, B) or pink sticks (C, D). Only polar hydrogen atoms are displayed. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

represented by Ser200, Glu327 and His440. Halfway up the active site gorge in acetylcholinesterase there is a constricted region where Phe330 is known to interact with the piperidine nitrogen of donepezil (Kryger et al., 1999). LASSBio-767 did not seem to interact with Phe330, although it was not bound very far from it (Fig. 4A). In contrast, LASSBio-822 made hydrophobic interactions with the enzyme by means of a close van der Waals contact between its *tert*-butyl function and Phe330 (Fig. 4B).

Close to the top (entrance) of the gorge are located the amino acid residues that compose the peripheral anionic site of acetylcholinesterase, such as Tyr70, Tyr121 and Trp279. The best posing obtained for LASSBio-767 showed hydrogen bonds with Tyr121 through the H atom pertaining to the protonated amino group of its piperidine moiety and also through the oxygen atom of its ester group (Fig. 4A). LASSBio-822 made a hydrogen bond with Tyr121 involving the two oxygen atoms from the *Boc* moiety (Fig. 4B). An additional hydrogen bond was observed with Glu73 through the oxygen atom of its terminal carbonyl group (Fig. 4B). Docking simulations were also run for the putative compound (−)-3-*O*-acetyl-cassine, which could have been formed from a small amount of (−)-cassine present in the plant extracts. The putative (−)-cassine derivative lacks two CH_2 groups in the aliphatic side chain but would also be expected to interact with the peripheral anionic site of *Torpedo* acetylcholinesterase, according to the simulations (data not shown).

Additional docking studies were performed with acetylcholine, using the top enzyme-inhibitor complexes obtained previously by docking procedures. The main goal here was to investigate if there would still be room for acetylcholine binding in the enzyme acylating site, close to Ser200. The results showed that, even with bound LASSBio-767 and LASSBio-822, acetylcholine would still fit into the gorge (Fig. 4C–D). This result reinforces the proposed molecular mechanism of inhibition for these compounds.

3.3. Effects in cholinergic amnesia: passive avoidance test

The effectiveness of the spectaline derivatives against cholinergic amnesia in mice was first evaluated through the passive avoidance behavior observed after training with an aversive stimulus. In the test session on the day after training, control mice avoided the electrified grid floor, remaining on the platform for a 14 times longer period (Fig. 5). Animals given the centrally acting muscarinic receptor antagonist scopolamine (1 mg/kg i.p.) 30 min before the training session showed impaired retention, with a step-down latency of 15.9±6.0 s (n=21). The anti-Alzheimer's disease drug, tacrine, was used at 1–10 mg/kg as a standard for the anti-amnestic effects. At 1 and

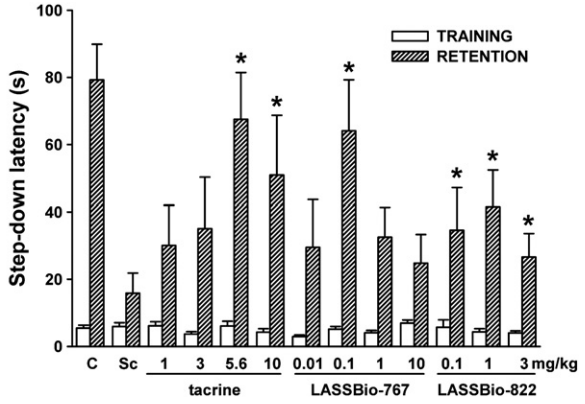

Fig. 5. Effect of tacrine, LASSBio-767, and LASSBio-822 on step-down passive avoidance. Results are expressed as the mean latency±S.E.M. from 7 to 22 Swiss male mice, to step off a platform in the training session (empty bars), and 24 h later, in the retention test session (hatched bars). Drugs or vehicle (i.p.) and scopolamine were injected 45 and 30 min before the test, respectively. Control group (C) received only vehicle. Asterisks represent significant differences ($P<0.05$) relative to the scopolamine-treated vehicle group (Sc), evaluated with the Mann–Whitney rank sum test.

3 mg/kg tacrine, given i.p. 15 min before scopolamine, the step-down latency increased relative to the group receiving scopolamine only, but the differences did not reach statistical significance. As we previously reported, tacrine was able to reverse the amnesia at 5.6 mg/kg (i.p.) (Viegas et al., 2005). At this dose of tacrine, the step-down latency reached 85% of control values. At the higher dose of 10 mg/kg, the step-down latency was also significantly different from that in the scopolamine group (50.9±17.8 s, $P<0.05$, $n=8$), but was only 64% of the control. Thus, the dose–response relationship for tacrine was apparently bell-shaped.

Compound LASSBio-767 was tested at a wide range of doses (0.01–10 mg/kg), given i.p. 15 min before scopolamine. Animals receiving 0.01 mg/kg LASSBio-767 showed a mean step-down latency of 29.5±14.2 s ($n=8$), which was larger but not significantly different from the scopolamine group. At 0.1 mg/kg LASSBio-767, the mean step-down latency was 64.1±15.2 s ($n=13$), demonstrating a marked anti-amnesic effect. However, higher doses of LASSBio-767 (1 and 10 mg/kg) were not significantly effective (Fig. 5). Compound LASSBio-822 effectively increased step-down latency at all doses tested (0.1–3 mg/kg), although the mean values (26.6 to 34.6 s, $n=8$–19) were smaller than those seen with tacrine 5.6 mg/kg or LASSBio-767 0.1 mg/kg. None of the compounds affected the step-down latency in the training session, compared to untreated controls (saline or DMSO 1%).

3.4. Effects in cholinergic amnesia: spatial learning test

Compound LASSBio-767 was elected for further testing in vivo, due to its slightly higher potency and convenience of synthesis compared to LASSBio-822. The spatial memory abilities of mice were assessed using the Morris water maze. The escape latencies for mice to reach the hidden platform using distant visual cues were determined on each training day. As expected, under control conditions, mice showed a progressive reduction of mean escape latencies from 86.2±5.5 s to 35.4±6.5 s ($n=21$), in the first and fifth days of testing, respectively (Fig. 6). When scopolamine was administered 30 min before the first trial in the maze, animals were no longer able to remember the position of the hidden platform. Thus, scopolamine-treated mice (1 mg/kg, i.p.) displayed latencies of 101.8±8.2 s and 103.5±8.4 s ($n=9$), on day 1 and day 5, respectively, significantly different from saline-treated mice (Fig. 6A). When tacrine was administered at 10 mg/kg 15 min before scopolamine, mice showed a marked reduction in escape latency from 114.3±3.6 to 59.4±13.7 s ($n=6$). This reversal of scopolamine-induced amnesia was not observed with doses of 1 and 5.6 mg/kg of tacrine (Fig. 6A). Compound LASSBio-767 administered i.p. at 1 mg/kg was also able to block the effect of scopolamine in the water maze, leading to a reduction of escape latencies from 102.3±10.6 to 53.4±13.1 s ($n=6$) between the first

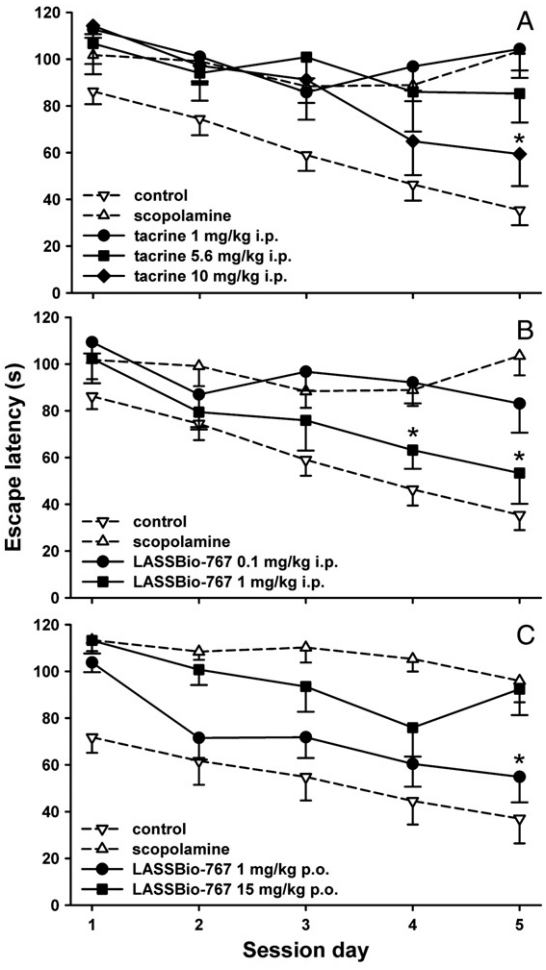

Fig. 6. Effect of tacrine and LASSBio-767 in the Morris water maze. (A) Mice treated with scopolamine 1 mg/kg had impaired learning, and showed a difference in escape latency after the second day compared with saline-treated mice. In the last day, scopolamine-treated mice showed reduced latencies when given tacrine 10 mg/kg, but not 1 or 5.6 mg/kg ($n=5$–21 mice). (B) Scopolamine-treated mice showed significantly reduced latencies in days 4 and 5 when given LASSBio-767 1 mg/kg i.p. ($n=6$–21 mice). (C) Orally administered LASSBio-767 1 mg/kg led to a reduced latency compared to vehicle (oral saline) in scopolamine-treated mice. At 15 mg/kg LASSBio-767 p.o., the effects were not significant ($n=7$–8 mice). Asterisks represent significant differences ($P<0.05$; Mann–Whitney rank sum test) relative to controls given vehicle and 1 mg/kg i.p. of scopolamine.

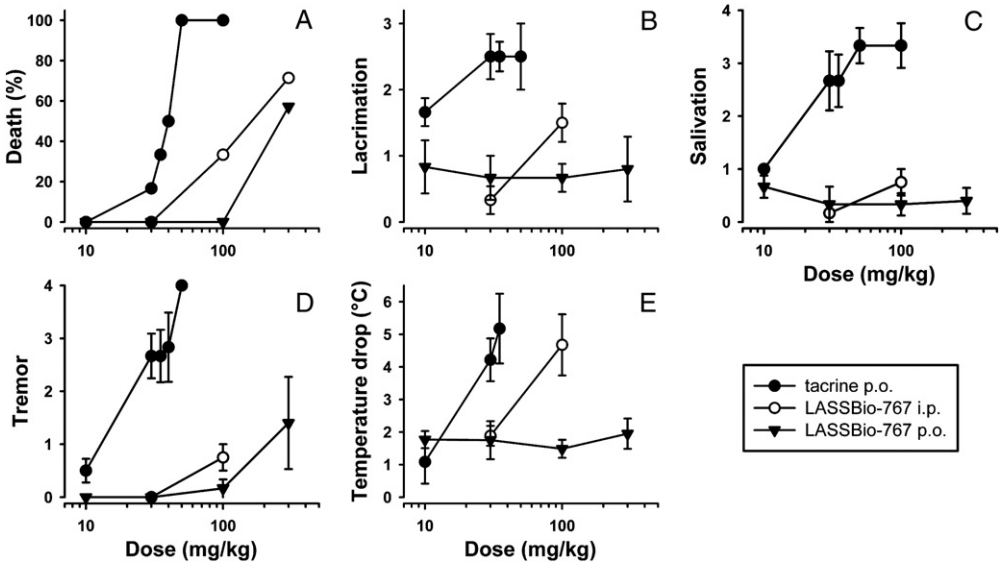

Fig. 7. Dose–response relationships for lethality and cholinergic side effects. The response to tacrine (p.o., filled circles) is shown for comparison with LASSBio-767 (i.p., empty circles, and p.o., filled triangles). Mice were observed for lethality (A), lacrimation (B), salivation (C), tremor (D) and hypothermia (E) during 6 h. In B–D, the values were averaged across animals at each time point and the largest mean is shown. In E, the differences between the initial (control) and the lowest temperature were calculated for each animal and then averaged. All temperature drops were significant ($P<0.05$, paired t test), except for tacrine 10 mg/kg ($n=4$–7 mice).

and fifth days, respectively (Fig. 6B). At 0.1 mg/kg, LASSBio-767 did not significantly reduce the escape latency compared to scopolamine, but the animals tended to use a shorter swim path leading to the platform (data not shown). This might reflect an improved retention of visual cues of the platform location, which are not retained in the case of animals treated with scopolamine alone. Therefore, LASSBio-767 antagonized the amnesia induced by scopolamine in the water maze at doses at least ten times lower than tacrine.

In order to examine if compound LASSBio-767 is effective when administered orally, doses of 1 and 15 mg/kg were given to mice (p.o.) 15 min before scopolamine, as described earlier. Similarly to i.p. administration, scopolamine-induced amnesia was offset by LASSBio-767 1 mg/kg p.o., with animals showing a marked reduction of escape latencies from 103.8±4.1 to 54.9±11.0 s ($n=9$) between the first and fifth days, respectively (Fig. 6C). At the higher dose of 15 mg/kg p.o., the compound was not effective; the escape latency of the LASSBio-767-treated animals in the last day of test was similar to that of scopolamine-treated (amnesic) animals. Therefore, LASSBio-767 was effective both by the intra-peritoneal and oral routes at the same dose.

3.5. Observations on side effects

None of the animals treated with compound LASSBio-767 (up to 10 mg/kg i.p. or 15 mg/kg p.o., $n>50$) for the memory tests showed cholinergic side effects during the 15-min period of observation that preceded the injection of scopolamine. Lethality, lacrimation, salivation, tremor and hypothermia induced by LASSBio-767 were measured for 6 h after a single i.p. or p.o. dose (up to 300 mg/kg) in naive mice. No deaths occurred at 30 mg/kg, but 57% (p.o.) and 71% (i.p.) died at 300 mg/kg. It must be noted that at this dose the compound was administered as suspension in saline and absorption may have been limited. All deaths occurred within the first hour. Hypothermia and tremor, which reflect central cholinergic activity, were observed with LASSBio-767 at 10 and 100 mg/kg, respectively (Fig. 7). However, while tremor was observed within the first hour, hypothermia lasted up to 4 h after administration. The maximal temperature drop was significant at all doses of LASSBio-767 (10–300 mg/kg, i.p. or p.o., $P<0.05$, paired t test). Salivation and lacrimation, two peripheral cholinergic side effects, were moderate even at the highest dose tested, and were also present for 3 to 4 h. Tacrine was more potent and generally more effective in inducing all cholinergic side effects, which also included diarrhea and splayed hindlimbs, and its LD_{50} was approximately 40 mg/kg p.o. (Fig. 7).

4. Discussion

The kinetic experiments presented herein established that the novel compounds LASSBio-767 and LASSBio-822 are reversible noncompetitive cholinesterase inhibitors. In agreement with that, molecular modeling of the enzyme-inhibitor complex suggested that neither of the compounds interacts with the catalytic triad. The experiments in vivo extended our previous preliminary report of single-dose experiments (Viegas et al., 2005) by showing that the selected compound LASSBio-767 is potent and effective in two paradigms of cholinergic amnesia in mice. Furthermore, we have now shown the effectiveness of LASSBio-767 administered by the oral route and its low cholinergic toxicity, which are essential to its assessment as a candidate drug for Alzheimer's disease.

Anti-cholinesterase compounds that mainly bind to the peripheral anionic site usually show linear noncompetitive inhibition against acetylcholine and acetylthiocholine, which

are cationic and bind to the peripheral anionic site on their way into the catalytic gorge (Bourne et al., 2006; Radic et al., 1991; Szegletes et al., 1999). Therefore, mutual exclusion at the peripheral site might explain the type of interaction observed between acetylthiocholine and the spectaline derivatives.

The acetylcholinesterase inhibitors in use for Alzheimer's disease have distinct molecular mechanisms of action. Galantamine and rivastigmine are competitive inhibitors, but while galantamine binds reversibly to the active site of acetylcholinesterase (Bar-On et al., 2002), rivastigmine acts pseudo-irreversibly, due to its carbamoylation of the active center serine (Schuh, 1976). In contrast, tacrine and donepezil are both reversible noncompetitive inhibitors of a mixed type (Nochi et al., 1995; Snape et al., 1999). Reversibility may be beneficial, because only reversible inhibitors lead to an up-regulation of CNS acetylcholinesterase and this is correlated with clinical improvement in AD patients (Darreh-Shori et al., 2006, and references therein). The crystal structure of the acetylcholinesterase-donepezil complex revealed multiple sites of interaction along the gorge, spanning from Trp84 near the catalytic site to Trp279 at the peripheral anionic site (Kryger et al., 1999). The dual interaction might explain the competitive and noncompetitive components of inhibition by donepezil, as well as its high potency. Like donepezil, LASSBio-767 is a piperidine derivative that probably binds along the gorge, according to our modeling experiments. However, in our best docking poses LASSBio-767 was unable to reach the bottom of the gorge and no strong interactions were found there. Docking simulations predicted two H bonds between this compound and Tyr121 at the peripheral anionic site. Binding to this site may lead to a steric blockade of the gorge that suffices to cause noncompetitive inhibition. Furthermore, with LASSBio-767 bound, there seems to be enough room left for substrate binding in the active site, thus explaining the lack of a significant competitive component of inhibition. Compound LASSBio-822 was similar to LASSBio-767 in its predicted binding mode in *Torpedo* acetylcholinesterase, as well as in IC_{50} (Viegas et al., 2005), K_i and kinetic profile in brain acetylcholinesterase. Any difference in acetyl-/butyrylcholinesterase selectivity is most likely due to distinct interactions of the compounds with butyrylcholinesterase. It is worth noting that our molecular docking simulations suggested that (−)-3-*O*-acetyl-cassine, a putative contaminant of LASSBio-767 samples, may also be an acetylcholinesterase inhibitor. Therefore, its contribution to the effects of LASSBio-767 cannot be ruled out at the moment.

Various memory-modifying pharmacological agents have been shown to produce non-monotonic (bell-shaped) dose–response curves (McGaugh, 1989). Cholinesterase inhibitors show bell-shaped dose–response curves in diverse models of memory impairment, including scopolamine-induced deficits (Bejar et al., 1999; Braida et al., 1996), lesions of central cholinergic pathways (Matsuoka et al., 1991; Sweeney et al., 1990), and APP23 transgenic mice (Van Dam et al., 2005). Direct cholinomimetics also show reduced anti-amnesic effects at higher doses (Flood et al., 1984; Matsuoka et al., 1991; Suzuki et al., 1995). This behavior was confirmed in our experiments with tacrine in the passive avoidance test and was clearly observed for LASSBio-767 both in passive avoidance and in the water maze (after oral treatment). Thus, the effectiveness of LASSBio-767 in the passive avoidance test was not detected in our previous screening because the single-dose of 1 mg/kg was too high (Viegas et al., 2005). Increasing the dose of tacrine beyond its optimum not only reduced effectiveness against the cognitive impairment but also led to marked cholinergic adverse effects. The main difference between tacrine and LASSBio-767 in this regard was that the latter did not show signs of peripheral cholinergic toxicity at doses 10–15 times above the best effective doses in the behavioral tests. Therefore, toxicity should be less of a concern than cognition-enhancing effectiveness for optimum dose titration of LASSBio-767.

The low acute toxicity of LASSBio-767 in rats and mice could be related to its mild selectivity for acetylcholinesterase versus butyrylcholinesterase. The ratio between the IC_{50}s (butyryl-/acetylcholinesterase) previously determined for LASSBio-767 in rat brain cholinesterases is around 20 (Viegas et al., 2005). There is convincing evidence that butyrylcholinesterase plays a significant role in the cholinergic toxicity of nonselective inhibitors, at least in rodents (Liston et al., 2004). In contrast with toxicity, the possible benefit of CNS butyrylcholinesterase inhibition in Alzheimer's disease has not been completely evaluated and some studies propose that nonselective inhibitors could offer a clinical advantage (Lane et al., 2006; Weinstock, 1999). In fact, current clinical evidence does not support any contention that selectivity is relevant to clinical efficacy, whereas data metanalysis is only slightly favourable to the acetylcholinesterase-selective inhibitors regarding tolerability (Birks, 2006).

Pharmacokinetic selectivity towards CNS cholinesterases is likely to be more relevant to the clinical tolerability of ChE inhibitors than their acetyl-/butyrylcholinesterase inhibitory potencies (Enz et al., 1993). Different inhibitors can vary considerably in this respect. In the present study, tacrine induced peripheral signs of cholinergic hyperactivity at doses only slightly higher than those effective in reversing amnesia, as previously described (Dronfield et al., 2000; Yoshida and Suzuki, 1993). The ratio between the ED_{50} for induction of salivation and central tremor is about 3 for donepezil in rats, 20 min after i.p. administration (Snape et al., 1999), and about 5 for galantamine in mice, after s.c. administration (Bores et al., 1996). Although mild hypothermia, lacrimation and salivation (but not tremor) were observed with LASSBio-767 10 mg/kg p.o., the ED_{50} for these cholinergic effects is probably above 30 mg/kg i.p. and even higher orally (Fig. 7). Considering that LASSBio-767 reversed scopolamine-induced amnesia at 0.1–1 mg/kg i.p. in two behavioral tasks, its CNS/peripheral potency ratio compares favorably with those of the ChE inhibitors in use for Alzheimer's disease.

Compound LASSBio-767 could owe part of its anti-amnesic effectiveness to interaction with additional molecular targets in the CNS. The 3-acetoxypiperidine moiety in natural (−)-3-*O*-acetyl-spectaline resembles acetylcholine and this originally inspired our interest in developing analogues and testing for ChE inhibition (Viegas et al., 2005). Indeed, 3-acetoxypiperidine itself is reported to be a substrate of acetylcholinesterase

(Lambrecht, 1980), and is also a direct cholinomimetic at muscarinic receptors (Lambrecht, 1976). The related 3-acetoxyquinuclidine aceclidine is also a muscarinic agonist (Ehlert et al., 1996; Ringdahl et al., 1982). Muscarinic agonists are well known for their cognition-enhancing properties and have been extensively investigated as possible Alzheimer's disease drugs (Koch et al., 2005). Even though the 3-acetoxypiperidine moiety of LASSBio-767 seems not to interact with the active site of cholinesterases, an interaction with the ACh binding pocket in muscarinic receptors cannot be ruled out. Therefore, a direct muscarinic effect could have contributed to the efficacy of LASSBio-767 against scopolamine-induced amnesia and this possibility is worth investigating. Like other peripheral anionic site ligands, the novel compounds may also have additional ACh-independent effects against neural cell death and β-amyloid aggregation (Johnson and Moore, 2006).

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References

Aldridge, W.N., 1950. Some properties of specific cholinesterase with particular reference to the mechanism of inhibition by diethyl *p*-nitrophenyl thiophosphate (E 605) and analogues. Biochem. J. 46, 451–460.

Bar-On, P., Millard, C.B., Harel, M., Dvir, H., Enz, A., Sussman, J.L., Silman, I., 2002. Kinetic and structural studies on the interaction of cholinesterases with the anti-Alzheimer drug rivastigmine. Biochemistry 41, 3555–3564.

Bartus, R.T., Dean III, R.L., Beer, B., Lippa, A.S., 1982. The cholinergic hypothesis of geriatric memory dysfunction. Science 217, 408–414.

Bejar, C., Wang, R.H., Weinstock, M., 1999. Effect of rivastigmine on scopolamine-induced memory impairment in rats. Eur. J. Pharmacol. 383, 231–240.

Birks, J., 2006. Cholinesterase inhibitors for Alzheimer's disease. Cochrane Database Syst. Rev. CD005593.

Black, S.E., Doody, R., Li, H., McRae, T., Jambor, K.M., Xu, Y., Sun, Y., Perdomo, C.A., Richardson, S., 2007. Donepezil preserves cognition and global function in patients with severe Alzheimer disease. Neurology 69, 459–469.

Blennow, K., de Leon, M.J., Zetterberg, H., 2006. Alzheimer's disease. Lancet 368, 387–403.

Bores, G.M., Huger, F.P., Petko, W., Mutlib, A.E., Camacho, F., Rush, D.K., Selk, D.E., Wolf, V., Kosley Jr., R.W., Davis, L., Vargas, H.M., 1996. Pharmacological evaluation of novel Alzheimer's disease therapeutics: acetylcholinesterase inhibitors related to galanthamine. J. Pharmacol. Exp. Ther. 277, 728–738.

Bourne, Y., Radic, Z., Sulzenbacher, G., Kim, E., Taylor, P., Marchot, P., 2006. Substrate and product trafficking through the active center gorge of acetylcholinesterase analyzed by crystallography and equilibrium binding. J. Biol. Chem. 281, 29256–29267.

Braida, D., Paladini, E., Griffini, P., Lamperti, M., Maggi, A., Sala, M., 1996. An inverted U-shaped curve for heptylphysostigmine on radial maze performance in rats: comparison with other cholinesterase inhibitors. Eur. J. Pharmacol. 302, 13–20.

Burns, A., O'Brien, J., Auriacombe, S., Ballard, C., Broich, K., Bullock, R., Feldman, H., Ford, G., Knapp, M., McCaddon, A., Iliffe, S., Jacova, C., Jones, R., Lennon, S., McKeith, I., Orgogozo, J.M., Purandare, N., Richardson, M., Ritchie, C., Thomas, A., Warner, J., Wilcock, G., Wilkinson, D., 2006. Clinical practice with anti-dementia drugs: a consensus statement from British Association for Psychopharmacology. J. Psychopharmacol. 20, 732–755.

Camacho, F., Smith, C.P., Vargas, H.M., Winslow, J.T., 1996. Alpha 2-adrenoceptor antagonists potentiate acetylcholinesterase inhibitor effects on passive avoidance learning in the rat. Psychopharmacology (Berl.) 124, 347–354.

Claussen, H., Buning, C., Rarey, M., Lengauer, T., 2001. FlexE: Efficient molecular docking considering protein structure variations. J. Mol. Biol. 308, 377–395.

Cumin, R., Bandle, E.F., Gamzu, E., Haefely, W.E., 1982. Effects of the novel compound aniracetam (Ro 13-5057) upon impaired learning and memory in rodents. Psychopharmacology (Berl.) 78, 104–111.

Darreh-Shori, T., Meurling, L., Pettersson, T., Hugosson, K., Hellstrom-Lindahl, E., Andreasen, N., Minthon, L., Nordberg, A., 2006. Changes in the activity and protein levels of CSF acetylcholinesterases in relation to cognitive function of patients with mild Alzheimer's disease following chronic donepezil treatment. J. Neural Transm. 113, 1791–1801.

Dronfield, S., Egan, K., Marsden, C.A., Green, A.R., 2000. Comparison of donepezil-, tacrine-, rivastigmine- and metrifonate-induced central and peripheral cholinergically mediated responses in the rat. J. Psychopharmacol. 14, 275–279.

Ehlert, F.J., Griffin, M.T., Glidden, P.F., 1996. The interaction of the enantiomers of aceclidine with subtypes of the muscarinic receptor. J. Pharmacol. Exp. Ther. 279, 1335–1344.

Ellman, G.L., Courtney, K.D., Andres, V., Featherstone, J., Featherstone, R.M., 1961. A new and rapid colorimetric determination of acetylcholinesterase activity. Biochem. Pharmacol. 7, 88–95.

Enz, A., Amstutz, R., Boddeke, H., Gmelin, G., Malanowski, J., 1993. Brain selective inhibition of acetylcholinesterase: a novel approach to therapy for Alzheimer's disease. Prog. Brain Res. 98, 431–438.

Ferri, C.P., Prince, M., Brayne, C., Brodaty, H., Fratiglioni, L., Ganguli, M., Hall, K., Hasegawa, K., Hendrie, H., Huang, Y., Jorm, A., Mathers, C., Menezes, P.R., Rimmer, E., Scazufca, M., 2005. Global prevalence of dementia: a Delphi consensus study. Lancet 366, 2112–2117.

Fillit, H.M., Smith Doody, R., Binaso, K., Crooks, G.M., Ferris, S.H., Farlow, M.R., Leifer, B., Mills, C., Minkoff, N., Orland, B., Reichman, W.E., Salloway, S., 2006. Recommendations for best practices in the treatment of Alzheimer's disease in managed care. Am. J. Geriatr. Pharmacother. 4 (Suppl 1), S9–S24.

Flood, J.F., Smith, G.E., Cherkin, A., 1984. Memory retention test performance in mice: improvement by chronic oral cholinergic drug treatment. Pharmacol. Biochem. Behav. 21, 169–173.

Harel, M., Schalk, I., Ehret-Sabatier, L., Bouet, F., Goeldner, M., Hirth, C., Axelsen, P.H., Silman, I., Sussman, J.L., 1993. Quaternary ligand binding to aromatic residues in the active-site gorge of acetylcholinesterase. Proc. Natl. Acad. Sci. U. S. A. 90, 9031–9035.

Houghton, P.J., Ren, Y., Howes, M.J., 2006. Acetylcholinesterase inhibitors from plants and fungi. Nat. Prod. Rep. 23, 181–199.

Jacobsen, J.S., Reinhart, P., Pangalos, M.N., 2005. Current concepts in therapeutic strategies targeting cognitive decline and disease modification in Alzheimer's disease. NeuroRx. 2, 612–626.

Johnson, G., Moore, S.W., 2006. The peripheral anionic site of acetylcholinesterase: structure, functions and potential role in rational drug design. Curr. Pharm. Des. 12, 217–225.

Koch, H.J., Haas, S., Jurgens, T., 2005. On the physiological relevance of muscarinic acetylcholine receptors in Alzheimer's disease. Curr. Med. Chem. 12, 2915–2921.

Kramer, B., Rarey, M., Lengauer, T., 1999. Evaluation of the FLEXX incremental construction algorithm for protein-ligand docking. Prot. Struct. Funct. Genet. 37, 228–241.

Kryger, G., Silman, I., Sussman, J.L., 1999. Structure of acetylcholinesterase complexed with E2020 (Aricept ®): implications for the design of new anti-Alzheimer drugs. Structure 7, 297–307.

Lambrecht, G., 1976. Structure and conformation-activity relationships of heterocyclic acetylcholine analogs. I. Muscarinic activity of 3-acetoxy-quinuclidine and 3-acetoxypiperidine enantiomers. Eur. J. Med. Chem. 11, 461–466.

Lambrecht, G., 1980. Structure-activity and conformation-activity relationships of cyclic acetylcholine analogs. XI. Acetylcholinesterase-catalyzed and base-catalyzed-hydrolysis of derivatives of 3-acetoxypiperidine and 3-acetoxythiacyclohexane. Arch. Pharm. (Weinh.) 313, 368–374.

Lane, R.M., Potkin, S.G., Enz, A., 2006. Targeting acetylcholinesterase and butyrylcholinesterase in dementia. Int. J. Neuropsychopharmacol. 9, 101–124.

Liston, D.R., Nielsen, J.A., Villalobos, A., Chapin, D., Jones, S.B., Hubbard, S.T., Shalaby, I.A., Ramirez, A., Nason, D., White, W.F., 2004. Pharmacology of selective acetylcholinesterase inhibitors: implications for use in Alzheimer's disease. Eur. J. Pharmacol. 486, 9–17.

Lleo, A., Greenberg, S.M., Growdon, J.H., 2006. Current pharmacotherapy for Alzheimer's disease. Annu. Rev. Med. 57, 513–533.

Matsuoka, N., Maeda, N., Ohkubo, Y., Yamaguchi, I., 1991. Differential effects of physostigmine and pilocarpine on the spatial memory deficits produced by two septo-hippocampal deafferentations in rats. Brain Res. 559, 233–240.

McGaugh, J.L., 1989. Dissociating learning and performance: drug and hormone enhancement of memory storage. Brain Res. Bull. 23, 339–345.

Morris, R.G., 1984. Developments of a water-maze procedure for studying spatial learning in the rat. J. Neurosci. Methods 11, 47–60.

Nochi, S., Asakawa, N., Sato, T., 1995. Kinetic study on the inhibition of acetylcholinesterase by 1-benzyl-4-[(5,6-dimethoxy-1-indanon)-2-yl] methylpiperidine hydrochloride (E2020). Biol. Pharm. Bull. 18, 1145–1147.

Nordberg, A., 2006. Mechanisms behind the neuroprotective actions of cholinesterase inhibitors in Alzheimer disease. Alzheimer Dis. Assoc. Disord. 20, S12–S18.

Radic, Z., Reiner, E., Taylor, P., 1991. Role of the peripheral anionic site on acetylcholinesterase: inhibition by substrates and coumarin derivatives. Mol. Pharmacol. 39, 98–104.

Raves, M.L., Harel, M., Pang, Y.P., Silman, I., Kozikowski, A.P., Sussman, J.L., 1997. Structure of acetylcholinesterase complexed with the nootropic alkaloid, (−)-huperzine A. Nat. Struct. Biol. 4, 57–63.

Ringdahl, B., Ehler, F.J., Jenden, D.J., 1982. Muscarinic activity and receptor binding of the enantiomers of aceclidine and its methiodide. Mol. Pharmacol. 21, 594–599.

Schuh, F.T., 1976. Molecular mechanism of action of galanthamine, an antagonist of nondepolarizing muscle-relaxants. Anaesthesist 25, 444–448.

Segel, I.H., 1993. Enzyme Kinetics: Behavior and Analysis of Rapid Equilibrium and Steady-State Enzyme Systems. John Wiley & Sons, New York.

Snape, M.F., Misra, A., Murray, T.K., De Souza, R.J., Williams, J.L., Cross, A.J., Green, A.R., 1999. A comparative study in rats of the in vitro and in vivo pharmacology of the acetylcholinesterase inhibitors tacrine, donepezil and NXX-066. Neuropharmacology 38, 181–193.

Suzuki, M., Yamaguchi, T., Ozawa, Y., Iwai, A., Yamamoto, M., 1995. Effect of YM796, a novel muscarinic agonist, on the impairment of passive avoidance response in senescence-accelerated mice. Pharmacol. Biochem. Behav. 51, 623–626.

Sweeney, J.E., Bachman, E.S., Coyle, J.T., 1990. Effects of different doses of galanthamine, a long-acting acetylcholinesterase inhibitor, on memory in mice. Psychopharmacology (Berl.) 102, 191–200.

Szegletes, T., Mallender, W.D., Thomas, P.J., Rosenberry, T.L., 1999. Substrate binding to the peripheral site of acetylcholinesterase initiates enzymatic catalysis. Substrate inhibition arises as a secondary effect. Biochemistry 38, 122–133.

Terry Jr., A.V., Buccafusco, J.J., 2003. The cholinergic hypothesis of age and Alzheimer's disease-related cognitive deficits: recent challenges and their implications for novel drug development. J. Pharmacol. Exp. Ther. 306, 821–827.

Van Dam, D., Abramowski, D., Staufenbiel, M., De Deyn, P.P., 2005. Symptomatic effect of donepezil, rivastigmine, galantamine and memantine on cognitive deficits in the APP23 model. Psychopharmacology (Berl.) 180, 177–190.

Viegas Jr., C., Bolzani, V.S., Barreiro, E.J., Young, M.C., Furlan, M., Tomazela, D., Eberlin, M.N., 2004. Further bioactive piperidine alkaloids from the flowers and green fruits of *Cassia spectabilis*. J. Nat. Prod. 67, 908–910.

Viegas Jr., C., Bolzani, V.S., Pimentel, L.S., Castro, N.G., Cabral, R.F., Costa, R.S., Floyd, C., Rocha, M.S., Young, M.C., Barreiro, E.J., Fraga, C.A., 2005. New selective acetylcholinesterase inhibitors designed from natural piperidine alkaloids. Bioorg. Med. Chem. 13, 4184–4190.

Weiner, S.J., Kollman, P.A., Case, D.A., Singh, U.C., Ghio, C., Alagona, G., Profeta, S., Weiner, P., 1984. A new force-field for molecular mechanical simulation of nucleic-acids and proteins. J. Am. Chem. Soc. 106, 765–784.

Weinstock, M., 1999. Selectivity of cholinesterase inhibition—Clinical implications for the treatment of Alzheimer's disease. CNS Drugs 12, 307–323.

Whitehouse, P.J., Price, D.L., Struble, R.G., Clark, A.W., Coyle, J.T., DeLong, M.R., 1982. Alzheimer's disease and senile dementia: loss of neurons in the basal forebrain. Science 215, 1237–1239.

Winblad, B., Kilander, L., Eriksson, S., Minthon, L., Batsman, S., Wetterholm, A.L., Jansson-Blixt, C., Haglund, A., 2006. Donepezil in patients with severe Alzheimer's disease: double-blind, parallel-group, placebo-controlled study. Lancet 367, 1057–1065.

Yoshida, S., Suzuki, N., 1993. Antiamnesic and cholinomimetic side-effects of the cholinesterase inhibitors, physostigmine, tacrine and NIK-247 in rats. Eur. J. Pharmacol. 250, 117–124.