Novel piperazine derivative PMS1339 exhibits tri-functional properties and cognitive improvement in mice

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Abstract

Amyloid-β-induced neuroinflammation plays a central role in the extensive loss of cholinergic neurons and cognitive decline in Alzheimer’s disease. The acetylcholinesterase (AChE) inhibitors are the first class of drugs used to enhance surviving cholinergic activities. However, their limited effectiveness following long-term treatment raises a need for new multi-target therapies. We report herein a novel piperazine derivative compound PMS1339 possesses multifunctional properties including anti-platelet-activating factor, AChE inhibition, Aβ aggregation inhibition and cognitive improvement. PMS1339 could significantly inhibit both mice brain AChE (IC50 = 4.41 ± 0.63 μM) and sera butyrylcholinesterase (BuChE, IC50 = 1.09 ± 0.20 μM). PMS1339 was also found to inhibit neuronal AChE secreted by SH-SY5Y cell line (IC50 = 17.95 ± 2.31 μM). Enzyme kinetics experiments performed on electric eel AChE indicated that PMS1339 acts as a mixed type competitive AChE inhibitor. Molecular docking studies using the X-ray crystal structure of AChE from Torpedo californica elucidated the interactions between PMS1339 and AChE: PMS1339 is well buried inside the active-site gorge of AChE interacting with Trp84 at the bottom, Tyr121 halfway down and Trp279 at the peripheral anionic site (PAS). Thioflavin T-based fluorimetric assay revealed the ability of PMS1339 to inhibit AChE-induced Aβ aggregation. In-vivo study indicated PMS1339 (1 mg/kg i.p.) reversed scopolamine-induced memory impairment in mice. Overall, these findings indicated that PMS1339 exhibits tri-functional properties in vitro and cognitive improvement in vivo, and revealed the emergence of a multi-target-directed ligand to tackle the determinants of Alzheimer’s disease.

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Key words: AChE inhibitor, Alzheimer’s disease, amyloid β, PAF antagonist, PMS1339.

Introduction

Alzheimer’s disease (AD) is a progressive neuro-degenerative disorder considered to be the leading cause of dementia affecting the elderly population. Clinically, this neuropathology is characterized by an insidious loss of memory and cognitive abilities in close connection with an impairment of brain cholinergic neurotransmission (Walsh & Selkoe, 2004).

Despite enormous research efforts devoted to AD so far, an effective treatment seems to be out of reach. The use of acetylcholinesterase (AChE) inhibitors to enhance surviving cholinergic activity is still the main therapy for Alzheimer’s patients. These compounds temporally aim to alleviate their disabilities in terms of improving cognition as well as behavioural and functional daily activities but do not stop the disease’s...
progression (Landmark & Reikvam, 2008; Onor et al. 2007; Raina et al. 2008). As a result, the classic single-target therapeutic approach solely based on the cholinergic hypothesis appears to be questionable and inappropriate to tackle a complex multifactorial syndrome such as AD, and other avenues are consequently being explored.

A new level of understanding about the molecular basis underlying the pathology of AD has highlighted its complexities and showed, along with loss of cholinergic neurons, the presence of additional hallmarks involving inflammation and oxidative stress which were reported to play a key role in AD pathogenesis and progression (Eikelenboom et al. 2006; Garcia-Alloza et al. 2009; Heneka & O’Banion, 2007; Uryu et al. 2002; Yao et al. 2004). Indeed, it is well established that all signs of inflammatory microglial and astroglial activation are present all around β-amyloid (Aβ) deposits and alongside axons of neurons with intracellular neurofibrillary tangles. Many converging lines of evidence suggest that platelet-activating factor (PAF), a potent pro-inflammatory mediator, is greatly implicated in those inflammatory events (Engelberts et al. 1991; Lo et al. 1997), particularly in brain injury (Bate et al. 2004; Bazan et al. 2002), thus promoting neuronal death and dementing disorders (Bate et al. 2006; Hershkowitz & Adunsky, 1996). As reported by Bulger et al. (2002), cytokine reduction occurs in a PAF-dependent manner.

Along with other investigators (Kwon et al. 2007; Rodriguez-Franco et al. 2006; Rosini et al. 2005, 2008; Yao et al. 2004; Zhu et al. 2009), we hypothesized that a more satisfactory clinical approach might be the design of compounds able to act simultaneously at different levels of the neurodegenerative process; these compounds should meet the innovative approach ‘one molecule, multiple targets’. In this regard, our research focused on the discovery of drugs that inhibit both AChE and PAF activities with the purpose not only of restoring acetylcholine (ACh) levels in the brain of AD patients but also of preventing the inflammatory process.

Previously, we have published the synthesis and preliminary in-vitro pharmacology of a series of 2,5-disubstituted tetrahydrofuran derivatives designed as dual PAF and AChE inhibitors (Le Texier et al. 1996). Among them, the promising compound PMS777 was shown to inhibit AChE activity, reverse scopolamine-induced dementia in mouse models, prevent PAF-induced neurotoxicity (Li et al. 2006) and LPS-induced oxidative/inflammatory disturbances in a human neuroblastoma cell lines (Ezoulin et al. 2005). Continuing our efforts to design novel multi-acting drugs with improved AChE inhibitory activity and anti-PAF property, in the present study we describe a new candidate molecule (PMS1339, MW 658.9, Fig. 1), one of the promising piperazine derivatives synthesized, which shares some structural similarities with PMS777. This molecule encompasses the chemical properties required for the dual activities, i.e. the presence of a quinolinium moiety as an AChE catalytic anionic site-binding element, and a piperazine moiety as a PAF receptor antagonism element connected to each other by a long methylene tether. The aim of this work was to further characterize the multifunctional properties of PMS1339 including anti-PAF, AChE inhibition, Aβ aggregation inhibition and cognitive improvement.

Methods

Animals

New Zealand White rabbits (2.5–3.0 kg) were provided by the Animal Center of Paris 7 University. Kunming mice (20–30 g) were purchased from Shanghai Jiao Tong University School of Medicine and were maintained on a 12-h light/dark cycle (lights on 06:00 hours) with free access to food and water. All studies were performed in accordance with the Guide for the Care and Use of Laboratory Animals as adopted and promulgated by the National Institutes of Health, USA.

Drugs and chemicals

PMS1339 was synthesized in our laboratory; tacrine (a mixed-mode inhibitor of AChE), the peripheral inhibitor propidium iodide and scopolamine were purchased from Sigma (USA) and used as reference compounds. Acetylthiocholine iodide (AcThCh), 5-butyrylthiocholine iodide (BTCh), 5,5′-dithio-bis-2-nitrobenzoic acid (DTNB), electric eel and recombinant human AChE were obtained from Sigma. Amyloid β (Aβ)40 peptide was purchased from Biosource (USA). Forebrain AChE and sera butyrylcholinesterase (BuChE) were obtained from Kunming mice. All other reagents were obtained from commercial sources.

Fig. 1. Chemical structure of PMS1339.
Inhibition of PAF-induced platelet aggregation

Blood from the carotid artery of New Zealand White rabbits was collected directly into 5-ml plastic syringes containing 3.8% (w/v) sodium citrate as anticoagulant to achieve a final volume proportion of 9:1 (blood-citrate). Platelet-rich plasma (PRP) was obtained from the supernatant fraction of blood after centrifugation at room temperature for 10 min at 800 rpm, and the platelet-poor plasma was obtained by centrifuging the remaining sedimentation for 10 min at 3000 rpm. The final cell count in PRP was adjusted to $4 \times 10^8$ platelets/ml with platelet-poor plasma.

Platelet aggregation was determined by the optical method of Born & Cross (1963) with an aggregometer at 37 °C under stirring (900 rpm). PRP was pre-incubated with vehicle or PMS1339 (0.1, 0.25, 0.5, 0.75, 1 μM) for 5 min at 37 °C. Then platelet aggregation was induced by the addition of PAF at a final concentration of 0.1 μM. Inhibition of aggregation at 5 min was calculated by comparing the extent of aggregation in the presence of PMS1339 with that in the presence of vehicle alone. The average inhibition rate for each concentration was calculated in order to determine the IC$_{50}$ value (the concentration required to inhibit platelet aggregation by 50%).

Cholinesterase inhibitory activity in vitro

AChE and BuChE activities were determined according to a modified Ellman’s method (Ellman et al. 1961) using mouse forebrain (AChE) and sera (BuChE) of Kunming mice. Briefly, mouse forebrain homogenates [1:9 w/v in 0.05 M phosphate buffer solution (PBS)] or sera (1:19 w/v in 0.05 M PBS) were pre-incubated with PMS1339 or tacrine for 20 min at 37 °C in 0.05 M PBS (pH 7.2), containing 0.25 mM DTNB. The substrates, 0.5 mM AcThCh or BTCh, were then quickly added. The reaction was terminated by the addition of 100 μl eserine (1 mM), and the colour production was measured spectrophotometrically at 412 nm.

The percent inhibition of the enzyme reaction in the presence of the inhibitors was determined by comparison with control, and the average inhibition rate for each concentration was calculated in order to deduce the IC$_{50}$ value (the inhibitor concentration required for 50% inhibition of AChE activity) for each test drug.

Determination of kinetic parameters

To estimate the kinetic parameters $K_m$ (Michaelis constant), apparent $K_m$ ($K_m$,app), $V_{max}$ (maximum velocity of reaction) and apparent $V_{max}$ ($V_{max}$,app), the enzyme activity of electric eel AChE was determined at substrate (AcThCh) concentrations ranging from 0.033 to 0.167 mM in the absence and in the presence of two concentrations of PMS1339 (0.33 and 1.33 μM). The $K_m$ and $V_{max}$ values for AChE were calculated by regression analysis of Lineweaver–Burk plots ($1/velocity$ vs. $1/[substrate]$).

Molecular modelling

To explore the binding mode of PMS1339 in AChE active site, a molecular modelling study was performed employing the docking protocol GOLD (Jones et al. 1997) on the basis of the X-ray crystallographic structure of Torpedo californica AChE (TcAChE).

Molecular simulations were performed on an R14000 SGI Fuel workstation with software package SYBYL 6.9 (Tripos Inc., USA). Standard parameters were used unless otherwise indicated. The coordinates of TcAChE structure were obtained from the donepezil (E2020)/TcAChE complex [Protein Data Bank (PDB; Berman et al. 2000) code 1EVE] (Kryger et al. 1999). Hetero-atoms and water molecules were removed from the PDB file, and all hydrogen atoms were added subsequently. The structure of PMS1339 was generated in SYBYL, and Gasteiger–Hückel partial charges were assigned. The resultant input ligand was obtained after 1000 steps of energy minimization using Tripos force field.

Molecular docking was performed using GOLD 3.0 (Cambridge Crystallographic Data Centre, UK) (Jones et al. 1997). The active site was defined as residues with at least one atom within a radius of 10 Å from any atom of E2020. Thirty genetic algorithm (GA) runs were performed. For each GA run, the default GA settings were used. All conformations that GOLD generated were evaluated with the scoring function X-SCORE 1.2.1 (Wang et al. 2002).

Inhibition of AChE-induced Aβ aggregation

Aβ40 peptide was dissolved from the lyophilized powder in hexafluoro-2-iso-propanol (HFIP) to make a solution of 2 mg/ml. HFIP was removed by evaporation under vacuum. Then Aβ was dissolved in anhydrous dimethyl sulfoxide (DMSO) to make a 2.3 mM stock solution.

For each experiment, 2 μl of the 2.3 mM Aβ-DMSO stock solution was added to a final volume of 20 μl of samples containing different concentrations of tested inhibitors and incubated for 48 h at room temperature. Aβ was diluted from the stock solution into 0.215 mM PBS (pH 8.0) (resulting in 10% residual DMSO) to make a final concentration of 230 μM Aβ.
For co-incubation experiments, aliquots (16 μl) of human recombinant AChE (Sigma-Aldrich) (final concentration 2.3 μM, Aβ/rHuAChE molar ratio 100:1) in the presence of 2 μl of the tested inhibitors at various concentrations were added. Blanks containing Aβ, rHuAChE, Aβ plus inhibitors at various concentrations in 0.215 M PBS (pH 8.0) were prepared. The final volume of each vial was also 20 μl. Inhibitor stock solutions (10 mM) were prepared in methanol.

Aggregation was followed by thioflavin T-binding technique. After incubation, the samples containing Aβ, Aβ plus rHuAChE or Aβ plus rHuAChE in the presence of inhibitors were diluted with 50 mM glycine-NaOH buffer (pH 8.5) containing 1.5 μM thioflavin T (Sigma-Aldrich) to a final volume of 2.0 ml. The fluorescence signal was monitored by a PerkinElmer LS45 fluorometer (excitation at 446 nm and emission at 490 nm). A time scan of fluorescence was performed during 300 s, and the intensity values were averaged after subtracting the background fluorescence from 1.5 μM thioflavin T and rHuAChE. The percentage inhibition of rHuAChE-induced aggregation due to the presence of increasing concentrations of the inhibitor was calculated by the following expression: 100 – (IFi/IFo × 100), where IFo and IFi are the fluorescence intensities obtained for Aβ or Aβ plus AChE in the presence and in the absence of inhibitor, respectively, after subtracting the fluorescence of respective blanks. Inhibition curves and linear regression parameters were obtained for compounds and the IC_{50} was extrapolated, when possible.

**In-vivo assessment of spatial memory**

The ability of PMS1339 to reverse scopolamine-induced spatial memory and recall deficits was evaluated in the Morris water maze as previously described (Terry, 2001). Briefly, a large black circular pool (140 cm diameter) was filled with water to a depth of 30 cm (maintained at 22 ± 1.0 °C). The pool was divided into four equal quadrants, and a black platform (9 cm diameter) was submerged ~1 cm below the surface of water in the centre of one quadrant (target quadrant). The platform was made invisible to the mice and remained in one location for the entire test. A high-resolution exview HAD camera (Shenzhen Hong Tianzhi Electronics Co. Ltd, China) was suspended over the centre of the pool, its images being monitored by a video-tracking system (Morris Water Maze Video Analysis System (DigBeh-MM), Shanghai Jiliang Software Technology Co. Ltd, China).

The male Kunming mice weighing 20–30 g were given four trials per day for four consecutive days, with a 30-s inter-trial interval. Each day, a trial was initiated by placing each mouse in the water facing the pool wall in one of the four quadrants. The daily order of entry into individual quadrants was randomized so that all four quadrants were used once every day. The mouse was allowed 90 s to locate the hidden platform. When successful, it was allowed a 30-s rest period on the platform, and the time spent in locating the platform was recorded as escape latency. If unsuccessful within the allotted time period, the mouse was assigned a latency of 90 s and then physically placed on the platform and also allowed a 30-s rest period. The escape latency and swim distance as well as swim paths were recorded by the video-tracking system.

On day 5, each mouse was subjected to a 60-s probe trial twice in which the hidden platform had been removed completely. The mouse was placed in the pool to swim for 60 s from the opposite and side position of the target quadrant respectively with a 30-s inter-trial interval. Time spent in the target quadrant and the proportions of swim distance in the target quadrant were determined by the analysis system, as well as total distance. Time percent and distance percent in the target quadrant were calculated, which were taken as a measure of spatial memory.

Scopolamine (0.5 mg/kg i.p.) was administered 20 min before the test once a day for all 5 d, and PMS1339 (0.1 and 1 mg/kg i.p.) was injected 10 min prior to scopolamine administration.

**Statistical analysis**

Data were expressed as mean ± S.E.M. Statistical significance of the observed differences was assessed by one-way analysis of variance (ANOVA). The statistical significance level was set at *p* < 0.05 or *p* < 0.01.

**Results**

**Inhibition of PAF-induced platelet aggregation**

*In vitro*, 0.1 μM PAF markedly induced rabbit platelet aggregation. PMS1339 significantly inhibited PAF-induced platelet aggregation in a concentration-dependent manner (Fig. 2), with an IC_{50} value of 332 ± 44 nM (*n* = 3). Tacrine, at the same concentrations, failed to exhibit any inhibition against PAF-induced platelet aggregation (data not shown).

**Cholinesterase inhibitory activities in vitro**

PMS1339 inhibited forebrain AChE and sera BuChE activities in mice in a concentration-dependent manner with an IC_{50} value of 4.41 ± 0.63 μM and
was 3.19 Å.

The nearest hydrophobic contact formed face-to-face hydrophobic interaction with the edge-to-edge hydrophobic interaction. The other contact formed a hydrogen bond with the nitrogen atom of Tyr121 side-chain located at the middle of the gorge. Distance between the hydrogen bond acceptor and donor was 2.61 Å.

Inhibition of AChE-induced Aβ aggregation

The preparation of the initial solutions of Aβ peptides is critical for the test and is meant to prevent any aggregation. HFIP alters the biological activity of Aβ by acting as a denaturant of proteins, breaking their secondary and tertiary structures and inducing α-helices. In 100% DMSO, Aβ appears to be monomeric (Snyder et al. 1994) and has no β-sheet character. Once diluted in PBS, the first step in Aβ self-assembly is the formation of β-sheet dimers from monomers. The residual DMSO has no significant effect on fibril growth (Shen & Murphy, 1995).

Our search for Aβ aggregation inhibition was prompted by the ability of PMS1339 to interact with the AChE peripheral anionic site that is well-known to induce Aβ aggregation. PMS1339 was found to inhibit rHuAChE catalytic activity with an IC50 value of 74.7 ± 6.3 nM (n = 6). The effects of PMS1339 have been compared to tacrine, a compound known as a mixed type AChE inhibitor, and also to propidium iodine, a purely non-competitive AChE inhibitor binding to the peripheral anionic site.

We first measured in-vitro rHuAChE-induced Aβ aggregation and it was shown that rHuAChE promotes Aβ aggregation by 155%. These data were results from two independent experiments. Control measurements showed PMS1339 and propidium exhibit no significant absorption at the wavelength of thioflavin excitation and have no direct effects on the fluorescence of this dye (data not shown). After 48 h incubation, PMS1339 significantly inhibited rHuAChE-induced Aβ aggregation displaying an apparent IC50 value of 45.1 µM. At 200 µM, PMS1339 showed an inhibition of 68.6% (Fig. 5). Propidium displayed an apparent IC50 value of 12.8 µM with an inhibition of 98% at 200 µM. Conversely, tacrine, at the same concentrations, did not show a significant inhibitory activity against the rHuAChE-induced Aβ aggregation (data not shown).

Reversal of spatial memory deficits in Morris water maze

During the first four consecutive training days, the escape latency and swim distance for animals to reach...
the platform gradually decreased over the training sessions (data not shown). In this experiment we chose the most appropriate dose of scopolamine (0.5 mg/kg i.p) to induce spatial memory deficits.

<p>| Table 1. Cholinesterase inhibitory activities of PMS1339 in forebrain (AChE) and sera (BuChE) of mice |
|--------------------------------------------------|--------------------------------------------------|---|---|</p>
<table>
<thead>
<tr>
<th>Drugs</th>
<th>IC_{50} (μM)</th>
<th>Selectivity IC_{50} BuChE/IC_{50} AChE</th>
</tr>
</thead>
<tbody>
<tr>
<td>PMS1339</td>
<td>17.95 ± 2.31</td>
<td>4.41 ± 0.63</td>
</tr>
<tr>
<td>Tacrine</td>
<td>4.63 ± 0.44</td>
<td>2.05 ± 0.28</td>
</tr>
</tbody>
</table>

AChE, Acetylcholinesterase; BuChE, butyrylcholinesterase. Each IC_{50} value represents the mean ± S.E.M. of the number of experiments indicated.

<p>| Table 2. Effect of PMS1339 on K_{m} and V_{max} of electric eel acetylcholinesterase activity |
|-----------------------------------------------|-----------------------------------------------|---|---|</p>
<table>
<thead>
<tr>
<th>PMS1339 (μM)</th>
<th>K_{m,app} (μM)</th>
<th>Increase (%)</th>
<th>V_{max,app} (μM/min)</th>
<th>Decrease (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>28.35 ± 3.00</td>
<td>0</td>
<td>94.32 ± 2.60</td>
<td>0</td>
</tr>
<tr>
<td>0.33</td>
<td>33.86 ± 0.44</td>
<td>16</td>
<td>70.80 ± 3.64</td>
<td>25</td>
</tr>
<tr>
<td>1.33</td>
<td>71.43 ± 2.20</td>
<td>152</td>
<td>32.43 ± 4.67</td>
<td>66</td>
</tr>
</tbody>
</table>

The values are the mean ± S.E.M. of three independent experiments.

As shown in Fig. 6a, PMS1339 (1 mg/kg) pre-treatment significantly increased scopolamine-induced reduction in time percent in the probe trial (p < 0.05, compared to scopolamine-treated mice). Similarly the same dose of PMS1339 also increased distance percent (p < 0.01, compared to scopolamine-treated mice) (Fig. 6b). The representative swim path for each tested group is presented in Fig. 6c. Compared to the control group, scopolamine-treated animals showed long and confused swim paths, which were improved by PMS1339.
Discussion

Advanced understanding of AD molecular basis revealed that its pathophysiology is complex and involves several different biochemical pathways. New targets have been identified such as Aβ-induced chronic neuroinflammation (Uryu et al. 2002; Yao et al. 2004). As a result of such findings, the therapeutic approach based only on the cholinergic hypothesis may need to be judiciously strengthened with additional non-anticholinergic functions. Further, despite modest benefits reached so far, clinical experience has shown that AChE inhibition remains a viable therapeutic approach to the palliative treatment of AD. Consequently, properly designed AChE inhibitors with the ability to prevent the inflammatory process should definitely lead to improved clinical outcomes. Given the role of PAF in the inflammation-induced neurodegeneration as well as the potency of AChE inhibitors to increase the availability of ACh at cholinergic synapses, we designed a new AChE inhibitor, which is a piperazine derivative compound hypothesized to combine AChE inhibition with anti-PAF activity.

In the present study we have demonstrated that PMS1339, contrary to tacrine, inhibits PAF-induced platelet aggregation. We interpret the potent blockade of PAF effects as evidence that PMS1339 may exert an inhibitory control over the Aβ-driven pathological cascade occurring in AD. Indeed, PAF was initially recognized as an essential component of the pathway that leads to neuronal death caused by Aβ (Bate et al. 2004, 2006). It is also interesting to note that both ginkgolide A (BN52020) and ginkgolide B (BN52021), known as PAF antagonists, were reported to provide an outstanding neuroprotection against Aβ toxicity. In addition, Ginkgo biloba extract Egb 761, which also contains ginkgolides, was shown to protect

Fig. 5. Determination of PMS1339 potency on human recombinant acetylcholinesterase (rHuAChE)-induced Aβ aggregation. Thioflavin T fluorescence of Aβ (230 μM) in 0.215 M PBS (pH 8.0) co-incubated with rHuAChE (2.3 μM) and PMS1339 (□) or propidium iodine (○) at increasing concentrations for 48 h, at room temperature. The values shown represent the mean ± S.E.M. of two independent experiments.

Fig. 6. Effects of PMS1339 (P) on time percent in target quadrant (a), distance percent in target quadrant (b) and swim path (c) in Morris water maze test in scopolamine (Scop)-treated mice. Scopolamine (0.5 mg/kg i.p.) was administered 20 min before the test once a day for all five trial days, and PMS1339 (0.1 and 1 mg/kg) was injected i.p. 10 min before scopolamine as well. Each value represents the mean ± S.E.M. (n = 12, * p < 0.05, ** p < 0.01), compared to scopolamine-treated group or control group.
Alzheimer’s patients against memory loss and cognitive disturbance (Bate et al. 2004; Kanowski & Hoerr, 2003; Mazza et al. 2006; Ramassamy et al. 2007).

Moreover, as shown in Table 1, PMS1339 exhibited AChE inhibitory activity in mice brain, as expected. It was found to be only 2-fold less potent than tacrine. PMS1339 also showed some abilities to inhibit AChE activity in a human neuronal-like cellular system (SH-SYSY cells). Interestingly, this result highlights its potency to exert an inhibitory effect on neuronal AChE.

The brain of mammals contains two major forms of cholinesterases (ChE): AChE and BuChE. In the healthy brain, AChE predominates and BuChE is considered to play a minor role in regulating brain Ach levels (Giacobini, 2003). Conversely, in the AD brain, BuChE activity progressively increases while AChE activity remains unchanged or progressively declines (Giacobini, 2003). To assess the selectivity profile of PMS1339 for ChE, we examined its effects on BuChE activity as well. Our results indicate that PMS1339 displays a modest selectivity for BuChE. Until recently, BuChE has been receiving considerable attention. Some controversies point to the interest of inhibiting BuChE which is responsible for peripheral cholinergic effects induced by AChE inhibitors (Liston et al. 2004). On the other hand, experimental studies, using drugs with enhanced selectivity for BuChE (cymselaine and analogues) and ChE inhibitors such as rivastigmine which have a dual inhibitory action on both AChE and BuChE, underline potential therapeutic benefits in AD and related dementias. Therefore the inhibition of both AChE and BuChE seems to be a promising approach (Darvesh et al. 2007; Eskander et al. 2005; Greig et al. 2005; Kamal et al. 2008). Thus, the observations emerging from our data suggest that by inhibiting both AChE and BuChE, PMS1339 should lead to improved clinical outcomes particularly in AD patients at advanced stages of the disease in which BuChE may replace AChE in hydrolysing brain ACh (Giacobini, 2003).

The present study not only highlights the AChE inhibitory activity of PMS1339 but also clarifies the mechanisms of action underlying this inhibition. Enzyme kinetics analysis helps to achieve this aim. Generally, examining Lineweaver–Burk plots of \frac{1}{V_{\text{max,app}}} vs. \frac{1}{[\text{substrate}]} as the inhibitor concentration increases, gives information about the type of inhibition. We observed both increasing slopes and increasing intercepts with higher PMS1339 concentrations which indicate that the kinetic behaviour causes \( K_m \) to increase and \( V_{\text{max}} \) to decrease as PMS1339 concentration increases. These data provide evidence that PMS1339 displays a mixed type inhibition, i.e. a combination of competitive and non-competitive inhibition and argue in favour of interactions of PMS1339 with both catalytic and peripheral binding sites of AChE.

To further understand the ligand–protein interactions that modulate the inhibitory activity of AChE, we performed molecular docking experiments. As shown in Fig. 4, it appears that PMS1339 interacts simultaneously with both catalytic and peripheral sites of AChE thanks to a linker of appropriate length showing a strong correlation with the observations we have from Lineweaver–Burk plots. Two highly conserved tryptophan residues are involved in these interactions: on the one hand the indole ring of Trp84, the principal element of the ‘anionic’ subsite of the catalytic site, with which the quaternary group of the choline moiety of ACh makes a cation–π interaction and on the other hand the indole ring of Trp279, the principal element of the peripheral ‘anionic’ site (PAS) at the entrance of the gorge, with which bisquaternary such as decamethonium and peripheral site ligands also make cation–π interactions (Silman & Sussman, 2005). PMS1339 was shown to align along the axis of the active-site gorge and interact with these two tryptophan residues. Contrary to ACh, the quaternary nitrogen group (quinolinium moiety) of PMS1339 interacts with AChE in the upper PAS showing greater affinity for Trp279 vs. Trp84. While the diphenylmethyl group and piperazine moieties are bound perfectly in the deep hydrophobic site. This finding seems somewhat intriguing because the quaternary nitrogen was initially predicted to interact with the active site. We also observed that the diphenylmethyl group and piperazine moiety, which are responsible for PAF antagonism, are also implicated in AChE inhibition. Our data suggest very interesting implications worthy of emphasis.

Biochemical studies indicated that AChE is involved in several secondary non-cholinergic functions such as accelerating Aβ peptide deposition and promoting Aβ fibril formation (Giacobini, 2003; Inestrosa et al. 2008; Silman & Sussman, 2005). Therefore, it has been speculated that the peripheral anionic binding site may be accountable for the aggregation-promoting action of AChE (Bartolini et al. 2003). Additionally, such an effect has been reported to be sensitive to drugs that block PAS of the enzyme (Castro & Martinez, 2006). As a result, in binding to the peripheral site PMS1339 may prevent Aβ fibrillogenesis. This hypothesis has naturally been tested to assess the potential anti-aggregating potency of PMS1339. In the co-incubation system, rHuAChE significantly
increased Aβ aggregation as previously reported. As expected, PMS1339 was found to prevent AChE-induced Aβ aggregation as determined by thioflavin T fluorescence, contrary to PMS777 (data not shown). This observation is consistent with the data obtained from the modelling study confirming the ability of PMS1339 to bind to AChE peripheral anionic site. The affinity of PMS1339 with PAS could thus be high to exert such an effect. This compound was shown to be about 2-fold less potent than propidium. The IC$_{50}$ value of propidium obtained for the blockade of rHuAChE-induced Aβ aggregation in our experimental conditions was comparable to that previously reported by Bartolini et al. (2003). Unlike PMS1339 and in accord with Bartolini’s report, tacrine, also known as a mixed type AChE inhibitor, did not show any significant inhibitory activity against rHuAChE-induced Aβ aggregation. This fact may be ascribed to the higher affinity of tacrine with the active site rather than with the peripheral one as suggested by Bartolini et al. (2003). These findings highlight a potential disease-modifying action for PMS1339 suggesting that novel non-selective AChE inhibitors could break the link between Aβ fibrillogenesis and its induced neurotoxicity, contrary to PMS777. Furthermore, PMS1339 has been demonstrated to be active in reversing scopolamine-induced memory impairment in mice as measured by decreases in time and distance percentages in probe trials in the Morris water maze, consistent with its potent AChE inhibition.

In conclusion, PMS1339 appears to be a compound able to interfere with different key targets of AD. Thus, it nicely meets the innovative approach ‘one molecule, multiple targets’. The new molecular structure of PMS1339 in comparison with PMS777 seems to have a deep influence on the inhibitory activities. The data obtained in this work clearly indicate that PMS1339 possesses a powerful AChE inhibitory activity with a mixed-mode competitive inhibition and displays a modest selectivity for BuChE in regard to AChE. Moreover, its higher anti-PAF property has been demonstrated thus expanding its biological profile and revealing potential additive pharmacological effects in terms of preventing inflammatory process, besides its capacity of ameliorating cholinergic level. Importantly, PMS1339 has been shown to interact strongly with the AChE peripheral anionic site and prevent rHuAChE-induced amyloid fibrillogenesis. Finally, PMS1339 reversed the scopolamine-induced memory impairment. Taken together, all these findings make this piperazine derivative compound a promising candidate for further studies as a disease-modifying agent and exciting prospects open up as it could interestingly offer a basis for the development of novel anti-amyloid compounds in connection with potential applications in the treatment of AD.

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Statement of Interest

None.

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