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Synthesis of the Alzheimer drug Posiphen into its primary metabolic products (+)-N¹-norPosiphen, (+)-N⁸-norPosiphen and (+)-N¹, N⁸-bisnorPosiphen, their inhibition of amyloid precursor protein, α -synuclein synthesis, interleukin-1 β , and cholinergic action

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Abstract

A major pathological hallmark of Alzheimer disease (AD) is the appearance in the brain of senile plaques that are primarily composed of aggregated forms of β -amyloid peptide (A β) that derive from amyloid precursor protein (APP). Posiphen (**1**) tartrate is an experimental AD drug in current clinical trials that reduces A β levels by lowering the rate of APP synthesis without toxicity. To support the clinical development of Posiphen (**1**) and elucidate its efficacy, its three major metabolic products, (+)-N¹-norPosiphen (**15**), (+)-N⁸-norPosiphen (**17**) and (+)-N¹, N⁸-bisnorPosiphen (**11**), were required in high chemical and optical purity. The efficient transformation of Posiphen (**1**) into these metabolic products, **15**, **17** and **11**, is described. The biological activity of these metabolites together with Posiphen (**1**) and its enantiomer, the AD drug candidate (–)-phenserine (**2**), was assessed against APP, α -synuclein and classical

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#This article is dedicated to our friend and collaborator Arnold Brossi (December 19, 1923 to July 16, 2011) whose lifetime work in the fields of medicinal and natural product chemistry aided in the development and continuing widespread use of numerous clinically valuable drugs, including the experimental Alzheimer drug Posiphen.

Conflicts of Interest

The authors declare that the AD experimental drugs Posiphen (**1**) and phenserine (**2**) were originally synthesized and developed by members of this collaborative team, but otherwise have no conflicts of interest regarding the contents of this manuscript.

cholinergic targets. All the compounds potently inhibited the generation of APP and α -synuclein in neuronal cultures. In contrast, metabolites **11** and **15**, and (–)-phenserine (**2**) but not Posiphen (**1**) or **17**, possessed acetylcholinesterase inhibitory action and no compounds bound either nicotinic or muscarinic receptors. As Posiphen (**1**) lowered CSF markers of inflammation in a recent clinical trial, the actions of **1** and **2** on proinflammatory cytokine interleukin (IL)-1 β human peripheral blood mononuclear cells was evaluated, and found to be potently inhibited by both agents.

Keywords

Acetylcholinesterase; Alzheimer disease; amyloid- β peptide (A β); amyloid- β precursor protein (APP); Posiphen; (+)-N¹-norPosiphen; (+)-N⁸-norPosiphen; (+)-N¹,N⁸-bisnorPosiphen; (–)-phenserine; α -synuclein; interleukin-1 β ; muscarinic receptor; nicotinic receptor; butyrylcholinesterase; Parkinson's disease

1. Introduction

Alzheimer's disease (AD), a progressive neurodegenerative condition resulting in memory loss and neuropsychiatric disturbances, is the most common form of dementia. It accounts for some 64% of all dementias and afflicts more than 30 million people worldwide [1,2]. This number is expected to escalate to in excess of 37 million by 2025. Its economic burden and impact on the quality of life of both patients and their caregivers are overwhelming [1]. Existing therapeutic options available for AD remain limited and numerous recent AD experimental drugs have failed to demonstrate efficacy [3]. The only available approved drugs are three cholinesterase inhibitors (ChEIs): donepezil (Aricept, Eisai/Pfizer), rivastigmine (Exelon, Novartis), and galantamine (Reminyl, Janssen), and memantine, a NMDA glutamate receptor antagonist (Namenda, Merz/Forest) that is increasingly administered in combination with one of the ChEIs [4,5]. Whereas these agents have demonstrated functional improvements in behavioral and cognitive measures in mild to moderate AD, such clinical improvements are recognized to be only marginal. These drugs in combination or alone appear to ameliorate disease symptoms, but do not impact disease progression and provide only minimal cost-benefit tradeoffs [6].

A key pathological hallmark of AD is the appearance within brain of senile plaques that are largely composed of aggregated forms of amyloid- β peptide (A β), a predominantly 38 to 42 amino acid length peptide that is derived from amyloid- β precursor protein (APP) following its proteolytic processing [2,7]. Numerous genetic associations between APP and familial AD (FAD) have focused APP into the center of AD research [7,8]. In general, FAD is accompanied by elevated levels of the longer A β ₄₂ form that readily aggregates to generate neurotoxic oligomers and fibrils that have been hypothesized to initiate a toxic cascade to eventually induce AD [2,7]. A further feature of AD is tau, a cytoplasmatic protein that binds to tubulin during its polymerization to, thereby, stabilize microtubules. Abnormal phosphorylation of tau results in the generation of aggregates (neurofibrillary tangles) that, likewise, are toxic to neurons [9]. These pathological changes, which include synaptic loss, brain atrophy and neuroinflammation, likely occur over one or two decades prior to outward loss of intellectual capacity [1,2,7]. Soluble forms of A β oligomers have been found to target

synapses, impair memory, drive tau phosphorylation, induce oxidative stress and cause significant toxicity in both neuronal cultures and the brain of preclinical animal models, and hence represent a major target for drug development in AD treatment [1,2,7,8].

Strategies to reduce A β levels in brain primarily fall into two approaches and include those to increase its clearance, as achieved by both active and passive monoclonal antibodies against A β , and to lower the initial rate of A β generation [2,7]. To achieve the latter, drug design has primarily focused on the discovery of agents to inhibit the activities of either β - or γ -secretases, that are involved in the cleavage of APP to yield A β [2,7]. An alternative method to achieve the same aim is to reduce the synthesis of APP and, thereby, lower all its proteolytic products, including A β as well as N- and C-terminal fragments of APP, N-APP and C-31, that have likewise been reported to be neurotoxic [2,7].

Posiphen ((+)-phenserine) (**1**) tartrate is a small molecular weight (mw 487.5), lipophilic (Log D value 2.2) experimental AD drug that reduces A β levels by lowering the rate of APP synthesis without toxicity [9–11]. APP levels are endogenously regulated at both the transcriptional and post-transcriptional levels. Post-transcriptionally, APP mRNA is efficiently translated and can be regulated at the level of its 5'-untranslated region (5'-UTR). Posiphen (**1**) acts to lower the rate of APP synthesis via this 5'-UTR [11–13]. This translates into a concentration-dependent reduction in both APP protein as well as A β ₄₂ levels by up to 50% in neuronal cultures and mice [10–12]. A key regulatory element within the 5'-UTR of APP mRNA is an iron response element (IRE) via which elevated brain iron concentrations and the proinflammatory cytokine interleukin-1 β (IL-1 β) are reported to up regulate [14], and Posiphen and (–)-phenserine to down regulate APP translation [11,13]. Importantly, this IRE stem loop structure is largely replicated within the 5'-UTR of α -synuclein mRNA [15] and similarly functions to confer iron dependent post-transcriptional control [16], thereby providing a means to potentially allow Posiphen (**1**) and (–)-phenserine (**2**) to lower α -synuclein levels; an important target in Parkinson's disease [17–19] for which drugs that impact disease progression also are lacking. Posiphen (**1**) has proved to be well tolerated in both rodents and dogs in preclinical toxicological studies and, likewise, was well tolerated in healthy elderly volunteers after oral dosing as well as in patients with mild cognitive impairment (MCI) [20]; widely considered to be an early prodromal stage of AD. In these MCI studies **1** effectively lowered brain APP levels, as assessed by time-dependent analysis of CSF before and after Posiphen (**1**) administration [20], providing proof of mechanism of the agent as an APP synthesis inhibitor in humans. Interestingly, it likewise lowered both total and phosphorylated tau levels and markers on neuroinflammation [20].

Although Posiphen (**1**) lacks cholinesterase inhibitory activity [10] its dose-limiting action in humans (nausea and vomiting at 160 mg single dose administration [20]) could theoretically be cholinergically mediated. Ex vivo hepatocyte incubation studies of Posiphen (**1**) indicate that it generates the same three primary metabolites across rodents, dogs and humans, and initial pharmacokinetic studies in rodents and humans [20] confirm the presence of these same primary metabolites: the N-demethylated (+)-N¹-norPosiphen (**15**), (+)-N⁸-norPosiphen (**17**) and (+)-N¹,N⁸-bisnorPosiphen (**11**). The reported acetylcholinesterase (AChE) inhibitory action of **15** (Table 1), in particular (IC₅₀ 63.4 $\pm$ 4.4 nM), may thereby provide Posiphen (**1**) indirect cholinergic action, mediated via its metabolism. By contrast,

the chirally pure and separate drug (–)-phenserine (**2**), the antipodal isomer of Posiphen (**1**) (Figure 1), provides anticholinesterase activity as its primary action (AChE IC₅₀ 24.0±6.0 nM) (Table 1) [21,22], but likewise reduces Aβ levels by lowering the rate of synthesis of APP in an equipotent manner to Posiphen (**1**) [10,11]. Additionally, compounds **1**, **2**, **11**, **15** and **17** could potentially possess muscarinic or nicotinic binding, which has not previously been measured but has been reported for (–)-physostigmine (**18**) [23–25]. (–)-Phenserine (**2**) has a lower maximally tolerated dose in humans (20 mg) [22] than Posiphen (**1**), reached phase 2/3 clinical trials in mild to moderate AD [26–28], and is currently being reformulated to optimize its clinical efficacy [29].

To support the clinical development of Posiphen (**1**) and elucidate its efficacy, the three primary metabolic products, (+)-N¹-norPosiphen (**15**), (+)-N⁸-norPosiphen (**17**) and (+)-N¹,N⁸-bisnorPosiphen (**11**) are required in quantity with high chemical and chiral purity. Our generation of these allowed a comparative assessment of their AChE inhibitory activities versus our initial appraisal following the original synthesis of the compounds more than a decade ago (Table 1). In addition, we report their action to lower APP as well as α-synuclein levels and describe their cholinergic binding activity and ability to lower the release of proinflammatory IL-1β.

2. Results and discussion

2.1. Chemistry

Posiphen (**1**) is a phenyl carbamoyl analogue of (+)-physostigmine, and hence all methods allowing synthesis of the natural product, physostigmine (**18**), are technically useful in the preparation of Posiphen (**1**). Physostigmine (**18**) [30], a major alkaloid from *Calabar* beans, was first synthesized by Julian et al. [31] in 1935. Since that time, new synthetic routes have been continuously developed and published [32].

Our initial synthesis of Posiphen (**1**) was published in 1988, based on a modified Julian's method [33,34]. In this, the separation of the enantiomers was accomplished by the introduction of a chiral center to form diastereomers, followed by chromatographic separation [35]. Alternatively, separation of the optical isomers can be achieved via the actions of D-camphorsulfonic acid and D-tartaric acid to provide the intermediate amine [31b]. Two further modified Julian's syntheses for Posiphen (**1**) have been described: (i) where separation of racemic mixtures into the enantiomers was achieved on a preparative scale by use of a chiral stationary phase [36] and (ii) where the introduction of a chiral center was accomplished by phase transfer asymmetric alkylation [37]. Each of these methods is valuable and practicable. However, in the event that synthesis of both **1** and **2** are desired concurrently, then the classical prime Julian method with modifications that permit separation of the (±)-intermediate amine by crystallization of salts of optically active organic acids to obtain both the (–)- and (+) enantiomers, is superior. In the event that Posiphen (**1**), alone, is required, then phase transfer would be preferable [37].

The total syntheses of N¹,N⁸-bisnorPosiphen (**11**) [38], N¹-norPosiphen (**15**) [37] and N⁸-norPosiphen (**17**) [39] were separately published during 1998, 2003 and 1997, respectively. With plenty of clinical grade Posiphen (**1**) in hand as an available starting material, it was

unnecessary to obtain **11**, **15** and **17** by total syntheses. The transformation of Posiphen (**1**) into these was therefore achieved as follows (Scheme 1, 2, 3), wherein the synthetic chemistry relating to Posiphen (**1**) and metabolites (**11**, **15** and **17**) is, for the first time, brought together in a single place.

Posiphen (**1**) was transferred into (+)-eseroline (**3**) fumarate, in line with a known procedure used to successfully convert (–)-physostigmine (**18**) into (–)-eseroline [40]. Specifically, a butanol solution of **1** containing a catalytic amount of sodium was refluxed under strict nitrogen protection for 1 h. An equivalent hot butanol solution of fumaric acid was then added and, after cooling, almost quantitative fumarate of **3** was precipitated and collected by filtration.

Compound **3** was reacted with methyl iodide in the presence of KOH in DMSO to provide a N¹-quaternary amine, **4**, which, without isolation, was heated with benzyl bromide at 100 °C and stirred for approximately 1 h to generate (+)-N¹-benzylnoresermethole (**5**). This method was first utilized to allow the conversion of (–)-physostigmine (**18**) into (–)-physoverine [41] in which the quaternary amine was replaced by an oxygen atom by reaction with NaOH. The analytical data of **5** were identical to its known antipodal, with the exception that the optical rotation value was the opposite [42].

Compound **5** was transformed into compound **8** by O-demethylation and N-demethylation according to a procedure used in the synthesis of N¹,N⁸-bisnorcymserine [43], with simplification. In this regard, the mixture of intermediates **6** and **7** was directly transferred into H¹-benzyl-N⁸-noreseroline (**8**) in the absence of any tedious chromatographic separation step.

Reaction of compound **8** with phenyl isocyanate generated the required OH addition product, carbamate **9**, or the unwanted NH addition compound, urea **10**, depending on the specific catalytic conditions utilized. Specifically, to an ether solution of **8**, a catalytic amount of Na was added, and then an equivalent of phenyl isocyanate was added with stirring at r.t.. Thereafter, the reaction mixture was continually stirred for 5 min, and a small amount of water was added to quench this reaction. Following isolation of the ether layer, routine work up provided the unwanted urea, **10**, as a foam (90%). Alternatively, to the THF solution of compound **8** an equivalent of NaOEt was added and stirred until all solid was dissolved. Thereafter, the same procedure (as described above) yielded the required N¹-norcarbamate (**9**) as a foam (90%).

Debenzylation of compound **9** was accomplished by catalytical hydrogenation. In detail, the 2-propanol solution of compound **9** with a catalytical amount of Pd(OH)₂/C was placed in a hydrogenation bottle that was mounted on a hydrogenation apparatus and shaken overnight at r.t. to provide debenzyl compound **11**. The alike procedure applied to unwanted **10** resulted in generation of product **12**. The treatment of **11** with D-tartaric acid provided the corresponding D-tartrate.

The transformation of compound **5** into N¹-norPosiphen (**15**) was accomplished by a known procedure [37]. Specifically, **5** was treated with BBr₃ in CH₂Cl₂ to generate (+)-N¹-

norbenzyl eseroline (**13**), which was subsequently reacted with equivalent phenyl isocyanate to provide phenyl carbamate (**14**). Debenzylation of compound **14** by catalytic hydrogenation, as described above, gave N¹-norPosiphen (**15**) that then was transferred into its D-tartrate by the same procedure described for compound **11**.

Posiphen (**1**) was oxidized by pyridinium dichromate (PDC) in CH₂Cl₂ to form N⁸-norformyl crabamate (**16**), which then was reacted with HCl (10%) to provide N⁸-norPosiphen (**17**), according to a known procedure for transformation of (–)-phenserine (**2**) into (–)-N⁸-norphenserine [39]. The D-Tartrate of **17** was obtained by treatment of base **17** with D-tartaric acid, following the procedure described above.

2.2. Cholinesterase inhibitory activity, cholinergic receptor binding, and inhibitory APP, α-synuclein and IL-1β action

Analyses of the IC₅₀ values (50% inhibitory concentration) required to inhibit human AChE are shown in Table 1 for clinical grade Posiphen (**1**) and the newly prepared compounds **11**, **15** and **17**, versus the literature values from our original syntheses via the Julian route more than a decade ago. Notably, AChE preparations were generated from a freshly drawn blood sample provided by the exact same donor from which these enzyme were obtained to analyse the original synthesized compounds. Immediately noticeable is the lack of activity of the measured Posiphen (**1**) sample against AChE, which is in accord with the lack of anticholinesterase action of (+)-physostigmine, versus the prior literature. A very minor contamination of 0.1% of the active chiral enantiomer, (–)-phenserine (**2**), within Posiphen (**1**) could readily account for the greater activity, albeit still low AChE action, in the literature value of **1**. Based on this, the measured IC₅₀ values of compounds **11**, **15** and **17** (Table 1) likely are a closer approximation of these agents' true activities against AChE. Specifically, the (+)-N¹-nor (**15**) and (+)-N¹,N⁸-bisnorPosiphen (**11**) metabolites demonstrated AChE activity of possible clinical relevance that can be compared to both (–)-phenserine (**2**) (IC₅₀ 18.6 nM) and donepezil (IC₅₀ 22 nM) [44], albeit metabolite **11** reaches only low levels in humans [20]. The measured AChE activity of **15** and **11** was slightly more potent versus data from our original syntheses (Table 1: measured vs. literature values), and the (+)-N⁸-Nor metabolite (**17**) was devoid of anticholinesterase action. Additionally, assessment against human butyrylcholinesterase (BChE) (not shown in Table 1), a sister enzyme to AChE that co-regulates brain acetylcholine levels and that may provide different actions in brain and prove a valuable target for AD [45–47] indicated a total lack of inhibitory action of Posiphen (**1**) and all metabolites (**11**, **15**, **17**) at this site, which is in accord with our original analyses [37,38].

In light of reports that (–)-physostigmine (**18**) [23–25] and, in particular, that (+)-physostigmine [48] possess cholinergic agonist actions, compounds **1**, **2**, **11**, **15** and **17** were assessed for binding to both muscarinic and nicotinic receptors subtypes (undertaken at the University of N Carolina, Chapel Hill, NC, Psychoactive Drug Screening Program). As shown in Table 2, the compounds lacked activity at all muscarinic (M1, M2, M3, M4, M5) and nicotinic (α2β2, α2β4, α3β2, α3β4, α4β2, α4β4) subtypes studied.

Interestingly, as assessed at a single concentration of 5 μ M in SH-SY5Y cell cultures, all three Posiphen primary metabolites (**11,15,17**), together with **1** and **2**, lowered secreted APP levels (Table 1), which is in accord with the ability of **1** and **2** to lower APP in SH-SY5Y human neuroblastoma cells at concentrations from 0.2 to 20 μ M as well as in mice following their systemic administration [10]. A similar assessment of the ability of (–)-phenserine (**2**), Posiphen (**1**) and metabolites (**11,15,17**) to lower APP as well as α -synuclein levels was hence undertaken in primary cortical neuron cultures obtained from wild-type mice and α -synuclein transgenic mice [49]. α -Synuclein is a ~15 kDa protein that can aggregate to form insoluble fibrils characterized by Lewy bodies in the pathogenesis of neurodegenerative α -synucleinopathies [17], including the prevalent movement disorder Parkinson's disease, in a manner similar to protein misfolding and aggregation of A β in AD [50]. Notably, α -synuclein shares overlap in its 5'UTR with APP, particularly with regard to the presence of a unique RNA stem loop iron responsive element that mediates regulatory actions on its translation [15,16]. At a concentration of up to 1 μ M **1, 2, 11, 15** and **17** induced a substantial decline (up to 70%) in both APP and α -synuclein protein levels in primary neuron cultures. Recent phase 1 Posiphen clinical trials in subjects with MCI in which levels of **1, 11, 15** and **17** were quantified in plasma and CSF suggest that concentrations in the order of 1–5 μ M may be readily achievable in brain [20] and can effectively lower APP levels.

In light of the reduction in markers of neuroinflammation induced by Posiphen's (**1**) within the CSF of clinical subjects with MCI [20] the abilities of **1** and **2** were assessed to inhibit the release of the proinflammatory cytokine IL-1 β from freshly obtained peripheral blood mononuclear cells (PBMCs), as this cytokine is a prime initiator of inflammation, is elevated in AD [51], and elevated IL-1 β levels are reported to heighten APP levels and its processing to A β [14]. In supernatants of PBMCs that were stimulated with phytohemagglutinin (PHA), IL-1 β levels were significantly elevated by approximately two-fold, as assessed by ELISA. Posiphen (**1**) and (–)-phenserine (**2**) (0.1 to 10 μ M) substantially (approx 50%) inhibited this IL-1 β elevation. As shown in Figure 2, the mRNA expression of IL-1 β was also assessed in freshly isolated PBMCs to define mechanisms responsible for the observed changes. Higher IL-1 β mRNA expression was observed in PBMCs following PHA challenge that was largely normalized by both Posiphen (**1**) and (–)-phenserine (**2**).

In synopsis, (+)-N¹-norPosiphen (**15**), (+)-N⁸-norPosiphen (**17**) and (+)-N¹, N⁸-bisnorPosiphen (**11**), generated by the described route, are currently being utilized to aid characterization as well as optimize the pharmacokinetics and pharmacodynamics of Posiphen (**1**) in animal and human studies to define its efficacy in the clinical treatment of AD. Posiphen (**1**), and particular metabolites possess a range of pharmacologic actions that include inhibition of APP, α -synuclein and IL-1 β generation.

3. Conclusion

Posiphen (**1**), the opposite enantiomer of the distinct and separate anticholinesterase experimental AD drug (–)-phenserine (**2**) [10,21,26–28], has recently completed phase 1 clinical trials and a proof of concept study in subjects with MCI and demonstrated brain entry and engagement of its primary target, APP. It appears to lower APP production in

subjects, as assessed within CSF, by as much as 50% [20]. Synthesis of Posiphen's three major metabolic products, (+)-N¹-norPosiphen (**15**), (+)-N⁸-norPosiphen (**17**) and (+)-N¹, N⁸-bisnorPosiphen (**11**) indicates in the current study that APP lowering activity is shared by all, and hence this action in humans is likely mediated by the composite actions of the primary drug and its metabolites. Furthermore, this activity extends to the PD pathological protein, α -synuclein, for all agents as well as to (–)-phenserine (**2**). Additionally, Posiphen (**1**), and its opposite enantiomer, **2**, limit IL-1 β generation. Neither Posiphen (**1**) and metabolites (**11**, **15**, **17**) nor (–)-phenserine (**2**) possess muscarinic or nicotinic binding. However, **11** and **15**, but not **1** or **17**, have AChE inhibitory action, potentially providing Posiphen (**1**) indirect cholinergic action, albeit that Posiphen (**1**) time-dependently remains the primary drug species following its oral administration to humans followed by the generation of **17**, **15**, and only low levels of **11** (representing the percentages of the total area under the plasma time dependent curve of 52.6%, 24.1%, 19.8% and 3.4%, respectively) [20]. A recent study demonstrates that Posiphen (**1**) and metabolites (**11**, **15**, **17**) as well as (–)-phenserine (**2**) possess neuroprotective/neurotrophic actions at concentrations of clinical relevance [52]. These, together with the biological activities of the compounds described herein, provide Posiphen (**1**) and metabolites as well as phenserine (**2**) interesting pharmacological profiles that may prove beneficial in AD, MCI and PD on a number of levels.

4. Experimentals

4.1. Chemistry

Melting points (uncorrected) were measured with a Fisher-Johns apparatus. ¹H NMR, and ¹³C NMR were recorded on a Bruker (Bellevue, MA) AC-300 spectrometer. MS (m/z) data were measured on an Agilent 5973 GC-MS (CI). Elemental analyses were performed by Atlantic Microlab, Inc. (Norcross, GA). All reactions involving non-aqueous solutions were performed under an inert atmosphere.

4.1.1. (3a*R*)-1,3a,8-trimethyl-1,2,3,3a,8,8a-hexahydropyrrolo[2,3-*b*]indol-5-ol (**3**)

—(+)-Posiphen (5g, 14.8 mmol) was dissolved in 40 ml of 1-butanol and a small piece of metal sodium (about 5 mg) added. After refluxing for 1 h in a nitrogen atmosphere, a hot butanol solution (32 ml) of fumaric acid (2g, 17.4 mmol) was added and stirred for 10 minutes. The reaction mixture was left in the refrigerator overnight for complete crystallization. Filtration gave crystal (+)-eseroline fumarate (4.66 g 95%): mp, [α]_D, ¹HNMR were identical with literature [23].

4.1.2. (3a*R*)-1-benzyl-3a,8-dimethyl-5-methoxy-1,2,3,3a,8,8a-hexahydropyrrolo[2,3-*b*]indole (**5**)

—Compound **3** (2.89 g, 13.2 mmol) was dissolved in DMSO (15 ml), and KOH powder (2.22 g, 39.6 mmol) was added. The reaction mixture was stirred and cooled by a water bath, and MeI (3.76 g, 26.5 mmol) was added and stirred for 0.5 h. Thereafter, a further portion of MeI (3.76 g, 26.5 mmol) was added and the reaction mixture was stirred at r.t. for an additional 0.5 h. Finally, all excess MeI was removed by vacuum evaporation. Benzyl amine (2.84 g, 26.5 mmol) was added to the reaction mixture, which then was placed into an oil bath and, during stirring, was heated to 100°C for 1 h.

Following cooling, 25 ml of water was added and the mixture was extracted by ether (3 x 20 ml). The extracted solution was washed with brine, dried over Na_2SO_4 and concentrated to provide a residue that was crystallized from MeOH to obtain crystalline compound **5** (3.26 g, 80%): mp $[\alpha]_D^{23} = +52.0^\circ$ ($c = 0.1$, CHCl_3). The ^1H NMR and MS were identical with that of its known enantiomer [42].

4.1.3. (3a*R*)-1-benzyl-3a-methyl-1,2,3,3a,8,8a-hexahydropyrrolo[2,3-*b*]indol-5-ol (8**)**—To a solution of **5** (3.08g, 0.01 mol) in CH_2Cl_2 (150 ml) was added NaHCO_3 (2 g). The mixture was stirred vigorously and cooled in an ice bath. Pyridinium dichromate (PDC) (7.52 g, 0.02 mol) was then added, and the mixture maintained with stirring over an ice bath for 2 h. The reaction mixture was, thereafter, filtered and the resulting solid was washed with CH_2Cl_2 (100ml). This combined CH_2Cl_2 solution was then washed with water (3 x 100 ml), dried over Na_2SO_4 and concentrated under vacuum. The residue was purified by column chromatography (silica gel, petroleum ether : ethyl acetate = 4 : 1) to remove unreacted **5**, as the fastest component, and pyridine, as slowest component, to generate a mixture of **6** and **7** (1.8g). Prepared TLC afforded a small amount of pure **6** and **7**, sufficient to acquire ^1H NMR and MS data that proved to be identical to that of their enantiomers reported in the literature [43]. This mixture of **6** and **7** was subsequently dissolved in CH_2Cl_2 (150 ml) and BBr_3 (10 g, 0.04 mmol) in CH_2Cl_2 (15 ml), and was added dropwise with stirring and cooling by a water bath. Thereafter, the mixture was stirred for 1 h at r.t., followed by evaporation of solvent and BBr_3 under vacuum. To this residue, MeOH (50 ml) was slowly added during stirring and cooling in a water bath, and the mixture was thereafter stirred for a further 0.5 h at r.t. Evaporation of solvent provided a residue that was then dissolved in H_2O (100 ml). To the water solution, ether (200 ml) and a saturated aqueous solution of Na_2CO_3 (100 ml) was then added. This mixture was stirred overnight until the entire solid complex was hydrolyzed and the base dissolved within the ether layer. This ether layer was separated, and the aqueous layer was then washed with fresh ether (2 x 100 ml). The combined ether solution was then dried over Na_2SO_4 , and concentrated under vacuum to provide a residue that next was dissolved into dry THF (100 ml). To this THF solution, NaBH_4 (1.4 g, 0.037 ml) was added and then stirred in excess of 5 h at r.t. until the reddish color of the reaction mixture gradually changed to a clear light yellow, and a single spot was evident under TLC. Thereafter, water (40 ml) was added and stirred for approximately 20 min to dissolve solid material. The mixture was subsequently extracted by ether (3 x 150 ml), and the combined extract was then dried over Na_2SO_4 . Evaporation of solvent gave product **8** as a foam (1.12 g, 40%). The ^1H NMR and MS of **8** proved to be identical with that of its known enantiomer [43] with the optical rotation providing the expected opposite value.

4.1.4. (3a*R*)-1-benzyl-3a-methyl-1,2,3,3a,8,8a-hexahydropyrrolo[2,3-*b*]indol-5-yl N-phenyl carbamate (9**)**—To the solution of compound **8** (280.4 mg, 1.0 mmol) in THF (8 ml), NaOAc (82 mg, 1.0 mmol) was added. After stirring for 5 minutes, phenyl isocyanate (107 mg, 0.9 mmol) was added dropwise. The mixture was then stirred at r.t. for 10 minutes, and 1.0 ml of water was added to quench the reaction. The reaction mixture was partitioned into ether and water, the organic layer was washed with water, brine, and dried over Na_2SO_4 . Evaporation of solvent gave a crude product that was directly chromatographed (silica gel, EtOAc / petroleum ether = 1/3) to give compound **9** (359mg, 90%) as a foam: ^1H NMR

(CDCl₃) δ 7.50–7.10 (m, 10H, Ar-H), 6.85 (s, 1H, C4-H), 6.78 (dd, J = 1.7, 8.5 Hz, 1H, C6-H), 6.50 (d, J = 8.5 Hz, 1H, C7-H), 4.54 (s, 1H, C8a-H), 3.85–3.90 (m, 2H, CH₂-Ph), 2.70–2.80 (m, 2H, C2-H₂), 1.90–1.80 (m, 2H, C3-H₂), 1.45 (s, 3H, C3a-CH₃) ppm; CI-MS (CH₄), m/z : 281(MH⁺-PhNCO), 120 (PhNCO+1).

4.1.5. (3a*R*)-1-benzyl-5-hydroxy-3a-methyl-*N*-phenyl-1,2,3,3a-tetrahydropyrrolo[2,3-*b*]indole-8(8a*H*)-carboxamide (10)—

To the solution of compound **8** (280.4 mg, 1.0 mmol) in ether (8 ml), a piece of metal Na (about 1 mg) was added. After stirring 5 minutes, phenyl isocyanate (107mg, 0.9 mmol) was added dropwise. The mixture was again stirred at r.t. for 10 minutes, and 1.0 ml of water was then added to quench the reaction. After washing the reaction mixture with water and brine, and drying over Na₂SO₄, evaporation of solvent provided a crude product that was directly chromatographed (silica gel, EtOAc / petroleum ether = 1/3) to give compound **10** (359 mg, 90%) as a foam: ¹HNMR (CDCl₃) δ 8.82 (s, 1H, 7.50–7.10, urea NH), 7.80–6.60 (m, 13H, Ar-H), 5.08(s, 1H, C8a-H), 4.10 and 3.90 (AB, J =16.6 Hz, 2H, CH₂-Ph), 2.90 (m, 2H, C2-H₂), 2.25 (m, 1H, C3-H_a), 2.00 (m, 1H, C3-H_b), 1.65 (s, 3H, C3a-CH₃) ppm; CI-MS (CH₄), m/z : 281(MH⁺-PhNCO), 120 (PhNCO+1).

4.1.6. D-Tartrate of (3a*R*)-3a-methyl-1,2,3,3a,8,8a-hexahydropyrrolo[2,3-*b*]indol-5-yl *N*-phenyl carbamate (11)—

A solution of compound **9** (100 mg, 0.25 mmol) in 2-propanol (5 ml) and Pd(OH)₂/C (10 mg) was placed into a hydrogenation bottle, which then was shaken in 40 psi at r.t. overnight. After the catalyst was filtered through celite, the filtrate was concentrated to provide a residue that was chromatographed (silica gel, CH₂Cl₂ / MeOH = 10 : 1) to generate compound **11** (62 mg, 80%). Compound **11** and an equivalent amount of D-tartaric acid then were dissolved in methanol, and evaporation of solvent gave a residue that was crystallized from ether to give the D-tartrate of compound **11**: m.p. 105–110 °C; [α]_D²³ = +66.6° (c = 0.1, acetone : H₂O = 1:1); ¹HNMR (CDCl₃) δ 10.02 (s, 1H, NH), 7.50 (d, J =8.5Hz, 2H, 2C2'-H), 7.30 (t, J =8.5Hz, 2H, 2C3'-H), 7.04 (s, 1H, C4-H), 7.00 (dd, J =1.7, 8.5Hz, 1H, C4'-H), 6.85 (d, J =8.5Hz, 1H, C6-H), 6.60 (d, J = 8.5 Hz, 1H, C7-H), 5.10 (s, 1H, C8a-H), 3.27 (m, 1H, C2-H_a), 2.75 (m, 1H, C2-H_b), 2.10 (m, 1H, C3-H_a), 2.04 (m, 1H, C3-H_b), 1.40 (s, 3H, C3a-CH₃) ppm. Anal. Calc. for C₁₈H₁₉N₃O₂·2C₄H₆O₆·H₂O·0.2Et₂O: C, 50.11; H, 5.49; N, 6.54. Found: C, 50.20; H, 5.80; N, 6.90.

4.1.7. D-tartrate of (3a*R*)-5-hydroxy-3a-methyl-*N*-phenyl-1,2,3,3a-tetrahydropyrrolo[2,3-*b*]indole-8(8a*H*)-carboxamide (12)—

Following a similar procedure involved in the generation of compound **11** from **9**, hydrogenative debenzylation of compound **10** provided compound **12** (80%) that then, likewise following the same procedure, was transferred to its D-tartrate: m.p. 132–136 °C; [α]_D²³ = –27.0° (c = 0.1, acetone : H₂O = 1:1); ¹HNMR (CDCl₃) δ 9.05 (s, 1H, NH), 7.45 (d, J =8.5Hz, 1H, C7-H), 7.30 (d, J =8.5Hz, 2H, 2C2'-H), 7.10 (dd, J =1.7, 8.7Hz, 2H, 2C3'-H), 6.80 (dd, J =1.7, 8.5Hz, 1H, C4'-H), 6.50 (s, 1H, C4-H), 6.40 (d, J =8.5Hz, 1H, C6-H), 5.15 (s, 1H, C8a-H), 2.90 (m, 1H, C2-H_a), 2.30 (m, 1H, C2-H_b), 1.85 (m, 1H, C3-H_a), 1.68 (m, 1H, C3-H_b), 1.22 (s, 3H, C3a-CH₃) ppm. Anal. Calc. for C₁₈H₁₉N₃O₂·1.6 C₄H₆O₆: C, 53.33; H, 5.25; N, 7.65. Found: C, 53.24; H, 5.20; N, 7.69.

4.1.8. D-Tartrate of (3a*R*)- 3a,8-dimethyl-1,2,3,3a,8,8a-hexahydropyrrolo[2,3-*b*]indol-5-yl N-phenyl carbamate (15)—Following a procedure within the literature [37], demethylation of compound **5** provided compound **14** (85%) that then was reacted with phenylisocyanate to generate compound **15** (90%). Utilizing the described methods to synthesize compound **11** from **9**, hydrogenative debenzoylation of **14** gave compound **15** (80%) that then was transferred in to its D-tartrate as similarly detailed above : m.p. 126–127 °C; $[\alpha]_D^{23} = +56.8^\circ$ ($c = 0.4$, EtOH); $^1\text{H NMR}$ (CDCl_3) δ 10.02 (s, 1H, NH), 7.55 (d, $J = 8.5\text{ Hz}$, 2H, 2C2'-H), 7.35 (t, $J = 8.5\text{ Hz}$, 2H, 2C3'-H), 7.10 (s, 1H, C4-H), 7.10 (m, 1H, C4'-H), 6.95 (d, $J = 8.5\text{ Hz}$, 1H, C6-H), 6.55 (d, $J = 8.5\text{ Hz}$, 1H, C7-H), 4.88 (s, 1H, C8a-H), 3.27 (m, 1H, C2-H_a), 2.75 (m, 1H, C2-H_b), 2.15 (m, 1H, C3-H_a), 2.04 (m, 1H, C3-H_b), 1.45 (s, 3H, C3a-CH₃) ppm. Anal. Calc. for $\text{C}_{19}\text{H}_{21}\text{N}_3\text{O}_2 \cdot 2.4\text{C}_4\text{H}_6\text{O}_6 \cdot \text{H}_2\text{O}$: C, 48.96; H, 5.17; N, 5.99. Found: C, 48.99; H, 4.99; N, 6.38.

4.1.9. D-Tartrate of (3a*R*)-1,3a-dimethyl-1,2,3,3a,8,8a-hexahydropyrrolo[2,3-*b*]indol-5-yl N-phenyl carbamate (17)—In accord with a procedure within the literature [39], oxidation of posiphen (**1**) gave compound **16** (35%) that then was reacted with HCl (10%) to provide compound **17** (70%). As described above, this then was transferred into its D-tartrate: m.p. 119–120°; $[\alpha]_D^{23} = +46.7^\circ$ ($c = 0.4$, EtOH); $^1\text{H NMR}$ (CDCl_3) δ 10.05 (s, 1H, NH), 7.52 (d, $J = 8.5\text{ Hz}$, 2H, 2C2'-H), 7.32 (t, $J = 8.5\text{ Hz}$, 2H, 2C3'-H), 7.10 (m, 1H, C4'-H), 7.00 (s, 1H, C4-H), 6.80 (d, $J = 8.5\text{ Hz}$, 1H, C6-H), 6.55 (d, $J = 8.5\text{ Hz}$, 1H, C7-H), 4.60 (s, 1H, C8a-H), 2.95 (m, 1H, C2-H_a), 2.75 (m, 1H, C2-H_b), 2.02 (m, 2H, C3-H₂), 1.40 (s, 3H, C3a-CH₃) ppm. Anal. Calc. for $\text{C}_{19}\text{H}_{21}\text{N}_3\text{O}_2 \cdot 1.5\text{C}_4\text{H}_6\text{O}_6$: C, 54.75; H, 5.51; N, 7.66. Found: C, 54.67; H, 5.88; N, 7.42.

4.2 Quantification of biological action

4.2.1. Anticholinesterase activity—The anticholinesterase activity of compounds **1,2,11,15** and **17** was assessed by quantifying their capacity to inhibit the ability of freshly prepared human AChE and BChE to enzymatically cleave their respective selective substrates, acetyl-(β -methyl)thiocholine and *s*-butyrylthiocholine (0.5 mmol/L) (Sigma Chemical Co., St. Louis, MO) [37–39]. Samples of AChE and BChE were prepared from freshly collected human erythrocytes and plasma, respectively. Compounds were dissolved in and then were diluted in 0.1 M Na₃P₀₄ buffer (pH 8.0) in half-log concentrations to provide a final concentration range that spanned 0.3 nM to 10 μM .

For the preparation of BChE, freshly collected blood was centrifuged (10,000 *g*, 10 min, 4° C) and plasma was separated and diluted 1:125 with 0.1 M Na₃P₀₄ buffer (pH 7.4). An absence of haemolysis was verified within the plasma to ensure no contamination with AChE. For AChE preparation, erythrocytes were washed five times in isotonic saline, lysed in 9 volumes of 0.1 M Na₃P₀₄ buffer (pH 7.4) containing 0.5% *Triton-X* (Sigma) and thereafter were diluted with an additional 19 volumes of buffer to a final dilution of 1:200.

Analysis of anticholinesterase activity was undertaken by utilizing a 25 μL sample of each enzyme preparation at their optimal working pH, 8.0 in 0.1 M Na₃P₀₄ buffer (0.75 mL total volume). **1,2,11,15** and **17**, alongside (–)-physostigmine (**18**) as an external control, were preincubated with enzymes (30 min, r.t.) and then were incubated with their respective

substrates and with 5,5'-dithiobis-2-nitrobenzoic acid (25 min, 37° C). The substrate/enzyme interaction was immediately halted by the addition of excess (–)-physostigmine (**18**) (1×10^{-5} M) and production of a yellow thionitrobenzoate anion was then measured by spectrophotometer at 412 nm λ . To correct for nonspecific substrate hydrolysis, aliquots were co-incubated under conditions of absolute enzyme inhibition (achieved by 1×10^{-5} M (–)-physostigmine (**18**)), and the associated alteration in absorbance was subtracted from that observed throughout the concentration range of each test compound. All agents were analyzed on a minimum of three separate occasions, each in duplicate. The mean enzyme activity at each concentration of compounds **1,2,11,15** and **17** was then expressed as a percent of the activity in the absence of compound. This was transformed into a logit format (in which $\text{logit} = \ln(\% \text{activity}/100 \text{ minus } \% \text{activity})$) and then was plotted as a function of its log concentration. Anticholinesterase activity was determined as an IC_{50} , defined as the concentration of compound (nM) required to inhibit 50% of enzymatic activity, as determined from a correlation between log concentration and logit activity. Results deriving from correlation coefficients of $r^2 \geq 0.98$ were considered acceptable, any not achieving this threshold were repeated.

4.2.2. APP and α -synuclein activity—Human SH-SY5Y neuroblastoma cells were acquired from the American Type Culture Collection (Manassas, VA) and cultured as previously described [10,53,54]. Cells were grown in complete media (10% fetal calf serum, 2 mM glutamine in DMEM and an antibiotic cocktail (Invitrogen)) to 70% confluence. Thereafter, spent media was removed and replaced with fresh media (DMEM) containing experimental drug. Primary neurons cultures from the cortices of PAC-Tg(SNCA) and wild type mice embryos were prepared from embryonic day 15 to 18 mice as previously described [55]. The PAC-Tg(SNCA) transgenic mice express the human *SNCA* gene translated via the bona fide *SNCA* 5'UTR [49,54]. Cortices were removed, dissected free of meninges, and dissociated in 0.025% trypsin. Cortical cells were plated onto poly-L-lysine (50 $\mu\text{g}/\text{ml}$)-coated 12-well or 48-well plates (Nunc) at a density of 600,000 cells/ cm^2 in DMEM supplemented with 10% FCS, 5% HS, and 10 $\mu\text{g}/\text{ml}$ gentamycin sulfate. The neurons were allowed to adhere for 2–3 hr before the plating medium was replaced with Neurobasal supplemented medium (serum free and with B27 minus antioxidants, 500 μM glutaMAX and 10 $\mu\text{g}/\text{ml}$ gentamycin sulfate). Neuronal purity of cultures was in the order of 90%–95%. On the day of experiments the medium was replaced with fresh Neurobasal-supplemented medium and for all further experimentation the medium was serum-free. Cells were challenged with compounds **1,2,11,15** and **17** (SH-SY5Y cells 5 μM , primary neurons 1 μM – and lower concentrations) overnight and probed for either APP or α -synuclein expression, as described previously [10,54]. Western immunoblotting was undertaken with either monoclonal mouse-anti-APP (22C11; Millipore, Billerica, MA) or mouse monoclonal anti- α -synuclein (BD Transduction Laboratories, Lexington, KY), and monoclonal mouse-anti- β -actin (Sigma) was utilized as an internal control. Thereafter, blots were probed with the appropriate horseradish peroxidase-conjugated antibody (Pierce, Rockford, IL) and detected by ECL techniques (GE Healthcare, Piscataway, NJ).

4.2.3. Cholinergic receptor binding—The receptor binding profiles of **1,2,11,15** and **17** were generously provided by the National Institute of Mental Health's Psychoactive Drug

Screening Program (Contract # HHSN-271-2008-00025-C (NIMH PDSP). The NIMH PDSP is directed by Bryan L. Roth M.D., Ph.D at the University of North Carolina at Chapel Hill and Project Officer Jamie Driscoll at NIMH, Bethesda MD, USA). For experimental details please refer to the PDSP web site <http://pdsp.med.unc.edu/> (click on “Binding Assay”).

Briefly, binding to human muscarinic receptors, M1, M2, M3, M4, M5, was assessed in stably transfected cell lines in muscarinic binding buffer (50 mM Tris-HCl, pH 7.7) in quadruplicate using a protocol adapted from Ernsberger and colleagues [56]. For all muscarinic subtypes, [³H]QNB ([³H]quinuclidinyl benzilate) (final concentration 0.5 nM) was used as the radiolabel and atropine (10 pM to 10 μ M) as the reference compound in a 96-well plate format. Membrane fractions of cells expressing recombinant targets were added together with test compounds (10 μ M), 250- μ L reactions were incubated at room temperature (shielded from light) for 1.5 hours, that then were harvested by rapid filtration onto Whatman GF/B glass fiber filters pre-soaked with 0.3% polyethyleneimine using a 96-well Brandel harvester. Four rapid 500- μ L washes were performed with chilled standard binding buffer to reduce non-specific binding. Filters were placed in 6-ml scintillation tubes and allowed to dry overnight. Thereafter, 4 ml of EcoScint scintillation cocktail (National Diagnostics) was added to each tube that was capped, labeled, and counted by liquid scintillation counting.

In a similar manner, binding of test compounds (10 μ M) was assessed to human nicotinic receptors, α 2 β 2, α 2 β 4, α 3 β 2, α 3 β 4, α 4 β 2, α 4 β 4, in quadruplicate using an assay adapted from Xiao and colleagues [57]. For all nicotinic subtypes, [³H]-epibatidine (final concentration 0.5 nM) was used as the radiolabel and (–)-nicotine (10 pM to 10 μ M) as the reference compound.

4.2.4 Separation of peripheral blood mononuclear cells, challenge with PHA and assessment of IL-1 β release—The Separation and stimulation of PBMCs was performed as described elsewhere [58]. The PBMC fraction was obtained by density centrifugation of diluted blood (1 part blood to 1 part pyrogenfree saline) over Ficoll-Paque (GE Healthcare, EuroClone, Pero (MI) Italy). PBMCs were washed twice in saline and suspended in culture medium (RPMI-1640, Sigma) supplemented with 10% fetal calf serum, L-glutamine, and PES (Sigma, St. Louis, MO). The cells were counted and their concentration was adjusted to 1x 10⁶ cells/ polypropylene round-bottom tube (Becton Dickinson, Franklin Lakes, NJ). Cell viability, assessed by Trypan blue dye exclusion, was not significantly influenced by the cell culture conditions, and more than 98% of PBMCs were viable at the end of incubation. After a 24 hr incubation at 37°C, in the presence an absence of PHA (20 μ g/ml (Sigma/Aldrich) – a dose chosen for its ability to aspecifically induce release of proinflammatory cytokines) cell-free supernatants were removed and stored at –80°C until assay, and pelleted cells were similarly kept until analysis. IL-1 β concentration in culture supernatants was determined by a solid phase sandwich ELISA kit with monoclonal anti-human IL-1 β (Endogen, Woburn, MA, USA).

Total RNA was extracted from PBMC cell cultures using TRIzol reagent (Invitrogen, Life Technologies, Paisley, U.K.), according to the manufacturer’s protocol. The RNA

concentration was estimated by measuring the absorbance at 260 nm λ using a Bio-Photometer (Eppendorf AG, Hamburg, Germany), and RNA samples were kept frozen at -80°C until use. Purified RNA was electrophoresed on a 1% agarose gel to assess the integrity of the purified RNA. One microgram of RNA was reverse transcribed into cDNA using a High Fidelity Superscript reverse transcriptase commercially available kit (Applied Biosystems, Foster City, CA, USA), in accord with the manufacturer's instructions. mRNA/cDNA specific cytokine primer pairs were designed and PCR was performed, as described previously [58].

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Abbreviations

(SNCA)	α -Synuclein gene
(AChE)	Acetylcholinesterase
(AD)	Alzheimer's disease
(A β)	amyloid- β peptide
(APP)	amyloid- β precursor protein
(BChE)	butyrylcholinesterase
(ChEIs)	cholinesterase inhibitors
(DMSO)	dimethylsulfoxide
(EtOAc)	ethyl acetate
(FAD)	familial Alzheimer's disease
(FCS)	fetal calf serum
(IL-1 β)	interleukin-1 β
(IRE)	iron response element
(MS)	mass spectroscopy
(MeOH)	methanol
(MeI)	methyl iodide, also called iodomethane
(MCI)	mild cognitive impairment

(NMR)	nuclear magnetic resonance
(Pd(OH) ₂ /C)	Pearlman's catalyst
(PBMCS)	peripheral blood mononuclear cells
(PHA)	phytohemagglutinin
(PDC)	pyridinium dichromate
(QNB)	quinuclidinyl benzilate
(NaOEt)	sodium ethoxide
(THF)	tetrahydrofuran
(TLC)	thin layer chromatography
(5'-UTR)	5'-untranslated region
(IC ₅₀) value	50% inhibitory concentration

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Figure 1.
Chemical structures of Posiphen (**1**), (-)-phenserine (**2**) and (-)-physostigmine (**18**).

Figure 2.

human peripheral blood mononuclear cells (PBMCs) were isolated by Ficoll-Hypaque density gradient centrifugation, maintained in cell culture and were then challenged with PHA (20 μ g/ml). The mRNA levels of IL-1 β normalized to those 18S mRNA in the corresponding samples are shown. Additionally, not shown, IL-1 β levels were measured with commercially available ELISA kits, were elevated by PHA and ameliorated by Posiphen (1) and phenserine (2).

Scheme 1.

Scheme 2.

Scheme 3.

50% Inhibitory Concentration (IC₅₀, nM, ± SEM) of Compounds Versus Freshly Prepared Human Erythrocyte AChE and Plasma BChE, and Activity to lower APP and α-Synuclein Expression

Table 1

No.	Compound	AChE (IC ₅₀ nM)		APP I* (5 uM)	α-Synuclein (1 uM)
		Measured [#]	Literature [@]		
1	(+)-Posiphen	>10,000	3500 ± 55	+	+
2	(-)-Phenserine	18.6 ± 0.3	24.0 ± 6.0	+	+
11	(+)-N ¹ ,N ⁸ -BisnorPosiphen	83 ± 9.0	231 ± 23	+	+
15	(+)-N ¹ -NorPosiphen	46 ± 6.0	63.4 ± 4.4	+	+
17	(+)-N ⁸ -NorPosiphen	>10,000	5655 ± 607	+	+

[#] Measured IC₅₀ values were determined from human AChE derived from erythrocytes, according to References 37–44. (Mean of 3 separate assays performed in duplicate ± standard error of the mean).

[@] Literature IC₅₀ data of compounds **1**, **2** and **17** are from Reference 37, the data of compound **11** is from Reference 38, and the data of **15** is from Reference 37.

* A reduction of APP levels (APP I (inhibition)) was assessed by Western blot analysis (APP mAb: 22c11) normalized against β-actin expression in cell culture studies according to Reference 10 at 5 uM in PC12 cells, a well characterized neuronal-like cell line derived from a pheochromocytoma of the rat adrenal medulla. This activity was confirmed in E18 (embryonic day 18) primary neurons prepared according to Reference 54, with methodology applied from References 13 and 53 in relation to α-synuclein (α-Syn) expression (mouse monoclonal anti-α-synuclein).

N/A: not assessed.

Table 2

Percent Inhibition Induced by Compounds at the Human Muscarinic and Nicotinic Receptors. Synopsis: no compounds demonstrated significant interaction with muscarinic or nicotinic receptor subtypes at 10 μ M

No.	Compound	% Inhibition Muscarinic Binding (at 10 μ M test compound)					% Inhibition Nicotinic Binding (at 10 μ M test compound)						
		M ₁	M ₂	M ₃	M ₄	M ₅	α 2 β 2	α 2 β 4	α 3 β 2	α 3 β 4	α 4 β 2	α 4 β 4	
1	(+)-Posiphen	0	0	1.5	0	0	0	0	0	0.9	0.5	4.7	
2	(-)-Phenserine	0	7.6	3.1	0.3	6.5	3.4	0	6.1	12.3	0.1	8.3	
11	(+)-N ¹ ,N ⁸ -BisnorPosiphen	0	9.8	0	0	0	0	0.5	0.8	0	2.6	5.1	
15	(+)-N ¹ -NorPosiphen	0	0.1	0	30	1.0	0	0	0	0	0	2.4	
17	(+)-N ⁸ -NorPosiphen	0	0	4.5	0	0	0	0	0	0	0.2	1.1	

Data represent mean % inhibition (N = 4 determinations) for compounds tested at receptor subtypes.

Significant inhibition is considered as >50%, which was not found for any of the assessed compounds (1, 2, 11, 15 and 17), indicating that they do not bind the evaluated cholinergic receptors.