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From natural products to Alzheimer experimental therapeutics, eserine based drugs as symptomatic and disease altering agents

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Titolo
A partire da prodotti naturali per la terapia sperimentale nell'Alzheimer, farmaci a base di eserina come agenti in grado di alterare la malattia e la sintomatologia

Key words
Alzheimer's disease, eserine, physostigmine

Parole chiave
Malattia di Alzheimer, eserina, fisostigmina

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Summary
Based on the hexahydropyrroloindole backbone of the natural product and alkaloid, (-)-physostigmine (eserine) – a short-acting non-selective reversible cholinesterase inhibitor, three series of compounds were designed and developed to overcome its pharmacokinetic and pharmacodynamic shortfalls to provide experimental therapeutics for Alzheimer's disease (AD) and pharmacological tools to define brain function in health, aging and disease. Posiphen and analogues are amyloid-β precursor protein (APP) and amyloid-β peptide (Aβ) lowering agents that are cholinergically inert but generate metabolites with anticholinesterase action. (-)-Bisnorcymserine and analogues are potent, centrally active, reversible, selective butyrylcholinesterase inhibitors, whereas (-)-phenserine and analogues are similar but selective acetylcholinesterase inhibitors, and each additionally lowers APP and Aβ generation as a secondary action. Defining the clinical value of these agents in AD and related dementias is a key focus of current research.

Riassunto
Basandosi sullo scheletro esaidropirroloindolico del prodotto naturale e alcaloide (-)-fisostigmina (eserina), un inibitore reversibile non selettivo a breve durata d'azione della colinesterasi, sono state progettate e sviluppate tre serie di composti con duplice scopo: per superare le sue carenze farmacocinetiche e farmacodinamiche in modo da allestire terapie sperimentali per la malattia di Alzheimer (MA) e come strumenti farmacologici per definire le funzioni cerebrali in materia di salute, invecchiamento e patologia. Il Posiphen e analoghi, che promuovono l'abbassamento dei livelli della proteina precursore β-amiloide (APP) e del peptide β-amiloide (Aβ), sono colinergicamente inerti ma generano metaboliti con azione anticolinesterasica. (-)-Bisnorcymserina e analoghi sono potenti, attivi a livello centrale, reversibili, inibitori selettivi della butirilcolinesterasi, mentre (-)-fenserina e analoghi sono simili ma inibitori selettivi dell'acetilcolinesterasi, e ciascuno abbassa ulteriormente la produzione di APP e di Aβ come azione secondaria. Definire il valore clinico di questi agenti nella MA e nelle demenze correlate è un obiettivo chiave delle ricerche in corso.

Introduction

Natural products have been the wellspring of drugs and drug leads in medicine for over a thousand years. Indeed, some 61% of the 877 small molecule new chemical entities introduced as drugs worldwide between 1982 and 2002 can be traced to, or were inspired by, natural products (1). In the search for pharmacophores to support the development of new drugs for the treatment of Alzheimer's disease, the alkaloid and natural product, (-)-physostigmine (*eserine*) was chosen that derives from the Calabar bean, which is the seed of the vine, *Physostigma venenosum*, a leguminous plant native to tropical Africa. (-)-Physostigmine possesses an optically active hexahydropyrroloindole backbone (Fig. 1: A,B,C ring), to which is attached a methylcarbamate group that, together, provides the compound anticholinesterase activity. (-)-Physostigmine reversibly and unselectively potently inhibits both cholinesterase (ChE) forms present in mammals, acetylcholinesterase (AChE, EC 3.1.17) and butyrylcholinesterase (BuChE, EC 3.1.1.8). In contrast, the unnatural (+)-enantiomeric form lacks anticholinesterase activity (2). This, thereby, provides the unusual scenario of having a lead compound that provides the potential to design and generate not only selective inhibitors of the two cholinesterases forms, AChE and BuChE, but also cholinergically inert (+)-enantiomers to define and differentiate non-cholinergic actions of any generated compounds of pharmacological interest.

Alzheimer's disease – cardinal features

Alzheimer's disease (AD), a progressive, degenerative disorder of the brain, is the most common cause of dementia among the elderly. It is an age-dependent disease that impacts both developed and developing countries, and is characterized by an increasing impairment of memory that is accompanied by psychiatric disturbances. The major hallmarks of AD are a synaptic loss and abnormal protein deposition within brain, particularly of (i) toxic amyloid-β peptide (Aβ) that is derived from Aβ precursor protein (APP) by the action of β- and γ-secretase activities, as well as of (ii) neurofibrillary tangles (NFTs) comprised of hyperphosphorylated tau protein. The disease has a heterogeneous etiology with a high percent, termed sporadic AD, arising from unknown causes and a smaller fraction of early onset familial AD caused by mutations in one of several genes, such as from APP and presenilins (PS1, PS2) (3,4). A common feature of these numerous mutations is that they, albeit through different mechanisms, increase production of Aβ and, particularly, the longer more hydrophobic $A\beta_{42}$ form (3, 4). Soluble aggregates of Aβ, such as Aβ-derived diffusible ligands (ADDLs) (5) and the dodecameric (56 kDa) Aβ form (6), have been reported to target synapses, induce dysfunction and impair memory. These, features, together with processes involved in the generation and clearance of Aβ, in addition to neuroinflammation induced by abnormal Aβ and tau phosphorylation, are current targets in AD drug development.

The cholinergic system, AD and current treatment

Dysfunction and loss of cholinergic neurons that arise from the basal forebrain and terminate in the cortex and hippocampus are involved in the cognitive decline that occurs during aging as well as AD, and have developed into the "cholinergic theory of aging" (7). Impaired cortical cholinergic neurotransmission, characterized by the loss of classical cholinergic markers (e.g., choline acetyltransferase (ChAT), muscarinic and nicotinic acetylcholine (ACh) receptor binding as well as levels of ACh (8)) may not only substantially attribu-

te to cognitive impairment but additionally contribute to amyloid plaque pathology during AD by impacting the expression and processing of APP and, thereby, Aβ generation. In contrast, low levels of soluble Aβ both impair and inhibit cholinergic synaptic function as well as reduce ACh synthesis. Aβ additionally induces oxidative stress, up regulation of cytokines such as interleukin-1 (IL-1), attributing to neuroinflammation (9), over-expression of APP, and tau hyperphosphorylation (4), to provide a complex and self-propagating cycle of events that not only links together the cardinal features of AD but also drives disease progression.

The hydrolysis of synaptic ACh to end its physiologic action is central in the regulation of cholinergic neurotransmission and is achieved by the cholinesterase enzymes, AChE and BuChE, which cleave ACh to yield choline and acetic acid (10,11). Within brain, AChE is primarily associated with neurons, whereas BuChE is predominantly located in and secreted from glial cells (9, 10). However, particular neurons possess BuChE rather than AChE to cleave presynaptic ACh, with 10 to 15% of cholinergic neurons in human hippocampus and amygdala expressing BuChE rather than AChE (10,11). Until the recent design and development of safe, reversible and highly selective BuChE inhibitors that enter brain (Fig. 1), the role of BuChE in brain remained largely unknown.

Further to their role in normal cholinergic function, AChE and BuChE appear to play a part in the onset and progression of AD. AChE activity declines by some 45% in specific brain areas during AD progression, in line with ACh presynaptic loss. In contrast, BuChE activity concomitantly is elevated by up to 2-fold (11). A disproportion, hence, develops between reduced ACh synthesis/availability and one of its main hydrolysing enzymes, BuChE. Furthermore, both AChE and BuChE co-localize with the primary pathological features in AD brain, with amyloid plaques, dystrophic neurons and NFTs, and may interact physiologically. Indeed, recent studies suggest that AChE and BuChE have opposing roles to increase and reduce Aβ fibril formation (12), respectively, and thereby aggregation. Thus BuChE, in addition to and separate from AChE, represents a potential target for AD treatment (13).

Selective reversible inhibition of either AChE or BuChE

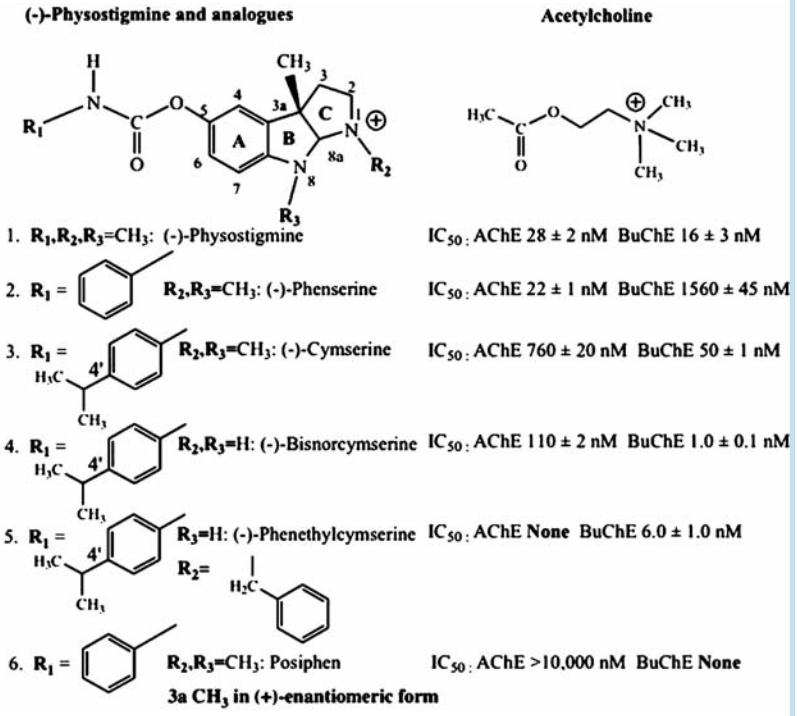
Characterization of the X-ray crystal structure of AChE and BuChE, together with site-directed mutagenesis, revealed that the active site involved in the hydrolysis of ACh was located at the bottom of a deep and narrow gorge, of some 20 Å, lined by conserved amino acids, that protrudes into both enzymes (10,11). The three-dimensional structure of both the gorge and binding domains in AChE and BuChE have been mapped and, although similar, can be differentiated based on differences in their amino acid sequence in critical areas, which permits drug design to exploit dissimilarities to design selective inhibitors. Notably, the gorge for BuChE is larger than AChE, as smaller aliphatic or polar residues in BuChE substitute six of the 14 bulky aromatic amino acid residues that delineate the active site gorge of AChE, particularly at the base of the gorge. The estimated gorge volumes are 500 $Å^3$ and 300 $Å^3$ for BuChE and AChE, respectively (11). This larger space within BuChE accounts for its more promiscuous binding and cleavage of substrates versus AChE, which is largely limited to ACh binding.

Extensive structure/activity relations together with pharmacokinetic/pharmacodynamic analyses resulted in the synthesis and choice of (-)-phenserine (Fig. 1) as an AChE-selective reversible inhibitor for clinical development. In rodents, the compound readily entered brain (brain/plasma ratio

10:1), and although rapidly disappearing from plasma and brain it achieved a long-term (half-life ($T_{1/2}$ 8.25 hr) inhibition of AChE with a selectivity of some 70-fold versus BuChE. It induced increases in brain extracellular levels of ACh, improved cognitive performance in multiple paradigms and was well tolerated in rodents and dogs (14, 15). The agent was tolerated in human studies in doses up to 15 mg BID, and showed efficacy in early AD clinical studies, but in larger AD studies, in which data proved more variable and there was a large placebo effect, differences between the treatment and placebo groups proved more difficult to achieve (15), albeit that efficacy was affirmed in a later smaller well-controlled AD study (16).

The development of (-)-cymserine from (-)-phenserine, by elongating the compound with a 4' (para) isopropyl substitution in the phenylcarbamate ring (Fig. 1), resulted in a compound with a small selectivity for BuChE over AChE of 15-fold. Extensive medicinal chemistry around (-)-cymserine generated a series of analogues with greater potency and selectivity. From this series, (-)-bisnorcymserine and (-)-phenethylcymserine (Fig. 1) were chosen for pharmacological profiling to define the role of BuChE in brain in health and disease. As indicated in Fig. 1,

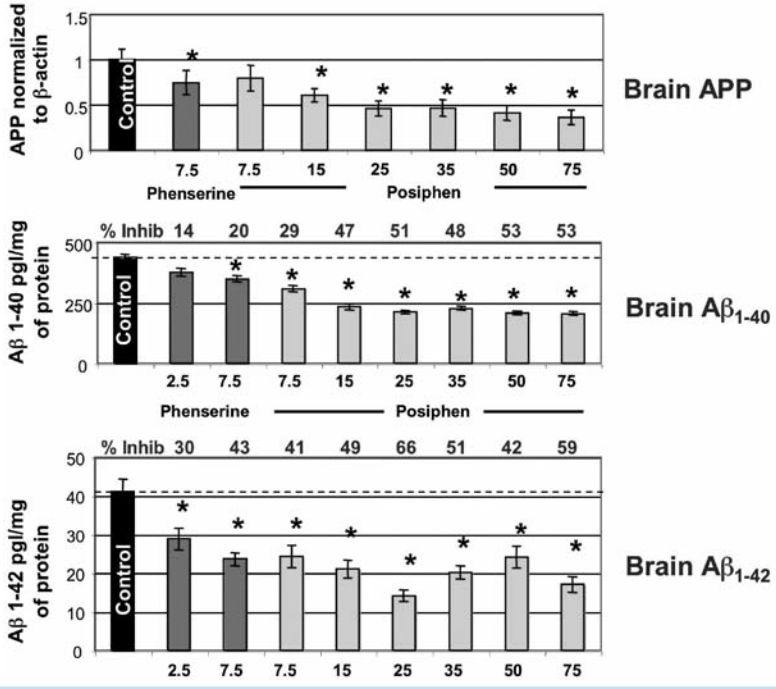
Figure 1 - Chemical structures and ChE inhibition (I) activities of (-)-physostigmine and phenylcarbamates. The methylcarbamate, (-)-physostigmine, is unselective between AChE and BuChE and short acting (in vivo ChE inhibition half-life ~30 min), whereas the unsubstituted phenylcarbamate, (-)-phenserine, is AChE selective (70-fold) and long-acting (in vivo ChE inhibition half-life ~8 h). Para- or 4'-isopropyl substitution on the phenylcarbamate, (-)-cymserine, generates a moderately BuChE selective (15-fold) and long-acting (in vivo ChE inhibition half-life ~8 h) inhibitor that can be made exquisitely potent and BuChE selective by modifications in the N1 and N8 positions to yield (-)-bisnorcymserine and (-)-phenethylcymserine. The unnatural (+)-enantiomeric form of phenserine, Posiphen, entirely lacks ChE action but potentially reduces APP and Aβ generation



these agents possessed high BuChE potency (1 nM and 6 nM) and selectivity (110-fold and absolute) versus AChE. (-)-Cymserine analogues, likewise, readily entered the brain (brain/plasma ratios up to 40:1) and induced a long-term selective BuChE reversible

inhibition ($T_{1/2}$ up to 11 hr) (17). Such actions elevated brain levels of ACh, augmented long-term potentiation (LTP – a cellular equivalent of memory) and resulted in improvements in cognitive performance in rodents (17); clearly demonstrating the ability of BuChE to co-regulate brain cholinergic activity. Interestingly, selective BuChE inhibition proved far better tolerated in animals than AChE selective inhibition or unselective inhibition of both ChE forms, using (-)-bisnorcymserine, (-)-phenserine, and (-)-physostigmine as pharmacological tools that share structural homology (15,17). Hence, (-)-bisnorcymserine has been moved through required toxicological studies to support its clinical use and assessment as an experimental therapeutic in AD – representing the first of a new class of potential drugs, 'centrally-active BuChE inhibitors', that will enter clinical assessment.

Figure 2 - Analysis of brain APP and Aβ levels from Posiphen and (-)-phenserine treated mice. Following once daily, 21 day consecutive i.p. administration of posiphen, phenserine or saline (control) to adult male C57BL/6 mice, animals were killed approx 90 min after the final dose and brain samples were immediately frozen (-70°C). Brain extracts were analyzed by Western blot with antibodies to APP (mAb 22c11) or β-actin (The latter was used as a control to normalize between animals). Ab levels were determined by ELISA, for full methodology see Ref. 20. Data are mean ± SEM (n=8-10)



Non-cholinergic actions and Posiphen

Early studies focused on elucidating molecular mechanisms underpinning AD, determined that (-)-phenserine, (-)-cymserine and analogues but, interestingly, not (-)-physostigmine lowered APP and Aβ levels in neuronal cell cultures (14). The generation of (+)-enantiomeric forms of each compound allowed assessment as to whether or not such actions were cholinergically mediated, as the (+)-isomers of each lack ChE inhibitory activity. APP/Aβ lowering actions were maintained in the chirally pure (+)- forms, and derived from the ability of the agents to lower the rate of synthesis of APP at a post-transcriptional level. This likely is mediated via several mechanisms, including action at the 5'-untranslated region (UTR) of APP mRNA (18), which is known to be involved in the up regulation of APP by Fe^{2+} and the inflammatory cytokine, IL-1 (19). Such actions translated into animals (20), allowing 50% reductions in brain levels of Aβ (Figure 2). The lack of direct anticholinesterase action of (+)-enantiomers allowed the safe use of dramatically higher doses, as the side-effect profile of AChE inhibitors is cholinergically mediated. Posiphen (Fig. 1) was chosen for clinical development, and early human studies indicate that it can be safely administered in doses up to 120 mg, substantially higher than (-)-phenserine (15 mg), to potentially achieve far more substantial

reductions of brain Aβ levels, oligomeric forms, as well other toxic elements generated by the misprocessing of APP. Posiphen is, hence, the first of a new class of drugs, 'APP synthesis inhibitors', to reach the clinic. Additionally, it is an 'indirect anticholinesterase'. Lacking AChE/BuChE activity, itself, it undergoes phase 1 metabolism (N-demethylation) to slowly generate N1- and N8- (+)-norPosiphen, and, thereafter, (+)-bisnorPosiphen, the former and latter of which possess clinically relevant anticholinesterase activity. We predict that slow onset of cholinergic activity, mediated via Posiphen's primary metabolites, will be better tolerated than a direct AChE inhibitor that more abruptly elevates brain ACh levels and will, likewise, provide cognitive improvement. The assessment of Posiphen in patients with mild to moderate AD will both define this as well whether lowering brain Aβ, by reducing the rate of APP synthesis, will change disease progression.

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