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POTENCIES AND SELECTIVITIES OF INHIBITORS OF ACETYLCHOLINESTERASE AND ITS MOLECULAR FORMS IN NORMAL AND ALZHEIMER'S DISEASE BRAIN*

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Eight inhibitors of acetylcholinesterase (AChE), tacrine, bis-tacrine, donepezil, rivastigmine, galantamine, heptyl-physostigmine, TAK-147 and metrifonate, were compared with regard to their effects on AChE and butyrylcholinesterase (BuChE) in normal human brain cortex. Additionally, the IC_{50} values of different molecular forms of AChE (monomeric, G_1, and tetrameric, G_4) were determined in the cerebral cortex in both normal and Alzheimer's human brains. The most selective AChE inhibitors, in decreasing sequence, were in order: TAK-147, donepezil and galantamine. For BuChE, the most specific was rivastigmine. However, none of these inhibitors was absolutely specific for AChE or BuChE. Among these inhibitors, tacrine, bis-tacrine, TAK-147, metrifonate and galantamine inhibited both the G_1 and G_4 AChE forms equally well. Interestingly, the AChE molecular forms in Alzheimer samples were more sensitive to some of the inhibitors as compared with the normal samples. Only one inhibitor, rivastigmine, displayed preferential inhibition for the G_1 form of AChE. We conclude that a molecular form-specific inhibitor may have therapeutic applications in inhibiting the G_1 form, which is relatively unchanged in Alzheimer's brain.

Keywords: Alzheimer's disease – acetylcholinesterase – butyrylcholinesterase – molecular forms

INTRODUCTION

AD is the most common age-related neurodegenerative disorder, with many cognitive and neuropsychiatric manifestations. Although numerous factors have been specified as causes of Alzheimer's disease (AD), a consistent finding is a depressed central cholinergic system, characterized by decreased levels of presynaptic cholinergic markers such as choline acetyltransferase (ChAT, EC 2.3.1.6) and acetylcholinesterase (AChE, EC 3.1.1.7) in postmortem brain tissue from AD patients as compared with age-matched controls [9, 14, 17]. A decrease of acetylcholine (ACh) in the brain of AD patients appears to be a critical element in producing dementia. The association of memory impairments with the cholinergic hypofunction has

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prompted considerable interest in cholinergic replacement therapy [6, 7]. The concept behind the use of cholinesterase inhibitors is the restoration of the cholinergic balance through elevation of the ACh levels and augmentation of the function of the remaining ACh receptors. AChE inhibitors approved by the U.S. Food and Drug Administration for the symptomatic treatment of patients with mild or moderate AD include tacrine, donepezil, rivastigmine and galantamine. It is well known that, as AD progresses, the levels of AChE in the brain are significantly reduced as compared with the levels in normal individuals, while the levels of butyrylcholinesterase (BuChE, EC 3.1.1.8) increase [9, 14, 17]. The inhibition of BuChE is therefore equally important.

It is well known that AChE exists in different molecular forms. These forms are divided into two classes: (1) globular forms consisting of monomer (G_1), dimer (G_2) and tetramer (G_4) structures with 1, 2 or 4 subunits, respectively, and (2) asymmetric forms containing a collagen-like tail possessing 4 (A_4), 8 (A_8) or 12 (A_{12}) catalytic subunits. Sedimentation analysis shows that in the human brain the most abundant AChE forms are G_4 (10 S) and G_1 (4 S) [15]. The G_4 form is functionally important for the degradation of ACh. Moreover, a selective loss of the membrane-associated G_4 form has been reported in the AD brain [1, 5, 19, 22], while the level of the G_1 form is relatively unchanged. However, less is known as to how inhibitors influence the different AChE molecular forms under normal and pathological conditions. In the experiments described below, the effects of eight pharmacologically important cholinesterase inhibitors were tested on AChE and BuChE and also on AChE molecular forms isolated from normal and AD postmortem brain tissues. We also wished to establish whether these inhibitors exert differential effects on the AChE molecular forms.

MATERIALS AND METHODS

The human brain samples were obtained from the Brain Bank of the Alzheimer's Disease Research Center (Szeged, Hungary). Following autopsy within 24 h after death, tissues were rapidly frozen and stored at –70 °C. The diagnoses were confirmed by histopathological methods. In the control brains, the diagnosis of a neurological disease was ruled out. Membrane-associated AChE was extracted from both the normal and the AD postmortem human frontal cortex [16]. Briefly: Tissues were homogenized in 10 vol. of 12.5 mM phosphate buffer, pH 7.4, and centrifuged at 100,000 × g for 1 h at 4 °C. The supernatant was discarded and the pellet was rehomogenized in the same buffer containing 0.5% Triton X-100. After centrifugation as above, the supernatant was used as membrane-associated AChE. Enzyme molecular forms were separated by ultracentrifugation on a linear 5–20% sucrose density gradient [16, 18] at 160,000 × g for 18 h at 4 °C in a Beckman SW 41 Ti rotor. Catalase from beef liver (11.3S) and bovine serum albumin (4.3S) were used as internal sedimentation markers. The molecular forms (G_4 and G_1) with the highest activities were pooled and used for enzyme inhibition studies. AChE and serum BuChE activ-

ities were determined by spectrophotometric [3] or, in the case of the AChE molecular forms, radiometric assays [8]. AChE activity was assayed in the presence of 0.1 mM tetraisopropyl pyrophosphoramide to inhibit BuChE. Serum BuChE activity was measured in the presence of 0.01 mM BW284c51 to inhibit AChE. The IC_{50} values of the inhibitors were calculated by linear regression of the log concentration versus inhibition functions (range 20–80% inhibition). Briefly, AChE was preincubated with different inhibitors for 30 min and the reaction was started by addition of the substrate acetylthiocholine or acetylcholine [acetyl-^{3}H] or, in the case of BuChE the substrate was butyrylthiocholine. All assays were performed in triplicate in 3–5 independent experiments. Protein concentration was measured with the Coomassie brilliant blue protein-binding method [2], using bovine serum albumin as standard. Statistical analysis was carried out by ANOVA.

Heptyl-physostigmine was from Mediolanum Farmaceutici (Milano, Italy), bis-tacrine was provided by Dr. Y. P. Pang (Mayo Clinic, Rochester, MN, USA) and TAK-147 was donated by Takeda Chem. Ind. (Osaka, Japan), and rivastigmine was from Novartis Pharma AG (Basel, Switzerland). Acetylcholine iodide ([acetyl-^{3}H], spec. act. 76 mCi/mM) was from NEN Life Sciences Products, Inc. (Boston, MA, USA). All other chemicals were of analytical grade and were obtained from commercial sources.

RESULTS

Effects of inhibitors on acetyl- and butyrylcholinesterase in normal brain cortex

The IC_{50} values of the following inhibitors were determined for AChE from the normal human brain cortex and for BuChE from the normal human serum: tacrine, bis-tacrine, donepezil, rivastigmine, galantamine, heptyl-physostigmine, TAK-147 and metrifonate (Table 1). The most selective AChE inhibitors, in decreasing sequence, were: TAK-147, donepezil and galantamine. Interestingly, bis-tacrine, a derivative of tacrine, was more potent in inhibiting AChE than BuChE. Bis-tacrine was identified as the compound with the lowest IC_{50} value among the inhibitors tested. For BuChE, the most specific inhibitors, in decreasing sequence were: rivastigmine, heptyl-physostigmine, tacrine and metrifonate. None of the inhibitors was absolutely specific for one type of cholinesterase (Table 1).

Effects of inhibitors on the acetylcholinesterase forms G_4 and G_1

The IC_{50} values of the following inhibitors were determined for both the G_1 and G_4 AChE molecular forms in the normal and the AD cortices: tacrine, bis-tacrine, donepezil, rivastigmine, galantamine, heptyl-physostigmine, TAK-147 and metrifonate (Table 2). There was no significant difference as concerns the IC_{50} values of

Table 1
In vitro inhibition of human brain acetyl- and serum butyrylcholinesterase activities

Compound	AChE	BuChE	Selectivity (BuChE/AChE)
Rivastigmine	4760±110	238±20	0.05
Heptyl-physostigmine	44±3	4.0±0.5	0.09
Tacrine	450±10	626±6.6	0.14
Metrifonate	1077±267	182±15	0.17
Bis-tacrine	0.0286±0.002	0.041±0.011	1.43
Galantamine	5000±170	59200±1700	11.8
Donepezil	323±126	12800±700	39.6
TAK-147	585±86	69000±7200	118

Inhibition of the cholinesterase activities was studied in the human brain cortex for AChE and in human sera for BuChE. IC_{50} values (nM) were determined and are reported as the means ±S.E. of 3–5 determinations.

Table 2
In vitro inhibition of human brain acetylcholinesterase molecular forms in normal and Alzheimer's disease brain cortices

Compound	G_1 form	
	Normal	AD
Rivastigmine*	5100±100	3500±100
Heptyl-physostigmine**	31.2±7.0	8.9±2.2
Donepezil	340±30	530±100
Tacrine	610±180	N.D.
Bis-tacrine	0.0298±0.0094	0.0182±0.0049
Metrifonate	1680±120	1800±170
TAK-147	720±20	N.D.
Galantamine	5130±630	N.D.
	G_4 form	
Rivastigmine*	14000±300	5600±400
Heptyl-physostigmine**	42.0±3.3	7.6±0.4
Donepezil	200±70	260±40
Tacrine	530±110	N.D.
Bis-tacrine	0.0300±0.0058	0.0160±0.0033
Metrifonate	1510±170	1760±370
TAK-147	590±10	N.D.
Galantamine	4960±450	N.D.

Inhibition of AChE activity was studied in normal and Alzheimer's disease brain cortices. IC_{50} values (nM) are reported as means ±S.E. of 3–5 determinations.

* $p < 0.001$ G_1 normal vs. G_1 AD; $p < 0.05$ G_4 normal vs. G_4 AD; $p < 0.01$ G_1 normal vs. G_4 normal; $p < 0.01$ G_1 AD vs. G_4 AD.

** $p < 0.01$ G_1 normal vs. G_1 AD; $p < 0.001$ G_4 normal vs. G_4 AD. One-way ANOVA.

AD = Alzheimer's disease.

N.D. = not determined.

the following inhibitors between the G_1 and G_4 forms in the normal and the AD brains: tacrine, bis-tacrine, donepezil, metrifonate, TAK-147 and galantamine. As regards AChE inhibition, bis-tacrine was the most potent. Among the inhibitors tested, only rivastigmine displayed preferential inhibition for the G_1 form in the normal human brain cortex. Interestingly, in the AD samples the AChE molecular forms were more sensitive to some of the inhibitors as compared with the normal samples, e.g. heptyl-physostigmine and rivastigmine.

DISCUSSION

In AD, cholinesterase inhibitors are currently used to increase the available ACh by preventing the hydrolysis of released ACh by inhibiting the enzymes AChE and BuChE. This therapeutic approach is based on attempts to correct the cognitive decline by manipulating the cholinergic neurotransmission. The main goal in the present work was to investigate the *in vitro* effects of different new-generation, pharmaceutically significant inhibitors on AChE and its molecular forms and BuChE in order to test their selectivities, and their therapeutic efficacies and to establish their potentials for use as palliative treatment of AD. Despite equivalent catalytic activity per active site for all the molecular forms [21], some of the AChE inhibitors displayed selectivity for certain molecular forms. It was found [11, 12] that heptyl-physostigmine preferentially inhibited the G_1 form. It has been demonstrated [20] that ethopropazine more strongly inhibited the G_1 form than the G_4 form. Enz et al. [4] reported that eptastigmine and rivastigmine are stronger inhibitors of G_1 than of G_4. A recent study has shown [23] that huperzine A preferentially inhibited the G_4 form, while tacrine and rivastigmine selectively inhibited the G_1 form in the rat brain cortex, hippocampus and striatum.

A selective loss of the membrane-associated AChE molecular form G_4 has been reported in the AD brain, while the G_1 form is relatively preserved. A possible molecular form-selective AChE inhibitor might therefore be useful for inhibition of the G_1 enzyme activity in order to increase the available ACh in the remaining cholinergic terminals in AD. Out of the eight, only one inhibitor, rivastigmine, preferentially inhibited the G_1 form of AChE. The question arises of the explanation of this type of selectivity, since the active sites for all of the molecular forms are identical [21]. A definite answer is not known. Perhaps the microenvironment of the enzyme active center, the hydrophobicities of the different molecular forms or the nature of the tissue lipids may play a role in determining the selectivity. Nevertheless, it has revealed [10] the abnormal localization and solubilization of AChE in the subcellular fractions in the AD brain, which may explain the different sensitivities of the enzyme to inhibitors under pathological conditions.

Several cholinesterase inhibitors are more specific for BuChE than for AChE. In AD, the BuChE activity in the brain is unchanged or slightly increased. Inhibitors for both AChE and BuChE may therefore play an equally important role as palliative treatment of this devastating disease. It emerged that tacrine, the first cholinesterase

inhibitor approved by the U.S. Food and Drug Administration, had unacceptable hepatotoxicity. Accordingly, this drug is no longer in widespread use. However, bis-tacrine, the parent compound of tacrine, is a new candidate for the treatment of AD [13]. Our data support the previous finding that bis-tacrine is a potent inhibitor (with an IC_{50} value of 10^{-11} M). Bis-tacrine might be used in a much lower dose than that of tacrine. Its therapeutic evaluation in AD is therefore highly recommended. Our data also suggest the use of rivastigmine as an AChE molecular form-selective inhibitor for therapeutic application.

An ideal cholinesterase inhibitor should be well tolerated, highly selective for the brain enzyme and its molecular forms, and region-specific (cerebral cortex and hippocampus), and it should have minimal effects on the peripheral cholinergic system and no organ toxicity. Finally, further compounds should be tested both *in vitro* and *in vivo* in the search for AChE molecular form-specific inhibitors for the treatment of AD.

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