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SHORT COMMUNICATION

Inhibition of Cholinesterase-associated Aryl Acylamidase Activity by Anticholinesterase Agents: Focus on Drugs Potentially Effective in Alzheimer's Disease

*Chiara Costagli and Alessandro Galli**

DIPARTIMENTO DI FARMACOLOGIA PRECLINICA E CLINICA, UNIVERSITÀ DI FIRENZE, V.G.B. MORGAGNI 65, 50134-FLORENCE, ITALY

ABSTRACT. The potency of a series of anticholinesterase (anti-ChE) agents and serotonin-related amines as inhibitors of the aryl acylamidase (AAA) activity associated with electric eel acetylcholinesterase (AChE) (EC 3.1.1.7) and horse serum butyrylcholinesterase (BuChE) (EC 3.1.1.8) was examined and compared with the potency of the same compounds as ChE inhibitors. Neostigmine, physostigmine, BW 284C51, (±)-huperzine A, E2020, tacrine, edrophonium and heptyl-physostigmine were, in that order, the most potent in inhibiting eel AChE-associated AAA activity, their inhibitor constant (K_i) values being in the range 0.02–0.37 μM. The rank order of the same compounds as AChE inhibitors basically paralleled that of AAA, although they were in general stronger on AChE (K_i = 0.001–0.05). The peripheral anionic site inhibitors propidium and gallamine were inactive on AChE-associated AAA. Serotonin and its derivatives were slightly stronger on AAA (K_i = 7.5–30 μM) than on AChE (K_i = 20–140 μM). Tacrine (IC_{50} = 0.03 μM), diisopropylfluorophosphate (IC_{50} = 0.04 μM), heptyl-physostigmine (IC_{50} = 0.11 μM), physostigmine (IC_{50} = 0.15 μM) and tetra-iso-propylpyrophosphoramide (iso-OMPA) (IC_{50} = 0.75 μM) were the most potent in inhibiting horse serum BuChE-associated AAA activity. Serotonin and related amines were very weak on BuChE-associated AAA activity. These results indicate that the inhibitory potencies of the active site anti-ChE agents on the AAA activity associated with eel AChE and horse serum BuChE are closely correlated with their action on the respective ChE. In addition, the efficacy of tacrine, E2020, heptyl-physostigmine and (±)-huperzine A in the treatment of Alzheimer's disease is unlikely to be related to the action of these drugs on ChE-associated AAA. BIOCHEM PHARMACOL **55**;10:1733–1737, 1998. © 1998 Elsevier Science Inc.

KEY WORDS. aryl acylamidase; acetylcholinesterase; butyrylcholinesterase; tacrine; anticholinesterase agents; serotonin

Abundant evidence indicates that ChE† have additional functions besides their well-known role in terminating the action of acetylcholine at the cholinergic synapses [1, 2 for reviews].

In 1976, Fujimoto [3] reported that purified AChE (EC 3.1.1.7) from *Electrophorus electricus* has AAA activity that is sensitive to serotonin, as well to ChE inhibitors. This report was followed by a series of other studies describing serotonin-sensitive AAA activity in purified AChE and BuChE preparations from brain and other sources in different animal species [4, 5]. Because every subsequent attempt to separate the two enzymatic activities has failed, the ability of both AChE and BuChE (EC 3.1.1.8) to hydrolyze aryl acylamides is now considered to be an intrinsic property of these enzymes [6].

The endogenous substrate for ChE-associated AAA is unknown at present; therefore, the physiological meaning of this enzymatic activity remains to be established [1]. Nevertheless, this novel catalytic function of ChE appears to be of considerable interest in many respects, particularly because ChE, or anomalous forms of these enzymes, are suspected to be involved in the pathogenesis of Alzheimer's disease on the basis of several experimental findings [7–9], and principally, on the basis of the claimed efficacy of the anti-ChE agents in this disease [10]. This point is strengthened by the observation that the ChE inhibitor tacrine, which is effective in the symptomatic treatment of Alzheimer's disease [11], inhibits the secretion of APP in various cultured cell lines: it is hypothesized that such an action could, in the long term, interfere with β-amyloid deposition

* Corresponding author: Dr. Alessandro Galli, Dipartimento di Farmacologia Preclinica e Clinica, Università di Firenze, Viale G.B. Morgagni 65, 50134-Firenze, Italy. Tel. (55)4237416; FAX (55)4361613; E-mail: galli@server1.pharm.unifi.it.

† *Abbreviations:* AAA, aryl acylamidase; AChE, acetylcholinesterase (EC 3.1.1.7); APP, β-amyloid precursor protein; ATCh, acetylthiocholine iodide; BuChE, butyrylcholinesterase (EC 3.1.1.8); BuTCh, butyrylthiocholine iodide; ChE, cholinesterase(s); DFP, diisopropylfluorophosphate; DTNB, 5,5′-dithio-bis-(2-nitrobenzoic acid); iso-OMPA, tetra-iso-propylpyrophosphoramide; K_i, inhibitor constant; o-NAA, 2′-nitroacetanilide.

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and slow down the disease process [12]. This effect is not mimicked by the potent ChE inhibitor physostigmine and, therefore, does not appear to be related to the anti-ChE action of the drug [13]. Although studies intended to investigate the presence of ChE-associated AAA within senile plaques or under other neuropathological conditions are, to our knowledge, lacking, it has been reported that in the histopathological structures of Alzheimer's disease, ChE are selectively inhibited by indoleamines [14]. This points out a resemblance to ChE-associated AAA and suggests a possible involvement of this enzymatic activity in the pathogenesis of this disease.

The fact that AAA, unlike ChE, is also sensitive to indoleamines suggests different structural requirements for interaction with the catalytic sites responsible for the two enzyme activities. To obtain information on this point, we compared the potencies of a number of anti-ChE agents and serotonin derivatives as AAA and ChE inhibitors on the same enzyme preparations. To our knowledge, a systematic study on this point has not yet been carried out. Within this ambit, it appeared of interest to also include drugs reported to be effective in ameliorating Alzheimer's disease symptomatology in the investigation. In fact, an inhibitory profile of these drugs which departed from that of the "classical" anti-ChE agents could provide an alternative explanation for their therapeutic efficacy and could indirectly suggest a role for ChE-associated AAA activity in Alzheimer's dementia.

MATERIALS AND METHODS

Chemicals

AChE from electric eel and DTNB were purchased from Boehringer Mannheim. (±)-Huperzine A was from Calbiochem. (RS)-1-benzyl-4-[(5,6-dimethoxy-1-indanon)-2-yl]-methylpiperidine hydrochloride (E2020) was a gift from Eisai Co. Ltd., Ibaraki, Japan. Heptyl-physostigmine tartrate was kindly donated by Mediolanum Farmaceutici, Milan, Italy. DFP was from Fluka. A stock solution of this compound was prepared in propylene glycol and stored at 4°. o-NAA was purchased from Avocado Research Chemicals Ltd., Heysham, U.K. BuChE from horse serum and all other chemicals were obtained from Sigma.

Enzyme Assays

AAA activity was measured basically according to Checler *et al.* [6]. o-NAA (10 mM) was incubated at 37° for 1 hr (AChE) or 2 hr (BuChE) in 0.5 mL of 0.1 M of potassium phosphate buffer, pH 8.0, containing 0.15–0.25 U of AChE from electric eel or 2–3 U of BuChE from horse serum in the absence or presence of the test compounds. Incubations were then stopped with 2.5 mL of 0.2 N of perchloric acid and extinction was measured at 410 nm against o-NAA blanks processed in the absence of enzyme. AAA activities were deduced from standard curves established with known amounts of 2-nitroaniline. The IC_{50} values were obtained from inhibition curves based on 4–6 different concentrations using the ALLFIT computer program [15]. K_i values were calculated according to Dixon [16] by using 5–6 different concentrations of the test compounds and 5 and 10 mM of o-NAA as substrate.

The inhibitory effects of the test compounds on electric eel AChE and horse serum BuChE were measured spectrophotometrically [17] using 0.5 mM of ATCh or 1 mM of BuTCh as substrates. The compounds were preincubated with the enzymes for 1 hr at room temperature before adding the substrate. The K_i values for AChE were measured according to Dixon [16], by using 0.125 and 0.5 mM of ATCh.

RESULTS AND DISCUSSION

In agreement with previous findings [3–5], the preparations of electric eel AChE and horse serum BuChE used in our experiments were able to hydrolyze o-NAA, although much less efficiently than choline esters: 1 U of AChE and BuChE hydrolyzed on average 0.45 and 0.005 μmol of o-NAA per hr at 37°, respectively. The K_m values for substrate-enzyme interaction, measured in three separate experiments in the presence of o-NAA varying between 0.625 and 10 mM, were 6.4 ± 1.4 and 5.2 ± 0.9 mM for AChE and BuChE, respectively.

The AAA activity of these enzymes was unlikely to be due to trypsin-like contaminants, because it was not inhibited by 10 μM of soybean trypsin inhibitor. Moreover, bovine pancreatic trypsin up to 0.25 mg/mL failed to hydrolyze o-NAA to any extent. The AAA activity of eel AChE was not inhibited by microbial leupeptin (up to 100 μM), bovine lung aprotinine (up to 1 μM), bestatin (up to 10 μM), potato carboxypeptidase inhibitor (up to 100 μg) or bacitracin (up to 100 units) (data not shown).

Eel AChE-associated AAA activity was shown to be sensitive to anti-ChE agents and serotonin-related amines (Fig. 1). Table 1 shows the IC_{50} and K_i values of a number of compounds belonging to these categories. For comparison, in Table 1 we also show the corresponding values for AChE activity obtained in parallel experiments. All tested compounds showed competitive or mixed competitive/noncompetitive behaviour; only AAA inhibition by heptyl-physostigmine was decidely noncompetitive. Neostigmine, physostigmine and 1,5-bis(4-allyldimethylammoniumphenyl)-pentan-3-one dibromide (BW 284C51) were the most potent in inhibiting the AAA activity of eel AChE, their K_i values being equal to 0.02 μM. They were followed in order by (±)-huperzine A, E2020, tacrine, edrophonium, heptyl-physostigmine and DFP, with K_i values in the range 0.05–0.6 μM. The selective anti-BuChE agents iso-OMPA and ethopropazine inhibited eel AChE-associated AAA to a negligible extent. The rank order of these compounds as AChE inhibitors basically paralleled that of AAA inhibition, although they were, in general, appreciably stronger on AChE (AAA/AChE K_i ratios in

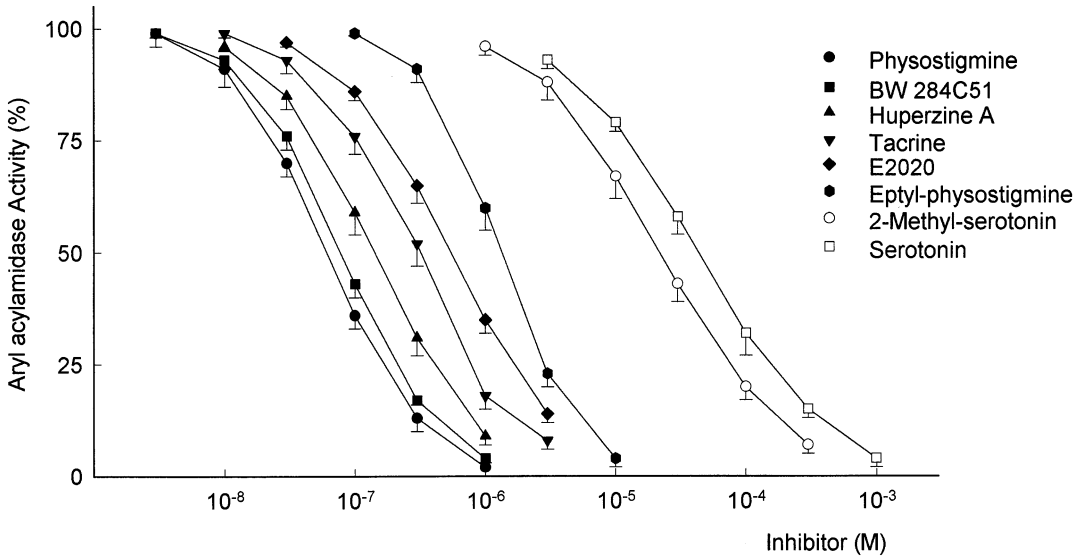

FIG. 1. Inhibition of electric eel AChE-associated AAA activity by increasing concentrations of anti-ChE agents and indoleamines. The enzyme assays were carried out in the presence of 10 mM of o-NAA as substrate. The points of the graph represent the means ± SEM of 3–4 separate determinations in duplicate.

the range 3.2–20). The correlation coefficients (r) between the IC_{50} and K_i values of these agents for the two activities were 0.822 ($P < 0.01$) and 0.787 ($P < 0.05$), respectively. Among the compounds tested, the inhibitory profiles of tacrine, E2020, heptyl-physostigmine and (±)-huperzine A, which were reported to be potentially effective in the treatment of Alzheimer's disease symptomatology [11, 18–20], were in line with those of the other anti-ChE agents and did not show any particular selectivity for either one of the two enzymatic activities (K_i ratios = 3.6–12.3). The peripheral anionic site inhibitors propidium [21], gallamine and decamethonium departed from this behaviour in that they were very weak, or altogether inactive, on AChE-associated AAA activity. This site of AChE, therefore, does not seem to be involved in the catalysis of aryl acylamide hydrolysis.

Unlike the compounds considered thus far, serotonin and related drugs were considerably more effective on AAA than on AChE (AAA/AChE K_i ratios < 1) (Table 1). However, the anti-AAA potency of these compounds was

TABLE 1. IC_{50} and K_i values of anti-ChE agents and serotonin-related compounds for the inhibition of AAA and AChE activities of electric eel AChE

	IC_{50} (M)			K_i (M)		
Compound	**AAA**	**AChE**	**Ratio***	**AAA**	**AChE**	**Ratio***
Physostigmine	0.06 ± 0.01	0.01 ± 0.002	6	0.02 ± 0.004	0.005 ± 0.001	4
Neostigmine	0.04 ± 0.004	0.005 ± 0.0002	8	0.02 ± 0.003	0.003 ± 0.0004	6.7
BW284C51	0.09 ± 0.01	0.004 ± 0.001	22	0.02 ± 0.004	0.001 ± 0.0002	20
Edrophonium	0.36 ± 0.05	0.30 ± 0.04	1.7	0.16 ± 0.03	0.05 ± 0.06	3.2
DFP	2.30 ± 0.40	0.55 ± 0.08	4.2	0.60 ± 0.10	0.05 ± 0.007	12
Tacrine	0.34 ± 0.03	0.03 ± 0.004	11.3	0.11 ± 0.02	0.01 ± 0.003	11
E2020	0.57 ± 0.07	0.03 ± 0.003	19	0.10 ± 0.02	0.009 ± 0.001	11.1
Heptyl-physostigmine	1.35 ± 0.22	0.12 ± 0.01	11.2	0.37 ± 0.04	0.03 ± 0.005	12.3
(±)-Huperzine A	0.16 ± 0.02	0.015 ± 0.002	10.7	0.05 ± 0.01	0.014 ± 0.002	3.6
Decamethonium	250 ± 35	5.0 ± 0.6	50	85 ± 7	1.5 ± 0.2	57
Gallamine	>1,000	200 ± 33	—	>1,000	31 ± 2.1	—
Propidium	>1,000	9.5 ± 1.5	—	>1,000	8.0 ± 1.0	—
Serotonin	50 ± 6	700 ± 65	0.07	30 ± 5	140 ± 25	0.2
2-Methyl-serotonin	22 ± 2	85 ± 12	0.26	7.5 ± 0.9	20 ± 3	0.4
α-Methyl-serotonin	25 ± 2	383 ± 17	0.06	14 ± 1.8	73 ± 20	0.19
N-Methyl-serotonin	28 ± 2	500 ± 30	0.06	10 ± 1	61 ± 12	0.16
Tetrahydro-β-carboline	36 ± 7	160 ± 10	0.22	21 ± 7	34 ± 9	0.6

IC_{50} values were calculated from inhibition curves using the ALLFIT computer program [15] and 10 mM of o-NAA (AAA) and 0.5 mM of ATCh (AChE) as substrates. K_i values were calculated from Dixon plots [16] using 5 and 10 mM of o-NAA (AAA) and 0.125 and 0.5 mM of ATCh (AChE) as substrates. The results are the means ± SEM of 3–4 separate determinations in duplicate.

*AAA/AChE ratio.

TABLE 2. IC_{50} values of anticholinesterase agents and serotonin-related drugs for AAA and BuChE activities of horse serum BuChE

Compound	IC_{50} (μM)		
	AAA	BuChE	Ratio*
Physostigmine	0.15 ± 0.02	0.03 ± 0.004	5
Neostigmine	0.97 ± 0.15	0.1 ± 0.01	9.7
iso-OMPA	0.75 ± 0.1	0.20 ± 0.03	3.7
Ethopropazine	2.55 ± 0.4	1.55 ± 0.3	1.6
DFP	0.04 ± 0.001	0.003 ± 0.0005	13.3
BW 284C51	35 ± 5	30 ± 5	1.2
Tacrine	0.03 ± 0.004	0.01 ± 0.001	3.75
E2020	36 ± 4	3.8 ± 0.3	9.5
Heptyl-physostigmine	0.11 ± 0.02	0.007 ± 0.002	15.7
(±)-Huperzine A	100 ± 9	87 ± 8	1.1
Serotonin	3,000 ± 200	>3,000	<1
2-Methyl-serotonin	>2,000	400 ± 52	>5
α-Methyl-serotonin	340 ± 27	400 ± 45	0.85
N-Methyl-serotonin	1,300 ± 240	>3,000	<0.43
1,2,3,4-Tetrahydro-β-carboline	>3,000	24 ± 4	>125

Values were calculated from inhibition curves using the ALLFIT computer program [15] and 10 mM of o-NAA (AAA) and 1 mM of BuTCh (BuChE) as substrates. The results are the means ± SEM of 3–4 separate determinations in duplicate.
*AAA/BuChE ratio.

considerably lower (K_i = 7.5–30 μM) than that of the classic active site AChE inhibitors. This observation points to a difference in the catalytic sites of the enzyme responsible for the two activities and indicates that these can be differentiated pharmacologically. Among the several amines tested in this work, only the serotonin methyl derivatives and 1,2,3,4-tetrahydro-β-carboline were slightly stronger than serotonin itself on AAA, but they were still considerably weaker than the anti-ChE agents in this action. Other serotonin-related amines such as 5-acetyl-serotonin, 5-methoxy-serotonin, 5-hydroxy-tryptophan, 5-hydroxy-tryptophol, kynurenine, kynuramine, harmane, norharmane, 6-hydroxy-1,2,3,4-tetrahydro-β-carboline and 6-methoxy-1,2,3,4-tetrahydro-β-carboline inhibited AChE-associated AAA activity to a negligible extent (IC_{50} > 1,000 μM).

Most of the compounds tested on eel AChE were also assayed on AAA activity associated with horse serum BuChE (Table 2). In this case, only the IC_{50} values were measured. Tacrine (IC_{50} = 0.03 μM), heptyl-physostigmine, physostigmine, iso-OMPA, neostigmine and ethopropazine, in that order, were the most potent inhibitors of this AAA among the compounds tested. The selective AChE inhibitors BW 284C51, E2020 and (±)-huperzine A were relatively weak on the AAA activity of horse serum BuChE. The anti-AAA potency of all these compounds, including those reported to be potentially effective in senile dementia [11, 18–20], strictly correlated with their potency as BuChE inhibitors ($r = 0.97$; $P < 0.01$). Serotonin and related drugs were rather weak on horse serum BuChE-associated AAA. In addition, the correlation between the action of these compounds on the two activities was not straightforward.

In conclusion, the results of this study have shown that the inhibitory potencies of the anti-ChE agents on the AAA activity associated with eel AChE and horse serum BuChE are strictly correlated with their action on the respective ChE, suggesting that the catalytic sites responsible for the two activities are largely overlapping. Tacrine and the other anti-ChE drugs reported to be effective against some symptoms of Alzheimer's disease did not depart from this behaviour. It appears unlikely, therefore, that the therapeutic effectiveness of these drugs is related in some way to their action on ChE-associated AAA. This work also indirectly confirms the results of previous studies, largely based upon biochemical purification steps, indicating that the AAA activity of ChE is intrinsically associated with these enzymes and is not due to contaminating peptidases or amidases. The possible physiological role of this enzymatic activity remains to be clarified.

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