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A Long-Lasting Cholinesterase Inhibitor Affecting Neural and Behavioral Processes

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BRUFANI, M , C. CASTELLANO, M MARTA, A OLIVERIO, P G PAGELLA, F PAVONE, M POMPONI AND P L RUGARLI *A long-lasting cholinesterase inhibitor affecting neural and behavioral processes* PHARMACOL BIOCHEM BEHAV **26**(3) 625–629, 1987 —A series of analogues of physostigmine were prepared with the aim of investigating their inhibitory effects on acetylcholinesterase in the treatment of Alzheimer's disease One of the isomers prepared was evaluated for its anticholinesterase activity in vivo, acute toxicity, and some behavioral effects This compound was a competitive inhibitor of the enzyme and was found to antagonize the stimulating effect produced by scopolamine on locomotor activity and to facilitate memory consolidation

Physostigmine derivatives Cholinergic mechanism Memory Aging

IN recent years a number of drugs increasing brain acetylcholine (ACh) have been used in clinical practice in the therapy of different neurological diseases [19]. Particular interest has been aroused in the role of ACh in the brain since decay in the cholinergic systems has been suggested as a possible cause of Alzheimer's disease [3, 10, 31], a disorder of aging Though other explanations have been suggested in order to explain this disease, which is spreading rapidly in industrialized countries, the cholinergic hypothesis seems to be central in the Alzheimer's pathogenesis [8,23]

Initial studies attempted to increase ACh synthesis by treatment with ACh precursor choline or lecithin [9,20], but the results were almost uniformly negative [3,11] Current attention is centered on the use of the cholinesterase inhibitor physostigmine for possible symptomatic therapy of this disease [12, 17, 29] However, the therapeutic applications of physostigmine are reduced by its high toxicity, adverse side effects, short half-life in the body, narrow effective dose range and low bioavailability [13, 21, 24]. As an alternative to this classic acetylcholinesterase inhibitor, a search is made for physostigmine derivatives characterized by reduced toxicity and reduced activity at the peripheral level

A series of physostigmine analogues have been synthesized, in which the methylcarbamic group has been substituted with alkylcarbamic groups [4] having from 1 to 16 carbon atoms. All these compounds should have a higher lipophilicity and consequently a different distribution in the body and different pharmacokinetics.

The present study focuses in particular on the results obtained with one of these lipophilic derivatives pyrrolol [2,3-b]heptylcarbamate (ester),(3aS-cis) (*Chem Abstr*), the "heptyl" (Fig. 1), which increases cholinergic function at the brain level and results in behavioral modifications suggesting a possible therapeutic use of this compound in Alzheimer's disease [28]

METHOD

Alkyl analogues of physostigmine were prepared in two steps using a vacuum procedure [1,2]. The first step consisted in the alkaline hydrolysis of physostigmine with sodium methoxide The second involved conversion of the eserolinate to the desired derivative with the corresponding isocyanate, using benzene as solvent [28].

The heptyl was salycilated and dissolved in bidistilled water with carboxymethyl cellulose sodium salt (2%). The heptyl vehicle was used for control injections. The subjects were tested at about 90 days of age. In view of the large number of physostigmine derivatives screened in prelimi-

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TABLE 1

ACETYLCHOLINESTERASE (AChE) INHIBITION IN THE BRAIN AND SERUM 10 MIN AFTER IP ADMINISTRATION OF DIFFERENT DOSES OF PHYSOSTIGMINE AND HEPTYL TO RATS

	Dose mg/kg IP	No	AChE Inhibition (%) Mean ± SE Brain	Serum	ID_{50} (mg/kg) IP Brain	Serum
Phys.	0 10	4	19 58 ± 11 43	19 55 ± 8 29		
	0 15	4	39 18 ± 4 32	32 93 ± 11 34	0 19	0 20
	0 20	4	53 70 ± 8 30	53 68 ± 2 41		
Hept	0 50	4	25 55 ± 11 64	20 85 ± 5 64		
	1.00	4	50 00 ± 1 32	59 40 ± 1 99	1 39	1 10
	2 00	4	53 62 ± 10 51	67 42 ± 4 29		
	4 00	4	72 35 ± 5 92	79 42 ± 2 89		

Control enzyme activity is 8 38 ± 0 76 moles acetylcholine hydrolyzed/min/g brain and 0 37 ± 0 03 moles acetylcholine hydrolized/min/ml/serum (N=10)
Phys physostigmine; Hept heptyl derivative

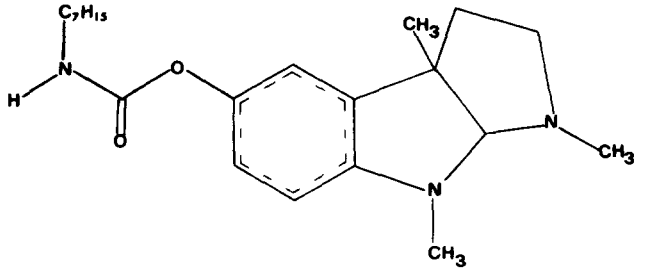

FIG 1 Heptyl pyrrolo[2,3-b]indol-5-ol,3,3a,8,8a-hexahydro-1,3a, 8-trimethyl-heptylcarbamate (ester),(3aS-cis)

nary experiments, the enzymatic assays were conducted in rats rather than in mice because of their greater brain weight. Male rats (n=62) belonging to the Wistar strain, weighing 180–200 g, were used. For histological, electrocorticographic and behavioral measures a total of 226 male mice belonging to the DBA/2 strain were used, since this strain has already been used in a number of learning studies and in pharmacogenetic experiments dealing with cholinergic function [27]. Finally, 300 mice were used for the toxicity measures.

Enzymatic Assays

In order to test physostigmine and heptyl effects on cerebral and serum AChE activity in vivo, the animals were intraperitoneally injected with drug solutions or with vehicle as control. Rats were decapitated 10, 60 and 120 min after treatment. Serum was collected and the brain was isolated in ice. AChE activity was measured in the brain according to the method described by Ellman *et al* [16] and the percent of enzyme inhibition in control and in the treated groups was calculated Cholinesterase activity in serum was also determined.

ID_{50} values and duration of the inhibitory activity, respectively, were evaluated in two different experiments.

Histology

The pharmacohistochemical procedure used for acetylcholinesterase (AChE) [5] enabled detailed visualization of AChE-containing neurons to be obtained. This technique is based on the use of organic phosphoric esters such as diisopropylfluorophosphate (DFP) For this experiment 50 animals were utilized

Electrocorticographic Activity (ECoG)

The mice (n=16) were anesthetized with pentothal and implanted with 4 cortical electrodes in the fronto-parietal area as previously described [6]. One hour elapsed between electrode implantation and recording; during this period the mice were kept at a temperature of about 30°C. Each mouse was placed in a 20×10 cm electrically shielded plastic cage The cortical electrodes were connected to an 8 channel Beckman Dynograph Polygraph and the ECoG was recorded for 30 min

Exploratory Activity

The locomotor activity was measured with a toggle-floor box (24.5×9.0 cm) as previously described [26] The number of crossings from one side of the box to the other was recorded automatically, and constituted the mouse's score Different groups of eight mice were tested 5 minutes after intraperitoneal drug and vehicle administration. The test session lasted 25 min

The results were statistically evaluated by one-way analysis of variance and Duncan multiple range test.

Passive Avoidance

The test and the experimental procedure utilized were similar to those previously described [7]. The apparatus consisted of a 20×20×20 Lucite box with black walls and a grid floor The time taken by the mouse to enter the box with all 4 feet (step-through latency) was recorded When the animal had entered the box, the opening was closed by a hand-operated sliding door, and the mouse received a 50 Hz scrambled footshock of 0 7 mA through the grid floor for 1 sec. It was then removed to its home cage to await testing. Test procedures were the same as during training, except

TABLE 2

ACETYLCHOLINESTERASE (AChE) INHIBITION IN THE BRAIN AND IN SERUM AFTER IP ADMINISTRATION OF EQUIACTIVE DOSES OF PHYSOSTIGMINE AND HEPTYL, AT VARIOUS TIME INTERVALS (10–60–120 MIN) AFTER DOSING

	Dose mg/kg IP	% AChE Inhibition at Minutes Mean ± SE (n=4) 10 min Brain	10 min Serum	60 min Brain	60 min Serum	120 min Brain	120 min Serum
Phys	0 2	53 70 ±8 30	53.68 ±2 41	12 47 ±2 83	9 82 ±6 83	3 28 ±1 99	2 58 ±1 49
Hept	1 0	50 00 ±1 32	59 40 ±1 99	28 46 ±9 56	41 15 ±4 29	22 45 ±2 54	21 53 ±2 31
		Statistical Comparison					
	t	0 3744	1 7323	1 6038	3 8844	5 9410	6 8938
	p	NS	NS	NS	<0 02	<0 005	<0 001

TABLE 3

SPONTANEOUS ACTIVITY MEAN NUMBER OF CROSSINGS IN THE TOGGLE FLOOR BOX

Treatment	mg/kg	n
Vehicle		41 0
Heptyl	0 3	44 6
Heptyl	3 0	36.3
Heptyl	5 0	13 8*
Scopolamine	1 0	103 7†
Heptyl + Scopolamine	3 0 1 0	68 0*

*$p<0$ 05, †$p<0$ 01 (ANOVA one-way and Duncan multiple range test)

TABLE 4

EFFECTS OF HEPTYL ON PASSIVE AVOIDANCE BEHAVIOR IN DBA/2 MICE

Treatment	mg/kg	24 hr	48 hr
Vehicle		104 6	65 3
Heptyl	0 5	116 3	71 1
Heptyl	1 0	163.8*	139 0*
Heptyl	2 5	170 8*	147 6*
Heptyl	5 0	180.0*	180 0*

*$p<0$ 01 (ANOVA one-way and Duncan multiple range test)
Mean step-through latencies on the test day 24 and 48 hour after training

that no footshock was administered Different groups of eight mice were intraperitoneally injected with drug or vehicle immediately after training and tested 24 and 48 hours later. In addition, a group of mice was injected with heptyl (2 5 mg/kg) 120 minutes after training, and another group received no footshock but was injected with the drug immediately after training. The results were statistically evaluated by one-way analysis of variance and Duncan multiple range test

RESULTS

Enzymatic Assays

Table 1 indicates that a treatment with physostigmine and heptyl resulted in a dose dependent reversible inhibition of brain and serum AChE activity Thus it is evident that higher doses of heptyl are necessary in order to produce comparable levels of AChE inhibition compared with physostigmine. The inhibitory activity was of the same order of magnitude in the two tissues. Physostigmine was more powerful also when the inhibitory activity of the two compounds was expressed in terms of ID_{50} Brain ID_{50} were 0.19 and 1.39 mg/kg for physostigmine and heptyl, respectively, serum ID_{50} were 0.20 and 1.10 mg/kg, respectively. The duration of the inhibitory activity of heptyl was longer than that of physostigmine, as shown in Table 2. The percent AChE inhibition 120 min after the heptyl injection was 22.45 and 21.53 while 60 min following the injection of physostigmine only 12.47% and 9 82% of AChE inhibition, respectively at brain and serum level, was observed.

Toxicity

In the mouse the LD_{50} of heptyl is 35 mg/kg. This dose is about 60 times lower than that of physostigmine, whose LD_{50} is 0 6 mg/kg. The LD_{100} of heptyl is 45 mg/kg.

Histology

A pharmacohistochemical procedure was employed to test possible acetylcholinesterase functional differences in the brain.

The histological analysis used to visualize AChE containing neurons showed that heptyl (20 mg/kg), injected 15 min before brain perfusion, resulted in a clear-cut fading of brain cholinergic areas. The effects of the higher dose of heptyl (30 mg/kg) were still evident one hour after the injection Thus a

clear visualization of AChE containing neurons was obtained, while less intense colorimetric changes were evidenced by using different doses of physostigmine (0 3 and 0.4 mg/kg)

Electrocorticographic Activity

Heptyl resulted in a modification of electrocorticographic activity An analysis of the ECoG recordings indicated changes typical of increased cholinergic function [22] There is an evident increase in amplitude, and a decrease in the frequency of the ECographic rhythms following the administration of 10 mg/kg of heptyl.

Exploratory Activity

At doses ranging between 0.1 and 0 3 mg/kg heptyl did not affect the spontaneous activity of mice. Table 3 shows that higher doses resulted in depressant effects on activity

Heptyl (3.0 mg/kg) resulted in an antagonism of the stimulating effect produced on locomotor activity by scopolamine (1 0 mg/kg) This finding indicates that increased cholinergic function (due to inhibition ACh hydrolysis) antagonizes the behavioral stimulation related to the anticholinergic effect of scopolamine [25]

Passive Avoidance

Table 4 indicates that step-through latencies of mice injected posttrial with different doses of heptyl were significantly modified Longer step-through latencies were evident 24 or 48 hours following the posttrial injection of heptyl, indicating that the drug acts on consolidation mechanisms by facilitating memory. No significant difference was observed between the performances of mice injected with heptyl 120 minutes after training and those of the control group The mean step-through latencies of the mice that had not received footshock on the training day did not differ significantly from controls

DISCUSSION

The main result emerging from the present research is that the compound tested, the heptyl, exerts its anticholinesterase activity at doses which are significantly less toxic than physostigmine. In fact the LD_{50} of heptyl is 35 mg/kg, while the same lethal effect is evident following 0 6 mg/kg of physostigmine Moreover, making physostigmine more non-polar by lengthening the alkyl chain to seven carbons, although decreasing its inhibitory properties, plays a decisive role in determining a powerful long-lasting inhibition of both brain and serum AChE activity in both rats and mice. Within the first species a significant AChE inhibition at the brain and serum level is found also 120 min after the treatment, while no effect of physostigmine is evident 60 min after its injection When mice are considered the pharmacohistochemical procedure indicates that a significant fading of brain cholinergic areas is still detectable one hour after the injection of 30 mg/kg of heptyl, while less intense colorimetric changes are evident following different doses of physostigmine (0 3–0 4 mg/kg)

At the behavioral level the action of heptyl on the brain results in an antagonism of the stimulating effects of scopolamine, a well known anticholinergic agent, on the locomotor activity of mice Moreover the clear-cut modifications of the ECoG activity following the administration of 10 mg/kg of heptyl also indicate a typical increase in cholinergic functions The most interesting result in terms of behavioral modifications is probably the effect of heptyl on passive avoidance learning, which may be interpreted in terms of enhanced consolidation processes [14] Though a number of neurochemical manipulations affecting neurotransmitters and neuromodulators produce significant changes in memory functions [18], the present findings on heptyl, indicating a positive effect of this cholinergic agent on passive avoidance acquisition, are interesting in terms of the relationships between memory loss and a pathological decrement of cholinergic function in the elderly Since the major brain alteration occurring in Alzheimer's disease is the generalized loss of cholinergic neurons, a therapeutic approach to the memory impairment characterizing this disease could be attempted through the use of long-lasting cholinergic agents that are less toxic than physostigmine, such as heptyl

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