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Effect of MDL 73,745 on acetylcholine and biogenic amine levels in rat cortex

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Abstract

We postulate that the effect of cholinesterase inhibitors to ameliorate the cholinergic deficit in Alzheimer's disease is related to their ability to maintain long-lasting, non-toxic steady-state levels of acetylcholine in cortex. We investigated the effect of the cholinesterase inhibitor, MDL 73,745 (2,2,2-trifluoro-1-(3-trimethylsilylphenyl)ethanone), on the extracellular levels of acetylcholine, norepinephrine, dopamine and 5-hydroxytryptamine in the cerebral cortex of the rat by high-performance liquid chromatography coupled with electrochemical detection. The drug significantly increased acetylcholine levels above the baseline at 2 and 10 mg/kg s.c., but not at the 1 mg/kg dose. At both 2 and 10 mg/kg, there was a good correlation between cholinesterase inhibition and acetylcholine increase in cortex. At the 2 and 10 mg/kg doses, the maximal cholinesterase inhibition was 64% and 77%, respectively, and the increase in acetylcholine release was 481% and 1016%, respectively. Norepinephrine and dopamine, but not 5-hydroxytryptamine levels, were also significantly increased by the 10 mg/kg dose. The increases of norepinephrine and dopamine levels reached a maximum of 124% and 370%, respectively, and continued for a period of at least 8 h. Cholinergic side-effects were most marked at the 10 mg/kg dose but were also noticeable at the 2 mg/kg dose in the form of fasciculations, tremor and splay.

Keywords: MDL 73,745; Microdialysis; Cholinesterase; Acetylcholine; Norepinephrine; Dopamine; 5-HT (5-hydroxytryptamine, serotonin)

1. Introduction

MDL 73,745, a trimethylsylilated aromatic trifluoromethylketone, is a representative of a new class of cholinesterase inhibitor based on the concept of transition state analog inhibitors (Fig. 1). The compound combines the reactivity of a trifluoromethylketone group responsible for a strong interaction at the active site of acetylcholinesterase with the highly lipophilic property of a trimethylsilyl residue to increase brain penetration. In vitro studies have shown that the compound is a slow-tight-binding inhibitor of acetylcholinesterase of different sources with a slow start of its effect and a slow rate of dissociation which lead to K_i values in the picomolar range (Hornsperger et al., 1994). MDL 73,745 selectively inhibits acetylcholinesterase versus other esterases, thus 60 times higher concentrations are needed to inhibit rat brain butyrylcholinesterase as compared to acetylcholinesterase. Following subcutaneous administration in the rat (10 mg/kg) the compound produces a long-lasting inhibition of brain acetylcholinesterase and a significant increase in acetylcholine extracellular levels (Giacobini et al., 1992; Hornsperger et al., 1994). In animal experiments, MDL 73,745 has shown low toxicity as compared to other cholinesterase inhibitors with only minimal effects on heart rate and blood pressure. It enhances working memory performance in a T-maze delayed, reinforced alteration model and improves retention in a passive avoidance task (Hornsperger et al., 1992). In relation to physostigmine and tetrahydroaminoacridine, MDL 73,745 can be classified as a

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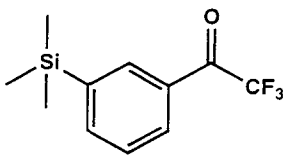

Fig. 1. MDL 73,745 (2,2,2-trifluoro-1-(3-trimethylsilylphenyl)-ethanone).

second generation cholinesterase inhibitor (Becker and Giacobini, 1988).

MDL 73,745 has been recently evaluated for safety, tolerance and pharmacological activity in normal male volunteers (Schleman et al., 1994). It was well tolerated up to single oral doses of 300 mg. A dose-dependent inhibition of red blood cell acetylcholinesterase was observed (Schleman et al., 1994).

In this investigation we have used a modified high-performance liquid chromatography coupled with electrochemical detection assay to measure intracortical fmole acetylcholine levels with a microdialysis system which does not use a cholinesterase inhibitor in the probe (Messamore et al., 1993a,b; Cuadra et al., 1993, 1994). This is a necessary condition in order to study the effect of systemically administered cholinesterase inhibitor without interference from a second cholinesterase inhibitor. Using this technique, we can measure simultaneously acetylcholine and other biogenic amines in the same cortical dialysate.

There is evidence of an interaction between the cholinergic and the monoaminergic system in the control of cognitive, mainly attentional cortical function (Riekkinen et al., 1990). Cuadra et al. (1993, 1994) found that physostigmine and heptylphysostigmine differentially modify the release of acetylcholine and other biogenic amines (norepinephrine, dopamine) in rat cortex in vivo. In addition, the positive clinical effect of cholinesterase inhibitor such as tetrahydroaminoacridine has been related to stimulation of both cholinergic and monoaminergic systems (Åhlin et al., 1991; Alhainen et al., 1993). Therefore, we have studied the effect of MDL 73,745 on extracellular levels of acetylcholine as well as other biogenic amines in rat cortex.

2. Materials and methods

Microdialysis probes were constructed by modifying the technique of Robinson and Whishaw (1988). The dialysis membrane was 6 mm long and 0.25 mm wide.

2.1. Surgery

Male Sprague-Dawley rats (225–350 g) were anesthetized with sodium pentobarbital (62.5 mg/kg i.p.) and placed in a Kopf stereotaxic frame with the incisor bar set 3.3 mm below the interaural line. The skull was trephined at a point 1.7 mm anterior to the bregma and tangential to the sagittal suture. The microdialysis probe was inserted at an angle of 54° to a depth of 5.6 mm and permanently secured with bone screws and dental cement. The skin of the rat was sutured. Following surgery, the rats were housed individually in large acrylic bowls and were allowed a minimum of 16 h recovery time before the start of the microdialysis experiment.

2.2. Microdialysis procedure

One day after surgery, the microdialysis probe was connected to a 2-channel swivel (Instech Labs., Plymouth Meeting, PA, USA) and perfused with Ringer's solution (NaCl 147 mM; KCl 4.0 mM; $CaCl_2$ 2.3 mM) at a flow rate of 1.0 μl/min. After a 120-min equilibration period only Ringer's solution with no cholinesterase inhibitors was perfused through the microdialysis probe, microdialysis samples were collected with a refrigerated fraction collector (CMA/170, Carnegie-Medicin Associates/Microdialysis, Sweden) every 20 or 30 min into vials containing 2 μl acetic acid (0.1 N) to prevent oxidation of monoamines. Under these conditions, acetylcholine is very stable for at least 24 h. MDL 73,745 was provided by Marion Merrell Dow. It was dissolved in 0.5% Tween-80 solution. After collecting 4–5 vials (pre-injection point = baseline), the vehicle (0.5% Tween-80, 1 ml/kg) or MDL 73,745 (1, 2 or 10 mg/kg) was administered s.c. Five or six animals were used in each treatment group. There were two vehicle groups, one for the acetylcholine assay and one for the monoamine assay.

2.3. Acetylcholine assay

The high-performance liquid chromatography system consisted of a Sekisui (Eicom Corp., Kyoto, Japan) dual-piston pump (LCP-320) and electrochemical detector (ECD-120) with platinum electrode (WE-PT) coupled to an autosampler (CMA-220, Carnegie-Medicin Associates/Microdialysis, Sweden). The PRP-1 guard column (polymeric reversed phase, Hamilton Co., Reno, NV, USA) was placed before the 4.1 × 150 mm PRP-1 analytical column (polymeric reversed phase, 10 μM particles, Hamilton Co., Reno, NV, USA). An acetylcholine/choline enzyme reactor (BAS, West Lafayette, IN, USA) preloaded with acetylcholinesterase and choline oxidase enzymes was placed between the analytical column and the detector. The analytical column was kept in a water bath at room temperature. The electrode potential was set at +500 mV versus an Ag/AgCl reference electrode. Peaks were recorded on a Hewlett-Packard (HP 3390A) inte-

grator. Acetylcholine in brain dialysates was quantified by comparing peak heights in the samples to a standard curve.

The mobile phase was made of 0.15 M Na_2HPO_4, 2.0 mM tetramethylammonium hydroxide, 0.3 mM disodium ethylenediaminetetraacetate, 0.005% reagent microbicide, and 5.0 μM octanesulfonic acid sodium salt, pH 8.0. The mobile phase was pumped at a rate of 0.6 ml/min. A lowest level of 50 fmol acetylcholine could be reliably detected.

2.4. Monoamine assay

Norepinephrine, dopamine and 5-hydroxytryptamine concentrations were measured on a Coulochem II with electrochemical detector (ESA, Bedford, MA, USA) equipped with dual porous graphite electrodes (Cuadra et al., 1994). The potential was set at +320 mV. A guard cell set at a potential of +400 mV preceded an HR-80 (high resolution) analytical column (3 μM octa decyl silane, 8.0 cm × 3 mm). The peaks were displayed, integrated and stored by means of a Kontron Instrument Data System 450. Quantification was made by comparing peak heights of the samples to a standard curve.

The mobile phase was 75 mM NaH_2PO_4; 1.0 mM sodium dodecyl sulphate; 100 μM disodium ethylenediaminetetraacetate; 1.48 mM triethylamine; 13% methanol and 15% acetonitrile; pH 5.6, filtered and pumped through the system at a flow rate of 1.0 ml/min by a Shimadzu LC-10AD pump (ESA, Bedford, MA, USA).

2.5. Cholinesterase activity assay

Male Sprague-Dawley rats (258 ± 4.1 g) were decapitated after receiving (s.c.) 0.5% Tween-80 solution (vehicle, 1 ml/kg) or MDL 73,745 (2 or 10 mg/kg) at 30 min, 1 h, 2 h, 4 h, 7 h, 9 h time points, the entire brain was removed and homogenized. Cholinesterase activity was assayed in whole brain homogenates of drug-treated rats by using the radiometric method of Johnson and Russell (1975), and compared to the activity of brain from Tween-80-treated rats. Acetylcholine was used as a substrate at a final concentration of 50 mM (Hallak and Giacobini, 1986). Studies of cholinesterase activity following various cholinesterase inhibitors show only small differences when measured in whole brain as compared to frontal cortex (DeSarno et al., 1989).

2.6. Statistical analysis

The data are presented as means ± S.E.M. One-way analysis of variance followed by Tukey's multiple range test was used to compare means between two or more different treatment groups. The paired t-test was used to compare the differences between baseline and post-injection levels of acetylcholine, norepinephrine, dopamine and 5-hydroxytryptamine levels within the same treatment group. The Student independent t-test was used to compare the differences of cholinesterase inhibition between the vehicle (0.5% Tween-80) and drug-treatment at the same time point. A P value of 0.05 or less was considered significant.

3. Results

3.1. Effect of MDL 73,745 on extracellular levels of acetylcholine in rat cortex

Following s.c. administration of 2 and 10 mg/kg doses, the drug significantly increased the acetylcholine level up to 481 ± 180% and 1052 ± 412%, respectively (mean ± S.E.M., $n = 5$) above the baseline (average of five samples prior to injection = 100%). Significant differences were also seen between the groups receiving 2 or 10 mg/kg and the vehicle group (0.5% Tween-80), but not for the 1 mg/kg dose (Fig. 2 upper). At both 2 and 10 mg/kg doses, there was a good correlation between cholinesterase inhibition and acetylcholine increase in cortex; maximal cholinesterase inhibitions were 64% and 77%, respectively. At the 10 mg/kg dose, acetylcholine was significantly increased during a period of 7 h after a single administration. The peak time of acetylcholine level was 60 min (Fig. 2 lower).

Cholinergic side-effects (fasciculations, tremor and splay) were observed during a period of 180 ± 20 min for the two highest doses. At the 10 mg/kg dose, side-effects were most marked in the form of fasciculations, tremor and splay. They were noticeable but less pronounced at the 2 mg/kg dose but were not seen at the 1 mg/kg dose.

Table 1 compares the effects of MDL 73,745 with that of four cholinesterase inhibitions being tested in Alzheimer's therapy (physostigmine, heptylphysostigmine, (L)-Huperzine-A) and tetrahydroaminoacridine (on extracellular acetylcholine, cholinesterase inhibition and cholinergic side-effects). MDL 73,745 (10 mg/kg) shows a more prolonged effect (7 h) on acetylcholine release than physostigmine (1.5 h) and tetrahydroaminoacridine (3 h). Cholinergic side-effects are also somewhat more pronounced following MDL 73,745 than after systemic (L)-Huperzine-A (Zhu and Giacobini, 1995).

3.2. Effect of MDL 73,745 on extracellular levels of norepinephrine, dopamine and 5-hydroxytryptamine in rat cortex

Following 10 mg/kg s.c. administration of MDL 73,745, norepinephrine and dopamine reached a maxi-

mum increase of 124% and 370% above the baseline, respectively. Both norepinephrine (Fig. 3 upper) and dopamine (Fig. 3 lower) levels were significantly ($P < 0.05$) higher than the baseline. Norepinephrine and dopamine levels were maintained constantly higher for a period of at least 8 h after the administration. There was no change of 5-hydroxytryptamine level (not shown).

Administration of 0.5% Tween-80 vehicle (1 ml/kg s.c., $n = 6$) produced no significant changes in the levels of norepinephrine and dopamine (Fig. 3). There was no change of 5-hydroxytryptamine above the basal level (not shown).

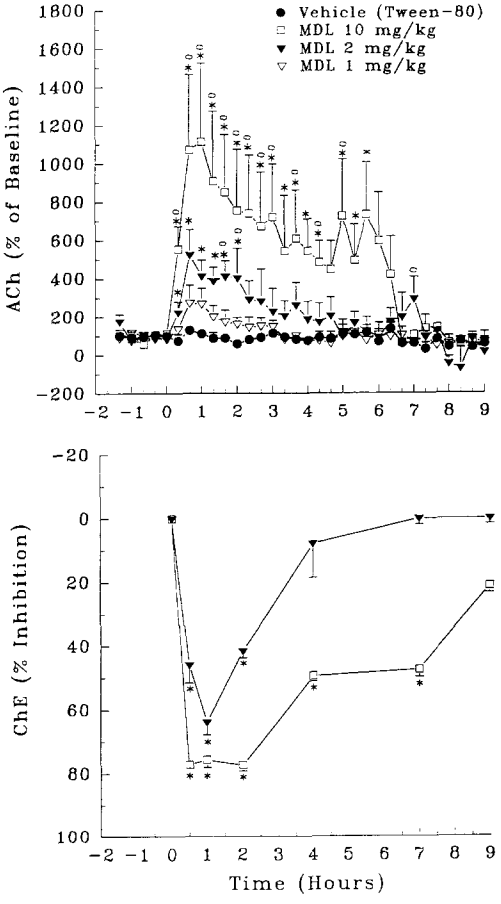

Fig. 2. Upper panel: Effect of three different doses of MDL 73,745 (0.5% Tween-80, ●; 10 mg/kg, □; 2 mg/kg, ▼; 1 mg/kg, ▽) (s.c.) on extracellular levels of acetylcholine in microdialysis samples from cerebral cortex of conscious, freely moving rats. MDL 73,745 and 0.5% Tween-80 vehicle were given at time zero. Each point is presented as percent of basal level (average of five samples prior to injection = 100%) and shown as mean ± S.E.M.; the baseline values are: 5.9 ± 1.5 nM for 0.5% Tween-80; 4.9 ± 1.2 nM for 10 mg/kg; 4.5 ± 1.0 nM for 2 mg/kg; and 6.0 ± 1.9 nM for 1 mg/kg; ($n = 5$). $^{*}P < 0.05$ compared to baseline by paired t-test; $^{\circ}P < 0.05$ compared to vehicle by one-way ANOVA followed by Tukey's multiple range test. Lower panel: Whole brain cholinesterase inhibition following the same dose of MDL 73,745 (10 mg/kg, □; 2 mg/kg, ▼; $n = 3$). $^{*}P < 0.05$ compared to the Tween-80 group by Student independent t-test.

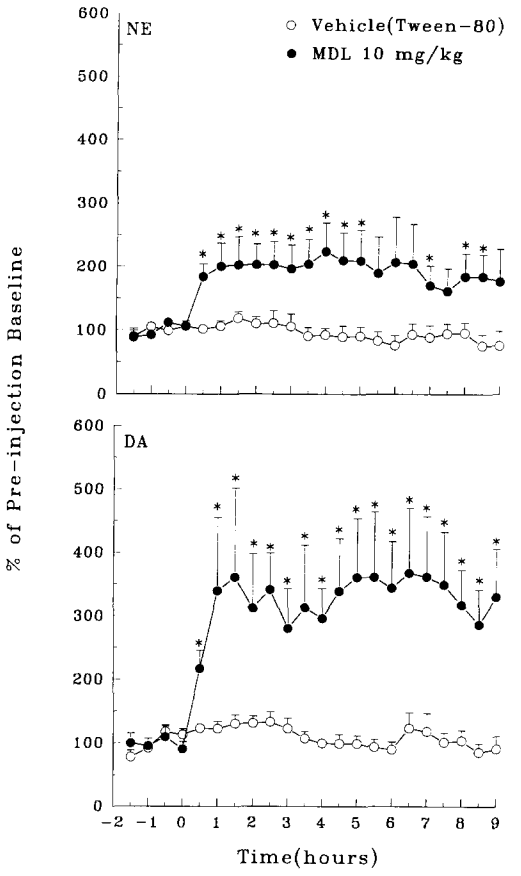

Fig. 3. Effect of MDL 73,745 (10 mg/kg, ●; 0.5% Tween-80, ○) (s.c.) on extracellular levels of norepinephrine (upper panel) and dopamine (lower panel) in microdialysis samples from cerebral cortex of conscious, freely moving rats. MDL 73,745 and 0.5% Tween-80 vehicle were given at time zero. Each point is presented as percent of basal level (average of five samples prior to injection = 100%) and shown as mean ± S.E.M.; the baseline values are: norepinephrine: 1.7 ± 0.4 nM for MDL 73,745; 1.8 ± 0.2 nM for 0.5% Tween-80; dopamine: 0.9 ± 0.5 nM for MDL 73,745; 1.2 ± 0.2 nM for 0.5% Tween-80 ($n = 6$). $^{*}P < 0.05$ compared to baseline by paired t-test.

4. Discussion

Cholinesterase inhibitors are, so far, the only drugs which have demonstrated clinical efficacy in the treatment of Alzheimer's disease (cf. Giacobini, 1994). Preclinical pharmacological characterization of these compounds is critical in order to develop derivatives or analogues with sustained effects on cerebral acetylcholinesterase and acetylcholine and reduced side-effects. Heptylphysostigmine is an example of a derivative of physostigmine that, because of its pharmacological characteristics, is a more promising therapeutic candidate than its parent compound (DeSarno et al., 1989; Cuadra et al., 1994).

Microdialysis studies, with a very few exceptions, have been performed using cholinesterase inhibitor such as neostigmine or physostigmine in the probe in order to increase the sensitivity of the acetylcholine assay. This produces inaccurate measurements of acetylcholine release, particularly when a second cholinesterase inhibitor is administered simultaneously via a systemic route (Messamore et al., 1993a,b). To avoid interference, the use of a cholinesterase inhibitor in the probe was excluded in this study (Cuadra et al., 1994). Our results show that acetylcholine basal levels can be measured by the sensitive high-performance liquid chromatography-electrochemical detection method developed in our laboratory.

Our results show that MDL 73,745 single-dose administration significantly increases extracellular acetylcholine levels in rat cortex above basal levels for a period of at least 6 h. This increase in cortical acetylcholine is related to the inhibition of acetylcholinesterase in brain; however, acetylcholinesterase activity recovers more slowly, particularly at a higher dosage of the inhibitor. Acetylcholinesterase activity is still 21% inhibited 9 h after 10 mg/kg MDL 73,745 administration while acetylcholine levels have returned to basal values.

Comparison of pharmacological effects of cholinesterase inhibitor in vivo may be a useful indication of their efficacy. The effect of acetylcholinesterase inhibition on extracellular acetylcholine levels measured with the microdialysis system varies strongly among various drugs (Messamore et al., 1993b). Peak concentrations of acetylcholine vary between 27 and 67 nM, while maximal acetylcholinesterase inhibition varies between 23% and 90% (Table 1). Physostigmine and tetrahydroaminoacridine elicit a stronger effect on acetylcholine levels in cortex at a lower acetylcholinesterase inhibition than both heptylphysostigmine and MDL 73,745. However, the duration of the acetylcholine increase is longer for heptylphysostigmine, (L)-Huperzine-A (Zhu and Giacobini, 1995) and MDL 73,745 than for physostigmine and tetrahydroaminoacridine (Table 1). This may constitute a therapeutic advantage.

Cholinergic side-effects also vary in intensity among drugs and seem to be generally related to high levels of acetylcholine rather than to cholinesterase inhibition (Messamore et al., 1993b; Giacobini and Cuadra, 1994) (Table 1). With MDL 73,745, cholinergic side-effects were most marked at the 10 mg/kg dose in the form of fasciculations and tremor.

In previous studies, in order to study the cholinesterase inhibitor effect on neurotransmitter levels in the same region of the brain where acetylcholine release was being monitored, the drugs were administered directly via the microdialysis probe (Cuadra et al., 1994). The fact that the intensity of symptoms of cholinergic toxicity was lower after local administration suggested that high acetylcholine levels in cerebral cortex may not be related to the severe side-effects seen after systemic injection.

After systemic administration of MDL 73,745, we observed that the increase in acetylcholine levels was accompanied by a significant norepinephrine and dopamine increase. However, only the highest dose (10 mg/kg) elicited significant changes. 5-Hydroxytryptamine levels were not elevated following any dose of MDL 73,745. This is consistent with the effects of other cholinesterase inhibitor including physostigmine, heptylphysostigmine and (L)-Huperzine-A on cortical neurotransmitters (Cuadra et al., 1994; Zhu and Giacobini, 1994).

Biochemical, behavioral and electrophysiological studies have shown close interactions between cholinergic and noradrenergic systems (Decker and McGaugh, 1991). Norepinephrine decreases the release of acetylcholine from cholinergic terminals in cortex (Vizi, 1980; Moroni et al., 1983). This effect is mediated both directly via α-adrenoceptors on cholinergic terminals and indirectly via norepinephrine modulation of GABA release (Beani et al., 1986). There is also evidence that norepinephrine and acetylcholine interact with each

Table 1
Comparison of effects of cholinesterase inhibitor on rat cortex

Drug	Dose (mg/kg)	Acetylcholine max. % increase	Peak acetylcholine conc. (nM)	Time to peak (min)	Acetylcholine increase duration (h)	Max. acetylcholinesterase inhibition	Duration time to peak (min)	Acetylcholinesterase inhibition (h)	Cholinergic side-effects
Physostigmine [a]	0.3	1100	67	30	1.5	50	10	2	+++
Heptylphysostigmine [a]	5	1000	65	90	10	90	60	27	++
MDL 73,745	10	1016	37	60	6	77	60	9	++
Tetrahydroaminoacridine [a]	5	500	27	90	3	50	60	10	+
(L)-Huperzine-A [b]	0.3	129	34	60	4	23	60	> 6	++ [c]

+++ = splay, tremor, fasciculations; ++ = tremor, fasciculations; + = fasciculations; [a] Messamore et al., 1993b; [b] Zhu and Giacobini, 1995 (in press); [c] facial-forelimb seizures when injected in the probe.

other, influencing learning and memory (Santucci et al., 1991). The interaction between acetylcholine and norepinephrine seems to be reciprocal, as acetylcholine also is able to regulate norepinephrine function. It is possible that the norepinephrine elevation seen in our experiments could down-regulate acetylcholine levels and decrease the therapeutic effect of these drugs.

In frontal cortex, dopamine is involved in the control of cognitive function (Brozoski et al., 1979). The participation of dopamine in learning and memory function is suggested by the role of dopamine in attention and reward mechanisms (Wise, 1978; Beninger, 1983). Cholinergic agonists increase dopamine turnover and release in vivo (Xu et al., 1989) and muscarinic antagonists impair the performance of cognitive tests and reduce dopamine turnover in frontal cortex (Memo et al., 1988). There is also evidence that dopamine affects cholinergic neurons. The activity of cortically projecting neurons in the nucleus basalis is regulated in an excitatory manner by central dopaminergic neurons (Day and Fibiger, 1992). The elevation of dopamine levels observed in our study, and the reciprocal interaction of dopamine and acetylcholine, suggest an additive therapeutic effect.

In conclusion, MDL 73,745 produced a long-lasting inhibition of cholinesterase and increased the levels of acetylcholine, norepinephrine, and dopamine in rat cortex and had fewer cholinergic side-effects than physostigmine. It also enhances working memory performance in a T-maze and improves retention in a passive avoidance task. Therefore, MDL 73,745 may constitute a potentially useful new cholinesterase inhibitor drug for the treatment of patients with Alzheimer's disease.

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