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Evaluation of acute bis(7)-tacrine treatment on behavioral functions in 17-day-old and 30-day-old mice, with attention to drug toxicity

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Abstract

Bis(7)-tacrine was evaluated for efficacy on memory retention in mice 17 days of age and 30 days of age. The tests used were a passive-avoidance response test and a measure of spontaneous motor activity. Also, possible drug-induced hepatotoxicity and acute drug toxicity were evaluated. Behavioral studies were performed using a step-through task and an open-field test with a 24-h interval between training and evaluation tests. Bis(7)-tacrine (0.06−20 μmol/kg) was subcutaneously injected 30 min prior to the first session of both test types. During the training session of the step-through task, bis(7)-tacrine treatment reduced (by 46%, $P<0.01$) the number of avoidable electric shocks (footshocks) only at a high dose of 20 μmol/kg in mice 17 days of age, but dose-dependently decreased the number of footshocks (10–56%, $P<0.001$) in mice 30 days of age. Bis(7)-tacrine treatment at all doses tested did not produce any detectable changes in retention latency in mice 17 days of age, but the drug significantly prolonged retention latency at low doses (1.25 and 2.50 μmol/kg), and not high doses (5–20 μmol/kg), in mice 30 days of age. In the open-field test, bis(7)-tacrine decreased spontaneous motor activity in the acquisition session only at a high dose of 20 μmol/kg in mice 17 days of age and 30 days of age (by 28 and 45%, respectively), but did not affect spontaneous motor activity in the recall session. Bis(7)-tacrine treatment at a dose of 20 μmol/kg produced a more potent hepatotoxic effect in mice 30 days of age than in mice 17 days of age, ($P<0.05$), and the drug caused acute toxicity with comparable potencies in mice of both age groups. In conclusion, mice 30 days of age seemed to be more sensitive than mice 17 days of age to bis(7)-tacrine-induced cognitive function enhancement and hepatotoxicity. Bis(7)-tacrine appears to be more potent and more selective as a cognitive function-enhancing agent than tacrine.

Keywords: Bis(7)-tacrine; Passive-avoidance response; Open-field memory; Spontaneous motor activity; Hepatotoxicity; Acute toxicity

1. Introduction

Tacrine, a reversible acetylcholinesterase (AChE) inhibitor, was found to improve cognitive function in naturally aged mice and in mice subjected to experimental models of aging (both groups of animals showed learning and memory deficits). Tacrine also improved cognitive function in young cognitively impaired rats (Marighetto et al., 2000; Van Dam et al., 2003; Sabolek et al., 2004). This drug is the first AChE inhibitor approved for treatment of Alzheimer's disease (AD), and is widely used as a cognition-enhancing agent (Wagstaff and McTavish, 1994; Qizilbash et al., 1998; Giacobini, 1998). However, clinical studies showed that cognitive function was improved in only 30–40% of patients receiving tacrine treatment for several months (Knapp et al., 1994; Farlow et al., 1992; Davis et al., 1992). Furthermore, the prolonged use of tacrine has proved to be hepatotoxic in about 30% of patients, who also suffered from other side effects (Salmon et al., 2001; Watkins et al., 1994). Experimental studies showed that acute hepatotoxicity may also be caused by a single, high, dose of tacrine (Stachlewitz et al., 1997). Given that the adverse effects of tacrine are likely a consequence of poor drug

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selectivity for AChE, the search for novel AChE inhibitors with high selectivity and potency has therefore been an area of intense interest.

Bis(7)-tacrine (1,7-N-heptylene-bis-9,9′-amino-1,2,3,4-tetrahydroacridine), derived from tacrine (9-amino-1,2,3,4-tetrahydroaminoacridine), is a second generation inhibitor of AChE. Bis (7)-tacrine was found to be 1000-fold more potent than tacrine in the inhibition of rat brain AChE, and 10,000-fold more selective for AChE than butyrylcholinesterase (Carlier et al., 1999; Patani et al., 2005; Wang et al., 1999a). Bis(7)-tacrine improved cognitive function in amnesic animals (Wang et al., 1999b; Liu et al., 2000). In the present study, we examine the effects of acute bis(7)-tacrine treatment on the passive-avoidance response, spontaneous motor activity, hepatoxicity and acute toxicity in mice 17 days of age or 30 days of age that were presumably free of cognitive deficits and behavioral abnormalities.

2. Materials and methods

2.1. Animal care

Male ICR mice 17 days of age (13−15 g) or 30 days of age (23−25 g) were used. Animals were purchased from Vital River Lab Animal Co. Ltd. (grade II, Certificate No: SCXK-2002-0003), and eight animals were housed in each cage. Mice were maintained on a 12 h light/dark cycle (light from 07:00 to 19:00) at 20−22 °C, with relative humidity of 50−55%, and were given food and water *ad libitum* in an animal care facility. All experimental protocols were approved by the University Committee of Research Practice of the Beijing University of Chinese Medicine.

2.2. Drug treatment

Bis(7)-tacrine hydrochloride was synthesized according to the method of Carlier et al. (1999). Elemental analysis confirmed that the compound was in the dehydrated form, and had a purity >99.5%, as assessed by HPLC. The drug was dissolved in distilled water and administered by subcutaneous injection. Control mice received the vehicle (10 ml/kg). Previous studies have shown that the inhibition of AChE in rat brain tissues reached a maximum extent 30 min after bis(7)-tacrine was administered (Wang et al., 1999a). In behavioral studies, therefore, mice of both age groups received bis(7)-tacrine or vehicle subcutaneously 30 min prior to the training session in the step-through task or the acquisition session in the open-field test. No drug was given before the retention session in the step-through task or the recall session in the open-field test. Animals in various experimental groups were randomized according to a Latin square design. Experiments were carried out between 08.00 and 13.00.

2.3. Passive avoidance response

Passive-avoidance responses of mice 17 days of age or 30 days of age were assessed in a step-through task experiment. The step-through apparatus was contained in a box with two symmetrical compartments [14 cm (long)×5 cm (wide)×8 cm (high)]. The compartments were separated by a dark board which had a door 3 cm in diameter near the floor. The grid floors of the two chambers consisted of copper bars (0.3 cm in diameter, separated by 1 cm distances center-to-center) that could be electrified with 1.5 mA in the dark, but not in the light (i.e., safe) compartment, to produce unscrambled electric shocks. The experiment began with a training session in which each mouse, with the head facing away from the door, was gently put inside the safe compartment of the conditioning box, in which two 40 W bulbs hung 200 cm above the chamber. Upon stepping from the safe room to the dark compartment, the mouse would receive an electric shock (footshock). In response to the punishment, the mouse would return to the safe compartment and avoid subsequent entry to the dark compartment. During the training session, the number of footshocks for each mouse was recorded during a 5 min session and the animal was then returned to its home cage. The retention session was conducted 24 h after the training session. Each mouse was placed in the safe room (as in the training session) and the time elapsed (the latency time) before crossing from the safe place to the electrified chamber was recorded in a 3 min session. If the mouse did not cross to the dark compartment within 3 min, the session was ended and a score of 180 was given. The drug-induced enhancement of the passive avoidance-response learning was indicated by a reduced number of footshocks required for training, and prolonged retention latency, when compared with untreated controls.

2.4. Spontaneous motor activity

The extent of spontaneous motor activity within a fixed time period, in mice 17 days of age or 30 days of age, was determined by an open-field test. The apparatus consisted of a rectangular chamber [38 cm (long)×20 cm (wide)×24 cm (high)], with the field being illuminated by two 40 W light bulbs hanging at 200 cm above the center of the field. In an experiment, each mouse was gently placed in the field for 5 min on two consecutive days. The first placement and the second placement are termed the acquisition session and the recall session, respectively. In each session, when a mouse was put in the chamber, spontaneous motor activity was automatically measured immediately after placement, for 5 min, using an Activity Meter (MK-ANIMEX, Muromachi Kikai Co., Japan). In the open-field test, drug-induced inhibition of spontaneous motor activity was indicated by a reduction in activity, when compared with an untreated control, during the acquisition session. Open-field memory was indicated by a decrease in spontaneous motor activity during the recall session, relative to the response noted in the acquisition session.

2.5. Hepatotoxicity assessment

Mice 17 days of age and 30 days of age received bis(7)-tacrine subcutaneously at 5 μmol/kg or 20 μmol/kg, and orbital blood samples were obtained 6 h post-dosing. A previous study indicated that maximum elevation of plasma alanine amino-transferases (ALTs) activity was observed 6 h after bis-tacrine

administration (Pan et al., 2002). Serum samples were obtained by centrifuging the whole blood at 2000 ×*g* at 4 °C, for 8 min. Serum ALT and aspartate aminotransferases (AST) activities were measured using an assay kit from Zhongsheng Beikong Bio-technology and Science Inc. (Beijing, China).

2.6. Acute toxicity assessment

In the acute toxicity test, bis(7)-tacrine was administered subcutaneously at increasing doses (41–80 μmol/kg) to mice either 17 days of age or 30 days of age, and the mortality rate was determined within 24 h post-dosing. LD_{50} values were then estimated using the Probit method.

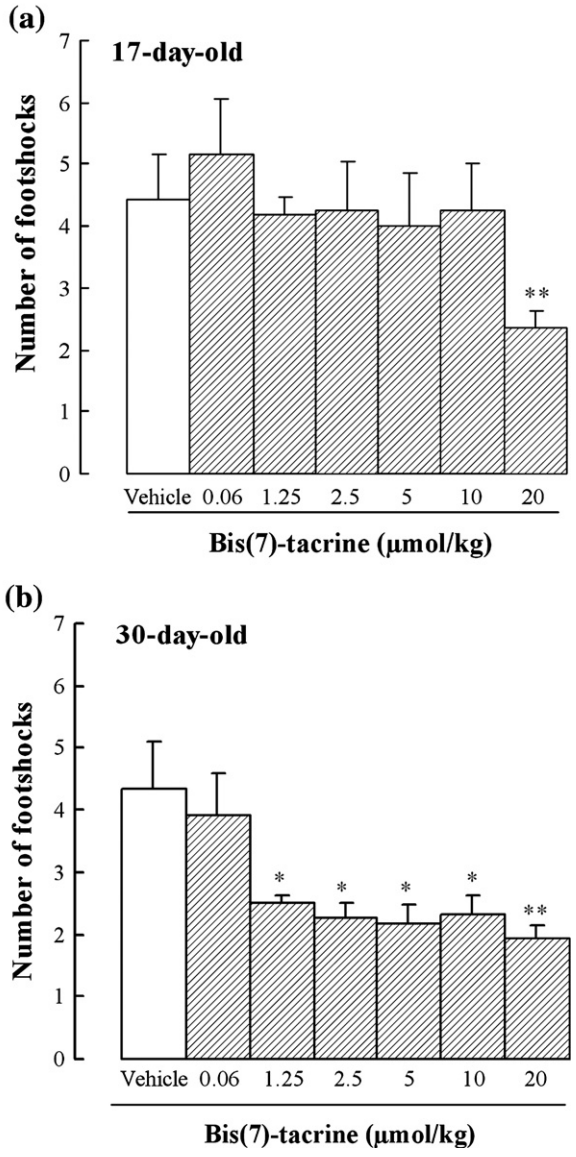

Fig. 1. Effects of bis(7)-tacrine treatment on the number of footshocks suffered in the passive-avoidance test in mice aged 17 days (a) or 30 days (b). Mice received bis(7)-tacrine subcutaneously at increasing doses (0.06–20 μmol/kg), or received vehicle only, 30 min prior to the training session. The numbers of footshocks within 5 min in each mouse during the training session were recorded. Each bar represents the mean±SEM, with n=11. *$P<0.05$, **$P<0.01$ vs. the respective vehicle-treated group, using one-way ANOVA followed by Dunnett's test.

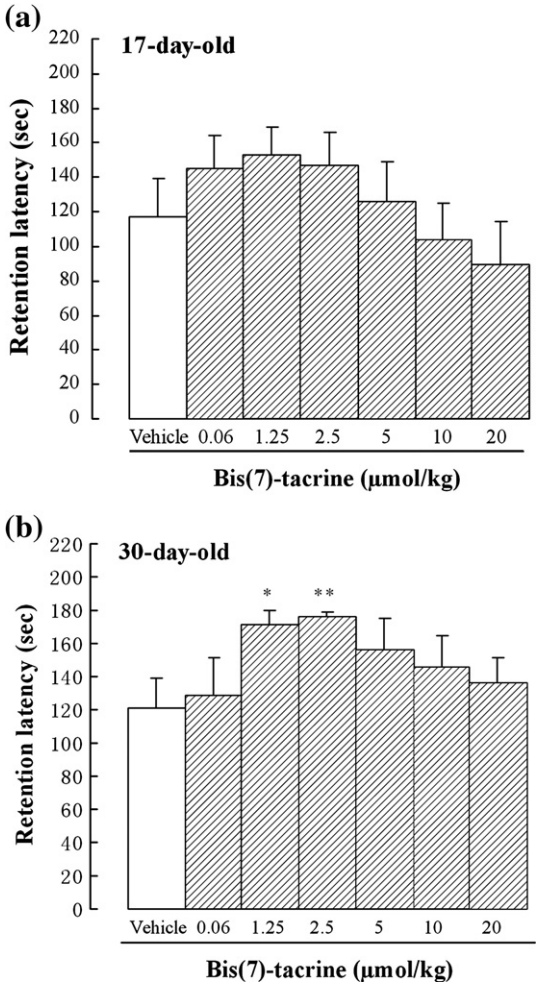

Fig. 2. Effects of bis(7)-tacrine treatment on retention latency in the passive-avoidance response in mice aged 17 days (a) or 30 days (b). Mice were treated as described in Fig. 1. The retention session was conducted 24 h after the training session and the retention latency was recorded within 3 min. Each bar represents the mean±SEM, with n=11. *$P<0.05$, **$P<0.01$ vs. the respective vehicle-treated group, using one-way ANOVA followed by Dunnett's test.

2.7. Statistical analysis

Values given are means±SEM. Data were analyzed either by one-way analysis of variance (ANOVA), followed by Dunnett's multiple-range test, or by Student's *t* test for paired comparisons, using SPSS12.0 software. Inter-group differences were considered significant when $P<0.05$.

3. Results

3.1. Passive-avoidance response

As shown in Fig. 1, bis(7)-tacrine treatment caused a decrease in the number of footshocks (by 46%, $P<0.01$) only at a dose of 20 μmol/kg in mice 17 days of age (Fig. 1a). In mice 30 days of age, bis(7)-tacrine treatment (1.25–20 μmol/kg) reduced the number of footshocks in a dose-dependent manner (by 10–56%, $P<0.001$) (Fig. 1b).

Table 1
Effects of bis(7)-tacrine treatment on spontaneous motor activity in open-field tests in mice aged 17 or 30 days

Bis(7)-tacrine (μmol/kg)	Acquisition session		Recall session	
	17-day-old	30-day-old	17-day-old	30-day-old
Vehicle	390±20	488±27$^{\#\#}$	235±11†††	297±37†††
2.5	342±21		235±24†††	
5.0	401±18	486±27	273±17†††	312±12†††
10	344±30	428±30	274±22	363±12†††
20	282±35*	270±31***	200±18†	270±27

Mice received bis(7)-tacrine subcutaneously at increasing doses (2.5−20 μmol/kg, or vehicle, 30 min prior to the acquisition session. The spontaneous motor activity in each mouse was monitored for 5 min in the acquisition session and recall session, with the latter being conducted 24 h after the former. Values given are means±SEM, with $n=11$. *$P<0.05$, ***$P<0.001$ vs. vehicle-treated controls, using one-way ANOVA followed by Dunnett's test. $^{\#\#}P<0.01$ vs. vehicle-treated 17-day-old mice; $^{\dagger}P<0.05$, $^{\dagger\dagger\dagger}P<0.001$ vs. the corresponding group in the acquisition session, using Student's t test.

During the retention session, bis(7)-tacrine treatment (0.06−2.50 μmol/kg) tended to increase retention latency in mice 17 days of age, but none of these changes attained statistical significance ($P>0.05$), when compared to vehicle-treated controls (Fig. 2a). In contrast, retention latency was significantly prolonged (up to 45%, $P<0.01$) by bis(7)-tacrine treatment at doses of 0.06–2.50 μmol/kg in mice 30 days of age, compared to vehicle-treated controls (Fig. 2b). Bis(7)-tacrine treatment at high doses (5–20 mmol/kg) gradually shortened retention latency in both mice both 17 days of age and 30 days of age. There was no significant difference in the average latency for entry into the dark compartment during the training session when vehicle and bis(7)-tacrine-treated mice groups were compared (data not shown).

3.2. Spontaneous motor activity

During the acquisition session, the number of motor activities was larger (25%, $P<0.01$) in vehicle-treated mice 30 days of age than in mice 17 days of age. Bis(7)-tacrine treatment decreased spontaneous motor activity (by 28% and 45% in the 17-day-old and 30-day-old mice, respectively, compared to control animals) during the acquisition session only when used at a high dose of 20 μmol/kg. Spontaneous motor activity counts recorded in the recall session were reduced by 40% ($P<0.001$) and 39% ($P<0.001$) from responses of vehicle-treated (control) mice 17 days of age, or 30 days of age, respectively, when data from the acquisition session were compared. There were no detectable changes in recall session spontaneous motor activity in mice treated with bis(7)-tacrine, when compared with vehicle-treated controls (Table 1).

Table 2
Bis(7)-tacrine-induced hepatic injury in mice aged 17 or 30 days

Group	Dose (μmol/kg)	ALT (U/L)	AST (U/L)
17-day-old			
Vehicle	0	25±2	112±12
Bis(7)-tacrine	5	28±1	101±15
	20	57±6***	212±23***
30-day-old			
Vehicle	0	27±1	103±10
Bis(7)-tacrine	5	25±2	120±14
	20	65±7***	293±28*** $^{\#}$

Mice subcutaneously received bis(7)-tacrine at doses of 5 or 20 μmol/kg. Control animals received vehicle only. Six hours after drug or vehicle administration, serum alanine aminotransferases (ALTs) and aspartate aminotransferase (ASTs) activity were measured. Each bar represents a mean±SEM, with $n=10$. ***$P<0.001$ vs. vehicle-treated control; $^{\#}P<0.05$ vs. 17-day-old mice treated with the same dose of bis(7)-tacrine, using Student's t test.

Table 3
Acute toxicity of bis(7)-tacrine in mice aged 17 or 30 days

	Dose (μmol/kg)	Mortality rate (%)	
		17-day-old	30-day-old
Bis(7)-tacrine	41.0	0	–
	51.2	30	10
	64.0	70	60
	80.0	–	100

Mice were divided into groups of 10 animals and subcutaneously received bis(7)-tacrine at increasing doses of 41–80 μmol/kg. The mortality rate in each group was determined within 24 h post-dosing. LD_{50} values (17-day-old=57.7±5.7 μmol/kg; 30-day-old=61.0±4.7 μmol/kg) were estimated by the Probit method.

3.3. Hepatotoxicity

Bis(7)-tacrine treatment at a dose of 20 μmol/kg, but not 5 μmol/kg, caused significant increases in serum ALT (128 and 141%, respectively) and AST (89 and 184%, respectively) activities in mice 17 days of age and mice 30 days of age 6 h post-dosing. The serum AST activity in mice aged 30 days was significantly higher than that of mice aged 17 days ($P<0.05$) (Table 2).

3.4. Acute toxicity

The LD_{50} values of bis(7)-tacrine were 57.7±5.7 μmol/kg (or 32.6±3.2 mg/kg) and 61.0±4.7 μmol/kg (or 34.5±2.7 mg/kg), respectively, in mice aged 17 days or 30 days (Table 3).

4. Discussion

The assessment of passive-avoidance response is commonly used for the pharmacological evaluation of cognitive function enhancers. The step-through task adopted in the present study provided several (not just one) occasions within 5 min for mice to enter the compartment with punishment. In the context of cognitive psychology, memory is categorized into three types: sensory memory, short-term memory, and long-term memory. In this context, the number of footshocks received during the 5 min training session in the step-through task is a functional measure of sensory or short-term memory, whereas the latency recorded during the retention session measures long-term memory function. Nevertheless, if a drug treatment regimen suppresses spontaneous motor activity and/or increases sensitivity to pain, false-positive cognitive function enhancement will occur. Bis(7)-tacrine treatment at low doses (1.25–

2.50 μmol/kg), however, reduced the number of footshocks, increased retention latency, but did not induce motor dysfunction. It is thus likely that the drug has a specific cognitive function-enhancing action. On the other hand, the decrease in the number of footshocks after bis(7)-tacrine treatment at a high dose of 20 μmol/kg, accompanied by reduced spontaneous motor activity, may well be caused by drug-induced hypopraxia. The lowest effective dose of bis(7)-tacrine for enhancement of the passive-avoidance response in mice was 1.25 μmol/kg. Given that the effective doses of bis(7)-tacrine for inhibition of AChE in rat brain tissues were 9.5–38 μmol/kg (Wang et al., 1999a), it is possible that the enhancement of the passive-avoidance response by bis(7)-tacrine may not be related to the inhibition of AChE in the brain. In this context, several studies have shown that bis(7)-tacrine caused various biological effects. Bis(7)-tacrine regulated calcium channels (Fu et al., 2006), inhibited nitric oxide synthase (Li et al., 2006), and blocked N-methyl-D-aspartate and GABA(A) receptors (Li et al., 2005, 1999), while also inhibiting AChE.

The pharmacological assessment of the cognitive effect caused by AChE inhibitors is often associated with an inverted U-shaped dose-response curve. This suggests that the cognitive function-enhancing effect of the drug is diminished, or memory is suppressed, when the drug is administered at doses higher than optimal for the enhancement of cognitive function (Riekkinen et al., 1991; Braida et al., 1996; Ou et al., 2001). In this regard, it has been shown that tacrine, when administered at the optimal doses for reduction of the number of footshocks in the step-through task (20 μmol/kg or 40 μmol/kg in mice aged 17 days or 30 days, respectively), caused a reduction in retention latency, indicative of impairment in long-term memory (Pan et al., 2006). While the inverted U-shaped dose-response curve of the bis(7)-tacrine-induced change in retention latency was apparent in mice aged either 17 days or 30 days, bis(7)-tacrine treatment, at the dose (20 μmol/kg) optimal for reducing the number of footshocks in mice aged either 17 days or 30 days, did not produce any significant negative effects on retention latency in the step-through task. The decrease in spontaneous motor activity during the recall session of the open-field test is a manifestation of open-field memory (Pan, 1995, 1992). In this regard, the microinjection of tacrine or nicotine into the core of the nucleus accumbens after the first exposure to the open field was found to enhance open-field memory (Schildein et al., 2000, 2002). In the present study, bis(7)-tacrine treatment at doses up to 20 μmol/kg did not enhance or suppress open-field memory in mice aged either 17 days or 30 days. Consistent with this observation, when tacrine or bis(7)-tacrine were intraperitoneally administered at 20 μmol/kg prior to the acquisition session in the open-field test, the spontaneous motor activity in the recall session increased significantly only in tacrine-treated mice. This indicates that tacrine, but not bis(7)-tacrine, impaired open-field memory (data not shown). Taken together, tacrine seems to be more potent than bis(7)-tacrine in suppressing the processes involved in the formation of passive-avoidance long-term memory and open-field memory.

Previous studies from our laboratory have shown that tacrine-induced enhancement in cognitive/behavioral function and associated hepatotoxicity were more potent in mice aged 17 days than in mice aged 30 days. In the present study, however, the mice aged 30 days were more sensitive than mice aged 17 days to bis(7)-tacrine-induced enhancement in passive-avoidance responses, and associated hepatotoxicities. While the biochemical mechanisms underlying developmental changes in sensitivity to bis(7)-tacrine remain to be determined, these may be related to the numbers of brain cholinergic receptors, and/or the development of drug metabolism, influencing drug availability. Consistent with this suggestion, experiments have shown that the number of cholinergic receptors in neonatal rat brain increases during development, with the number of receptors reaching an adult level around 35 days after birth (Zhu et al., 2000; Daws and Overstreet, 1999). Moreover, other studies have shown that while bis(7)-tacrine was 140-fold more potent than tacrine in inhibiting AChE in rat cortex homogenates *in vitro*, bis(7)-tacrine was only 6.3-fold more potent than tacrine in suppressing the activity of AChE in rat cortex *in vivo* (Wang et al., 1999a). In addition, the LD_{50} value of bis(7)-tacrine was found to be about one half that of tacrine when the drugs were subcutaneously administered (Table 3, Pan et al., 2006). Conceivably, a proportion of bis(7)-tacrine molecules may be broken into two molecules of tacrine by *in vivo* metabolism, resulting in a decrease in bioavailability of the more potent bis(7)-tacrine in brain tissues. The higher sensitivity of mice aged 30 days, compared to mice aged 17 days, to bis(7)-tacrine-induced cognition enhancement may therefore be related to developmental changes in *in vivo* metabolism of the drug. In this regard, the sensitivity of muscarinic receptor-stimulated phosphoinositide metabolism in the brain was found to be higher in immature than adult rats (Balduini et al., 1987, 1990).

In summary, bis(7)-tacrine treatment at doses of 1.25–10 μmol/kg was more effective in enhancing the passive-avoidance response than in inhibiting spontaneous motor activity, in mice aged 30 days. The cognitive function-enhancing effect of bis(7)-tacrine at a high dose (20 μmol/kg) was associated with motor dysfunction and hepatotoxicity. The mice aged 30 days seemed to be more sensitive than mice aged 17 days to bis(7)-tacrine-induced cognitive function enhancement and hepatotoxicity. These data, together with our previous report (Pan et al., 2006), suggest that bis(7)-tacrine is a more potent and selective cognitive function-enhancing agent than is tacrine.

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