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Inhibitory effects of huperzine B on cholinesterase activity in mice

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KEY WORDS huperzine B; tacrine; cholinesterase inhibitors; cholinesterases; acetylcholinesterase; brain; cerebral cortex

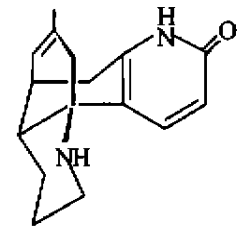
ABSTRACT

AIM: To determine the anticholinesterase properties of huperzine B (Hup B) and compare with tacrine *in vitro* and *in vivo*. **METHODS**: Spectrophotometry was used to determine ChE activity. **RESULTS**: Hup B showed much more selective inhibition to acetylcholinesterase (AChE) than tacrine. The IC_{50} ratios of Hup B and tacrine for butyrylcholinesterase (BuChE): AChE were 65.8 and 0.54, respectively. Hup B ig exhibited higher efficacy on the inhibition of brain AChE than that of tacrine. Tacrine was more effective in the inhibition of serum BuChE in mice with severe concomitant peripheral adverse effects than Hup B. A single ig dose of Hup B produced steady state of AChE inhibition in 4 h. **CONCLUSION**: Hup B exhibits higher selectivity and efficacy in the inhibition of AChE, and lower toxicity in mice than tacrine.

INTRODUCTION

Shortly after the discovery that cholinergic system is involved most consistently and most severely in Alzheimer's disease (AD)[1], attempts began to ameliorate the cognitive dysfunction characteristic of AD with agents intended to enhance the central cholinergic transmission. So far, cholinesterase inhibitors (ChEI) are the only drugs demonstrating clinical efficacy in the treatment of AD. Among these compounds, tacrine showed clinical efficacy in 20 % - 30 % of AD patients[2] and has been approved by FDA (USA) for the symptomatic treatment of AD. However, it has met with limited success owing to the dose-limiting liver toxicity[3] and severe cholinergic side effects. In view of these problems, searching for ChEI with relatively safer and greater therapeutic effects in the treatment of AD is still ongoing.

Huperzine B (Hup B), a new *Lycopodium* alkaloid isolated from Chinese herb *Huperzia serrata* (Thunb) Trev[4], is a potent, reversible ChEI exhibiting more selective inhibition on AChE than those of galanthamine *in vitro*. In behavioral studies, Hup B improved memory retention and memory retrieval in adult and aged mice and reversed the disruption of memory retention induced by scopolamine, $NaNO_2$, electroconvulsive shock and cycloheximide in mice, meanwhile it exhibited less peripheral side effects compared with galanthamine and physostigmine[5]. The preliminary results of Hup B showed definite advantages as compared with the first generation of ChEI. The aim of this



Huperzine B

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study was to provide further comparative data on Hup B and tacrine by testing their anti-ChE activities *in vitro* and *in vivo*.

MATERIALS AND METHODS

Mice Kunming strain mice (n = 240, 28.5 g ± s 2.0 g) of either sex were supplied by Shanghai Experimental Animal Center, Chinese Academy of Sciences (Grade Ⅱ, Certificate № 005).

Materials Hup B {$[\alpha]$ = − 54.2 ℃ (c 0.203, MeOH), mp 270 − 271 ℃, purity >98 %} was prepared by Department of Phytochemistry of this Institute. Acetylthiocholine iodide (ACh), sodium dodecyl sulfate (SDS), and Coomassie brilliant blue were purchased from Fluka Chemie. Tacrine, butyrylthiocholine iodide (BuChE), 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB), tetraisopropyl pyrophosphoramide (isoOMPA), electric eel, bovine erythrocyte, human erythrocytes AChE and horse serum BuChE were purchased from Sigma Chemical Co. Other chemicals were reagent grade.

Preparation of samples Mice were dosed ig or ip with several dosages of Hup B or tacrine and decapitated at various time intervals. The brains were rapidly dissected on ice. The right frontal cortex and the left hemisphere were homogenized in 50 (wt/vol) volumes of ice-cold sodium phosphate buffer (75 mmol · L^{-1}, pH 7.0). Before testing, the homogenate was preincubated with isoOMPA 0.1 mmol · L^{-1}, a selective inhibitor of butyrylcholinesterase (BuChE) activity. The blood was collected from the orbital venous plexus of mice. The serum was obtained after centrifugation (3000 × g, for 10 min) and diluted with ice-cold sodium phosphate buffer (1∶12, vol/vol).

ChE assay AChE and BuChE activities were measured by the spectrophotometric method[6], ACh 0.3 mmol · L^{-1} or BuCh 0.4 mmol · L^{-1} were used as substrates for the assay of AChE and BuChE, respectively. The mixture, including substrates, sodium phosphate buffer (0.1 mmol · L^{-1}, pH 7.4) 1 mL, and enzyme 0.1 mL, was incubated in a total volume of 4 mL at 37 ℃ for 8 min. The reaction was terminated by adding 3 % SDS 1 mL, then 0.2 % DTNB 1 mL was added to produce the yellow anion of 5-thio-2-nitrobenzoic acid. The color was measured spectrophotometrically at 440 nm. All samples were assayed in duplicate.

Protein assay Protein concentrations were measured by the Coomassie blue protein-binding method[7] using bovine serum albumin as standard.

Statistical analysis Data were expressed as μmol substrate hydrolyzed · min^{-1} · g^{-1} (protein) or % inhibition (*vs* saline control) ± s. Statistical analysis was performed with t-test.

RESULTS

Inhibition of AChE and BuChE activities *in vitro* Hup B and tacrine both produced a marked concentration-dependent inhibition of AChE. The results, expressed as IC_{50} values, were listed on Tab 1.

Tab 1. IC_{50} (nmol · L^{-1}) of huperzine B and tarcine on cholinesterase activities *in vitro*. $\bar{x} \pm s$ for triplicate.

ChEI	Huperzine B	Tacrine
BuChE		
rat serum	169 300 ± 5 900	50.2 ± 1.7
horse serum	26 330 ± 6 300	10.95 ± 0.01
AChE		
rat cortex	2 572 ± 149	92 ± 6
rat RBC membrane	2 714 ± 100	96 ± 4
electric eel	1 017 ± 42	39.41 ± 0.14
bovine RBC membrane	2 738 ± 132	118 ± 10
human RBC membrane	2 391 ± 58	139 ± 7

Regarding to the ChE inhibition, Hup B was

less potent than tacrine *in vitro*. However, Hup B inhibited BuChE at a much higher concentration than that needed for inhibition of AChE. On the basis of the IC_{50} ratios of rat serum BuChE : rat frontal cortex AChE, Hup B (65.8) was much more selective in AChE inhibition than tacrine (0.54).

Inhibition of ChE activity *in vivo* Changes of ChE activity at 30 min in the hemisphere, frontal cortex and serum of mice were tested following ig administration of 3 dosages of Hup B and tacrine. Dose-dependent inhibition of ChE occurred in both cases. Inhibition (24 %) of AChE was observed in the frontal cortex at 40 $\mu mol \cdot kg^{-1}$ (*vs* saline control, $P < 0.01$) of Hup B. The anti-AChE potencies of Hup B in the frontal cortex of mice were eight times more potent than that of tacrine based on the dosage of molecular weights (Tab 2). Tacrine at 80 $\mu mol \cdot kg^{-1}$ inhibited 17 % of the BuChE activity ($P < 0.05$ *vs* saline control). However, Hup B inhibited BuChE only at 60 $\mu mol \cdot kg^{-1}$.

Tab 2. Anti-cholinesterase effects of huperzine B and tacrine. AChE and BuChE activity were expressed as μmol substrate hydrolyzed · $min^{-1} \cdot g^{-1}$ (protein). $\bar{x} \pm s$. $^bP < 0.05$, $^cP < 0.01$ *vs* saline.

ChEI $\mu mol \cdot kg^{-1}$	AChE Whole brain 8 – 12 mice	AChE Cortex 8 – 12 mice	BuChE Serum 4 – 8 mice
Saline	104 ± 8	44 ± 4	77 ± 12
Huperzine B			
60	78 ± 6[c]	32.5 ± 2.2[c]	62 ± 6[b]
40	85 ± 4[c]	33.0 ± 2.3[c]	66 ± 3
20	91 ± 5[c]	37 ± 7	72 ± 6
Tacrine			
160	85 ± 6[c]	36 ± 3[b]	58 ± 7[c]
120	89 ± 4[c]	37 ± 4	60 ± 6[c]
80	92 ± 4[c]	41 ± 3	64 ± 8[b]

Hup B exhibited similar efficacy on ChE inhibition via either ig administration or ip injection (Tab 3), while tacrine showed more potent inhibition via ip injection ($P < 0.01$ *vs* ig route). It implies that ig bioavailability of Hup B is better than that of tacrine.

Tab 3. The effect of ig or ip administration of huperzine B 40 μmol · kg^{-1} and tacrine 120 μmol · kg^{-1} on the ChE activity. $\bar{x} \pm s$. $^aP > 0.05$, $^cP < 0.01$ *vs* ig route.

ChEI	AChE Whole brain 8 – 10 mice	AChE Cortex 6 – 8 mice	BuChE Serum 4 – 8 mice
Huperzine B			
ig	18 ± 4	24 ± 8	14 ± 4
ip	19 ± 5[a]	21 ± 3[a]	18 ± 13[a]
Tacrine			
ig	14 ± 4	14 ± 8	21 ± 8
ip	24 ± 5[c]	30 ± 10[c]	59 ± 10[c]

Values were expressed as percentage of control. Basal saline control AChE values were 44 ± 4 (cortex) and 104 ± 8 (whole brain) μmol substrate hydrolyzed · $min^{-1} \cdot g^{-1}$ (protein) ($n = 12$), basal saline control BuChE values of serum was 77 ± 12 μmol substrate hydrolyzed · $min^{-1} \cdot g^{-1}$ (protein) ($n = 8$).

Time course of ChE inhibition after ig medication Following ig a single dose of Hup B (40 $\mu mol \cdot kg^{-1}$), AChE inhibition in the brain hemisphere and frontal cortex reached the maximum in 120 min, and recovered to 50 % of the maximal AChE inhibition at 240 min (Fig 1). The inhibition of BuChE was greater by tacrine than by Hup B, however, of AChE, the vice versa. In case of Hup B, the BuChE activity recovered to control level at 6 h, whereas still about 15 % inhibition existed in case of tacrine.

Side effects Side effects were severe for tacrine and weak for Hup B. Besides fasciculation, other characteristic symptoms of cholinergic hyperactivity such as salivation, lacrimation and myasthenia were clearly visible following ig administration of tacrine, whereas Hup B (40 –

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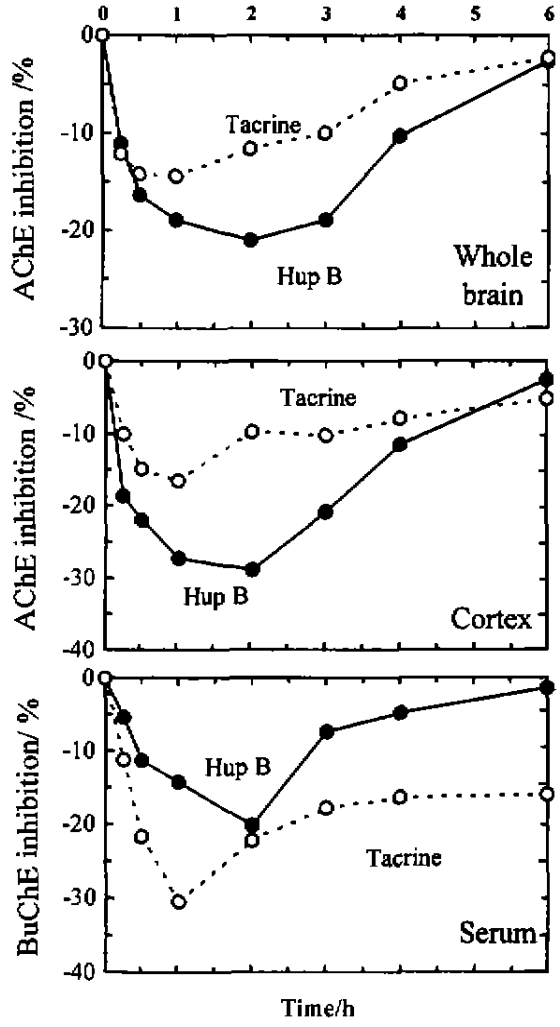

Fig 1. ChE inhibitions after ig administrations of Hup B 40 μmol·kg^{-1} or tacrine 120 μmol·kg^{-1} in 4 – 10 mice. ChE was expressed as μmol substrate hydrolyzed · min^{-1} · g^{-1} (protein). Basal saline control AChE values were 104 ± 5 (whole brain, 12 mice) and 49 ± 8 (cortex, 12 mice), basal saline control BuChE values of serum was 70 ± 7 (8 mice).

60 μmol·kg^{-1}) only caused slight fasciculation in mice.

DISCUSSION

In vitro, the selectivity of Hup B was about 120 times higher than that of tacrine according to the IC_{50} ratio of BuChE (rat serum) : AChE (rat cortex). There is evidence that the inhibition of BuChE concurred with AChE may contribute to the peripheral side effects[8]. Hence, drugs with high selectivity to AChE might be better drugs for the treatment of AD than non-selective ones. Cholinergic side effects were observed in parallel with the enzyme assay in mice. Hup B induced less peripheral adverse effects than tacrine. The results obtained with Hup B proved that the disadvantage of ChEI might be overcome by improving selective inhibition of AChE and thereby decreasing the peripheral cholinergic effects.

In vivo, the relative potency of inhibition of Hup B to AChE activity was about 8 times as potent as that of tacrine on basis of the molecular weights after ig administration. The AChE inhibition induced by Hup B was almost similar following ip and ig administration, however, tacrine ip showed much higher potency than ig administration. The hepatotoxic potential of tacrine may be inherent in the aminoacridine structure and its metabolites *in vivo*[3,9-11]. The results indicate that via oral route tacrine decomposes lots in liver causing hepatotoxicity and low bioavailability.

In conclusion, Hup B is a highly selective inhibitor of AChE. In animals, Hup B possesses a number of advantages over tacrine: stronger efficacy on brain AChE, higher oral bioavailability, and lower peripheral cholinergic side effects. Hup B would be a promising candidate in AD therapy.

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石杉碱乙的抗胆碱酯酶作用

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关键词 石杉碱乙；他克林；胆碱酯酶抑制剂；胆碱酯酶类；乙酰胆碱酯酶；脑；大脑皮质

目的：测试石杉碱乙的抗胆碱酯酶作用并与他克林进行比较. **方法**：比色法用于测定胆碱酯酶活性. **结果**：石杉碱乙和他克林对 BuChE 和 AChE 抑制的 IC_{50}值的比率分别为 65.8 和 0.54. 石杉碱乙对乙酰胆碱酯酶有更强的选择性抑制作用. 石杉碱乙灌胃对脑内 AChE 的抑制作用明显强于他克林. 而他克林对 BuChE 的抑制作用强于石杉碱乙，并有严重副反应. 单次灌胃石杉碱乙在 4 小时内对脑内 AChE 产生较为稳定的抑制作用. **结论**：与他克林比较石杉碱乙是乙酰胆碱酯酶的高选择性抑制剂，灌胃时药效高，毒性低.

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