

Effects of synthetic (-)-huperzine A on cholinesterase activities and mouse water maze performance

LIU Jing, ZHANG Hai-Yan, TANG Xi-Can¹, WANG Bin², HE Xu-Chang², BAI Dong-Lu²
(¹State Key Laboratory of Drug Research; ²Department of Synthetic Chemistry,
Shanghai Institute of Materia Medica, Chinese Academy of Sciences, Shanghai 200031, China)

KEY WORDS cholinesterase inhibitors; cholinesterases; huperzine A; cerebral cortex; erythrocyte membrane; scopolamine; memory; maze learning

AIM: To compare the effects of synthetic and natural (-)-huperzine A (Hup A) on cholinesterase and mouse water maze performance.

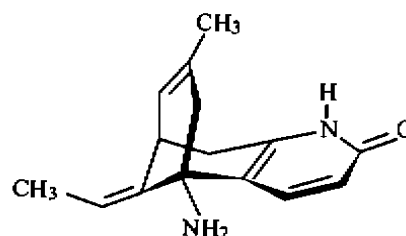
METHODS: Spectrophotometry was used to determine cholinesterase activity. Mouse water maze was used to evaluate nootropic effect.

RESULTS: The IC_{50} of synthetic Hup A for acetylcholinesterase (AChE) of rat cortex and rat erythrocyte membrane determined *in vitro* were 64.7 (52.6 - 79.5) and 53.9 (43.6 - 66.6) $nmol \cdot L^{-1}$, respectively, and for butyrylcholinesterase of rat serum was 53.6 (44.9 - 63.8) $\mu mol \cdot L^{-1}$. Synthetic Hup A 0.12 - 0.48 $mg \cdot kg^{-1}$ ig produced a dose-dependent inhibition of brain AChE in mice. Synthetic Hup A 0.05 $mg \cdot kg^{-1}$ ig attenuated scopolamine-induced impairment of spatial memory. The efficacy of synthetic Hup A was the same as natural Hup A.

CONCLUSION: Synthetic Hup A yielded an *in vitro* and *in vivo* pharmacological profile of activities similar to that of natural Hup A.

The cholinergic hypothesis of Alzheimer's disease (AD) was proposed after the consistent findings of the severe depletion of the cholinergic special enzymes, and the good correlation between the extent of the cholinergic neuronal loss and the impairment of cognitive function^[1]. These findings resulted in a number of strategies designed to improve cholinergic function. The use of cholinesterase inhibitors (ChEI) in the treatment of AD has been the most encouraging^[2]. However, the short duration of action, the low bioavailability, and the frequent

side effects of the first generation ChEI limited the clinical values^[3]. (-)-Huperzine A (Hup A) is a novel alkaloid isolated from *Huperzia serrata* (Thunb) Trev, a Chinese folk medicine^[4]. Preclinical and clinical studies demonstrated that natural Hup A was the drug of choice for the treatment of AD, due to its high selective inhibition toward AChE, longer duration of action, high oral bioavailability, and fewer side-effects^[5,6]. Three research groups have reported the total synthesis of (-)-Hup A successfully^[7-10], but there was rare data about the pharmacological activity of synthetic (-)-Hup A up to date. Recently Hup A was synthesized by the authors. In this paper, the effects of synthetic Hup A on ChE activities and scopolamine-induced memory deficits were studied.



(-)-Huperzine A

MATERIALS AND METHODS

Animals Kunming strain mice [$n = 110$, ($28.5 \pm s 2.0$) g] and Sprague-Dawley rats [$n = 30$, ($250 \pm s 5$) g] of either sex were supplied by Shanghai Experimental Animal Centre, Chinese Academy of Sciences (Grade Clean, Certificate No 005).

Materials Synthetic Hup A $\{[\alpha]_D^{10} = -173.0$ ($c = 1.04$, $CHCl_3$), mp ($229 - 231$) $^{\circ}C$, purity $> 98\%$ } was prepared by the authors. Natural Hup A (purity $> 98\%$) was prepared by Department of Phytochemistry in this institute. Acetylthiocholine iodide (s-ACh), sodium dodecyl sulfate (SDS), and Coomassie

¹ Correspondence to Prof TANG Xi-Can. Phn 86-21-6431-1833, ext 405.
Fax 86-21-6437-0269. E-mail xctang@server.shmc.ac.cn
Received 1998-06-19 Accepted 1998-06-26

brilliant blue (G250) were purchased from Fluka Chemie. *S*-butyrylthiocholine iodide (*s*-BuChE), 5, 5'-dithio-bis(2-nitrobenzoic acid) (DTNB), and tetraisopropyl pyrophosphoramidate (isoOMPA) were purchased from Sigma Chemical Co. Other chemicals were reagent grade.

Cholinesterase preparation Rat frontal cortex homogenate was used as AChE source. To prepare brain AChE, rat cortex was dissected on ice and homogenized in sodium phosphate buffer $0.75 \text{ mmol} \cdot \text{L}^{-1}$, and kept at $-30 \text{ }^{\circ}\text{C}$.

Rat erythrocyte membrane and serum were used as the sources of AChE and BuChE, respectively. To prepare erythrocyte AChE, rat blood was collected in a heparinized tube and centrifuged at $3000 \times g$ for 15 min. The plasma was diluted 1:4 with sodium phosphate buffer $5 \text{ mmol} \cdot \text{L}^{-1}$, pH 7.4, containing NaCl $0.15 \text{ mmol} \cdot \text{L}^{-1}$. The erythrocytes were washed 3 times in saline followed by lysis in 50-fold volumes of sodium phosphate buffer $8.45 \text{ mmol} \cdot \text{L}^{-1}$, pH 8.0. After 2-h stirring, the lysed cells were centrifuged at $12\,000 \times g$ at $4 \text{ }^{\circ}\text{C}$ for 15 min. The erythrocyte membranes were washed 3 times and then diluted with Tris-HCl buffer $10 \text{ mmol} \cdot \text{L}^{-1}$, pH 7.4. Serum and erythrocyte membranes were stored at $-30 \text{ }^{\circ}\text{C}$ for ChE assays.

ChE activity assay AChE and BuChE activities were measured by the spectrophotometric method⁽¹¹⁾: *s*-ACh ($0.3 \text{ mmol} \cdot \text{L}^{-1}$) or *s*-BuCh ($0.4 \text{ mmol} \cdot \text{L}^{-1}$) were used as specific substrates for the assay of AChE and BuChE, respectively. The mixture was incubated at $37 \text{ }^{\circ}\text{C}$ in a total volume of 4 mL for 8 min⁽¹²⁾. The reaction was terminated by adding 3% SDS 1 mL, then 0.2% DTNB 1 mL was added to produce the yellow 5-thio-2-nitrobenzoic acid, which was measured at $\lambda = 440 \text{ nm}$ for 30 min after initiation. All samples were assayed in duplicate.

Preparation of samples Mice were decapitated 30 min after ig synthetic or natural Hup A. Control mice were given saline. The brains were dissected on ice into frontal cortex and one hemisphere. Each brain region was homogenized in 50 (wt/vol) volumes of ice cold sodium phosphate buffer ($75 \text{ mmol} \cdot \text{L}^{-1}$, pH 7.0). The blood was collected from the orbital venous plexus of male mice. The serum was

obtained after centrifugation ($3000 \times g$, for 10 min) and diluted with ice cold sodium phosphate buffer (1:12).

Protein assay Protein concentration was measured by the Coomassie blue protein-binding method⁽¹³⁾ using bovine serum albumin as standard.

Behavioral test The plastic water maze ($80 \text{ cm} \times 50 \text{ cm} \times 20 \text{ cm}$, Fig 1) was used to assay performance. Mice [$n = 134$, weighing ($22.0 \pm s 1.5$) g] were kept in a 12-h light-dark cycle and given food and water *ad lib*. The mouse was placed on the water maze, with the nose towards the wall of the starting point and trained to find the platform. Following the period of 2-d habituation, each mouse received 3 training sessions daily for 7 d. Mice were trained to a criterion of finding the platform within 20 min and < 2 errors of entering non-exit. Once a mouse reached the criterion, training was reduced to one session daily until all mice reached the criterion. Synthetic and natural Hup A were dissolved in saline. Trained mice were randomly assigned to subgroups. Synthetic and natural Hup A were administered ig 45 min and scopolamine was injected ip 30 min before behavioral testing. The number of errors and the time of reaching platform were recorded.



Fig 1. Mouse water maze apparatus. S: starting point. P: the platform.

Statistical analysis Data were expressed as % inhibition (*vs* saline control) $\pm s$. Statistical analysis was performed with *t*-test. Data of behavior test were expressed as $\bar{x} \pm s$. ANOVA followed by Duncan's multiple-range test was used for comparison between the groups.

RESULTS

Inhibitory effects on AChE and BuChE

in vitro The anti-ChE activity of synthetic Hup A was compared with natural Hup A by using rat cortex homogenate, rat erythrocyte membrane, and rat serum. Synthetic Hup A was tested over a concentration range from $10.3 \text{ nmol} \cdot \text{L}^{-1}$ – $517 \text{ } \mu\text{mol} \cdot \text{L}^{-1}$. IC_{50} for AChE and BuChE inhibition revealed that synthetic Hup A showed the same anti-AChE activity potency compared with natural Hup A. They inhibited BuChE at much higher concentration than needed for inhibition of AChE (Tab 1).

Tab 1. Inhibitory effects of synthetic (-)-Hup A and natural Hup A on cholinesterase activities *in vitro*.

$n = 1$ homogenate from 30 rats for triplicate experiments. BuChE = butyrylcholinesterase (rat serum). AChE (c) = acetylcholinesterase (rat cortex homogenate). AChE (e) = acetylcholinesterase (rat erythrocyte membrane).

* $P > 0.05$ vs natural Hup A.

(-)-Hup A	$\text{IC}_{50}/\text{nmol} \cdot \text{L}^{-1}$ (95 % confidence limits)		
	BuChE	AChE(c)	AChE(e)
Synthetic	53 627 ^a (44 933 – 63 803)	64.7 ^a (52.6 – 79.5)	53.9 ^a (43.5 – 66.6)
Natural	54 398 (45 588 – 64 709)	61.4 (49.8 – 75.6)	57.5 (46.3 – 71.1)

Inhibitory effects on AChE and BuChE

in vivo Inhibition of ChE activity at 30 min in the whole brain, cortex, and serum was tested following ig administration with 3 doses of synthetic Hup A. There was a dose-dependent inhibition of ChE by synthetic and natural Hup A. The inhibitory effects of synthetic and natural Hup A on cortex AChE were stronger than those on whole brain AChE. The results indicated that synthetic and natural Hup A exhibited the same activity on ChE inhibition (Tab 2).

Effects on scopolamine-induced memory deficits Mice injected scopolamine ($4.5 \text{ mg} \cdot \text{kg}^{-1}$ ip) showed increased errors of entering non-exit and prolonged the path to find the platform ($P < 0.01$ vs control group). Synthetic Hup A (0.05 and $0.1 \text{ mg} \cdot \text{kg}^{-1}$ ig) and natural Hup A (0.05 and $0.1 \text{ mg} \cdot \text{kg}^{-1}$ ig) all attenuated the scopolamine-induced deficit (Fig 2).

Tab 2. Anti-cholinesterase effects of synthetic (-)-Hup A and natural Hup A in mice. Values were expressed as % inhibition (vs saline control) $\pm s$. Basal saline control values of whole brain and cortex were 1818 ± 70 , and 870 ± 100 A values/g protein, respectively ($n = 14, 7$ mice). Basal saline control value of serum was 1443 ± 138 A values/g protein ($n = 12$ mice). ^b $P < 0.05$, ^c $P < 0.01$ vs saline group.

(-)-Hup A $\text{mg} \cdot \text{kg}^{-1}$, ig	AChE		BuChE
	Whole brain ($n = 10$ mice)	Cortex ($n = 5$ mice)	Serum ($n = 4$ mice)
Synthetic			
0.48	28 ± 7^c	36 ± 7^c	29 ± 9^c
0.24	19 ± 5^c	31 ± 6^c	16 ± 5
0.12	14 ± 8^c	17 ± 5^b	9 ± 6
Natural			
0.48	27 ± 7^c	37 ± 10^c	27 ± 9^c
0.24	16 ± 4^c	27 ± 8^c	16 ± 13
0.12	11 ± 7^c	17 ± 11^b	11 ± 16

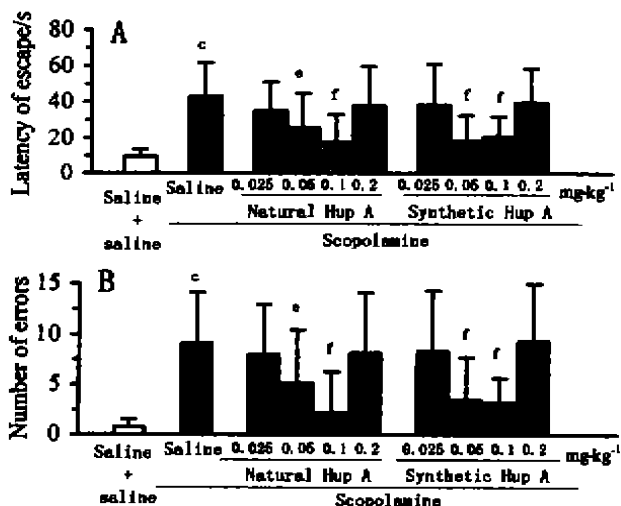


Fig 2. Effects of synthetic (-)-Hup A and natural Hup A on scopolamine-induced spatial performance deficits in the mouse water maze, A) the time to find the platform, B) the number of entering non-exit. $n = 12$ mice, $\bar{x} \pm s$. ^c $P < 0.01$ vs saline + saline ($n = 12$ mice). ^{*} $P < 0.05$, [†] $P < 0.01$ vs saline + scopolamine ($n = 26$ mice).

DISCUSSION

Recently (-)-Hup A was synthesized by the authors by means of asymmetric palladium-catalyzed bicycloannulation of β -keto ester with 2-methylene-1, 3-propanediol diacetate in the presence of chiral ligands as the key step. In contrast to the reports in the literature^[7-10], our synthetic approach is more practical in respect of the chemical yield and optical yield. It made

possible to provide enough quantities of the compound to carry out pharmacological studies. The results reported in this paper demonstrated that synthetic and natural (-)-Hup A exerted the same efficacy on cholinesterase inhibition and improving scopolamine-induced memory deficit. The naturally occurring (-)-Hup A has been showed to be a promising agent for the palliative treatment of patients with AD^[5,6]. The yield of natural Hup A is low, and the successful synthesis of (-)-Hup A would provide one more way to meet the requirement of clinical use.

REFERENCES

- 1 Bartus RT, Dean RL, Beer B, Lippa AS. The cholinergic hypothesis of geriatric memory dysfunction. *Science* 1982; 217: 408-17.
- 2 Pomponi M, Giacobini E, Brufani M. Present state and future development of the therapy of Alzheimer disease. *Aging* 1990; 2: 125-53.
- 3 Winbald B, Adem A, Backman L, Nordberg A, Elinder F, Arhem P. Cholinesterase inhibitors in Alzheimer's disease: Evaluation of clinical studies. In: Berker R, Giacobini E, editors. *Cholinergic basis for Alzheimer therapy*. Boston: Birkhäuser; 1991. p 238-43.
- 4 Liu JS, Zhu YL, Yu CM, Zhou YZ, Han YY, Wu FW, et al. The structure of huperzine A and B, two new alkaloids exhibiting marked anticholinesterase activity. *Can J Chem* 1986; 64: 837-9.
- 5 Tang XC. Huperzine A (Shuangyiping): a promising drug for Alzheimer's disease. *Acta Pharmacol Sin* 1996; 17: 481-4.
- 6 Xu SS, Cao ZX, Weng Z, Du ZM, Xu WA, Yang JS, et al. Efficacy of tablet huperzine-A on memory, cognition, and behavior in Alzheimer's disease. *Acta Pharmacol Sin* 1995; 16: 391-5.
- 7 Yamada F, Kosikowski AP, Reddy ER, Pang YP, Miller JH, McKinney M. A route to optically pure (-)-huperzine A: molecular modeling and *in vitro* pharmacology. *J Am Chem Soc* 1991; 113: 4695-6.
- 8 Chen WP, Yang FQ. Asymmetric total synthesis of optically active huperzine A. *Chin J Med Chem* 1995; 5: 10-7.
- 9 Kaneko S, Yoshino T, Katoh T, Terashima S. An enantioselective synthesis of natural (-)-huperzine A via cinchona alkaloid-promoted asymmetric Michael reaction.

- Heterocycles 1997; 46: 27-30.
- 10 Kaneko S, Yoshino T, Katoh T, Terashima S. A novel enantioselective synthesis of the key intermediate (-)-huperzine A employing asymmetric palladium-catalyzed bicycloannulation. *Tetrahedron: Asymmetry* 1997; 8: 829-32.
- 11 Ellman GL, Courtney KD, Andres V Jr, Featherstone RM. A new and rapid colorimetric determination of acetylcholinesterase activity. *Biochem Pharmacol* 1961; 7: 88-95.
- 12 Wang YE, Yue DX, Tang XC. Anti-cholinesterase activity of huperzine A. *Acta Pharmacol Sin* 1986; 7: 110-3.
- 13 Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* 1976; 72: 248-54.

413-416

合成左旋石杉碱甲对胆碱酯酶活性和小鼠水迷宫操作的作用

R 971

刘 橙, 章海燕, 唐希灿¹, 王 斌², 何煦昌², 白东鲁² (中国科学院上海药物研究所新药研究国家重点实验室, ²合成化学室, 上海 200031, 中国)

关键词 胆碱酯酶抑制剂; 胆碱酯酶; 石杉碱甲皮层; 红细胞膜; 东莨菪碱; 记忆; 迷宫学习

目的: 比较合成和天然左旋石杉碱甲(Hup A)对胆碱酯酶的抑制作用以及对东莨菪碱所致记忆障碍的改善作用. 方法: 比色法用于测定胆碱酯酶活性, 小鼠水迷宫用于评价促智作用. 结果: 合成 Hup A 抑制大鼠皮层和红细胞膜乙酰胆碱酯酶的 IC₅₀值分别为 64.7 (52.6-79.5), 53.9 (43.6-66.6) nmol·L⁻¹, 抑制大鼠血清丁酰胆碱酯酶的 IC₅₀为 53.6 (44.9-63.8) μmol·L⁻¹. 灌服合成 Hup A (0.12-0.48 mg·kg⁻¹), 量效依赖性抑制小鼠体内的胆碱酯酶. 行为实验证明, 合成 Hup A (0.05 mg·kg⁻¹) 明显地改善东莨菪碱所致的记忆损害. 合成 Hup A 具有与天然 Hup A 相等的效力. 结论: 合成 Hup A 与天然 Hup A 具有相似的药理活性.