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Effects of isovanihuperzine A on cholinesterase and scopolamine-induced memory impairment

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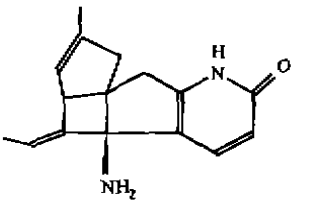
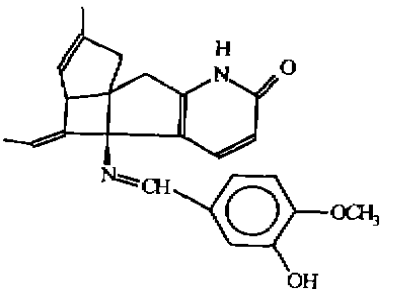
AIM: To study the effects of isovanihuperzine A (IVHA) on cholinesterase and scopolamine-induced memory deficit. **METHODS:** AChE and BuChE activities were determined by the colorimetric method of Ellman. The K_i value was determined by the plotting method of Lineweaver and Burk. In a behavioral test, rats were trained to perform a radial arm maze task using a partially baited procedure. **RESULTS:** The anti-AChE activity of IVHA was comparable to huperzine A (Hup-A), and was more potent than those of physostigmine and galanthamine with an IC_{50} value of 0.11 $\mu mol \cdot L^{-1}$. IVHA was a mixed competitive type with a K_i value of 32 $nmol \cdot L^{-1}$. It bound to AChE in a reversible manner. IVHA at a dose of 0.2 $mg \cdot kg^{-1}$ ip significantly reversed scopolamine-induced working memory and reference memory impairments in radial arm maze. **CONCLUSION:** IVHA is a new potential reversible AChE inhibitor and merits further study as a cognitive enhancer.

KEY WORDS huperzine A; isovanihuperzine A; physostigmine; galanthamine; cholinesterase inhibitors; scopolamine; isoflurophate; cognition disorders; memory

The degeneration of cholinergic neurons of the brain observed in Alzheimer's disease (AD)[1] and in aging[2] is considered an important pathogenetic element of dementia and of age-associated cognitive impairments. Cholinesterase inhibitors (ChEI) enhancing cholinergic transmission by slowing the degradation of ACh have been suggested to be a potential treatment of these impairments. Physostigmine (Phys) tetrahydroaminoacridine (THA) and other ChEI have been well investigated for memory-enhancing effects in animals and patients with AD[3]. However, there is no therapeutic ChEI that has been shown to be both effective and safe in the treatment of AD[4]. Huperzine A (Hup-A) is a new *Lycopodium* alkaloid which was first isolated from Chinese herb *Huperzia*



huperzine A



isovanihuperzine A

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serrata (Thunb) Trev by Chinese[5]. It is a potent and selective AChE inhibitor[6] with better therapeutic index than those of Phys and THA[4,7]. Clinical trials have indicated that Hup-A was a promising candidate of ChEI in the treatment of AD[4].

Therefore, it is worthwhile to study the structure-activity relationships and explore further structural requirements. Huperzine derivatives have been synthesized in this institute[8,9] and other laboratories[10]. Isovanihuperzine A (IVHA), one of the most potent derivatives of Hup-A, was found in this institute. In this paper, effects of IVHA on ChE activities and task of spatial memory were reported.

MATERIALS

Rats Sprague-Dawley rats (♂, $n=35$) weighing 255±*s* 23 g were housed individually at 20—22 ℃ on a 12-h light/dark cycle and kept on a restricted diet of standard laboratory chow with water available *ad lib*.

Apparatus The plastic radial arm maze was elevated 70.5 cm above the floor, and had an octagonal center platform with 8 arms radiating from the center. The platform was 51.5 cm in diameter, and each arm was 61 cm long and 12 cm wide. Plexiglas wall was 10 cm high extending along the length of each arm. Food wells, located 3 cm from the distal end of each arm, were 1 cm deep and 2 cm in diameter.

Chemicals Acetylthiocholine iodide (s-ACh), 5,5'-dithiobis 2-nitrobenzoic acid (DTNB), physostigmine salicylate, and isoflurophate were purchased from Fluka Chemie. Tetraisopropyl pyrophosphoramide (isoOMPA) was purchased from Sigma Chemical Company. Butyrylthiocholine iodide (s-BuCh) was a product of Koch-Light Lab, UK. Sodium lauryl sulphate (SDS) was bought from BDH, UK. Galanthamine hydrobromide (Gal) was a product of Shanghai Hai Pu Pharmaceutical Works. Scopolamine hydrochloride was a product of Chengdu № 1 Pharmaceutic Factory. Hup-A (colorless powder, purity > 98 %) and IVHA (light yellow powder, purity > 98 %) were prepared in our laboratory.

Enzyme source Rat hippocampus and frontal cortex homogenate are used as the brain AChE source. To prepare brain AChE, rat was decapitated and the hippocampus and frontal cortex were dissected on ice and homogenized in 0.05 mmol · L^{-1} sodium phosphate buffer, then kept at −30 ℃.

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 3 000×*g* for 15 min. The plasma was diluted 1:4 with 0.2 mmol · L^{-1} sodium phosphate buffer, pH 7.4, containing NaCl 0.15 mmol · L^{-1}. The erythrocytes were washed 3 times in saline followed by lysis in 50-fold volumes of 8.45 mmol · L^{-1} sodium phosphate buffer, pH 8.0. After 2 h, the lysed cells were centrifuged at 10 000 × *g* for 15 min. The erythrocyte membrane were washed 3 times and then diluted with 10 mmol · L^{-1} Tris-HCl buffer, pH 7.4.

Serum and erythrocyte membrane were stored at −30 ℃ for ChE and total protein assays[11].

METHODS AND RESULTS

Inhibitory effects on AChE and BuChE

The hippocampus or frontal cortex homogenate was thawed and suspended in 10 mmol · L^{-1} edetic acid phosphate buffer, pH 7.4. The homogenate was diluted until activity was linear with tissue concentration. The homogenate was preincubated for 5 min with iso-OMPA (0.1 mmol · L^{-1}), a selective inhibitor of BuChE activity.

AChE and BuChE activities were determined in 25 mmol · L^{-1} sodium phosphate buffer, pH 7.4, using the spectrophotometric method[12]. s-ACh (0.3 mmol · L^{-1}) and s-BuCh (0.4 mmol · L^{-1}) were used as specific substrates for the assay of AChE and BuChE, respectively. The mixture was incubated at 37 ℃ for 8 min. The reaction was terminated by adding 1 ml of 3 % SDS, then 1 ml of 0.2 % DTNB was added to produce the yellow anion of 5-thio-2-nitro-benzoic acid. The production of the yellow thionitrobenzoate anion

was measured at 440 nm for a period of 30 min after initiation of the reaction.

The enzyme activity was expressed as a % of the activity observed in the absence of inhibitor. The IC_{50} was defined at the concentration of inhibitor necessary to yield a 50 % inhibition of enzyme activity.

The anti-AChE activity of IVHA was comparable to that of Hup-A, and was 2 and 18 times more potent than those of Phys and Gal, respectively. In contrast, IVHA was the least potent against BuChE among the ChEI tested. The ratio of IC_{50} of IVHA for BuChE : AChE was comparable to Hup-A and much greater than that of Phys or Gal (Tab 1).

Tab 1. Inhibitory effects of IVHA and reference compounds on rat serum BuChE and erythrocyte membrane AChE *in vitro*. Number in parenthesis refers to slope value of enzyme activity *vs* IC_{50} value.

ChEI	$IC_{50}/\mu mol \cdot L^{-1}$		Ratio of IC_{50} (BuChE/AChE)
	BuChE	AChE	
IVHA	107.1 (0.90)	0.11 (0.72)	973.6
Huperzine A	63.1 (0.87)	0.06 (0.75)	1051.7
Physostigmine	1.3 (0.78)	0.23 (0.77)	5.6
Galanthamine	13.5 (0.85)	1.99 (0.72)	6.8

AChE from rat hippocampus, cerebral cortex, and erythrocyte membrane had the same qualitative sensitivity to IVHA and Hup-A. The IC_{50} of hippocampus AChE and frontal cortex AChE were, respectively, 91.2 and 83.3 $nmol \cdot L^{-1}$ for IVHA and 60.1 and 56.2 $nmol \cdot L^{-1}$ for Hup-A.

Inhibition mechanism The erythrocyte membrane AChE was incubated with the inhibitor and the inactivation was monitored by determining the remaining activity at indicated times (0, 1, 3, 5, and 7 min after incubation). 100 % was defined as enzyme activity observed in the absence of inhibitor. The AChE activity did not exhibit progressive decrease with the prolongation of incubation with IVHA. Its inhibitory manner was reversible and different from that of isoflurophate (Fig 1).

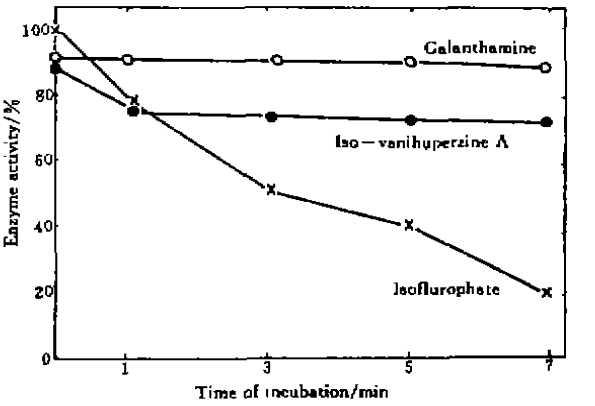

Fig 1. Inhibition of rat erythrocyte membrane AChE by IVHA 0.07 $\mu mol \cdot L^{-1}$, Gal 1.05 $\mu mol \cdot L^{-1}$, and isoflurophate 1.36 $\mu mol \cdot L^{-1}$.

IVHA was incubated with erythrocyte membrane AChE at 37 ℃ for 3 min. The mixture was washed 5 times with 3 ml of Tris-buffer (50 $mmol \cdot L^{-1}$, pH 7.4) and spun at 20 000 × *g*. The AChE activity recovered to 94.7 % of the control. These results show that IVHA combines to AChE in a reversible way.

The K_i value for IVHA was determined on the plotting method of Lineweaver-Burk plot[7], which demonstrated the lines of 1/OD *vs* 1/[S] in the presence of IVHA 20 and 40 $nmol \cdot L^{-1}$ intersected the line of the uninhibited enzymatic reaction in the area on the left between the vertical and horizontal axes (Fig 2). Secondary plot of 1/OD versus the inverse of the substrate concentration was also made to distinguish between mixed and simple competitive modes of inhibition of ChE. Thus the inhibition for IVHA was apparent of mixed type. The K_i value was 32 $nmol \cdot L^{-1}$.

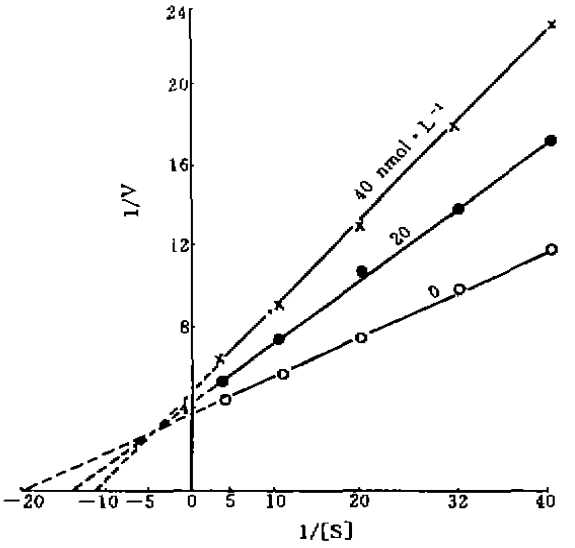

Fig 2. Typical Lineweaver-Burk plots for inhibition of rat erythrocyte membrane AChE by IVHA.

Effect of IVHA on scopolamine-induced memory deficits The rats' spatial memory abilities were assessed in the radial maze task. Rats received one training session daily. Only 4 predetermined arms were used as the baited arms. The baiting pattern remained the same throughout the experiment but varied from rat to rat. The rat was placed on the central platform and allowed to move freely until either all 4 baited arms were chosen or 10 min had elapsed. A correct response was defined as the first entry to a baited arm. Three types of error were recorded. Reentry to a baited arm was regarded as a working memory (WM) deficit. First entry to an unbaited arm was considered as error in reference memory (RM) and reentry to an unbaited arm was regarded as a deficit of both WM and RM (WRM). Rats were trained to a criterion of at most one error over 4 consecutive trials.

Scopolamine (125 $\mu g \cdot kg^{-1}$, ip 30 min before test) increased the number of 3 types of errors (Tab 2). IVHA (200 $\mu g \cdot kg^{-1}$, ip 30 min before test) attenuated the scopolamine-induced deficit (one-way ANOVA followed by Duncan's multiple-range test).

Tab 2. Effect of IVHA on scopolamine-induced disruption of radial maze performance in rats. $n = 8$. $\bar{x} \pm s$. $^bP < 0.05$, $^fP < 0.01$ *vs* saline. $^eP < 0.05$, $^iP < 0.01$ *vs* scopolamine. RM: reference memory; WM: working memory; WRM: working and reference memory.

Ip dose $\mu g \cdot kg^{-1}$		Number of errors RM	WM	WRM
Saline		0.3±0.6	0.1±0.3	0
Scopolamine	125	2.0±1.1^f	2.0±1.3^e	0.7±1.2^b
Scopolamine +IVHA	125 200	0.7±0.8^i	0.2±0.4^i	0^e

DISCUSSION

Although a number of analogs of Hup-A have been synthesized to date, none of the compounds have shown any AChE inhibitory activity rivaling that of Hup-A[8-10]. In present studies, IVHA has a number of similar character as its parent compound. First is its high potency against AChE. Rats brain AChE and erythrocyte AChE are similar with respect to their inhibition sensitivity to IVHA and Hup-A. As a second character, IVHA possesses a high selectivity of AChE action, which may be a useful property for a centrally action drug. As a third advantage, IVHA is a reversible AChE inhibitor. These findings indicate that the stucture of Hup-A can be changed while retaining biological activity when introducing isovani group on the $-NH_2$ group.

In radial arm maze task, IVHA exerted an ameliorating effect on the amnesia induced by scopolamine. Its efficacy was comparable to Hup-A[13]. This result provided strong evidence of the potency and efficacy of this novel compound as an AChE inhibitor capable of attenuating a specific cholinergic deficit.

In summary, potential AChE inhibition, lower toxicity than Hup-A (unpublished data), as well as ameliorating effect on scopolamine-induced memory deficit indicate that IVHA deserves further study as a novel ChEI.

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石杉碱类似物异香兰石杉碱甲对胆碱酯酶的抑制作用和东莨菪碱导致的记忆障碍的影响

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目的：研究石杉碱类似物异香兰石杉碱甲（IVHA）对胆碱酯酶的抑制作用和东莨菪碱导致的记忆障碍的影响。**方法**：乙酰胆碱酯酶和丁酰胆碱酯酶活力用 Ellman 比色法测定。抑制常数 K_i 值和抑制机制用 Lineweaver 和 Burk 的双倒数作图法测定。行为测试用八臂迷宫装置。**结果**：IVHA 的抗 AChE 作用稍弱于 Hup-A。它对红细胞膜 AChE 的 IC_{50} 为 0.11 $\mu mol \cdot L^{-1}$。作用强于 Phys 和 Gal。属混合型抑制剂，K_i 值为 32 $nmol \cdot L^{-1}$。不同于异氟磷，与 AChE 为可逆性结合。ip 给与 IVHA 0.2 $mg \cdot kg^{-1}$ 翻转东莨菪碱导致的八臂迷宫记忆障碍。**结论**：IVHA 是一个新的强效可逆胆碱酯酶抑制剂，值得进一步研究。

关键词 石杉碱甲；异香兰石杉碱甲；毒扁豆碱；加兰他敏；胆碱酯酶抑制剂；东莨菪碱；异氟磷；认识障碍；记忆