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Pharmacological Profile of Huperzine A, a Novel Acetylcholinesterase Inhibitor from Chinese Herb

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INTRODUCTION

Alzheimer's disease (AD) is a multifaceted neurodegenerative disorder characterized by progressive deterioration of memory and cognition. The disease will continue to constitute a major burden upon social and medical care systems due to the rising mean age of the population. The key symptoms of AD are primarily caused by cholinergic dysfunction. A significant correlation has been found between a decrease in cortical cholinergic activity and the deterioration of mental test scores in patients with AD (35). Current efforts to develop an effective drug treatment for AD are based in large part upon the consistent finding that patients with this disorder suffer from marked reduction of cholinergic neuronal function, resulting in a deficiency in acetylcholine (ACh) concentration in the central nervous system (6,7,12,55). Cholinergic enhancement strategies have been at the forefront of efforts to pharmacologically palliate the cognitive impairments. Among the various therapeutic approaches investigated to enhance cholinergic transmission, cholinesterase inhibitors (ChEIs) are the first group of compounds showing some promise in the treatment of AD. To date, tacrine, donepezil, galanthamine, and ENA 713 are available for the treatment of AD, and several new ChEIs are being studied (13,22). However, the clinical usefulness of ChEIs has been limited by their short half-lives and excessive side effects caused by activation of peripheral cholinergic systems, as well as hepatotoxicity, which is the most frequent and important side effect of tacrine therapy (11,21,38). To

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obtain better therapeutic benefit in AD the search for a long-acting ChEI which exerts minimal side effects in the clinic is still ongoing (13).

(–)-Huperzine A (HupA, Fig. 1) was originally discovered from the Chinese folk medicine *Qing Ceng Ta* (*Huperzia serrata*), which has been used in China for centuries to treat contusion, strain, swelling, schizophrenia, etc. (30) (Fig. 2). HupA, a novel *Lycopodium* alkaloid that is chemically unique in comparison with other agents under study for AD, is a reversible, potent, and selective acetylcholinesterase (AChE) inhibitor. Its potency and duration of AChE inhibition rival those of physostigmine, galanthamine, donepezil, and tacrine (49,54). HupA has been found to be an effective cognition enhancer in a number of different animal species (43). Clinical trials conducted in China have demonstrated that HupA induced significant improvements in memory of aged subjects and patients with AD without any remarkable side effects (60,61). HupA appears to meet the criteria for an ideal AChE inhibitor for the symptomatic treatment of AD. The present review will describe the pharmacological properties of HupA and its clinical studies.

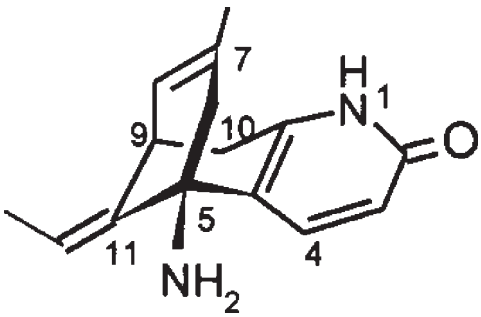

Fig. 1. [5*R*-(5,9,11E)]-5-amino-11-ethylidene-5,6,9,10-tetrahydro-7-methyl-5,9-methanocycloocta [b] pyridin-2(1H)-one

Fig. 2. Chinese herb *Huperzia serrata* (*Qing Ceng Ta*).

ANTICHOLINESTERASE EFFECTS AND INHIBITION MECHANISM

The cholinesterase (ChE) inhibition of HupA has been evaluated *in vitro* and *in vivo* using a spectrophotometric method (10) with minor modifications. For assay of AChE or butyrylcholinesterase (BuChE) activity, a reaction mixture of 4 mL containing acetylthiocholine iodide (0.3 mM) or butyrylthiocholine iodide (0.4 mM), 1 mL sodium phosphate buffer (0.1 mM), the test compound (0.1–0.5 mL), and enzyme (0.1–0.2 mL) was incubated at 37°C for 8 min. The inhibitory effects of HupA on AChE and BuChE compared with other ChE inhibitors are listed in Table 1. HupA inhibited the activity of AChE in the rat cortex beginning at 10 nM. The 50% inhibitory concentration (IC_{50}) of HupA for AChE was estimated to be 82 nM. The inhibition of AChE activity induced by HupA was more pronounced than that induced by tacrine, physostigmine, or galanthamine but less pronounced than that induced by donepezil (3,54). In contrast, HupA inhibited BuChE at much higher concentration than donepezil, while tacrine was more potent toward BuChE. HupA has the highest specificity for AChE. HupA exerted inhibitory effects on different AChE to a similar extent but interestingly was a weaker inhibitor of human serum BuChE (Table 2).

TABLE 1. Effects of HupA and other cholinesterase inhibitors on AChE activity in the rat cortex and BuChE activity in rat serum in vitro

Cholinesterase inhibitor	IC_{50}* (μM)		Ratio of IC_{50} (BuChE/AChE)
	AChE	BuChE	
HupA	0.082	74.43	907.7
Physostigmine	0.251	1.26	5.0
Galanthamine	1.995	12.59	6.3
Donepezil	0.010	5.01	501.0
Tacrine	0.093	0.074	0.8

* The cortex homogenate was preincubated for 5 min with iso-OMPA 0.1 mM. The rate of color production was measured spectrophotometrically at 440 nm. Data from refs. 3,54.

TABLE 2. Inhibitory effects of HupA on cholinesterase activities in vitro

Cholinesterase	IC_{50} (μM)	
	AChE	BuChE
Rat cortex	0.082	
Rat erythrocyte membrane	0.087	
Bovine erythrocyte membrane	0.090	
Human erythrocyte membrane	0.079	
Pig caudate nuclei	0.126	
Rat serum		74.4
Horse serum		117.3
Human serum		1259.0

Data are partially from ref. 54.

Significant inhibition of AChE activity was demonstrated in the cortex, hippocampus, striatum, medial septum, medulla oblongata, cerebellum, and hypothalamus of rats that were sacrificed 30 min following the administration of HupA at several dose levels compared with saline control. (4,44,46) There clearly was a dose-dependent inhibition of AChE in the brain region by HupA. In contrast to the inhibition of AChE activity *in vitro*, the relative inhibitory effect of oral HupA on cortex AChE was found to be about 24- and 180-fold, on an equimolar basis, as potent as donepezil and tacrine, respectively (49). Correlated to the dosage of AChE inhibition, however, only donepezil and tacrine produced significant BuChE inhibition in serum. Tacrine was a more potent inhibitor of serum BuChE than of brain AChE (Table 3). HupA i.p. exerted similar anti-ChE efficacy in rats as observed following oral administration, while tacrine produced a greater inhibition of both brain AChE and serum BuChE (Fig. 3). At the doses of 0.03 μM (8 μg) and 0.06 μM (16 μg), HupA significantly inhibited brain AChE activity 30 min after intraventricular injection; the inhibitory potency of HupA was less than that of donepezil, but stronger than that of tacrine (4) (Fig. 4). These findings indicate that the inhibitory potency of HupA differs from that of donepezil and tacrine following different routes of administration. HupA, in contrast to donepezil and tacrine, has higher bioavailability and more easily penetrates the blood brain barrier.

Maximal AChE inhibition in rat whole brain was reached at 60 min and maintained for 360 min following oral administration of HupA, 1.5 μmol/kg (0.36 mg/kg). Peak inhibitions in cortex and serum were observed at 30–60 min. Cortex AChE inhibition by HupA exceeding 10% was maintained for 15–240 min. BuChE activity recovered to the control

TABLE 3. Anticholinesterase activities of oral HupA, donepezil, and tacrine in rats

ChEI	Dose mg/kg (μmol/kg)	AChE Inhibition (%) (n = 6) cortex	hippocampus	striatum	BuChE Inhibition (%) serum (n = 3)
HupA					
	0.36 (1.5)	20 ± 6[1]	17 ± 3[1]	18 ± 4[1]	18 ± 10
	0.24 (1.0)	16 ± 6[1]	15 ± 3[1]	16 ± 8[1]	16 ± 14
	0.12 (0.5)	10 ± 6[1]	8 ± 7	13 ± 10[2]	7 ± 12
Donepezil					
	6.66 (16)	18 ± 6[1]	12 ± 5[1]	12 ± 8[2]	33 ± 7[1]
	5.00 (12)	11 ± 6[1]	10 ± 4[1]	10 ± 6[2]	22 ± 11
	3.33 (8)	9 ± 11	6 ± 8	8 ± 6	8 ± 10
Tacrine					
	28.2 (120)	20 ± 6[1]	11 ± 10[2]	11 ± 10[2]	52 ± 5[1]
	21.1 (90)	8 ± 6[1]	9 ± 6	8 ± 41[1]	40 ± 20[2]
	14.1 (60)	7 ± 7	2 ± 2	2 ± 5	24 ± 17

[1]$P < 0.01$, [2]$P < 0.05$ vs. saline group. Values are expressed as percent inhibition (vs. saline control) ± S.D. Basal saline control values of cortex, hippocampus, striatum are 1360 ± 70, 1540 ± 150, 9390 ± 880 *A* values/g protein, respectively (n = 14). Basal saline control value of serum is 23 ± 5 *A* values/g protein (n = 7). Data from ref. 49.

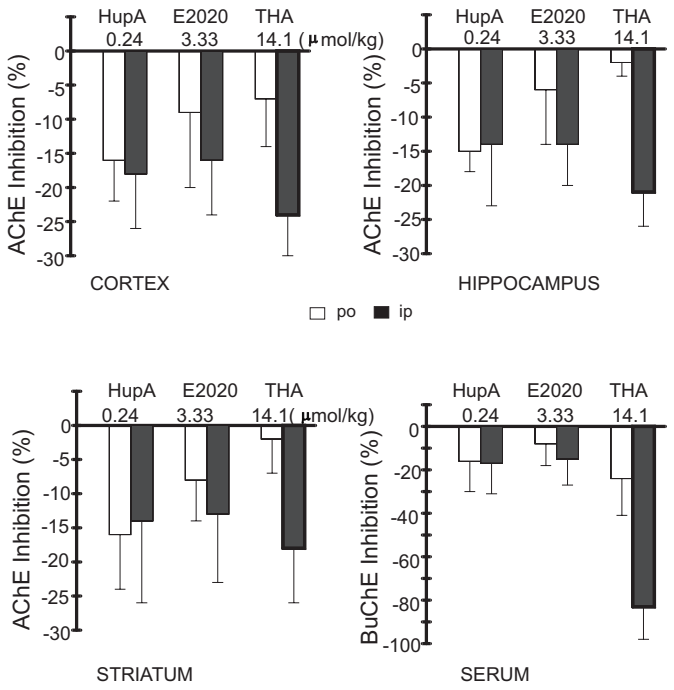

Fig. 3. Comparative effects of HupA, donepezil (E2020), and tacrine (THA) on cholinesterase inhibition in rats. Values are expressed as percent inhibition (vs. saline control) ± S.D. $n = 4–12$. Data from ref. 49.

Fig. 4. Comparison of HupA, donepezil, and tacrine on AChE inhibition in rats. Rats were killed 30 min after i.c.v. injection of inhibitors. Values are expressed as percent inhibition (vs. saline control) ± S.D. $n = 4–12$. $^{*}P < 0.05$, $^{**}P < 0.01$ vs. control. Data from ref. 4.

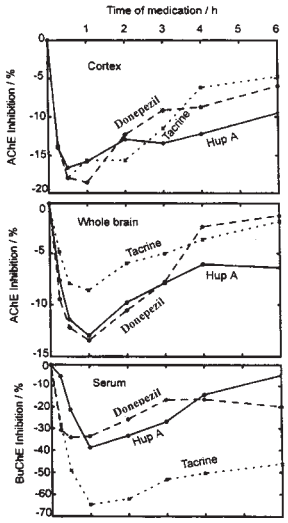

Fig. 5. Time course of ChE inhibition following oral administration of HupA (1.5 µmol/kg), donepezil (16 µmol/kg), and THA (120 µmol/kg) in rats. Values were expressed as percent inhibition vs. saline control. $n = 4–6$. Data from ref. 49.

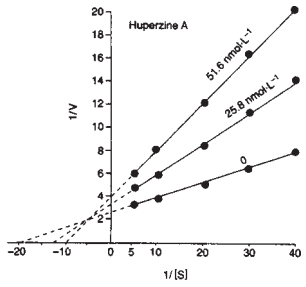

Fig. 6. Double-reciprocal plots of rat erythrocyte membrane AChE after HupA *in vitro*. AChE was incubated with acetylthiocholine iodide (0.3 mM) and 25 mM phosphate buffer (pH 7.4) for 8 min at 37°C. Data from ref. 54.

level at 360 min after the use of HupA, whereas donepezil and tacrine inhibited BuChE by 20 and 46%, respectively (49) (Fig. 5).

Repeated doses of HupA showed no significant difference in AChE inhibition as compared to that of a single dose, indicating that no tolerance to HupA occurred (24,49).

Figure 6 shows the Lineweaver–Burke representation of the inhibition of rat erythrocyte membrane AChE by HupA. The plot indicates a mixed competitive type of inhibition, because the intersection of the lines occurred in the second quadrant. The K_i values of HupA and the other ChEIs are listed in Table 4. HupA was about 4-fold and 9-fold as potent as tacrine and galanthamine, respectively, and was about half as potent as donepezil (3,54).

The rat erythrocyte membrane AChE activity did not exhibit progressive decrease with prolonged incubation with HupA *in vitro* (Fig. 7), and the AChE activity recovered to 94% of the control after being washed 5 times, indicating that the inhibitory action of HupA was reversible and different from that of isoflurophate (DFP) (54).

Over the past decade, the inhibitory mechanisms for AChE by HupA have been extensively studied utilizing kinetic (1,54), computer-aided docking (34), and X-ray crystallography approaches (37). In particular, the 2.5 Å resolved crystal structure of a *Torpedo* AChE–HupA complex demonstrated the "ingenious design" of the natural alkaloid (40) to bind more tightly and specifically to the

TABLE 4. Apparent inhibition constants of rat erythrocyte membrane AChE after HupA, donepezil, tacrine, and galanthamine in vitro

ChEI	Inhibitory Pattern	K_i (nM)
HupA	mixed competitive	24.9
Donepezil	noncompetitive	12.5
Tacrine	noncompetitive	105.0
Galanthamine	competitive	210.0

Data from refs. 3,54.

enzyme than do other known AChE inhibitors such as tacrine and edrophonium. Furthermore, the refined structure clearly identifies the principal protein–ligand interactions responsible for the efficacy of the inhibitor upon binding to AChE (37). The principal interactions include (Fig. 8): direct and strong hydrogen bonds between the carbonyl group of HupA and the hydroxy oxygen of Tyr 130 (located at the peripheral site of the enzyme), as well as between the ethylidene methyl group and the main-chain oxygen of His 440 (a modality of the catalytic triad); indirect hydrogen bonds, mediated by one or two

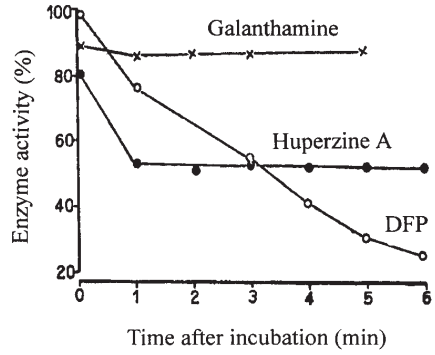

Fig. 7. Rat erythrocyte membrane AChE activity after incubation with HupA, galanthamine, and DFP. AChE was incubated with acetylthiocholine iodide (0.3 mM) and 25 mM phosphate buffer (pH 7.4) for 8 min at 37°C. Data from ref. 54.

Fig. 8. Schematic figure showing the principle interactions between a *Torpedo* AChE and HupA. Reproduced with permission from ref. 37.

water molecules, between HupA and residues of the enzyme which constitute the active center (e.g., the ring nitrogen of HupA is hydrogen-bonded to carboxylic oxygen of Glu 199, while the -NH_3^+ group is bonded to hydroxy oxygen of Tyr 121); the cation–π interactions induced upon inhibitor binding between the -NH_3^+ group of HupA and the aromatic rings of Trp 84 and Phe 330 at the choline site (it should be noted that other reversible AChE inhibitors such as tacrine and edrophonium bind to this same site) (19); and a large number of hydrophobic interactions which are established between a carbon atom of HupA and the various oxygen, nitrogen, or carbon atoms of the amino acid residues comprising the enzyme.

Computer-assisted docking studies and the solution of high-resolution crystal structure data for the AChE–HupA complex provide a valuable platform for the rationalization of the higher selectivity of the inhibitor, as well as the distinct thermodynamic stability of the complex. For example, HupA can form an extra hydrogen bond with Tyr 337 within the choline site that exists only in the mammalian homolog of AChE, but not in *Torpedo* enzyme and BuChE (39,42). This particular interaction may be largely responsible for the much stronger inhibitory property of HupA for mammalian AChE than for the other two enzymes. In addition, the peptide flip between Gly 117 and Gly 118 (induced only by the binding of HupA) may explain why HupA possesses a longer residence time than other commonly used anticholinesterase agents (1).

EFFECTS ON NEUROTRANSMITTER LEVELS

Compared with tacrine and physostigmine, which have been used already in experimental therapy of AD, HupA displays the longest-lasting increase in ACh level. ACh levels in the whole brain increased linearly from 10% at 5 min to 40% above control at 60 min following intramuscular injection of HupA, 0.5 and 2 mg/kg, and the elevation of ACh level was slower than the onset of AChE inhibition (44). HupA produced a more prolonged increase of ACh levels than tacrine, heptylphysostigmine, physostigmine, or metrifonate; the rise of ACh levels lasted for at least 6 h after the administration of HupA. An inverse relationship was seen between ACh levels and AChE activities in the frontal cortex (9,46) and whole brain (44) (Fig. 9). There was considerable regional selectivity in ACh levels after HupA administration. Increases in ACh level varied from area to area: maximal increases were observed at 60 min in the frontal and parietal cortex, intermediate increases at 30 min in the hippocampus and at 5 min in the medulla oblongata, and only slight increases at 30 min in the striatum. The increase of ACh levels in the cortex was seen even following i.p. injections of HupA at doses as low as 0.1 and 0.3 mg/kg (to a maximum of 54 and 129%, respectively). HupA, perfused through a microdialysis probe, produced a maximal increase in ACh levels of 3090% (68). Considering that ACh level is particularly low in the cerebral cortex of patients with AD (2), this particular regional specificity produced by HupA may constitute a therapeutic advantage. HupA did not alter choline levels or the activity of choline acetyltransferase (ChAT) in any region assayed the rat brain (24,46), suggesting that the increase of ACh levels by HupA was not likely to be mediated through an increase in the rate of ACh synthesis.

HupA (1–100 μM) *in vitro* did not significantly alter the electrically evoked fractional release of [^{3}H]ACh from rat cortical slices (44), which contrasted with the decreased release seen with tacrine, physostigmine, and metrifonate (17).

Brain norepinephrine (NE) and dopamine (DA) levels increased significantly following either systemic administration of HupA or local administration of HupA through microdialysis (68). The increase in ACh level was 10 times greater than the increase of NE and 6 times greater than the increase of DA. These effects may be involved in memory improvement through HupA since there is evidence of interactions between cholinergic and monoaminergic systems in the control of cognition (8). No effect of HupA on 5-HT levels was found in rat cortex.

Fig. 9. Time course of AChE inhibition and ACh levels in whole rat brain following i.m. injection of HupA 2 mg/kg. Values were expressed as mean percent inhibition of AChE activity or percent increase of ACh ($n = 4$ per group). Data from ref. 44.

EFFECTS ON CHOLINERGIC PARAMETERS

HupA significantly increased the amplitude of muscle contraction induced by stimulating nerve *in vitro* and *in vivo*. As anticurare agent HupA was much more potent than neostigmine following oral adminstration (62). The vertebrate neuromuscular junction where ACh is released in a quantal manner is an alternative experimental model to measure the level of ACh release and the mean life of ACh molecules. To study the effect of HupA on cholinergic transmission at mouse neuromuscular junction *in vitro*, isolated mouse phrenic nerve-hemidiaphragm preparations were used with the conventional intracellular recording technique. HupA 1 μM increased the amplitude, time-to-peak, and half-life of miniature end-plate potentials (MEPP) of muscle fiber (25). HupA had no effect on resting membrane potentials of muscle fiber, indicating that the effects of HupA may not be mediated through a postsynaptic mechanism. HupA did not increase the mean quantal content of end-plate potentials and the frequency of MEPP. Thus, the possibility of presynaptic action can be excluded. In contrast to donepezil and tacrine, neither the appearance of giant MEPP nor slow MEPP was changed by HupA, suggesting that non-specific promoting effects on terminal ACh release is unlikely. Therefore the facilitating effect of HupA on neuromusclar ACh transmission is likely to be mediated by AChE inhibition (26).

In a study of toad paravertebral ganglia (PVG) using intracellular recording techniques (66), it was also found that there was no change in membrane potential and input resistance during superfusion of HupA 0.3 or 1 μM for 15 min. HupA, 0.3 or 1 μM, increased the rate of orthodromic action potential evoked by preganglionic stimulation and still exerted potential action at high concentrations (50 or 100 μM), in contrast to physostigmine (32) and tacrine (50). HupA increased exogenous ACh- but not carbachol-induced depo-

Fig. 10. Effects of HupA on exogenous Ach potential and carbachol potential in toad paravertebral ganglia neurons. A. ACh potential induced by pressure ejection (triangular arrow, 0.1 mM, 20 ms, 200 kPa) was enhanced by HupA. B. Carbachol-induced depolarization was not much affected by HupA. Downward deflections represent electrotonic potentials induced by hyperpolarizing current pulses (0.2 nA, 40 ms). The resting potentials of cell A and B were –50 and –45 mV, respectively. Data from ref. 6.

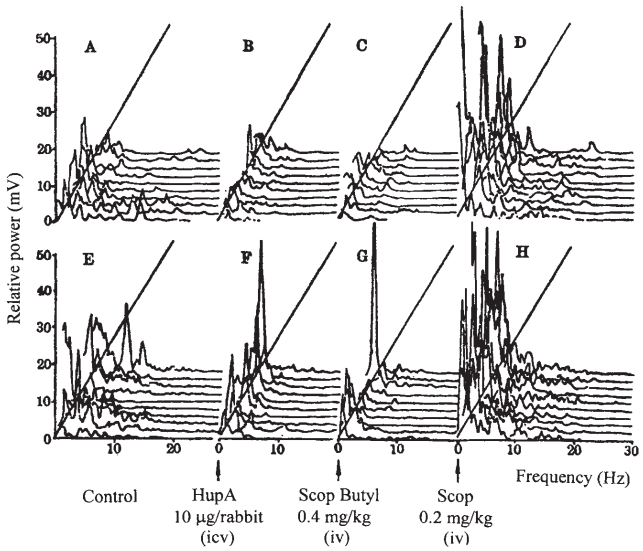

Fig. 11. Antagonistic effects of scopolamine butylbromide (Scop Butyl) and scopolamine (Scop) to HupA on EEG power spectral arrays in rabbit. A, B, C, D: frontal cortex; E, F, G, H: hippocampus. Data from ref. 15.

larization (Fig. 10). These findings indicated that the facilitating effect of HupA on ACh transmission was mainly mediated by its anti-AChE activity. A non-specific mechanism for HupA in its facilitation of transmitter release can be excluded.

In conscious rabbits, HupA (0.1 mg/kg, iv) produced an altered EEG which showed a decrease of lower frequency components, the total EEG power in cortical area, and the dominant frequency transferring from delta rhythm to theta rhythm in the hippocampus. These effects are cholinergic in nature and can be reversed by scopolamine but not by scopolamine butylbromide, which cannot pass the blood-brain barrier (14,15) (Fig. 11).

HupA is a potent inhibitor of the high-affinity transport of choline. In the hippocampus, high-affinity choline transport was reduced by 28% after 10 i.p. doses of HupA,

0.5 mg/kg, and there was no effect in the striatum (24). This effect is probably mediated through a regulatory control of high-affinity choline transport in response to ACh increases following ChE inhibition rather than by directly acting on the transporter since the effect of HupA was completely reversible with time and not mediated through a direct interaction with the transporter.

Studies on the displacement of [^{3}H]QNB- and [^{3}H](–)nicotine-specific binding showed that HupA had little direct effect on cholinergic receptors compared to tacrine and heptylphysostigmine (9,44). The concentration required to display 20% specific binding was 20 μM for [^{3}H](–)nicotine and 160 μM for [^{3}H]QNB, indicating lower concentrations of HupA have a stronger displacing effect on [^{3}H](–)nicotine- than on [^{3}H]QNB-specific binding (Fig. 12). A stronger effect of a low-dose HupA on central nicotinic receptors may constitute an additional therapeutic advantage in the treatment of AD. The low level of ACh synthesis in the cortex of a patient with AD may maintain presynaptic nicotinic receptors in an active state with no desensitization (33). In such a state, nicotinic receptors may become more sensitive to stimulation by HupA.

Fig. 12. Displacement of [^{3}H]QNB and [^{3}H](–)nicotinic specific binding in rat cortex by various concentrations of HupA (50 nM to 600 μM); n = 4–5. Data from ref. 44.

NEUROPROTECTIVE ACTION

In cultures of cells from the cortex, hippocampus, and cerebellum of rat embryos, HupA (100 μM) did not affect neuronal cell survival but reduced neuronal cell deaths caused by toxic levels of glutamate (100 μM). HupA reduced glutamate-induced calcium mobilization but did not affect the increase in intracellular free calcium channel induced by exposure to high KCl or the calcium activator Bay-K-8644 (48). These results suggested that HupA might act on glutamate receptors to exert its neuroprotective effects.

It recently has been reported that HupA acts as an NMDA receptor antagonist in the cerebral cortex (52). HupA (0.1–300 μM) reversibly inhibited NMDA-induced (100 μM) current in acutely dissociated hippocampal pyramidal neurons in a concentration-dependent manner with an IC_{50} of 45.4 μM (Fig. 13). In extensively washed crude synaptic membrane, HupA inhibited [^{3}H]dizocilpine binding in a concentration-dependent manner with an IC_{50} of 0.49 μM. In the presence of L-glutamate, the IC_{50} of HupA was 12.3 μM for the inhibition of [^{3}H]dizocilpine binding. The binding assay results clearly demonstrated that HupA acts directly on the NMDA receptor. Neuronal cell deaths caused by overstimulation of glutamate receptors have been proposed to be the final common pathway for various neurodegenerative diseases such as AD (27). Thus, HupA may be used as a preventive agent to slow down or block the pathogenesis of AD at an early stage, if excitotoxicity caused by overstimulation of the glutamate receptor is involved in the pathogenesis of this disease.

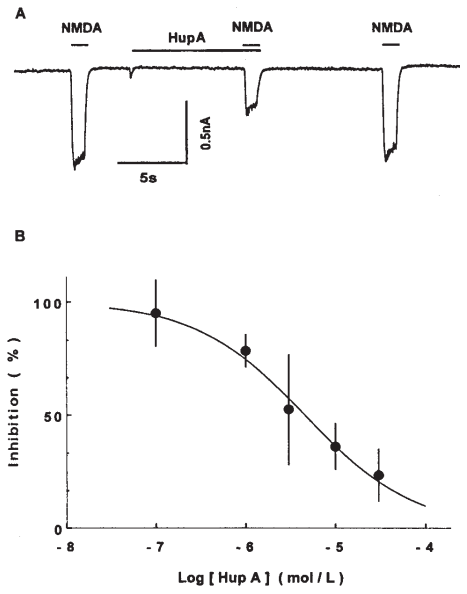

Fig. 13. A. HupA reversibly inhibited NMDA-induced current in acutely dissociated hippocampal pyramidal neurons. Both concentrations of NMDA and HupA were 100 μmol/L. B. The concentration–response curve of HupA inhibiting NMDA-induced current (n = 2–5 cells). Neurons were prepared from 7–14-day-old SD rats using a modified method of protease digestion. Data from ref. 52.

It has been reported that synthetic (+/–)-HupA is a better prophylactic drug than carbamates against organophosphate toxicity (16). Preincubation of fetal bovine serum AChE with a sufficient amount of HupA to inhibit > 90% of the enzyme activity substantially prevented irreversible phosphorylation of the enzyme by potent nerve agents. Approximately 45% and 60% of AChE active sites were protected even after 2 h of incubation with a 4-fold molar excess of soman and sarin, respectively, over AChE. Pretreatment with HupA (0.5 mg/kg, ip) increased the LD_{50} of soman in mice by 2-fold for at least 6 h, while physostigmine was effective in conferring a protective ratio of 1.5 for only 1.5 h after injection in mice, suggesting that HupA is likely to provide a safe and long-lasting prophylactic treatment against nerve agent toxicity in humans.

HupA pretreatment prevented soman-induced seizures and ensured the survival of all guinea pigs for 1 d after intoxication. The hippocampal tissue was free of any neuronal damage (23). The anticonvulsant efficacy of HupA may be related to its antimuscarinic activity and indirect desensitization of nicotinic receptors in numerous brain regions, particularly the hippocampus. In addition, HupA might act as an NMDA-receptor antagonist (52) to prevent soman-induced seizures.

AMELIORATING EFFECTS ON MEMORY IMPAIRMENT

HupA has been found to be an effective cognition enhancer in a number of different animal species. Enhancement of learning and memory performance following the administration of HupA 0.01–0.5 mg/kg was documented in various rodent models, such as the passive footshock avoidance (31,45,47,69,70), escape task of water maze (29), and spatial discrimination of radial arm maze (3,18,51,56–58), as well as delayed response performance in monkeys (63). Beneficial effects were seen not only in intact adult rodents, aged rodents (31), and monkeys (63), but also in cognitively impaired rodents and monkeys, such as scopolamine-, electroshock-, cycloheximide-, $NaNO_2$-, or CO_2-treated (31) and cholinergically lesioned rats (3,18,56,57) (Fig. 14). Inverted U-shaped dose-response curves typical of cognition enhancers were found with HupA. The durations of improve-

Fig. 14. Effects of AChE inhibitors on AF64A-induced working memory deficits in a partially baited radial maze paradigm. $^{**}P < 0.01$ vs. nonlesioned group, $^{++}P < 0.01$ vs. AF64A-lesioned, saline drug control. Data from ref. 3.

ments induced by oral HupA on learning and memory retention processes were longer than those induced by physostigmine, galanthamine, and tacrine, respectively (47). HupA significantly reversed memory deficits induced by scopolamine in young adult monkeys on delayed response task and increased choice accuracy in aged monkeys; these improvements remained for about 24 h after a single injection of HupA 10 μg/kg (63) (Fig. 15). The oral and i.p. administrations of HupA, 0.2 mg/kg, produced approximately the same results, indicating that orally HupA has a higher efficacy than tacrine or donepezil given by the same route (Fig. 16). These improvements were more pronounced on working memory than on reference memory (51). This effext may benefit AD patients because the cognitive deficit in memory of recent events is more severe in AD. HupA, 0.25 mg/kg, p.o., 1 × 8 d, was as potent as by acute administration in attenuating scopolamine-induced amnesia in rats (58), indicating no significant tolerance to HupA in cognitive improvement. This finding is consistent with the inhibition of AChE (24,49).

Fig. 15. Effects of HupA on delayed response tasks in aged monkeys ($n = 4$). Performance was measured 20 min (A), 24 h (B), and 48 h (C) after i.m. injection of HupA, respectively. The number of trials correct on saline was subtracted from the number of trials correct on HupA; this difference score was then multiplied by 3.3% as each trials constitutes 3.3% of the total number of trials: [(number correct HupA – number correct saline) × 3.3%]. Values in the figure represent mean ± S.E.M. $^{*}P < 0.05$ vs. saline control. Data from ref. 63.

Fig. 16. Comparison of HupA, donepezil, and tacrine on the scopolamine-induced working memory disruption of the partially baited redial maze performance in rats. AChE inhibitor was administered either orally or i.p. 30 min before the behavioral testing ($n = 8$). $^{++}P < 0.01$ vs. saline ($n = 21$). $^{*}P < 0.05$. $^{**}P < 0.01$ vs. saline + scopolamine ($n = 29$). Data from ref. 51.

Compared with other AChEIs, HupA has a clearly superior safety/efficacy ratio (Table 5). HupA did not show any significant affinity for muscarinic receptors and was devoid of pre- and postsynaptic actions as well as ChAT inhibition, suggesting that the improvements in cognition with HupA were primarily due to inhibition of brain AChE.

PHARMACOKINETIC STUDIES

The pharmacokinetics of HupA have been studied in rodents and healthy volunteers. HupA was absorbed rapidly, distributed widely in the body, and eliminated at a moderate rate (Table 6). The blood levels of HupA following i.v. or p.o. [^{3}H]HupA in rats declined in two phases, the distribution phase and elimination phase. The oral bioavailability was 96.9%. In mice, the radioactivities were highest in the kindey and liver. The majority of the radioactivity was excreted in the urine 24 h after i.v. [^{3}H]HupA. Only 2.4% was recovered from the feces. Paper chromatograms of urine revealed that [^{3}H]HupA was excreted partially as prototype and its metabolite (53). Autoradiographic study of the mouse showed that HupA was present in all regions of the brain, but was particularly concentrated in frontoparietal cortex, striatal cortex, hippocampus, and nucleus accumbens after i.v. injection (46).

TABLE 5. Comparison of the efficacies and toxicities of cholinesterase inhibitors in mice

ChEI	Memory enhancement (μmol/kg, p.o.)	Acute LD_5 (μmol/kg, p.o.)
HupA	0.83	17.31
Physostigmine	1.09	6.14
Galanthamine	5.43	71.96
Tacrine	68.17	199.83

Retention memory was assayed by step-down passive avoidance performance.

TABLE 6. Pharmacokinetic parameters of [^{3}H]HupA 13.9 mEq/kg in each group of 3 rats

Parameters	i.v. (mean ± S.D.)	i.g. (mean ± S.D.)
α (min^{-1})	0.107 ± 0.016	0.08 ± 0.04
β (min^{-1})	0.006 ± 0.003	0.004 ± 0.001
K_a (min^{-1})	–	0.16 ± 0.07
$T_{1/2\alpha}$ (min)	6.6 ± 1.1	10 ± 6
$T_{1/2\beta}$ (min)	149 ± 96	203 ± 53
$T_{1/2Ka}$ (min)	–	5.1 ± 3
K_{12} (min^{-1})	0.047 ± 0.02	0.05 ± 0.03
K_{22} (min^{-1})	0.05 ± 0.04	0.024 ± 0.004
K_{10} (min^{-1})	0.014 ± 0.006	0.013 ± 0.006
V_c (L/kg)	1.6 ± 0.9	2.4 ± 0.7
V_d(L/kg)	3.6 ± 1.0	7.8 ± 2.3
Cl_p (L/(kg·min))	0.020 ± 0.006	0.028 ± 0.014
AUC (10^{-7} × (dpm × min)/ml)	2.6 ± 0.9	1.8 ± 0.8
T_{max} (min)	–	21 ± 12
C_{max} (dpm/mL)	–	98,569 ± 12,153

$F = Cl_{p.ig} \cdot AUC_{ig}/Cl_{p.iv} \cdot AUC_{iv} = 96.9\%$. Data from ref. 53.

In young healthy volunteers, HupA plasma levels were determined by reverse phase HPLC with a spectrophotometric detector. The time course of plasma concentrations conformed to a one-compartment open model with a first-order absorption following oral administration of HupA 0.99 mg. HupA was rapidly absorbed and widely distributed *in vivo* (36) (Table 7). The half-life of HupA was at least 4–17 times longer than that of tacrine or physostigmine (20).

TOXICOLOGICAL STUDIES

A series of studies have been conducted to evaluate the toxicity of HupA in mice, rats, rabbits (62), and dogs. Dose–response curves for salivation indicated that HupA was less potent than other ChE inhibitors (62). The characteristic symptoms of cholinergic hyperactivity were less severe for HupA in rats compared with donepezil and tacrine; oral

HupA, 0.48 mg, did not produce fasciculation or other cholinergic signs (49). The LD_{50} doses of HupA were 4.6 mg (p.o.), 3.0 mg (s.c.), 1.8 mg (i.p.) and 0.63 mg (i.v.) in mice. Atropine exerted a significant antagonistic effect on the toxicity induced by HupA (Table 8). Subacute toxicity studies have been conducted in rats, rabbits, and dogs; the results showed that no histopathological changes were found in liver, kidney, heart, lung, and brain in rats (1.5 mg/kg, p.o.) and dogs (0.6 mg/kg, i.m.) after administration of HupA for 180 d. No teratogenic effect was detected in mice (0.019–0.38 mg/kg, i.p.) or rabbits (0.02–0.2 mg/kg, i.m.) after the administration of HupA.

CLINICAL TRIALS

To date, several clinical studies with HupA have been reported. Favorable efficacy of HupA was demonstrated in the treatment of 447 patients suffering from age-related memory dysfunction or dementia in China (28,59–61,64,67). An early study conducted on 100 patients with pobable AD oral HupA (0.15–0.25 mg, t.i.d.) showed significant improvement in all rating scores evaluated by the Buschke Selective Reminding task.

TABLE 7. Pharmacokinetic parameters of HupA after 0.99 mg p.o. (tablet) in 6 healthy volunteers

Parameter	mean ± S.D.
K_a (min^{-1})	0.061 ± 0.017
K_e (min^{-1})	0.0025 ± 0.0006
$T_{1/2Ka}$ (min)	13 ± 5
$T_{1/2Ke}$ (min)	288 ± 63
T_{max} (min)	80 ± 9
C_{max} (μg/L)	8.4 ± 0.9
T_{lag} (min)	25.4 ± 1.8
V_d/F (L/kg)	0.108 ± 0.008
AUC (mg/(L · min))	4.1 ± 1.2

HupA concentrations in plasma were determined by reverse phase HPLC. Data from ref. 36.

TABLE 8. Antidotal effect of atropine on HupA intoxication

Dose (mg/kg, i.p.)	No. of mice	
	test	death
	HupA	
7.0	10	10
	Atropine + HupA	
0.5 + 7.0	10	2
2.5 + 7.0	10	0

Atropine was administered 30 min before HupA.

An inverted U-shaped dose–response curve for memory improvement was observed (47,64,65).

A more comprehensive clinical study has been conducted in 191 patients who met AD criteria of NINCDS-ADRDA and DSM-III-R at 11 mental hospitals in China (41,59,61). The results from a double-blind, placebo-controlled, parallel-group, 8-w study conducted with 103 patients confirmed the efficacy of HupA in improving cognitive performance (Table 9). The changes of oxygen free radicals in the HupA treatment group showed marked improvement (59).

The most frequently occurring side effects with HupA were related to its cholinergic property. The incidence of adverse events such as dizziness, nausea, and diarrhea with HupA 0.2 mg was comparable to that observed with placebo control. No liver and kidney toxicity was detected (59,60).

The efficacy of HupA has recently been tested in 34 pairs of junior high school students who had complained of memory inadequacy by using double-blind and matched-pair method. HupA at a dose of 0.1 mg twice per day for 4 w increased the scores for 'accumulation,' 'recognition,' 'association,' 'factual memory,' and 'number of recitation' factors, but not the 'understanding' factor (41).

In another study, 99% of 128 patients with myasthenia gravis showed controlled or improved clinical manifestations of the disease. The duration of action of HupA lasted 7 ± 6 h, and side effects were minimal compared with neostigmine (5).

SUMMARY

HupA, a novel alkaloid isolated from the Chinese medicinal herb *Huperzia serrata*, is a reversible, potent, and selective inhibitor of AChE. Compared with other well-known AChEIs, such as physostigmine, galanthamine, tacrine, and even donepezil, HupA has

TABLE 9. Therapeutic efficacy of oral tablet HupA in patients with AD

	Placebo (n = 53)	Huperzine A (0.2 mg, n = 50)
Memory quotient		
Before trial	47.9 ± 21.5	55.8 ± 21.1
8-w trial	51.6 ± 25.6[*1]	64.4 ± 26.2[*1,*2]
Mini-Mental State Scale (MMSE)		
Before trial	14.4 ± 4.7	16.0 ± 5.0
8-w trial	14.9 ± 6.4	18.9 ± 6.2[*1,*3]
Hachinski ischemic scale		
Before trial	15.6 ± 5.3	16.1 ± 5.6
8-w trial	15.4 ± 6.7	19.7 ± 6.5[*1,*3]
Activity of daily living scale		
Before trial	30.7 ± 9.3	32.6 ± 9.6
8-w trial	31.9 ± 0.7	29.1 ± 9.3[*1]

[*1]$P < 0.01$ vs. before trial; [*2]$P < 0.05$, [*3]$P < 0.01$ vs. placebo group. Patients were randomly divided into two groups given four tablets twice a day. Double-blind trial. Data from ref. 60.

better penetration through the blood-brain barrier, higher oral bioavailability, and longer duration of AChE inhibitory action. HupA exhibited memory-enhancing efficacy in a broad range of animal models of cognitive impairment. Double-blind and placebo-controlled clinical trials have demonstrated that HupA produced significant improvements in memory deficiencies in aged patients and patients with AD. Furthermore, both animal and clinical safety testings showed that HupA was devoid of unexpected toxicity, particularly the dose-limiting hepatotoxicity induced by tacrine. These encouraging preclinical and clinical findings suggest that HupA is a highly promising candidate for clinical development as a symptomatic treatment for AD and other memory disorders related to a central cholinergic deficiency.

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