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# Progress in studies of huperzine A, a natural cholinesterase inhibitor from Chinese herbal medicine[1]

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## Key words

## Abstract

Huperzine A (HupA), a novel alkaloid isolated from the Chinese herb *Huperzia serrata*, is a potent, highly specific and reversible inhibitor of acetylcholinesterase (AChE). Compared with tacrine, donepezil, and rivastigmine, HupA has better penetration through the blood-brain barrier, higher oral bioavailability, and longer duration of AChE inhibitory action. HupA has been found to improve cognitive deficits in a broad range of animal models. HupA possesses the ability to protect cells against hydrogen peroxide, β-amyloid protein (or peptide), glutamate, ischemia and staurosporine-induced cytotoxicity and apoptosis. These protective effects are related to its ability to attenuate oxidative stress, regulate the expression of apoptotic proteins Bcl-2, Bax, P53, and caspase-3, protect mitochondria, upregulate nerve growth factor and its receptors, and interfere with amyloid precursor protein metabolism. Antagonizing effects of HupA on *N*-methyl-*D*-aspartate receptors and potassium currents may also contribute to its neuroprotection as well. Pharmacokinetic studies in rodents, canines, and healthy human volunteers indicated that HupA was absorbed rapidly, distributed widely in the body, and eliminated at a moderate rate with the property of slow and prolonged release after oral administration. Animal and clinical safety tests showed that HupA had no unexpected toxicity, particularly the dose-limiting hepatotoxicity induced by tacrine. The phase IV clinical trials in China have demonstrated that HupA significantly improved memory deficits in elderly people with benign senescent forgetfulness, and patients with Alzheimer disease and vascular dementia, with minimal peripheral cholinergic side effects and no unexpected toxicity. HupA can also be used as a protective agent against organophosphate intoxication.

## Introduction

Alzheimer disease (AD) is a progressive neurodegenerative disorder associated with a global impairment of higher mental function, and presenting an impairment of memory as the cardinal symptom[1]. Histopathological hallmarks of the disease are the extracellular deposition of amyloid β-peptide (Aβ) in senile plaques, the appearance of intracellular neurofibrillary tangles (NFT), a loss of cholinergic neurons, and extensive synaptic changes in the cerebral cortex, hippocampus and other areas of brain essential for cognitive functions. 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[2]. Based on the cholinergic hypothesis of AD, 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 (ChEI) are the first group of compounds that have shown some promise in the treatment of AD. The most significant therapeu-

tic effect of ChEI in AD treatment is to stabilize cognitive function at steady level during at least a 1 year period in approximately 50% of patients. Most clinical studies show that in a certain percentage of AD patients (approximately 20%) cognitive function can be stabilized for a period of up to 24 months. In addition, the AD patients who do not respond to therapy with one ChEI can be switched to a second one with a 50% rate of success. To date, four ChEI, tacrine, donepezil, galanthamine and rivastigmine have been approved by the US Food and Drug Administration for the treatment of AD, and several new ChEI are being studied[3–6]. However, the clinical usefulness of ChEI 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[7–9]. To obtain better therapeutic benefit in the treatment of AD, the search for a long-acting ChEI that exerts minimal clinical side effects is still ongoing[5].

(–)-Huperzine A (HupA), a novel *Lycopodium* alkaloid, is isolated from the Chinese medicinal herb *Huperzia serrata* (Qian Ceng Ta; Figure 1). The herb has been used in China for centuries in the treatment of such conditions as contusions, strains, swelling, and schizophrenia. HupA, a compound that is chemically unique in comparison with other
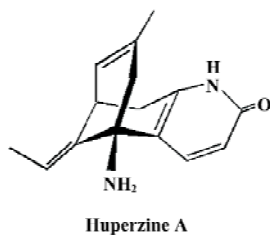
agents under study for AD, is a reversible, potent, and selective acetylcholinesterase (AChE) inhibitor. Its potency and duration of AChE inhibition rival those of tacrine, galanthamine, donepezil, and rivastigmine[10–13]. HupA has been found to improve cognitive deficits in a broad range of animal models. The phase IV clinical trials conducted in China have demonstrated that HupA induces significant improvement in the memory of elderly people and patients with AD and vascular dementia (VD) without any notable side effects[14–17]. In this paper the pharmacological properties, pharmacokinetics, and toxicology of HupA, in addition to the clinical trials so far conducted using this agent, are reviewed.

## Effects on cholinesterase activity and inhibition mechanism

The cholinesterase inhibition by HupA has been evaluated *in vitro* and *in vivo*, using a spectrophotometric method[18] with minor modifications. For assay of AChE or butyrylcholinesterase (BuChE) activity, a reaction mixture of 4 mL containing acetylthiocholine iodide (0.3 mmol/L) or butyrylthiocholine iodide (0.4 mmol/L), 1 mL sodium phosphate buffer (0.1 mmol/L), the test compound (0.1–0.5 mL), and enzyme (0.1–0.2 mL) was incubated at 37 °C for 8 min.

*In vitro* and *in vivo* comparison studies with respect to AChE inhibition showed that the potency of HupA was similar or superior to the inhibitors currently being used in AD treatment (Table 1)[11–13,19–21]. Based on the 50% inhibitory concentration ($IC_{50}$), HupA was more potent than tacrine, physostigmine, galanthamine, and rivastigmine with respect to inhibition of AChE activity, whereas HupA was the least potent BuChE inhibitor among the inhibitors tested[11,21–23]. The $IC_{50}$ ratio of HupA for BuChE:AChE was much greater than those of the other 4 inhibitors. HupA exerted inhibitory effects on AChE from different sources to a similar extent but, interestingly, was a weaker inhibitor of human serum



**Figure 1.** The Chinese herb *Huperzia serrata* (Qian Ceng Ta).

**Table 1.** Comparative effects of five cholinesterase inhibitors on cholinesterase activity *in vitro*.

| | $IC_{50}$ (μM) | | Ratio of $IC_{50}$ | $K_i$ (nM) |
| | AChE[*] | BuChE[**] | BuChE/AChE | |
|---|---|---|---|---|
| HupA | 0.082 | 74.43 | 907.7 | 24.9 |
| Tacrine | 0.093 | 0.074 | 0.8 | 105.0 |
| Donepezil | 0.010 | 5.01 | 501.0 | 12.5 |
| Rivastigmine | 181.39 | 31.07 | 0.17 | – |
| Galanthamine | 1.995 | 12.59 | 6.3 | 210.0 |

[*]rat cortex; [**]rat serum. Data from ref 11, 22.

BuChE relative to BuChE from other sources. Studies using 8 and 4 cholinesterase isoenzymes, which were extracted from mouse and dog plasma, respectively, showed that HupA had a more selective inhibition on AChE isoenzymes, but had little or no inhibition on BuChE[24]. The better reversible effect of (±)-HupA on AChE was also demonstrated in studies with porcine intrinsic cardiac neurons, which express both AChE and BuChE[25].

The apparent inhibition constants ($K_i$ value) for AChE are in the nanomole range, which indicates that these inhibitors have high affinity for the enzyme. However, the doses of donepezil and tacrine used orally are much higher than that of HupA[26], which might be explained by their low bioavailability and/or by rapid metabolism.

AChE exists in multiple molecular forms that can be distinguished by their subunit associations and hydrodynamic properties[27,28]. In mammalian brain, the bulk of AChE occurs as a tetrameric, G4 form (10S) together with much smaller amounts of a monomeric, G1 (4S) form[29,30]. There is evidence that AChE inhibitors do not inhibit all forms equally well. Studies from our laboratory showed that HupA preferentially inhibited tetrameric AChE (G4 form), whereas tacrine and rivastigmine preferentially inhibited monomeric AChE (G1 form). Donepezil showed pronounced selectivity for G1 AChE in striatum and hippocampus, but not in cortex. Physostigmine showed no form-selectivity in any brain region. In cortex, the most potent inhibitors of G4 AChE were HupA and donepezil. The potent inhibitors of cortical G1 AChE were donepezil and tacrine. In hippocampus, HupA and physostigmine were the most potent inhibitors of G4 AChE, whereas donepezil and tacrine were the most potent against G1 AChE. In striatum, HupA and donepezil were the most potent against G4 AChE, and again donepezil was the most potent against G1 (Table 2). It is well known that approximately 60%–90% of G4 AChE is ectocellular, and is the major form for metabolizing ACh. The G4 AChE is the physiologically relevant form at cholinergic synapses, and its inhibition would be expected to prolong the action of ACh. The results mentioned above suggest that the use of AChE inhibitors in the treatment of AD must consider both form-specific and region-specific characteristics of AChE inhibition[12].

Significant inhibition of AChE activity was demonstrated in the cortex, hippocampus, striatum, medial septum, medulla oblongata, cerebellum, and hypothalamus of rats that were killed 30 min following the administration of HupA at several dose levels compared with the saline control[26,31,32]. There was a clearly dose-dependent inhibition of AChE in the brain region by HupA. In contrast to the inhibition of AChE activ-

**Table 2.** Effects of five acetylcholinesterase inhibitors on acetylcholinesterase G4 and G1 form in rats *in vitro*.

| | | $K_i$ (M) | | |
| | | Cortex | Hippocampus | Striatum |
| --- | --- | --- | --- | --- |
| Huperzine A | G4 | $7.0\pm3.5\times10^{-9**}$ | $5.0\pm0.6\times10^{-7*}$ | $1.1\pm0.1\times10^{-7**}$ |
| | G1 | $3.5\pm1.5\times10^{-7}$ | $8.4\pm0.9\times10^{-7}$ | $6.0\pm6.0\times10^{-7}$ |
| Donepezil | G4 | $4.0\pm1.5\times10^{-9}$ | $5.6\pm1.4\times10^{-6}$ | $5.2\pm0.9\times10^{-7}$ |
| | G1 | $3.5\pm1.2\times10^{-9}$ | $2.9\pm3.5\times10^{-9**}$ | $1.4\pm0.4\times10^{-10**}$ |
| Tacrine | G4 | $6.0\pm1.4\times10^{-8}$ | $5.4\pm4.3\times10^{-6}$ | $1.7\pm0.5\times10^{-6}$ |
| | G1 | $2.3\pm0.3\times10^{-8*}$ | $3.2\pm2.0\times10^{-9**}$ | $1.9\pm0.2\times10^{-8**}$ |
| Rivastigmine | G4 | $1.5\pm1.4\times10^{-3}$ | $3.5\pm0.5\times10^{-4}$ | $1.0\pm4.0\times10^{-4}$ |
| | G1 | $1.6\pm2.1\times10^{-5**}$ | $3.1\pm0.8\times10^{-5**}$ | $1.5\pm1.1\times10^{-5*}$ |
| Physostigmine | G4 | $9.7\pm1.9\times10^{-7}$ | $3.3\pm0.3\times10^{-8}$ | $6.6\pm1.1\times10^{-8}$ |
| | G1 | $5.8\pm1.2\times10^{-7}$ | $3.0\pm2.2\times10^{-8}$ | $3.6\pm1.2\times10^{-8}$ |

Data represent mean±SD. All assays were performed in triplicate for three independent experiments. $K_i$ values were derived from $IC_{50}$ values with the aid of the Cheng equation, using con-current estimates of substrate $K_m$ from double-reciprocal Lineweaver-Burk plots. $IC_{50}$ values were determined graphically from log concentration-inhibition curve from 20% to 80% of initial activity. $^*P<0.05$, $^{**}P<0.01$, significant difference between G4 and G1 forms. Data from ref 12.

ity *in vitro*, the relative inhibitory potency of oral HupA on cortex AChE was found to be approximately 24- and 180-fold, on an equimolar basis, that of donepezil and tacrine, respectively[19]. Correlated to the dosage of AChE inhibition, however, only donepezil and tacrine produced significant BuChE inhibition in serum[19]. Tacrine was a more potent inhibitor of serum BuChE than that of brain AChE. The inhibitory potency of HupA on AChE differs from that of donepezil and tacrine following different routes of administration. HupA exerted an almost similar anti-ChE efficacy in rats following oral and ip administration, whereas tacrine ip produced a greater inhibition both on brain AChE and serum BuChE. At doses of 0.03 μmol/L (8 μg) and 0.06 μmol/L (16 μg), HupA significantly inhibited brain AChE activity 30 min after intraventricular injection, which was less potent than donepezil, but more potent than tacrine[26]. These findings indicate that HupA, in contrast to donepezil and tacrine, has higher oral bioavailability and better penetrability through the blood-brain barrier.

AChE inhibition in rat whole brain reached a maximum at 60 min and was maintained for 360 min following oral administration of HupA, at a dose of 1.5 μmol/kg (3.6 mg/kg). Peak inhibition in cortex and serum was observed at 30–60 min, and inhibition exceeding 10% in the cortex was maintained between 15–240 min. The BuChE activity recovered to the control level at 360 min after administration of HupA, whereas

20% and 46% inhibition still existed for donepezil and tacrine, respectively[19]. The rapid decrease of AChE and BuChE activity seen in red blood cells and plasma, respectively, with HupA correlates with the short-lasting and mainly peripheral side effects[31]. Repeated oral doses (once daily for 8 d and 30 d) of HupA produced no significant difference in AChE inhibition as compared to a single dose, indicating that no tolerance to HupA developed[19,33].

The enantiomers of HupA differ greatly in their ability to inhibit AChE. At equivalent doses, (+)-HupA was much weaker than (–)-HupA in inhibiting AChE in NG108-15 cells[34]. Careful measurement of AChE inhibition in cell-free systems revealed that (–)-HupA was 49-fold more potent than (+)-HupA as shown both by $IC_{50}$ values and $K_i$ (Table 3). The natural isomer was also more selective for AChE, as shown by the 9-fold higher ratio of BuChE $IC_{50}$ *versus* AChE $IC_{50}$. Similar differences in anti-cholinesterase potency were seen when changes in ChE activity in whole brain, cortex and serum were compared 30 min after oral administration of (–)-HupA and (+)-HupA. Although both enantiomers of HupA produced a dose-dependent inhibition of ChE, (–)-HupA was approximately 50-fold more potent than (+)-HupA[34]. This difference in activity measured between the 2 enantiomers may be partially ascribed to the fact that the H-bond between the ethylidene methyl of (–)-HupA and His440 is absent in the (+)-HupA complex[35,36].

**Table 3.** Anti-cholinesterase effects of huperzine A enantiomers *in vitro*.

| | $IC_{50}$ (nM)[a] | | BuChE/AChE | $K_i$[b] |
| | BuChE[c] | AChE[d] | | |
|---|---|---|---|---|
| (–)-HupA | 53632 | 65 | 829 | 30 |
| (+)-HupA | 282910 | 3153 | 90 | 1480 |
| (±)-HupA | – | 300 | – | – |

[a] The concentration of inhibitor that yields a 50% inhibition of enzyme activity; [b] rat erythrocyte membrane; [c] rat serum; [d] rat cortical homogenate. Data from ref 34.

The mechanisms by which HupA inhibits AChE have been extensively studied by using kinetics[11,23,37], computer-aided docking studies[36,38] and X-ray crystallography approaches[35]. The Lineweaver-Burke plot representation of the inhibition of rat erythrocyte membrane AChE by HupA indicates a mixed competitive type of inhibition, because the intersection of the lines occurs in the second quadrant (Figure 2)[11,22,23]. A similar type of inhibition was found in porcine
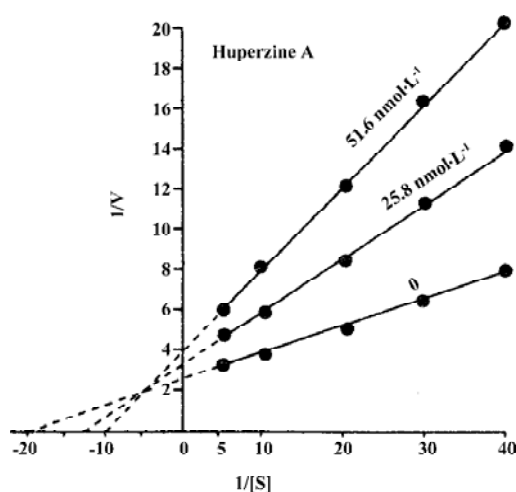


**Figure 2.** Typical Lineweaver-Burke plots for inhibition of rat erythrocyte membrane AChE by HupA. Data from ref 11.

brain immobilized AChE[23]. Rat erythrocyte membrane AChE activity did not exhibit progressive decrease with prolonged incubation with HupA *in vitro*, 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)[11,23].

Immense effort has been directed towards gaining insights into the binding between HupA and AChE since 1991, when the 3-D structure of the native *TcAChE* was determined by using both X-ray crystallography and molecular modeling. The 2.5 Å resolved crystal structure of a *Torpedo* AChE-HupA complex demonstrated the "ingenious design" of the natural alkaloid[39] to bind more tightly and specifically to the 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[35]. The principal interactions include: (i) direct and strong hydrogen bonds between the carbonyl group of HupA and the hydroxy oxygen of Tyr130 (located at the peripheral site of the enzyme), as well as between the thylidene methyl group and the main-chain oxygen of His440 (a modality of the catalytic triad); (ii) indirect hydrogen bonds, mediated by 1 or 2 water molecules, between HupA and residues of the enzyme that constitute the active center (eg the ring nitrogen of HupA is hydrogen-bonded to carboxylic oxygen of Glu199, whereas the $-NH_3^+$ group is bonded to hydroxyl oxygen of Tyr 121); (iii) the cation-π interaction induced upon binding between the $-NH_3^+$ group of HupA and the aromatic rings of Trp84 and Phe 330 at the choline site (it

should be noted that other reversible AChE inhibitors such as tacrine and edrophonium bind to the same site)[40]; and (iv) a large number of hydrophobic interactions that 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 resolution 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 Tyr337 within the choline site that exists only in the mammalian homologue of AChE, but not in *Torpe*do enzymes and BuChE[41,42]. This particular interaction may be largely responsible for the much stronger inhibitory effect of HupA on mammalian AChE than that on the other 2 enzymes. In addition, the peptide flip between Gly117 and Gly118 (induced only by the binding of HupA) may explain why HupA possesses a longer residence time than other commonly used anticholinesterase agents[37].

Dvir *et al* reported that the oxyanion holes of AChE composed of Gly117, Gly118, and Gly119 were disrupted by HupA. The carbonyl oxygens of HupA appear to repel the carbonyl oxygen of Gly117, thus causing the peptide bond between Gly117 and Gly118 to undergo a peptide flip[36]. The new conformation is stabilized by Gly117O making H-bonds with Gly119N and Ala201N, the other 2 functional elements of the 3-pronged oxyanion hole characteristic of AChE[36]. It has been suggested that the peptide flip itself is responsible for the low on-rates observed for AChE inhibition by (–)-HupA[35]. Its stabilization may contribute to the low rates of dissociation observed[37].

## Effects on cholinergic parameters

To study the effect of HupA on cholinergic transmission at mouse neuromuscular junctions *in vitro*, isolated mouse phrenic nerve-hemidiaphragm preparations were used with a conventional intracellular recording technique. HupA at a concentration of 1 μmol/L increased the amplitude, time-to-peak, and half-life of miniature end-plate potentials (MEPP) of muscle fiber[43]. HupA had no effect on resting membrane potentials, the mean quantal content of end-plate potentials and the frequency of MEPP of muscle fiber, indicating that the effects of HupA may not be mediated through presynaptic or postsynaptic mechanisms. In contrast to donepezil and tacrine, neither the appearance of giant MEPP nor slow MEPP was changed by HupA, ruling out the possibility of non-specific promoting effects on terminal ACh release[44].

In a study of toad paravertebral ganglia (PVG) using intracellular recording techniques[45], HupA, at concentrations of 0.3, or 1 μmol/L, did not change membrane potential or input resistance, but increased the rate of orthodromic action potential evoked by preganglionic stimulation, in contrast to physostigmine[46] and tacrine[47]. HupA increased exogenous ACh- but not carbachol-induced depolarization, indicating that the facilitating effect of HupA on ACh transmission is mainly mediated by its anti-AChE activity.

Neuronal nicotinic acetylcholine receptors (nAChR) are involved in cognition and may play a role in AD. Studies on nAChR in rat hippocampal CA1 interneurons in slices using patch-clamp techniques showed that HupA had no significant effect on either the amplitude or kinetics of α7 nAChR activated by ACh, but slowed the rate of recovery from desensitization through an indirect mechanism. For non-α7 receptors, HupA significantly increased the amplitude and decay phase for responses induced by ACh (but not carbachol), also through an indirect mechanism. The results suggested that AChEI were likely to be important regulators of cholinergic signaling in the hippocampus[48].

In isolated rat phrenic nerve-diaphragm preparation, HupA significantly increased the amplitude of muscle contraction induced by stimulating the nerve. The anticurare effect of HupA was found to be much more potent than that of neostigmine in anesthetized rat sciatic-tibialis preparation. The salivation induced by HupA was less potent than that induced by neostigmine[49].

HupA produced an altered electroencephalography (EEG) result in conscious rabbits, which showed a decrease in lower frequency components and the total EEG power in the cortical area, and the dominant frequency changed from delta rhythm to theta rhythm in the hippocampus. These effects are cholinergic in nature and can be reversed by scopolamine or atropine[49–51].

The acetylcholine potentiating action of HupA has been observed in the frog rectus abdominus muscle, rat phrenic nerve diaphragm preparation, guinea pig ileum, and human iris sphincter muscle. HupA has greater acetylcholine potentiating activity on vertebrate muscles than does physostigmine[49,52].

In the hippocampus, high-affinity choline transport was reduced by 28% after multiple ip doses of 0.5 mg/kg HupA[33]. Because the effect of HupA was completely reversible with time and not mediated through a direct interaction with the transporter, this effect was probably mediated through regulatory control of high-affinity choline transport in response to ACh increases following ChE inhibition rather than by directly acting on 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 with tacrine and heptylphysostigmine[31,53]. The concentration required to display 20% specific binding was 20 µmol/L for [$^3$H](−)nicotine and 160 µmol/L for [$^3$H]QNB, indicating that lower concentrations of HupA have a stronger displacing effect on [$^3$H](−)nicotine- than on [$^3$H]QNB-specific binding. 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[54]. In such a state, nicotinic receptors may become more sensitive to stimulation by HupA.

HupA at concentrations of 1–100 µmol/L tested *in vitro* did not affect the electrically evoked release of [$^3$H]ACh from rat cortical slices[31], which contrasted with the decreased release seen with tacrine, physostigmine, and metrifonate[55]. This finding suggests that HupA might not have exerted direct action on the cholinergic presynaptic receptors controlling ACh release.

## Effects on brain neurotransmitters

Compared with other inhibitors being used in the therapy of AD, HupA produced a more prolonged increase of ACh levels than did tacrine, donepezil, rivastigmine, physostigmine, or metrifonate after systemic administration[13,31,32,53,56]. There is considerable regional variation in the increase of ACh levels after HupA administration, with maximal increase seen in frontal and parietal cortex, and smaller increases in the striatum and cerebellum[31]. Considering that ACh level is particularly low in the cerebral cortex of patients with AD[57], this particular regional specificity produced by HupA may constitute a therapeutic advantage. A positive correlation was seen between ACh levels and AChE activity in the frontal cortex and whole brain[13,31,32,53]. Studies using microdialysis techniques in conscious, freely moving rats showed that HupA dose-dependently elevated the level of ACh in cortex and hippocampus. The time course of cortical AChE inhibition with HupA mirrored the increase of ACh at the same doses[13]. This result is consistent with the idea that the increase in extracellular ACh was due primarily to the inhibition of cortical AChE. In molar terms, HupA was 8- and 2-fold more potent than donepezil and rivastigmine, respectively, in increasing cortical ACh levels, with a longer-lasting effect (Figure 3)[13]. Tolerance or accumulation of increasing ACh level was not formed after multiple administra-
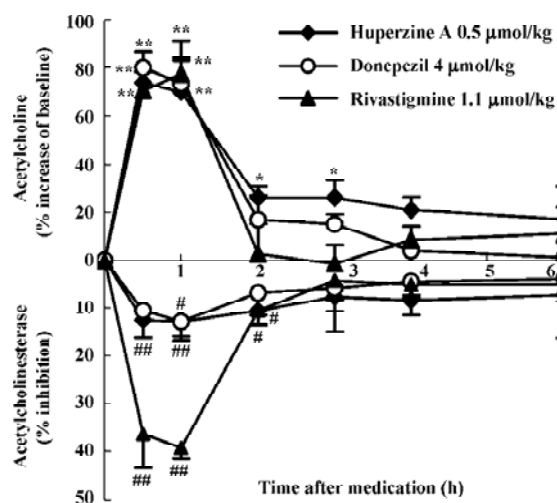


**Figure 3.** Comparative effects of HupA, donepezil, and rivastigmine on the cortex ACh levels and AChE activity in rats after ip injection. Results shown as mean±SEM. $^*P<0.05$, $^{**}P<0.01$ *vs* baseline; $^#P<0.05$, $^{##}P<0.01$ *vs* saline control. Data from ref 13.

tion of HupA (Liang YQ *et al*, unpublished data). HupA did not alter choline levels[32] or the activity of choline acetyltransferase (ChAT) in any region of the rat brain assayed[32,33], suggesting that the increase of ACh levels by HupA was not likely to be mediated through an increase in the rate of ACh synthesis.

Brain norepinephrine (NE) and dopamine (DA) levels increased significantly following either systemic administration of HupA or local administration of HupA through microdialysis, but 5-HT level was not affected[56]. HupA produced an 11- and 2-fold more potent increase in the DA level of the middle prefrontal cortex than donepezil and rivastigmine, respectively. The increasing effect of HupA on DA level was more potent than that on NE level (Liang YQ *et al*, unpublished data). Oxotremorine (a muscarinic agonist) and mecamylamine (a nicotinic antagonist) completely blocked the increasing effects of HupA on DA and NE levels, suggesting that ACh regulation by presynaptic ACh muscarinic receptors or nicotinic receptors accounts for the effect of HupA on DA and NE. These effects may be involved in the memory improvement effected by HupA (Liang YQ *et al*, unpublished data).

## Enhancing effects on cognition

HupA has been found to be an effective cognition enhancer in a number of different animal species. Enhancement of learning and memory performance was demonstrated in passive footshock avoidance[58–61], water maze escape

task[63,64], and spatial discrimination in a radial arm maze[10,65], as well as in delayed response performance in monkeys[66,67]. Beneficial effects were seen not only in intact adult rodents, aged rodents[58,64] and monkeys[66], but also in rodents cognitively impaired by scopolamine[10,31,50,60–63,68], AF64A[26,69], electroshock[61,68], cycloheximide[61], $NaNO_2$[61], $CO_2$[58,60], and *D*-galactose[70]. Inverted U-shaped dose-response curves typical of cognition enhancers were found with HupA. The duration of improvement induced by oral HupA on learning and memory retention processes were longer than those induced by physostigmine, galanthamine, and tacrine, respectively[71]. HupA has a higher efficacy than tacrine and donepezil given orally[19]. The improvement effected by HupA was more pronounced in working memory than in reference memory[10], which may benefit AD patients because the cognitive deficits in memory of recent events are more severe in AD. In aged rats, HupA could significantly reduce the latent period of finding the platform and increased the time in the probe quadrant in the Morris water maze performance[64]. In addition, HupA improved cognition in cholinergically lesioned rats[58,61] and the spatial working memory deficit induced by lesions in the nucleus basalis magnocellularis[72].

HupA reversed memory deficits induced by bilateral injection of scopolamine and muscimol (a $GABA_A$ agonist) into hyperstriatum ventrale in chicks. The improvement was observed from 30 min to 90 min, but not at 10 min after training, indicating that HupA participated in the modulation of intermediate-term memory and long-term memory formation in a passive avoidance task. This finding suggests that HupA improved memory formation not only by acting as a highly potent inhibitor of AChE, but also by antagonizing the effects mediated through the $GABA_A$ receptor[62]. It is well known that deficiencies in ACh and GABA content have been observed in the cortical regions of AD brains[73]. Anatomical evidence also suggests an important interaction between GABA and cholinergic neurons in the septum and hippocampus[74]. Thus, a drug that is able to enhance synaptic ACh and also antagonize the $GABA_A$ receptor could be ideal for treatment of AD.

To extend the antiamnesic effect of HupA to non-human primates, HupA was evaluated for its ability to reverse the deficits in spatial memory produced by scopolamine in young adult rhesus monkeys or those occurring naturally in aged monkeys using a delayed-response task[66]. HupA (0.01–0.1 mg/kg, im) improved the memory deficits induced by scopolamine in young adult monkeys. In aged monkeys, HupA (0.001–0.01 mg/kg, im) significantly increased choice accuracy in delayed response performance (Figure 4). The ben-
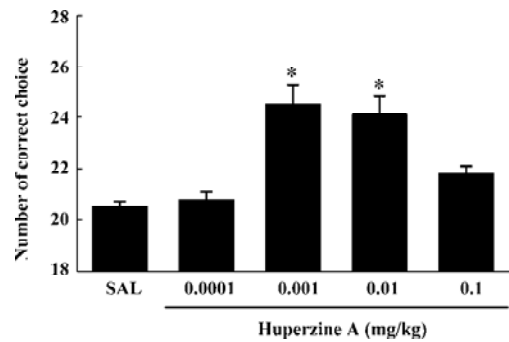


**Figure 4.** Effects of HupA in aged monkeys. Saline or HupA was administered im 20 min before testing. HupA produced a dose-related improvement in the delayed-response performance of aged monkeys ($n$=4). *$P$<0.05 *vs* saline (SAL) control. Values represent mean±SEM of number of trials correct out of a possible 30 trials. Data from ref 66.

eficial effects of HupA were long lasting. Monkeys retained improved performance for approximately 24 h after a single injection of HupA. Given that the NE and DA levels decreased significantly with aging in monkeys[75], the effect of HupA on memory may be associated with increased levels of NE and DA. HupA (0.01–0.1 mg/kg, im) also produced significant improvement on reserpine- (a catecholamine depleting agent) and yohimbine- (an $\alpha_2$-adrenoceptor antagonist) induced memory impairments in monkeys (Figure 5). These effects are primarily due to the increase of NE level by HupA, which might stimulate the $\alpha_2$-adrenoceptor in the prefrontal cortex to improve the delayed response performance[67].

Deposition of β-amyloid protein is considered a crucial event in initiating the neuritic and neuronal degeneration in AD, which mainly affects the areas involved in cognitive function, such as cortices, some limbic structures, and the forebrain nuclei projecting to those areas. Repeated icv infusion of β-amyloid protein-(1-40) induced marked amnesic effects along with signs of neurodegeneration and extracellular amyloid deposits throughout the frontoparietal cortex and hippocampus, which indicated that β-amyloid protein deposition in the brain was related to cognitive impairment, hypofunction of cholinergic neurons and neuronal death. Daily intraperitoneal administration of HupA for 12 consecutive days produced significant reversals of the β-amyloid-induced deficit in learning a water maze task (Figure 6). Treatment with HupA also attenuated the neuronal degeneration induced by Aβ1-40 in the cortex and hippocampus, indicating that neuroprotection is involved to some extent in the favorable effect of HupA on Aβ-induced memory deficits[76].
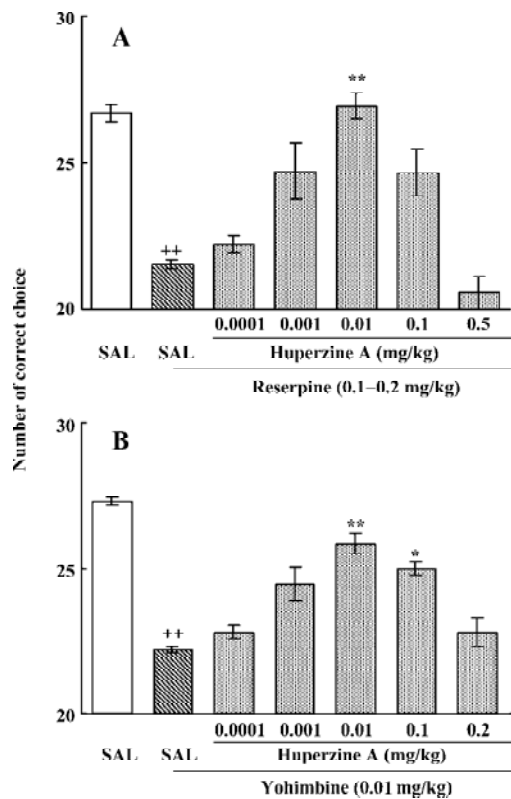
It is well-documented that *N*-methyl-*D*-aspartate

**Figure 5.** Effects of HupA on the deficit of delayed response performance induced by reserpine (A) or yohimbine (B) in young adult monkeys. Saline, reserpine, yohimbine or HupA was administered im 30 min before testing. HupA produced a dose-related improvement in the delayed response performance of young monkeys ($n$=4). Values represent the mean±SEM number of trials correct out of a possible 30 trials. [++]$P<0.01$ *vs* saline control, [*]$P<0.05$, [**]$P<0.01$ *vs* reserpine or yohimbine control. Data from ref 67.



**Figure 6.** HupA improves the memory deficit induced by icv infusion of Aβ1-40 (800 pmol×3) in rats. (A) The typical swimming-tracking path in Morris water maze; a, b, and c are the performance on the fourth training day; d, e, f are the performances of probe trial on the fifth training day; a and d: vehicle control; b and e: Aβ1-40-treated rat; c and f: rat treated with HupA 0.2 mg/kg plus Aβ1-40. (B) Mean latencies to escape from the water onto the hidden platform in fourth training day. (C) The time spent and swum distance in the target quadrant swimming 60 s without platform. Data expressed as means±SEM indicated by vertical bar. [+]$P<0.05$, [++]$P<0.01$ *vs* vehicle-treated group. [*]$P<0.05$, [**]$P<0.01$ *vs* Aβ1-40-treated group. Data from ref 76.

(NMDA)-receptor activation mediates the generation of long-term potentiation (LTP), a cellular process that underlies learning and memory[77,78]. There is evidence that the suppressive action of Aβ on LTP in both CA1 and dentate gyrus operates via an NMDA receptor-independent pathway that involves cholinergic terminals in the hippocampus. It is of some interest that HupA (1.0 μmol/L) was found to enhance LTP, but at a much lower dose (0.1 μmol/L) largely blocked the suppressive effects of Aβ on LTP induction[79,80], which might involve the mechanism of HupA against Aβ-induced cognitive deficits.

Apart from AD, the most common dementia in the elderly is vascular dementia (VD). This disorder, like AD, presents as a clinical syndrome of intellectual decline produced by ischemia, hypoxia, or hemorrhagic brain lesion. Rats with permanent bilateral ligation of the common carotid arter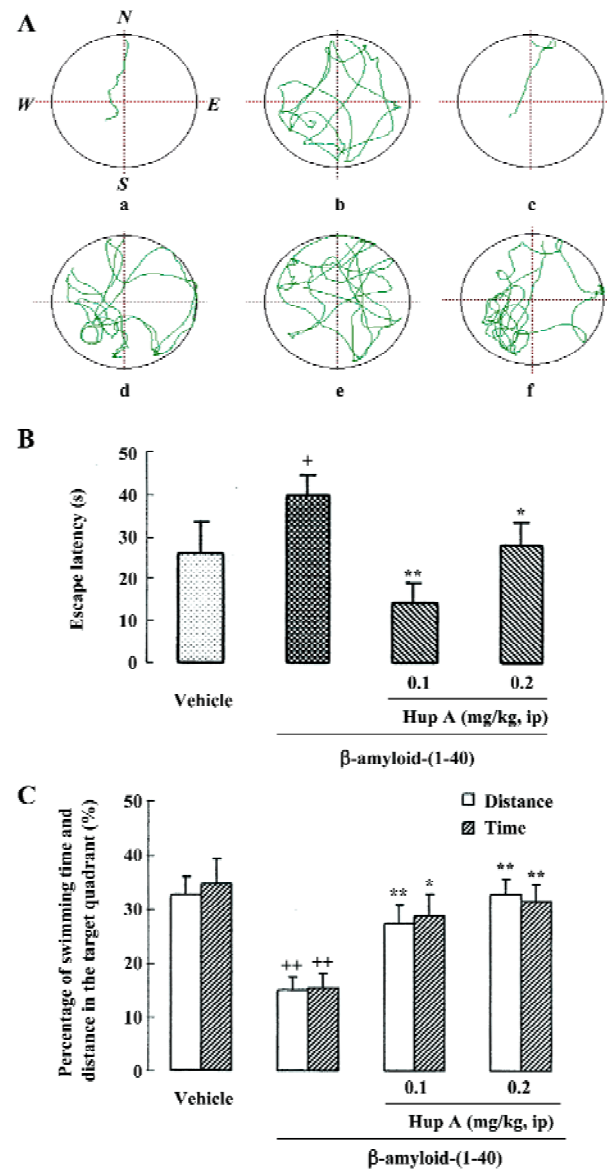ies exhibit learning and memory impairments and neuronal damage resembling those in VD. In these rats, daily oral administration of HupA produced a significant improvement of the deficits in learning the water maze task, beginning 28 d after

ischemia, along with approximately 33%–40% inhibition of AChE activity in the cortex and hippocampus[81]. Similar cognitive improvement of HupA was observed in a gerbil model of transient global ischemia[82]. The protective effects of HupA against hypoxic-ischemic (HI) brain injury were also found in neonatal rats. Unilateral HI brain injury was produced by ligation of the left common carotid artery followed by 1 h hypoxia with 7.7% oxygen in 7-d-old rat pups. After 5 weeks, HI brain injury in rat pups resulted in working memory impairments as shown by increased escape latency in a water maze and reduced time spent in the target quadrant. Rats treated with HupA at doses of 0.05 or 0.1 mg/kg ip for 5 weeks after HI injury performed better than saline-treated HI rats, and neuronal damage in the ipsilateral hemisphere was attenuated (Figure 7). These findings suggest that HupA might be beneficial in the treatment of HI encephalopathy in adults and neonates[83].
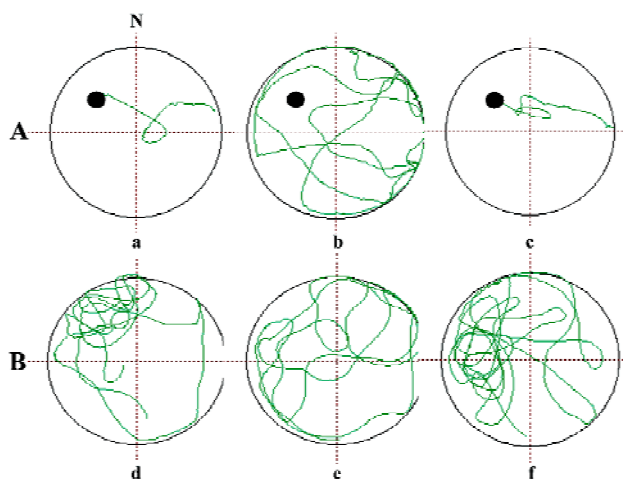


**Figure 7.** Representative swim paths in the Morris water maze. Each rat subjected to three trials per day for five consecutive days. Intraperitoneal administration of HupA or saline for 5 weeks after hypoxic-ischemic (HI) brain injury in neonatal rats. (A) The acquisitive performance on the fifth day. (B) The performance in the probe trial on the fifth day. (a, d) Sham-operated group ($n$=10); (b, e) saline-treated HI group ($n$=11); (c, f) HupA 0.1 mg/kg-treated HI group ($n$=12). Data from ref 83.

## Neuroprotective effects

Several neurodegenerative disorders such as AD, cerebral ischemia-reperfusion injuries and head injuries are thought to be related to changes in oxidative metabolism. Increased oxidative stress, resulting from free radical damage to cellular function, can be involved in the events leading to AD, and is also connected with lesions called tangles and plaques. Plaques are caused by the deposition of Aβ and are observed in the brains of AD patients[2,84–86]. Studies show that oxygen radicals initiate amyloid build-up, leading to neurodegeneration[85,87]. HupA has been found to protect against $H_2O_2$- and Aβ-induced cell lesion, decrease the level of lipid peroxidation, and increase antioxidant enzyme activities in rat PC12 and NG108-15 cell lines and primary cultured cortical neurons (Figure 8)[34,88–91]. Following 6 h exposure of the cells to $H_2O_2$ (200 µmol/L) or 48 h exposure to Aβ25-35 (1 µmol/L), a marked reduction in cell survival, activity of glutathione peroxidase (GSH-Px) and catalase (CAT), as well as increased production of reactive oxygen species (ROS) and malondial-dehyde (MDA) were observed. Pretreatment of the cells with HupA (0.1–10 µmol/L) 2 h before $H_2O_2$ or Aβ exposure significantly elevated cell survival. HupA reversed $H_2O_2$- and Aβ-induced decreases in GSH-Px and CAT activity, as well as causing increases in the production of ROS, MDA and superoxide dismutase (SOD).

Oxygen-glucose deprivation (OGD) for 30 min caused death in more than 50% of rat pheochromocytoma PC12 cells, along with major changes in morphology and biochemistry, including elevated levels of lipid peroxide, SOD activity and lactate concentration. Cells pretreated for 2 h with HupA (0.1, 1, or 10 µmol/L), however, had increased survival and reduced biochemical and morphologic signs of toxicity. HupA protected PC12 cells against OGD-induced toxicity most likely by alleviating disturbances of oxidative and energy metabolism[92].

In rat studies, intracerebroventricular infusion of β-amyloid1-40 (800 pmol×3) induced significant morphological injury and decreases in cortical ChAT activity. Daily ip administration of HupA for 12 consecutive days attenuated the loss of ChAT activity in the cerebral cortex and the neuronal degeneration induced by β-amyloid1-40[76].

MDA level and manganese-SOD (Mn-SOD) activity in hippocampus, cerebral cortex, and serum of aged male rats were 2.3–2.8 times and 1.8–2.8 times greater, respectively, than those of adult male rats. HupA (0.05 mg/kg, ig) markedly lowered the levels of MDA and the activities of Mn-SOD in aged male rats following 7–14 consecutive days of daily administrations[93]. A reduction of oxygen free radicals in the plasma and erythrocytes was also demonstrated in a clinical study[16].

In chronic cerebral hypoperfused rats, HupA restored the decrease in ChAT activity in the hippocampus, improved neuronal morphological damage, and restored SOD and lipid peroxides activities, as well as lactate and glucose concentrations to their normal levels[81]. Similar protective effects
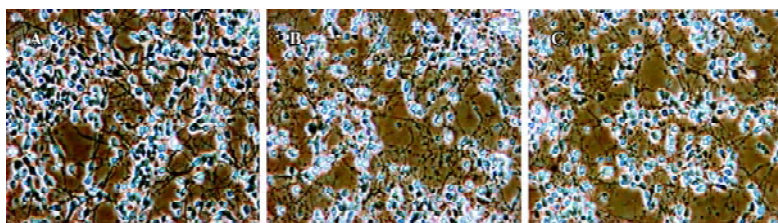
**Figure 8.** Attenuation of Aβ23-35-induced neuronal injury by HupA. Cultures after vehicle treatment (A), Aβ23-35 (20 μM) treatment for 24 h (B), and pretreatment with 1 μM of HupA for 2 h followed by exposure to Aβ23-35 for 24 h (C). Data from ref 91.

of HupA were observed in the studies of transient global ischemia in gerbils[82]. The protective effect of HupA on HI brain injury has also been found in neonatal rats[81]. A unilateral HI brain injury was produced by the ligation of the left common carotid artery (CCA) followed by 1 h hypoxia with 7.7% oxygen in 7-d-old rat pups. After 5 weeks, the HI brain injury in rat pups caused damage in the ipsilateral striatum, cortex and hippocampus, as well as a marked reduction in CA1 neuron density. These neuropathologic signs were attenuated by the administration of HupA at a dose of 0.1 mg/kg (Figure 9). These results raise the possibility that HupA may be potentially useful in treating HI encephalopathy in neonates.

In studies carried out to examine the stereoselectivity of the cellular protective effect induced by (–)-HupA, it was found that (–) and (+) HupA exerted similar potency in protecting against the cellular toxicity induced by Aβ25-35. This result contrasted with the stereoselectivity of cholinesterase inhibition *in vitro* and *in vivo* (Figure 10)[34]. The ability of HupA to inhibit the catalyzing activity is not parallel to its neuroprotective effect, implying that the cytoprotective effect of the 2 enantiomeric forms of HupA might relate to the non-catalytic action of AChE.

It was recently reported that HupA exerted a neuroprotective effect via modulating the intracellular $Ca^{2+}$ ($[Ca^{2+}]_i$) level together with the mRNA transcription of calmodulin (CaM) and calmodulin-dependent protein kinase II (CaMPK II) in hippocampal neurons[94]. Mice given repeated CCA ligation-reperfusion treatment showed a marked increase in $[Ca^{2+}]_i$, and a decrease in CaM and CaMPK II mRNA levels in hippocampal neurons. Daily oral administration of HupA (0.05 mg/kg) for 30 consecutive days significantly reversed the shift in $[Ca^{2+}]_i$, CaM and CaMPK II mRNA levels induced by ischemia.

The findings mentioned above indicate that HupA has protective effects against free radical-, ischemia- and Aβ-induced cell toxicity, which might be beneficial in the treatment of patients with AD and VD.

Apoptosis is the process by which neurons die during normal development and is also a feature of chronic and acute neurodegenerative diseases and stroke[95]. In accor-

dance with previous reports, studies from our lab demonstrate typical apoptotic changes when neuron-like cells are exposed to stressors such as $H_2O_2$[96], Aβ peptides[91], oxygen-glucose deprivation (OGD)[97], serum deprivation[98], the pro-
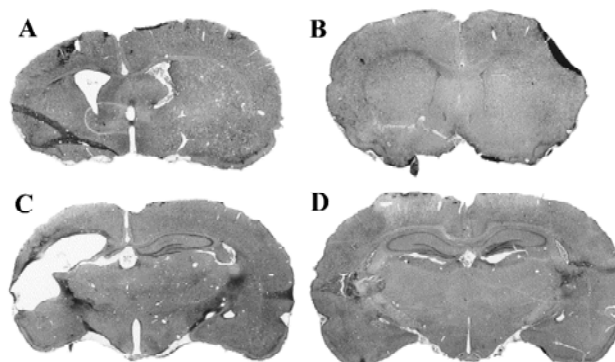


**Figure 9.** Photomicrographs of coronal brain sections stained with cresyl violet at the levels of the striatum (A, B) and the dorsal hippocampus (C, D) for representative saline-treated and huperzine A 0.1 mg/kg-treated rats. Intraperitoneal administration of huperzine A or saline for 5 weeks after hypoxic-ischemic (HI) brain injury in neonatal rats. Note the gross infarction and atrophy in left hemisphere of saline-treated HI rats (A,C) (*n*=11) and the subtle reduction in left hemisphere in huperzine A treated HI rats (B, D) (*n*=12). Data from ref 83.

tein kinase C (PKC) inhibitor staurosporine[99], and global ischemia[82]. These changes include DNA laddering (Figure 11), cell shrinkage, generation of nuclear apoptotic bodies, terminal deoxyribonucleo-tidyl transferase-mediated dUTP-digoxigenin nick end-labeling (TUNEL) positive staining (Figure 12), chromatin condensation (Figure 13), and other classic hallmarks of apoptosis (Figure 14)[91,96,97,99]. Such abnormalities are markedly relieved by HupA. Administration of HupA (0.1 or 0.2 mg/kg, ip, per day) for 12 consecutive days conferred substantial neuroprotection on rats that received icv injections of β-amyloid1-40 (800 pmol×3): the number of apoptotic-like neurons were markedly reduced[76]. In primary cultured neurons, preincubation with HupA at concentrations higher than 0.01 μmol/L led to a large dose-dependent attenuation of cell toxicity induced by Aβ25-35[91].
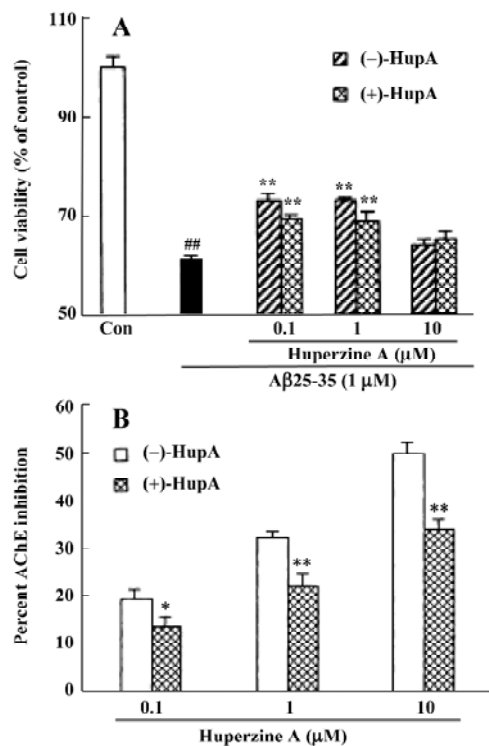
**Figure 10.** Protective effects against cytotoxicity induced by Aβ25-35 (A) and anti-cholinesterase effects (B) of (+)-HupA and (–)-HupA in NG108-15 cells. Values are means±SEM expressed as % inhibition (*vs* saline control). Basal saline control values in NG108-15 cells were 238.54±13.02 absorbance values/g protein (n=3). A, [##]*P*<0.01 *vs* control; [*]*P*<0.05, [**]*P*<0.01 *vs* Aβ group; B, [*]*P*<0.05, [**]*P*<0.01 *vs* (–)-HupA group. Data from ref 34.

Moreover, HupA (1 μmol/L) caused large reductions in the amounts of subdiploid DNA detected in a flow cytometry assay and weakened the ladder pattern on agarose gel electrophoresis, which is typically seen after exposure to Aβ
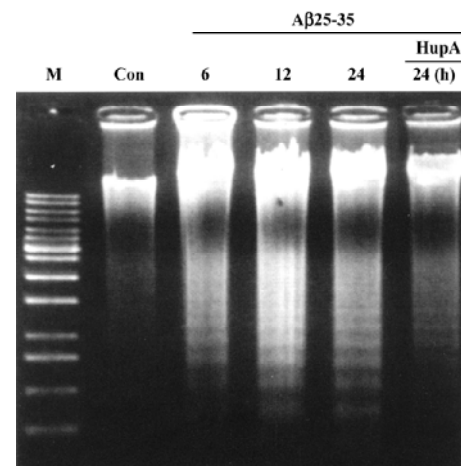


**Figure 11.** Reduction of Aβ25-35-induced DNA fragmentation by HupA. Neurons were treated with 20 μM of Aβ25-35 with or without 1 μM of HupA. Fragmented DNA was isolated by NucleoBond DNA and RNA purification kit, electrophoresed with agarose gel, and finally stained with ethidium bromide. M: DNA size marker; Con: control. Data from ref 91.

(Figure 11)[91]. The inhibition of ROS formation may involve the anti-apoptotic actions of HupA[91].

The cellular commitment to apoptosis is regulated by the Bcl-2 family of proteins. High levels of Bcl-2 expression will inhibit apoptosis. In contrast, an increased expression of P53 and Bax is associated with the initiation of apoptosis[100]. Treatment with HupA attenuated $H_2O_2$-, Aβ- and OGD-induced overexpression of mRNA and protein levels for c-jun, Bax and P53, and downregulated that of Bcl-2 to normal levels (Figure 15)[76,96,97].

In the mitochondrial-mediated cell death pathway, a key step is transient opening of the mitochondrial permeability transition (MPT), involving a non-specific increase in the
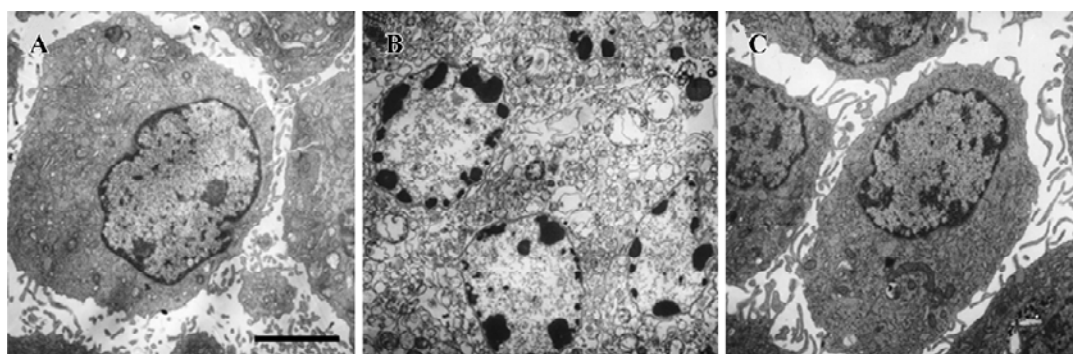


**Figure 12.** Ultrastructural characteristics of PC12 cells following $H_2O_2$ exposure with or without HupA by transmission electron microscopy. A, untreated control PC12 cells; B, PC12 cells exposed to 100 μM $H_2O_2$ for 12 h; C, PC12 cells exposed to 100 μM $H_2O_2$ in the presence of 1 μM HupA. Bar=1.0 μm. Data from ref 96.
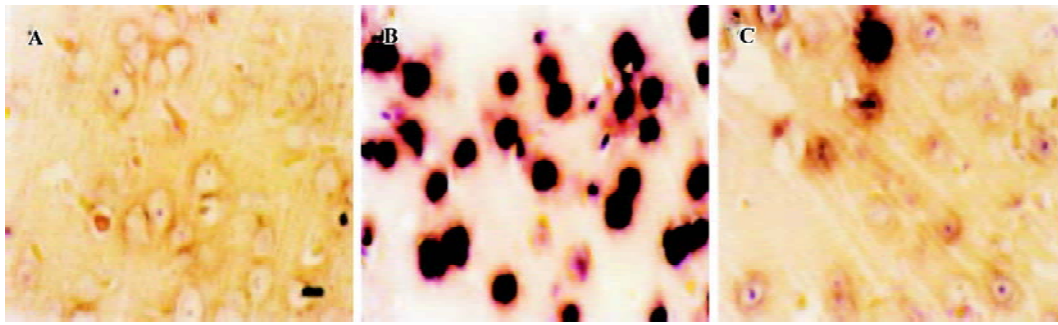
**Figure 13.** Apoptotic cell death induced by icv infusion of Aβ1-40 in the cortex in rats. DNA fragmentation was observed using the TUNEL method. Four rats in each group (*n*=7) were examined. A) Vehicle-treated rat; B) Aβ1-40-treated rats; C) Rats treated with Aβ1-40 and daily administration of HupA (0.2 mg/kg, ip) for 12 consecutive days. Scale bar=5 µm. Data from ref 76.
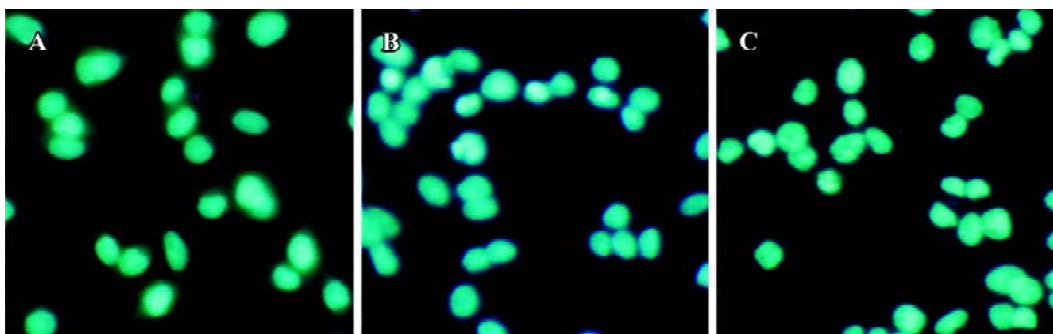


**Figure 14.** Nuclear morphology of apoptotic cells shown by Hoechst 33342 staining. A, control culture; B, culture treated with OGD 3 h and reoxygenation 24 h; C, pre-incubation for 2 h with 1 µM HupA, then treated with OGD 3 h and reoxygenation 24 h. Data from ref 97.

permeability of the inner mitochondrial membrane[101,102]. In this process, cytochrome c moves from the intermembrane space into the cytoplasm[103], where it binds to Apaf-1 (the molecular core of apoptosome, which executes mitochondria-dependent apoptosis). In the presence of dATP, this complex polymerizes into an oligomer known as the apoptosome. The apoptosome activates the protease, caspase-9, which in turn activates caspase-3. The cascade of proteolytic reactions also activates DNase, which leads to cell death[104]. Our recent results showed that PC12 cells, when pre-incubated with HupA at concentrations above 0.01 µmol/L, were markedly protected against apoptosis induced by Aβ, with a significant reduction in mitochondrial swelling and improved mitochondrial membrane potentials (Gao X *et al*, unpublished data).

A series of studies were carried out in our laboratory that focused on caspase activation in primary cultures of rat cortical neurons subjected to a variety of stresses. Measurements of caspase-3-like fluorogenic cleavage demonstrated that HupA (1 µmol/L) attenuated the Aβ25-35-induced increase in caspase-3 activity at 6, 12, 24, and 48 h[91]. Western blot analyses confirmed these results at the protein level.
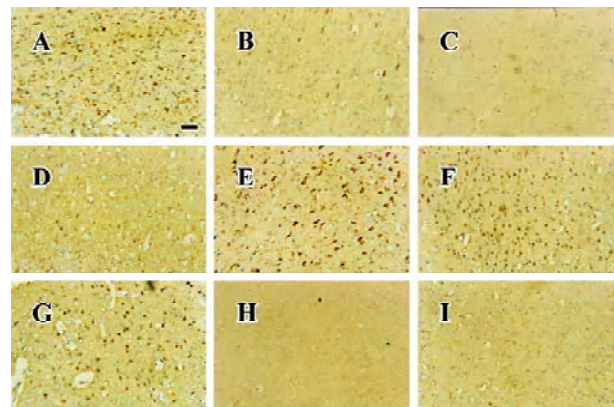


**Figure 15.** Effects of HupA on apoptotic related protein expression in rats. Rats were killed after fifth performance of water maze. HupA 0.2 mg/kg ip was administered once per day for 12 consecutive days. Shows are the representative photographs of Bcl-2, Bax and P53 immunostaining in the cortex. A, B, C: vehicle treated group; D, E, F: Aβ1–40-treated rats; G, H, I: rats infusion of Aβ1-40 and administration of HupA. A, D, G: the Bcl-2 expression in cortical region; B, E, H: Bax; and C, F, I: P53 in the same region. Scale bars 20 µm. Data from ref 76.

HupA also inhibited caspase-3 activation in models of apoptosis induced by serum deprivation and staurosporine treatment. The apoptosis induced by serum deprivation for 24 h was accompanied by enhanced caspase-3 activity and a release of mitochondrial cytochrome c into the cytosol[98]. HupA (0.1–10 µmol/L) improved neurons survival, inhibiting the rise in caspase-3 activity and protein expression (Figure 16)[98]. Likewise, cell survival was greatly enhanced when HupA (0.1–100 µmol/L) was introduced 2 h before 24 h exposure to 0.5 µmol/L staurosporine. Staurosporine-induced DNA fragmen-tation, upregulation of the pro-apoptotic gene *bax*, downregulation of the antiapoptotic gene *bcl*-2, and decrease in caspase-3 proenzyme protein level were all attenuated by HupA at dose of 1 µmol/L[99].

A potassium channel with delayed rectifier characteris-tics may play an important role in Aβ-mediated toxicity[105]. The upregulation of an outward $K^+$ current known as $I_k$ medi-ates several forms of neuronal apoptosis and might specifi-cally contribute to the pathogenesis of Aβ-induced neuronal death. Exposure to 20 µmol/L Aβ25-35 or Aβ1-42 is known to enhance the apoptosis-related current, $I_K$[106]. Expression of wild-type PS-1 or PS-2 increases outward $K^+$ current densi-ties in HEK293 cells relative to untransfected or mock-trans-fected cells[107]. These data raise the intriguing possibility that manipulations aimed at reducing outward $K^+$ current may provide an approach to reducing neuronal degenera-tion in patients with AD.

HupA reversibly inhibited the fast transient potassium current ($I_A$) in CA1 pyramidal neurons acutely dissociated from rat hippocampus. The effect was voltage-independent and insensitive to atropine. HupA slowed down the decay of $I_A$ and its recovery from inactivation and showed no effect on steady-state inactivation, but hyperpolarized the activa-tion curve of $I_A$ by 6 mV, suggesting that HupA may act as a blocker at the external mouth of the A channel[108]. In addition, HupA inhibited another important outward $K^+$ current, the sustained potassium current ($I_K$) in a voltage-dependent manner in acutely dissociated rat hippocampal neurons. The effect was insensitive to atropine. HupA hyperpolarized the activation curve of the current by 16 mV, and markedly pro-longed the decay time constant τ2[109]. Because outward $K^+$ current has proven very important in apoptosis induction, the reversible inhibitory effects of HupA on $I_A$ and $I_K$ might contribute to its anti-apoptotic effect.

In light of these findings, we can conclude that HupA, in addition to being a potent, highly specific and reversible inhibitor of AChE, possesses the ability to protect neurons against cytotoxicity and apoptosis induced by $H_2O_2$, Aβ, OGD, ischemia, serum deprivation and staurosporine. The protective and anti-apoptotic actions of HupA may involve inhibiting the production or the effects of ROS, improving energy metabolism, regulating apoptosis-related gene expression, protecting mitochondrial function, as well as modulating intracellular $Ca^{2+}$ concentrations and inhibiting outward $K^+$ currents. The neuroprotective effect of HupA is not correlated with its AChE inhibitory activity. These find-ings suggest that the therapeutic effects of HupA may be exerted via a multi-target mechanism.

## Protection of HupA against glutamate-induced cytotoxicity

Glutamate is the main excitatory neurotransmitter in the central nervous system (CNS), with important roles in neu-rotransmission and functional plasticity. Excitatory amino acid neurotransmitters are also involved in CNS pathology.
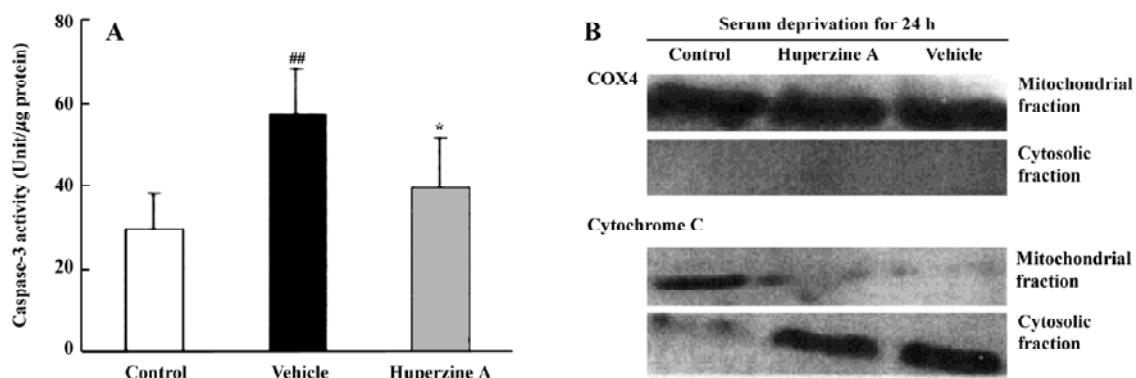


**Figure 16.** Effect of HupA on caspase-3 activity (A) and Western blot detection of cytochrome c and COX4 (B) in primary cortical neurons. A: Data expressed as means±SD. $^{##}P<0.01$ compared to control group. $^{*}P<0.05$ compared to serum deprivation group. B: The mitochondrial and cytosolic fractions of COX4 and cytochrome c under different treatments. Data from ref 98.

The deleterious effects of overstimulation with excitatory amino acids have been implicated in a variety of acute and chronic neurodegenerative disorders, including ischemic brain damage, AD and neuronal cell death[110–115]. Glutamate-mediated overactivation of receptors induces excessive $Ca^{2+}$ influx, which results in elevated intracellular $Ca^{2+}$ concentrations[116,117] with serious consequences such as necrosis and apoptosis[118]. Blockade of glutamate receptors prevents most of the $Ca^{2+}$ influx and neuronal cell death induced by glutamate exposure[119,120].

It has been reported that HupA protects against glutamate-induced toxicity. HupA (100 μmol/L) was found to decrease neuronal cell death caused by a toxic level of glutamate. In those experiments, HupA reduced glutamate-induced calcium mobilization but did not affect the increase in intracellular free calcium induced by exposure to high KCl or a calcium activator Bay-K-8644[121]. HupA dose-dependently inhibited the NMDA-induced toxicity in primary neuronal cells, most likely by blocking NMDA ion channels and inhibiting the subsequent $Ca^{2+}$ mobilization at or near the phencyclidine (PCP) and MK-801 ligand sites[122]. HupA reversibly inhibited NMDA-induced current in acutely dissociated rat hippocampal pyramidal neurons and blocked specific [$^3$H]MK-801 binding in synaptic membranes from rat cerebral cortex[123]. Of all AChE inhibitors tested, HupA is the most powerful both in protecting mature neurons and in blocking the binding of [$^3$H]MK-801. The effect was non-competitive, and showed neither "voltage-dependency" nor "use-dependency"[124]. HupA acts as a non-competitive antagonist of the NMDA receptors, via a competitive interaction with one of the polyamine binding sites[125]. It is interesting that natural (–)-HupA and synthetic (+)-HupA reduced the binding of [$^3$H]MK-801 with similar potency[126], indicating that HupA inhibits NMDA receptors in rat cerebral cortex without stereoselectivity. This result is in dramatic contrast with the stereoselective inhibition of acetylcholinesterase.

Neuronal cell death caused by overstimulation of glutamate receptors has been proposed as the final common pathway for a variety of neurodegenerative diseases, including AD. The ability of HupA to attenuate glutamate-mediated neurotoxicity may be one additional reason for considering this agent as a potential therapeutic for dementia and as a means of slowing or halting the pathogenesis of AD at an early stage[122].

## Effects on secretory amyloid precursor protein and protein kinase C-α

Aβ is a self-aggregating 39–43-amino-acid peptide that originates from a larger polypeptide that is known as the Alzheimer's amyloid precursor protein (APP). Alternate pathways for APP processing have been described: the non-amyloidogenic secretory pathway, which releases a soluble ectodomain (APPs) and prevents Aβ formation[127], and the endosomal-lysosomal pathway, which produces amyloidogenic products[128]. The amyloid hypothesis of AD[129,130], which is focused on the potential toxic role of an excessive production of Aβ, suggests that the aberrant metabolism of APP is a central pathogenetic mechanism for the disease.

Several factors can affect the secretory non-amyloidogenic pathway of APP. For example, the stimulation of phospholipase C (PLC)-coupled receptors, such as muscarinic m1 and m3, has been shown to potentiate the secretion of APPs in cell cultures. These effects are probably mediated mainly by PKC[131]. It has also been reported that several AChEI affect APP processing in addition to the catalytic function of AChE[6,132,133].

HupA can alter the disturbance of PKC and APPs induced by Aβ in both rats and the HEK293sw cell line[134]. The levels of APPs and PKCα were significantly decreased after infusion with Aβ$_{1-40}$ in rats. HupA caused a marked reduction of these changes (Figure 17), but had no significant effects on APPs or PKCα in normal rats. In HEK293sw cells, HupA increased the levels of APPs and PKCα, but had no effect on the levels of PKCδ and PKCε. These findings suggest that HupA may reduce APP processing through upregulating PKC, especially PKCα levels.

In an attempt to clarify the receptor mechanisms involved in such effects, we found that HEK293wt cells treated with scopolamine, a nonselective muscarinic antagonist, partly blocked the HupA-induced increase in the levels of APPs and PKC. In contrast, the nicotinic antagonist mecamylamine had no effect, suggesting that stimulation of the non-amyloidogenic secretory pathway of APPs metabolism by AChE inhibition is probably accomplished via an effect on the muscarinic receptors/PKC cascade (Yan H *et al*, unpublished data). Because PKC is a key enzyme in signal transduction, and because APPs itself has neuroprotective effects, modulating the levels of these 2 proteins by HupA may be beneficial in AD therapy.

## Effects on nerve growth factor

Nerve growth factor (NGF) is a member of the neurotrophin family that promotes the survival and outgrowth of central cholinergic neurons[135]. The decrease in trophic support for the neurons in the aging brain is associated with neuronal death and appearance of neurodegenerative disorders such
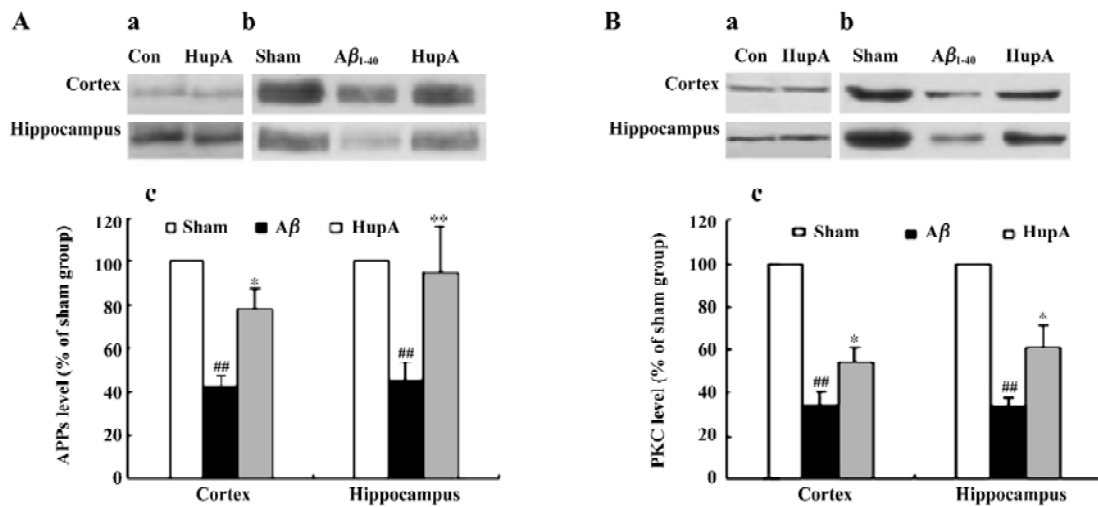
**Figure 17.** Effect of HupA on APPs (A) and PKC (B) level in cortex and hippocampus. (a, b) Western blot results of APPs and PKC in normal rats administered with saline/HupA(a) and in Aβ1-40 infused rats (b), respectively. The sham group received the same treatment as the Aβ group except Aβ1-40 infusion. (c) Quantitative summary of results in b. Values are the mean±SEM expressed as a percent of sham group value. Three separate experiments were performed. $^{##}P<0.01$ *vs* sham group. $^{*}P<0.05$, $^{**}P<0.01$ *vs* Aβ1-40-treated group. Data from ref 134.

as AD[136]. Accumulated data suggest that cholinergic mechanisms are involved in the regulation of NGF synthesis and release, and some AChE inhibitors are known to exert NGF-like activities by potentiating the neuritogenic effect of NGF[137]. HupA has been shown to increase neurite outgrowth from undifferentiated PC12 cells (Figure 18) and to enhance the expression and secretion of NGF, as well as increase p75[NTR] mRNA in primary astrocytes (Figure 19). These effects suggest the possibility that HupA increases the NGF-induced enhancement of neuron survival and function improvement, which is helpful in the rescue of injured neurons in neuro-degenerative disease. In addition to inhibiting AChE activity, AChE mRNA expression and protein levels were significantly upregulated after treatment with HupA. Accumulating evidence indicates that AChE may influence neurite outgrowth through a non-catalytic mechanism[138]. Hence, the effect of HupA on neurite outgrowth may be associated with the level of AChE expression[139].

Neurotrophic factors such as NGF not only promote the survival of responsive neurons but also protect them from oxidative injury. Our study with SH-SY5Ycells used $H_2O_2$ to generate an oxidative stress sufficient to cause cell loss along with a substantial decrease in the mRNA and protein levels of NGF, neurotrophin receptor p75 (p75[NTR]) and tyrosine kinase A (TrkA). HupA not only reduced the overt signs of cytotoxicity, but also preserved the expression of NGF and its receptors (Figure 20). These neuroprotective effects of HupA on $H_2O_2$-induced cytotoxicity were blocked by the

TrkA phosphorylation inhibitor K252a, and were antagonized by the mitogen-activated protein (MAP) /extracellular signal-regulated kinase (ERK) inhibitor PD98059 (Figure 21). These findings indicate that the NGF and TrkA receptor mediate key events required for the neuroprotective actions of HupA. Among the downstream signaling events triggered by the action of NGF at the TrkA receptor, activation of the MAP/ERK kinase pathway may be particularly important for the ability of HupA to protect SH-SY5Y cells against oxidative stress[140].

## Pharmacokinetics

The pharmacokinetics of HupA have been studied in rodents and healthy human volunteers. HupA is absorbed rapidly, distributed widely in the body, and eliminated at a moderate rate (Table 4). The levels of HupA in the blood following iv or *po* administration of [³H]HupA in rats declined in 2 phases, namely the distribution phase and elimination phase. The oral bioavailability was 96.9% in mice, with the highest radioactivities in the kidney and liver. The majority of the radioactivity was excreted in the urine 24 h after iv administration of [³H]HupA. Only 2.4% was recovered from the feces. Paper chromatograms of rat urine revealed that [³H]HupA was excreted partially as prototype and its metabolite[141]. Autoradiographic studies in mice showed that HupA was present in all regions of the brain, but was particularly concentrated in the frontoparietal cortex,
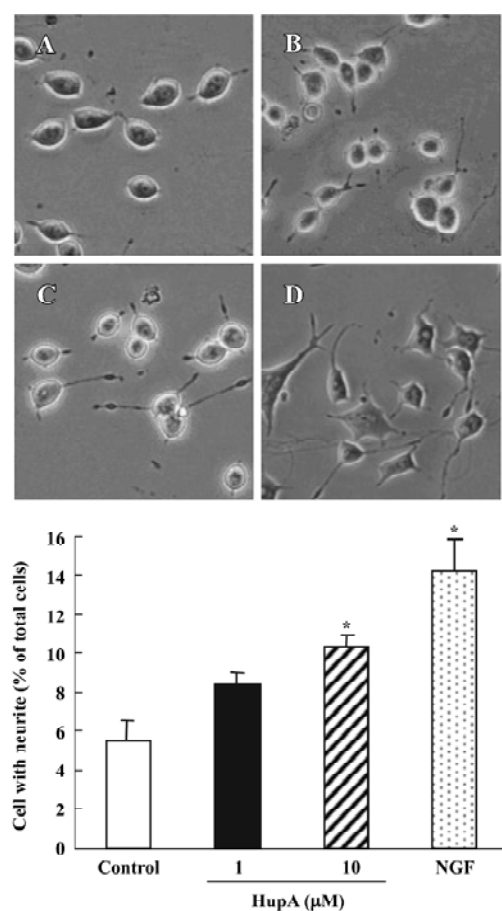
**Figure 18.** Effects of HupA on neurite outgrowth in PC12 cells. Top panels, representative phase contrast micrographs (200 magnification). PC12 cells were incubated for 48 h under control conditions (A), with 1 μM HupA (B), with 10 μM HupA(C), and with 2 ng/mL NGF (D). Bottom panels, quantitative effects of HupA on neurite outgrowth. Three independent experiments were carried out. $n$=8 for each independent experiment. Mean±SEM. $^*P<0.01$ *vs* control. Data from ref 139.



**Figure 19.** Effects of HupA on NGF and p75$^{\mathrm{NTR}}$ mRNA expression and NGF secretion in rat cortical astrocytes. (A) Representative RT-PCR pattern. Lane 1: control; Lanes 2–4: 10 μmol/L HupA incubated for 2, 4, and 6 h, respectively. The PCR products were normalized with reference to β-actin mRNA. (B) HupA enhances NGF protein level in rat cortical astrocytes. Astrocytes serum deprived for 24 h were incubated with vehicle or 10 μmol/L HupA for another 24 h. NGF protein level was determined directly in the culture medium by NGF-ELISA. $n$=9. Mean±SEM. $^*P<0.05$ *vs* control. Data from ref 139.

striatal cortex, hippocampus, and nucleus accumbens after iv injection[32].

The plasma concentration-time curves of HupA in dogs were determined by using the liquid chromatography- mass spectrum-mass spectrum (LC-MS-MS) method after the last intramuscular injection (10 μg/kg per day for 15 d) of a sustained-release formulation, and the mean $C_{\max}$ was 0.36±0.08 ng/mL, which occurred at 48.0±24.5 h. The mean plasma elimination half-life was 54.8±5.6 h, and the mean area under the plasma concentration versus time curve was 92.6±4.5 ng·h/mL[142]. A pharmacokinetic study using HupA transdermal patches in 6 beagle dogs showed that the HupA patches were able to deliver sustained or controlled drug release *in vivo*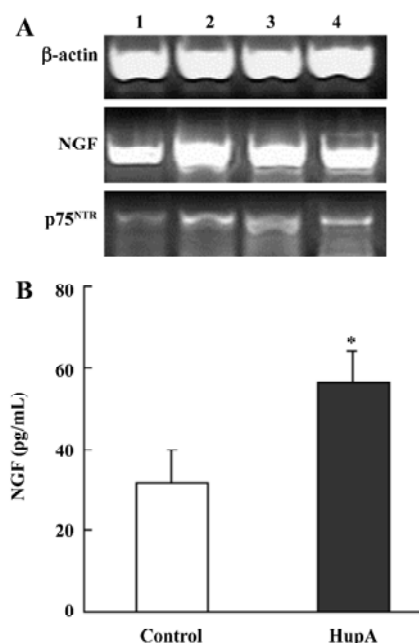. Following application of the first patch (4 mg per 20 cm²), HupA concentrations in serum increased for approximately 12–24 h, reaching an average maximum concentration of 3.4 ng/mL. Thereafter, blood concentrations were maintained up to 84 h during the period in which the patches were worn[143].

In young healthy volunteers, HupA levels in plasma were determined by reverse phase high performance liquid chromatography (HPLC) by using a spectrophotometric detector. The time course of plasma concentration conformed to a one-compartment open model with first-order absorption following oral administration of 0.99 mg HupA. HupA was rapidly absorbed and widely distributed *in vivo*[144]. The half-life of HupA was at least 4–17 times longer than that of tacrine or physostigmine[145]. A pharmacokinetic comparison between young and elderly human volunteers treated with 0.225 mg HupA was recently conducted. The areas under the blood level-time curve (AUC$_{0\rightarrow36}$) was 75.0 and 97.2 nmol/L·h in young and elderly volunteers, respectively. The mean maximum blood concentrations ($C_{\max}$) were 9.1 and 6.8 nmol/L, respectively, and the elimination half-lives ($T_{1/2}$) of
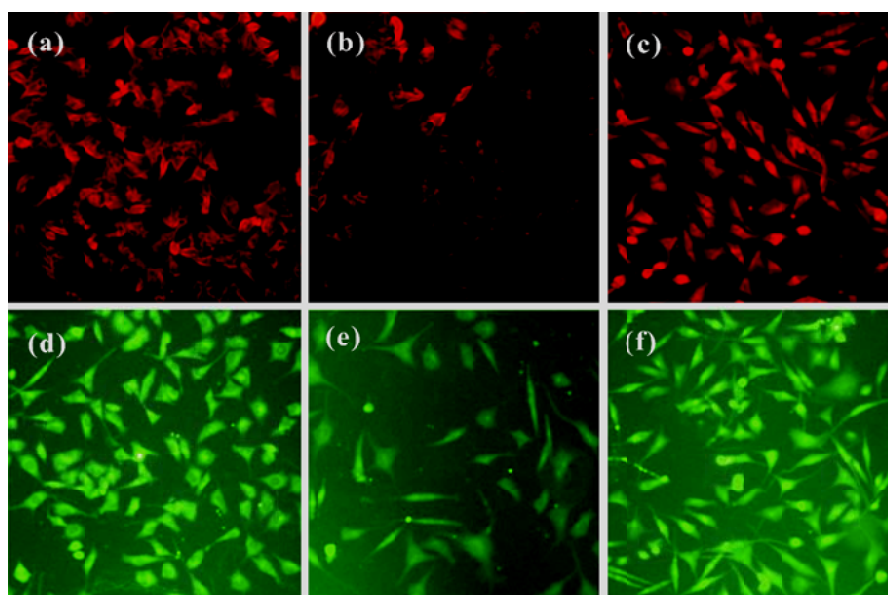
**Figure 20.** Effects of HupA on TrkA receptor and p75[NTR] receptor levels in SHSY5Y cells by immunofluorescence assay. The tests were performed at 24 h after incubation with $H_2O_2$. Preincubation with 10 μM HupA was conducted 2 h before the 200 μM $H_2O_2$ was added. Shows were immunofluorescence assay results. (a–c) TrkA receptor level; (d– f) p75[NTR] receptor level. (a, d) control; b,e $H_2O_2$ treatment; (c, f) HupA plus $H_2O_2$. The figure is representative of three experiments with similar results. Data from ref 140.

**Table 4.** Pharmacokinetic parameters of [³H]HupA 13.9 mEq/kg in each group of 3 rats. $F = Cl_{p.ig} \times AUC_{ig}/Cl_{p.iv} \times AUC_{iv} = 96.9\%$. Data from ref 141.

| Parameters | iv | ig |
|---|---|---|
| $\alpha$ (min$^{-1}$) | 0.107±0.016 | 0.08±0.04 |
| $\beta$ (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) | – | 98569±12153 |

HupA were 10.0 and 13.0 h, respectively. Thus the mean residence times (MRT$_{0–72}$) were 10.3 and 12.2 h, respectively (Chen Y *et al*, unpublished data). The elimination of HupA in the elderly volunteers was slower than that in the young volunteers. HupA was released in a slow and prolonged manner after oral administration.

To identify which cytochrome P450 (CYP) isoenzymes are involved in the metabolism of HupA, an *in vitro* study was performed with rat liver microsomes, and immunoinhibition and chemical inhibition methods. HupA metabolism was analyzed with HPLC and expressed as the HupA disappearance rate. The results showed that 76.2% of HupA metabolism was inhibited by a CYP1A2 antibody and 17.8% by a CYP3A1/2 antibody. The inhibitory effects produced by CYP2C11 and 2E1 antibodies were minor. The CYP1A2 substrate phenacetin produced an inhibitory effect of 70.3%. These data suggest that HupA metabolism in rat liver microsomes is mediated primarily by CYP1A2, with a probable secondary contribution by CYP3A1/2. CYP2C11 and 2E1 are probably not involved in HupA metabolism[146].

To predict possible drug interactions and confirm the safety of HupA as a medication, the effects of HupA on the activity and expression of CYP were examined. Liver microsomes and total mRNA were prepared from rats treated orally with 0, 0.1, 1, or 2 mg/kg HupA for 2 weeks. Phenobarbital, 3-methylcholanthrene (3-MC), ethanol, and dexamethasone were used as positive controls. No change in isoenzyme expression or catalytic activity was found in rats treated with 0.1 mg/kg HupA, but CYP1A2 activity and levels of CYP1A2 protein and mRNA were increased when treated with HupA at doses of 1 and 2 mg/kg, although they were minor when contrasted with 3-MC. HupA produced no effects on CYP2C11, CYP2B1/2, 2E1, or 3A. These results indicate that the activity and expression of liver CYP isoenzymes are not affected in rats treated with pharmacological doses of HupA, but may elicit a slight inductive response in CYP1A2 at a toxicological dose. The CYP1A2 induction produced by HupA is related to transcription enhancement[147].
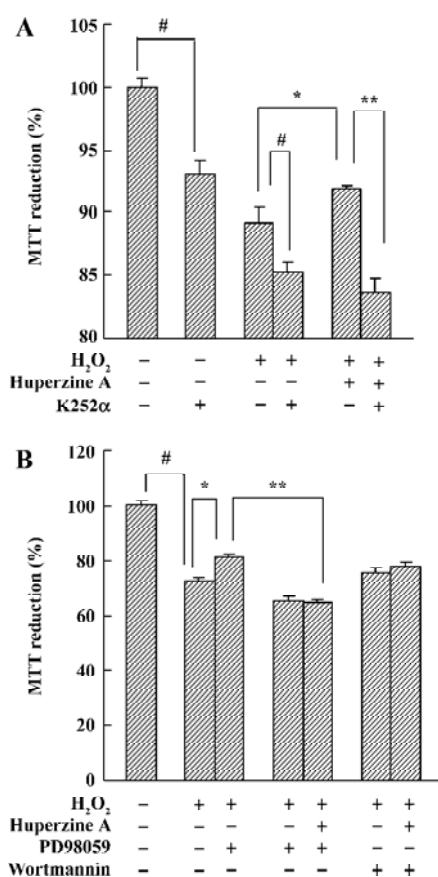
**Figure 21.** Effects of K252a, PD98059 and wortmannin [an inhibitor of phosphatidylinositol 3-kinase (PI3K)] on the protection of HupA against $H_2O_2$-induced cell injury in SHSY5Y cells. Cells were pretreated with 100 nmol/L K252a, 100 μmol/L PD98059, or 100 nmol/L wortmannin for 1 h before the addition of 10 μmol/L HupA. HupA was conducted 2 h before the 200 μmol/L $H_2O_2$ was added. After incubation with $H_2O_2$ for 24 h, cell viability was determined by MTT assay. The data are mean±SEM obtained from three independent experiments. (A) Effects of K252a. *$P<0.05$, $H_2O_2$ group *vs* $H_2O_2$+HupA group, **$P<0.01$, $H_2O_2$+HupA group *vs* $H_2O_2$+HupA+ K252a group. #$P<0.05$, K252a group *vs* control, $H_2O_2$ group *vs* $H_2O_2$+K252a group. (B) Effects of PD98059 and Wortmannin. *$P<0.05$, $H_2O_2$ group *vs* $H_2O_2$+PD98059 group. **$P<0.01$, $H_2O_2$+HupA group *vs* $H_2O_2$+HupA+PD98059 group. #$P<0.05$ $H_2O_2$ group *vs* control. Data from ref 140.

## Toxicology and detoxification

A series of studies have been conducted to evaluate the toxicity of HupA in mice, rats, rabbits, and dogs. Dose-response curves for salivation indicated that HupA was less potent than other ChE inhibitors[49]. The characteristic symptoms of cholinergic hyperactivity were less severe for HupA in rats compared with donepezil and tacrine; fasciculation or other cholinergic signs were not found with oral HupA at a

dose of 0.48 mg[19]. The LD$_{50}$ doses of HupA were 4.6 mg (*po*), 3.0 mg (sc), 1.8 mg (ip) and 0.63 mg (iv) in mice. Atropine exerted a significant antagonistic effect on the toxicity induced by HupA. Studies to evaluate subacute toxicity have been conducted in rats, rabbits, and dogs, in which no histopathological changes were found in liver, kidney, heart, lung, or brain in rats (1.5 mg/kg, *po*) or dogs (0.6 mg/kg, im) after administration of HupA for 180 d. No teratogenic effect was detected in mice (0.019–0.38 mg/kg, ip) or rabbits (0.02– 0.2 mg/kg, im) after the administration of HupA.

To examine the acute effects of HupA on rat liver, changes in liver coefficient, serum biochemistry, and histopathology were detected after a single dose. The cytotoxicity of HupA was also assessed by determining extracellular and intracellular amounts of lactate dehydrogenase in cultured hepatocytes. Similar to tacrine, HupA raised the liver coefficient and increased serum levels of aspartate aminotransferase and alanine aminotransferase. Unlike tacrine, however, acute administration of HupA did not induce histopathological changes in the liver. That atropine redressed the effects of HupA on the liver indicates that the acute effects of HupA on rat liver are not related to hepatotoxicity[148].

A study in mice also demonstrated that HupA did not perturb respiration at a dose inhibiting 40% of AChE, and at a lethal dose, did not affect any other enzyme important for respiration[149].

HupA has been tested as a prophylactic drug against poisoning with soman and other nerve gases with reasonable outcomes[150]. It works by protecting cortical AChE from soman inhibition and preventing subsequent seizures.

Several studies have reported that acute administration of HupA protects rodents against organophosphate (OP) intoxication without the typical cholinergic side-effects[150–152]. Similarly, in primates (Chinese rhesus monkeys), HupA by itself has been shown to protect animals against the toxic signs and lethality induced by the injection of 1.3 LD$_{50}$ of soman[151]. When compared with pyridostigmine (PYR)[153,154], the cumulative dose of soman needed to produce convulsions and epileptic activity was 1.5-fold greater in animals that received HupA compared with the group of primates pretreated with PYR. HupA also selectively inhibited red cell AChE activity, whereas PYR also inhibited plasma BuChE. This result was confirmed in a study with guinea-pigs[155]. PYR cannot protect against seizures and subsequent neuropathology induced by OP agents, because it does not penetrate into the brain. HupA, when combined with atropine methyl nitrate or without any supporting therapy acting on the CNS (atropine sulfate or benzodiazepine), prevented lethality and manifested anticonvulsant and central neuropro-

tective properties[155]. The superior protection offered by HupA appears to be related both to the selectivity of HupA for red cell AChE, which preserves the scavenger capacity of plasma BuChE for OP agents, and to its protective effect on cerebral AChE[154]. HupA is more stable than the carbamates used as pretreatment for OP poisoning. These prophylactic effects make HupA a potential protective agent against OP intoxication.

## Clinical trials

The efficacy and safety of HupA in AD patients have been evaluated by clinical trials in China. In an early double-blinded study conducted in 100 elderly patients with 17 probable cases of AD, HupA (0.03 mg, im) produced a significant improvement in all rating scores as evaluated by Buschke Selective Reminding performance[156]. A more comprehensive clinical study was conducted in 819 patients who met the AD criteria of National Institute for Communicative Disorders and Stroke-Alzheimer's Disease and Related Disorders Association (NINCDS-ADRDA) and Diagnostic and Statistic Manual of Mental Disorders-Third Edition-Revised (DSM-III-R) at 39 mental hospitals in China. After treatment with HupA at a dose of 0.03–0.4 mg/d, patients showed improvement in their memory, cognitive skills, and ability in their daily life. No severe side effects were found[14–16,157–170]. The results from a 12-week, double-blinded, randomized and placebo-controlled nationwide clinical trial with 202 patients with the diagnosis of possible or probable AD confirmed the efficacy of HupA in improving the results of cognitive tasks. In this study, their cognitive functions [measured with Mini-mental State Examination Scale (MMSE) and Alzheimer's Disease Assessment Scale-Cognitive Subscale (ADAS-Cog)], non-cognitive functions (measured with mood and behavior-ADAS-non-Cog) and activity of daily living (ADL) were all improved significantly at week 6, and further improved at week 12 after patients had been treated with HupA at a dose of 0.1–0.2 mg, bid[17]. The results of a clinical trial with HupA carried out over a longer time period were reported by Jiang *et al*, and showed that HupA at a dose of 0. 15 mg twice per day significantly improved the cognition of 33 AD patients at 12, 24, and 48 weeks, with no statistical difference among the 3 time points[164].

Combined therapy with HupA plus other medicine or mental training also showed favorable clinical results. In a placebo-controlled clinical trial with 24 AD patients and 35 VD patients, subjects showed significant favorable differences in MMSE and ADL scores after treated with HupA (0.15 mg, twice per day) plus nicergoline (20 mg, twice per day), HupA plus nicergoline and conjugated estrogen, or HupA alone; combined treatment was better than HupA alone[171]. The superiority of combined therapy over HupA alone was also suggested by the marked improvement in MMSE, clinical dementia rating (CDR) and ADL scores from a clinical trial, in which 30 female AD patients were treated with 2 mg nilestriol once every fortnight, and 0.1 mg HupA twice per day for 24 weeks[172]. In a group of 43 patients with mild to moderate AD, American Association of Mental Deficiency (AAMD) and ADL scores were significantly improved after treatment with HupA (0.1 mg, twice per day) combined with training in daily life activities for 8 weeks[173]. After 22 patients with AD and 38 with VD who were treated for 8 weeks with HupA (0.1 mg, twice per day) complemented with a mental stimulation program consisting of reminiscence, reality orientation and remotivation, Hasegawa dementia scale (HDS), CDR and ADL scores were significantly improved[174]. These results suggest that combined treatment using HupA might be a reasonable means in the clinical therapy.

The therapeutic effects of HupA on VD were evaluated early in 1991 by conducting a randomized, matched and double-blinded study involving 56 patients with multi-infarct dementia (MID)[175]. Using a self-controlled design, marked improvement in memory deficits were observed after 0.15–0.45 mg HupA given orally for 4 weeks[176,177]. Patients with AD (23 cases) or VD (41 cases) were treated with HupA for 8 weeks, and memory deficiency and recognition decline was markedly improved in both AD and VD[178]. Yin *et al* reported that 39 patients who met the DSM-R criteria for mild to moderate vascular dementia, showed a significant increase in AAMD, MMSE and ADL scores after treated with 0.1 mg HupA twice per day for 8 weeks[179]. A similar result was reported in a trial involving 20 patients[180]. Moreover, HupA has been shown to be more effective than pyritinol in the treatment of MID[181]. Wang *et al* reported that the CDR and ADL scores of VD patients who met the DSM-IV criteria were markedly improved in MMSE after treatment for 6 months with HupA[20]. HupA also showed efficacy in improving cognitive deficiency in endemic cretins[182], and urinary incontinence in patients with cerebral stroke[183].

A survey involving 50 middle-aged and elderly patients with different degrees of dysmnesia demonstrated the efficacy of HupA in improving verbal recall, retention and retrieval in patients with mild and moderate dysmnesia. Values of total recall, long-term retrieval, long-term storage, consistent long-term retrieval and unreminded recall were markedly increased when patients were treated with 0.1 mg HupA orally twice daily for 2 weeks[184]. In a study with conducted in

children who had language delay and other developmental conditions, treatment with 0.05 mg HupA twice daily for more than 3 months improved language delay by a total of 67.56% [185].

Zhang *et al* reported that HupA could improve memory function and neurotransmission in patients with mild and moderate traumatic brain injury. Thirty patients were treated with traditional therapeutic (0.8 g piracetan and 20 mg nimodipine, twice per day, combined with function convalescence training), and other 30 patients were treated with 0.1 mg HupA bid besides traditional therapeutic. Both groups showed significant improvement in memory and cognition after both 1 and 3 months, and the improvement in memory and cognition in patients treated with HupA was more dramatic than the improvement in patients treated with traditional therapeutics[186].

It is well documented that people with schizophrenia have neurocognitive impairments across multiple domains, including impairments in motor functioning, various aspects of attentional abilities, executive functions and memory functioning. Ma *et al* have recently studied the effect of HupA on memory disorders in schizophrenic patients. Sixty patients with schizophrenia, who were at the rehabilitation stage, were divided into groups that received HupA or a placebo, and 30 non-schizophrenic people were used as controls. After 12 weeks of treatment with 0.2–0.4 mg HupA, memory functions were significantly improved[187]. Similar results were also reported by Fang *et al*[188] and Yang[189].

HupA has also shown some efficacy in ameliorating sleeping. It was reported that alternating HupA treatment with clonazepam treatment in the day and night, respectively, markedly extends sleeping duration, modifies the rhythm of sleep, and improves the quality of sleeping in patients with chronic insomnia. With this treatment regime, the dosage of clonazepam can be decreased gradually[190].

Several clinical studies have shown that HupA is effective for the treatment of benign senescent forgetfulness (BSF)[156,175,191–193], in addition to AD and VD. Seventy four percent of patients treated with HupA (0.15 mg, bid) for 4 weeks showed improvement in memory quotient (MQ) and Wechsler memory scale (WMS) scores[15,194–197]. The improving effect of HupA was studied in 34 pairs of junior high school students who had complained of memory inadequacy. HupA at a dose of 0.1 mg, twice per day for 4 weeks, increased the scores for "accumulation", "recognition", "reproduction", "association", "tactual memory", and "number of recitation" factors, but not the "understanding" factor[170].

In the early clinical studies using HupA for the treatment

of myasthenia gravis (MG), it was found that 99% of 128 patients with MG had their clinical manifestations controlled or improved with HupA treatment. The duration of action of HupA lasted for 7±6 h, and the side effects were minimal compared with neostigmine[198]. Xia *et al* reported similar improving effects in 63 MG patients treated with im HupA at a dose of 0.2 mg twice per day[199].

## Summary

AD is a multi-causal and multi-factorial progressive neurodegenerative disease with complicated pathogenesis, thus it is likely that multiple drugs or drugs with poly-pharmacological activities will be the best therapeutic approaches to address the varied pathological aspects of the disease. Based on the characteristic cholinergic deficits in AD, AChE inhibitors are still the drugs of choice for the symptomatic therapy of AD. As a potent and reversible AChE inhibitor, HupA has attracted attention because, relative to other well-known AChE inhibitors, it has greater potency, higher selectivity with respect to its AChE inhibitory effect, and marked memory-enhancing efficacy in a broad range of animal models of cognitive impairment, and in patients with AD, VD, and other cognitive problems. Interestingly, new data from our lab show that HupA, besides inhibiting the hydrolysis of synaptic ACh, has neuroprotection, APP metabolism modulation and NGF-like neurotrophic activities, indicating that the non-cholinergic effects of HupA could play important roles for the treatment of neurodegenerative disease through interfering with the key factors of the disease. These encouraging preclinical and clinical findings suggest that HupA is a promising candidate for the treatment of neurodegenerative diseases such as AD and VD, and is very likely to exert its therapeutic effects via a multi-target mechanism, which therefore provides us with a large amount of exciting research to carry out.

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