Neuroprotective Effects of Huperzine A

A Natural Cholinesterase Inhibitor for the Treatment of Alzheimer’s Disease

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Huperzine A  •  Alzheimer’s disease  •  Acetylcholinesterase  •  Cholinesterase inhibitor  •  Cognitive enhancer  •  Neuroprotection  •  Oxidative stress  •  Beta-amyloid  •  Amyloid precursor protein  •  Cerebral ischemia  •  Apoptosis  •  Apoptotic-related gene  •  Mitochondria  •  Glutamate  •  NMDA receptor  •  Potassium current

Abstract
Huperzine A (HupA), isolated from Chinese herb Huperzia serrata, is a potent, highly specific and reversible inhibitor of acetylcholinesterase. It has been found to reverse or attenuate cognitive deficits in a broad range of animal models. Clinical trials in China have demonstrated that HupA significantly relieves memory deficits in aged subjects, patients with benign senescent forgetfulness, Alzheimer’s disease (AD) and vascular dementia (VD), with minimal peripheral cholinergic side effects compared with other AChEIs in use. 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, and interfere with APP metabolism. Antagonizing effects on NMDA receptors and potassium currents may contribute to the neuroprotection as well. It is also possible that the non-catalytic function of AChE is involved in neuroprotective effects of HupA. The therapeutic effects of HupA on AD or VD are probably exerted via a multi-target mechanism.

Alzheimer’s 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.

To date, the cause and the mechanism by which neurons die in AD remain unclear, but Aβ has been established as a crucial factor in AD pathogenesis. Aβ deposition may cause neuronal death via a number of possible mechanisms, including oxidative stress, excitotoxicity, energy depletion, inflammation and apoptosis. Despite this multifactorial etiology, genetics plays a key role in...
The disease progression. However, environmental factors (e.g., cytokines, neurotoxins) may be even more important in the development and progression of AD. Several lines of evidence support the involvement of oxidative stress [2, 40]. Oxidative damage, mediated by reactive oxygen species (ROS) generated following cell lysis, oxidative bursts, or an excess of free transition metals, has been hypothesized to play a pivotal role in AD neurodegeneration. On the other hand, postmortem studies provide direct morphological and biochemical evidence that some neurons in the AD brain degenerate via an apoptotic mechanism. Thus, it is not surprising that a number of different intervention therapies are currently being researched to address distinct aspects of the disease.

Huperzine A (HupA) is a novel Lycopodium alkaloid with recognized medicinal properties. In China, the folk medicine Huperzia serrata (Qian Ceng Ta) (fig. 1) is a source of HupA; it has been used for centuries in the treatment of contusions, strains, swelling, schizophrenia, etc.

Attenuating Oxidative Stress

Protection of HupA against Hydrogen Peroxide and β-Amyloid Protein-Induced Injury by Attenuating Oxidative Stress

Several neurodegenerative disorders such as AD, cerebral ischemia-reperfusion and head injury 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 [7, 40, 45, 48]. Studies show that oxygen radicals initiate amyloid build-
Fig. 1. Chinese herb *Huperzia serrata* (Qian Ceng Ta).

Fig. 5. Anti-apoptotic mechanism of HupA. Black real line arrows = apoptotic-inducing pathway; red real line arrows = affecting sites of HupA; red dash arrows = speculative pathways; (−) = inhibiting; (+) = promoting.
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.

In rat studies, an intracerebroventricular (i.c.v.) infusion of β-amyloid 1–40 (800 pmol × 3) induced a significant cognitive deficit, morphologic signs of injury and a decrease of cortical choline acetyltransferase activity [64]. Daily i.p. administration of HupA for 12 consecutive days produced partial reversal of the β-amyloid-induced deficit in learning a water maze task. This treatment ameliorated the loss of choline acetyltransferase activity in cerebral cortex and the neuronal degeneration induced by β-amyloid 1–40 [64]. HupA also reduced the level of lipid peroxidation and superoxide dismutase in the hippocampus, cerebral cortex and serum of aged rats [49]. In a rat model of chronic cerebral hypo-perfusion, HupA significantly reduced the increases in SOD and lipid peroxide while restoring lactate and glucose to their normal levels [61]. A clinical study also demonstrated a reduction of oxygen free radicals in plasma and erythrocytes from AD patients [77]. These findings indicate that HupA has protective effects against free radical and Aβ-induced cell toxicity, which might be beneficial in the treatment of patients with various kinds of dementia.

Experience with HupA enantiomers has shown that the neuroprotective properties have no relation to anticholinesterase potency. Thus, preincubation with (+)-HupA or (−)-HupA (0.1–10 μM) protected cells with similar potency against Aβ toxicity and similar enhancement of survival [86]. This result contrasted with the stereoselectivity of cholinesterase inhibition in vitro and in vivo, in which (−)-HupA is approximately 50-fold more potent than (+)-HupA. In another study, we examined drug effects on the apoptosis induced by incubation with Aβ25–35 and on the increase of AChE activity accompanyng this reaction. We observed that inhibiting the hydrolyzing activity of AChE without decreasing AChE expression itself did not attenuate the Aβ25–35 induced apoptosis [85]. In other words, the ability of HupA to block the catalytic activity of AChE did not parallel its neuroprotective effect. Therefore, the cytoprotective effect of HupA enantiomers may relate to some kind of non-catalytic actions on AChE, or to actions on other cellular targets.

Anti-Apoptotic Effect of HupA

Apoptosis is the process by which neurons die during normal development and is also a feature of chronic and acute neurodegenerative diseases and stroke [82]. The cellular commitment to apoptosis is regulated by the Bcl-2 family of proteins. High levels of Bcl-2 expression will
**Fig. 3.** Effects of HupA on H₂O₂-induced expression of bcl-2, bax and p53 in PC12 cells by RT-PCR. Cells were exposed to 100 μM H₂O₂, and total RNA was extracted after the indicated recovery period and then subjected to RT-PCR. The PCR products were normalized by β-actin mRNA. Lane 1: non-treated intact control; lanes 2–5: 0, 2, 6 and 12 h after 30 min H₂O₂-treatment, respectively. Lanes 6–9 represent the same time points as lanes 2–5 but preincubated with 1 μM HupA before H₂O₂ exposure.

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inhibit apoptosis. In contrast, an increased expression of P53 and Bax is associated with the initiation of apoptosis [29]. In accordance with previous reports, studies from our lab demonstrate typical apoptotic changes when neuron-like cells are exposed to stressors such as H₂O₂, Aβ peptide, oxygen-glucose deprivation (OGD), serum deprivation, or the PKC inhibitor, staurosporine. These changes include DNA laddering, cell shrinkage, the generation of nuclear apoptotic bodies, TUNEL positive staining, and other classic hallmarks of apoptosis (fig. 2) [63, 71, 87, 94]. Such abnormalities are markedly relieved by HupA. For example, in rats that received i.c.v. injections of β-amyloid1–40 (800 pmol × 3), administration of HupA (0.1, 0.2 mg/kg, i.p.) for 12 consecutive days gave substantial neuroprotection in the brain. This treatment greatly reduced the number of apoptotic-like neurons and partly reversed the down-regulation of bcl-2 and up-regulation of Bax and P53. Anti-apoptotic effects of HupA were also found in primary cultured neurons. Preincubation with HupA at concentrations higher than 0.01 μM led to a large, dose-dependent attenuation of cell toxicity induced by Aβ25–35 (20 μM). Moreover, HupA (1 μM) caused large reductions in the amounts of subdiploid DNA detected in a flow cytometry assay and weakened the ladder pattern on agarose gel electrophoresis, typically seen after exposure to Aβ (fig. 2).

The anti-apoptotic actions of HupA may involve inhibition of the production or the effects of ROS [71]. We found that preincubation of PC12 cells with HupA before exposure to H₂O₂ substantially reduced apoptosis, by any of several measures. The same treatment also attenuated H₂O₂-induced overexpression of bax and p53, while restoring bcl-2 to normal levels (fig. 3) [63]. HupA was also effective in the OGD paradigm. Exposure to OGD for 3 h followed by reoxygenation for 24 h triggers apoptosis characterized by chromatin condensation, nucleus fragmentation and DNA laddering, accompanied by altered levels of mRNA for c-jun, p53, bcl-2 and bax. In this model, HupA significantly attenuated apoptosis and reduced the up-regulation of c-jun and bax as well as the down-regulation of bcl-2 [94].

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 permeability of the inner mitochondrial membrane [25, 28, 53]. In this process, cytochrome c moves from the intermembrane space into the cytoplasm [5] where it binds to another factor (Apaf-1). 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 DNAases, which leads to cell death [83].

When PC12 cells were pre-incubated with HupA at concentrations above 0.01 μM, there was a marked neuroprotection against apoptosis induced by β-amyloid, with a significant reduction in mitochondrial swelling and an improvement in mitochondrial membrane potentials [unpubl. data of this lab]. Pretreatment of rat cortical neurons with HupA (0.01–10 μM) significantly elevated cell survival and reduced all signs of apoptosis resulting from exposure to Aβ25–35.

Further studies 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 μM) attenuated an Aβ25–35-induced increase in caspase-3 activity at 6, 12, 24, and 48 h [71]. Western blot analyses confirmed these results at the protein level. HupA also inhibited caspase-3 activation in models of apoptosis by serum deprivation.
and staurosporine treatment. The apoptosis induced by 24 h of serum deprivation was accompanied by enhanced caspase-3 activity and a release of mitochondrial cytochrome c into the cytosol [97]. HupA (0.1–10 μM) improved neuronal survival in this model, inhibiting the rise in caspase-3 activity and protein expression [97]. Likewise, cell survival was greatly enhanced when HupA (0.1–100 μM) was introduced 2 h before a 24-hour exposure to 0.5 μM staurosporine. Incubation with HupA at dose of 1 μM also reduced staurosporine-induced DNA fragmentation, up-regulation of the pro-apoptotic gene, bax, down-regulation of the anti-apoptotic gene, bcl-2, and decrease in caspase-3 proenzyme protein level (fig. 4) [87].

A potassium channel with delayed rectifier characteristics may play an important role in Aβ-mediated toxicity. The up-regulation of an outward K+ current known as \( I_k \) mediates several forms of neuronal apoptosis and could contribute to the pathogenesis of Aβ-induced neuronal death. Exposure to a 20-μM concentration of Aβ25–35 or Aβ1–42 is known to enhance the apoptosis-related current, \( I_k \) [81]. Interestingly, HupA will reversibly inhibit the fast transient current, \( I_A \), and the sustained potassium current, \( I_k \), in CA1 pyramidal neurons acutely dissociated from rat hippocampus [33]. Such effects might contribute to this agent’s anti-apoptotic effect.

In light of these findings and the effects of HupA on apoptosis-related genes, we propose that HupA blocks apoptosis by antagonizing the mitochondrial-dependent caspase pathway, directly or indirectly (fig. 5). The effects of HupA on the intrinsic caspase-3 pathway might be downstream consequences of altered expression of bcl-2 family genes. Functionally, bcl-2 is a potent cell death suppressor, whose over-expression can prevent cell death in response to a variety of stimuli. It is well known that Bcl-2 suppresses apoptosis by inhibiting cytochrome c release from the mitochondria. On the other hand, bax is a death-promoting factor, whose translocation to the mitochondrial membrane leads to a loss of mitochondrial membrane potential and increases mitochondrial permeability. Increased mitochondrial permeability results in the release of cytochrome c followed by activation of caspase-3 [21]. We consider it likely that HupA owes some of its anti-apoptotic effects to an effective antagonism of the up-regulation of bax and the down-regulation of bcl-2, which impairs mitochondria-dependent caspase pathway. At present, however, direct effects of HupA on cytochrome c and caspase-3 and other possible targets are not excluded.
**Effects of HupA 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 termed 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 [15], and the endosomal-lysosomal pathway, which produces amyloidogenic products [24]. The amyloid hypothesis of AD [17, 46] is focused on the potential toxic role of an excessive production of Aβ and 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 APP in cell cultures. These effects are probably mediated mainly by protein kinase C (PKC) [43]. It has also been reported that several anticholinesterases affect APP processing in addition to the catalytic function of AChE [18, 42].

Our own studies showed that HupA could alter APP processing in the brains of rats given i.c.v. infusions of Aβ1–40, and in otherwise untreated human embryonic kidney 293 (HEK293sw) cells [88]. In the Aβ treated rats, levels of APPs and PKCα were significantly decreased by treatment with Aβ1–40. These decreases were much reduced by 12 consecutive days of HupA treatment (0.2 mg/kg, i.p.), but HupA in normal rats caused no change in either APPs or PKCα. In normal HEK293sw cells, on the other hand, the levels of APPs and PKCα rose progressively during an 18-hour exposure to HupA (1 µM). However, no significant alternations in the levels of PKCβ and PKCε were found after HupA treatment. Taken together these findings suggest that HupA may affect the processing of APP by up-regulating PKC, especially PKCα.

In an attempt to clarify the receptor mechanisms involved in such effects, we treated HEK293wt cells with cholinergic receptor antagonists [unpubl. data]. The non-selective muscarinic antagonist, scopolamine, partly blocked the HupA-induced rise in levels of APPs and PKCα. By contrast the nicotinic antagonist, mecamylamine, had little effect. These results suggest that muscarinic ACh receptors may mediate, at least in part, the effects of HupA on the regulation of APPs and PKCα in HEK293sw cells.

Our recent results provide the first demonstration that HupA can reduce the disturbance of PKC and APPs both in rats and in an isolated cell line. The effect of HupA to enhance non-amyloidogenic processing of APP and elevate APPs levels likely depends on the activation of muscarinic receptors and the PLC/PKC cascade. A number of biological activities such as cell proliferation, promotion of cell-substratum adhesion, neurite outgrowth and the prevention of intracellular calcium accumulation and cell death have been attributed to APPs [47]. Since PKC is a key enzyme in signal transduction, and since APPs itself has neuroprotective effects, modulating the levels of these two proteins by HupA may well be beneficial in AD therapy (fig. 6).

**Protection of HupA against Hypoxic-Ischemic and Glutamate Induced Brain Injury and Cytotoxicity**

Apart from AD, the most common dementia in the elderly is VD. This disorder, like AD, presents a clinical syndrome of intellectual decline produced by ischemia, hypoxia, or hemorrhagic brain lesion. Cerebral ischemia in rats with permanent bilateral ligation of the common carotid arteries (CCA) provides a useful model of VD, in which to investigate the effects of HupA. These animals experience a significant reduction of cerebral blood flow and exhibit learning and memory impairments and neuronal damage resembling those in VD. Daily oral administration of HupA (0.1 mg/kg) to such rats for 14 days produced significant improvement in the learning of a water maze task. Simultaneously there was marked recovery from the decrease in choline acetyltransferase activity in hippocampus and a restoration of SOD, lipid peroxide, lactate and glucose to normal levels [61]. Similar protection was also observed in gerbils given subchronic oral doses of HupA (0.1 mg/kg, twice daily for 14 days) following 5 min of global ischemia [96].

An in vitro model of neuronal ischemia is the rat pheochromocytoma PC12 cell treated with OGD for 30 min. In our hands, this treatment causes death in more than 50% of the cells in culture, along with major changes in morphology and biochemistry, including elevated levels of lipid peroxide, SOD activity and lactate. Cells pretreated for 2 h with HupA (0.1, 1 and 10 µM), however, showed 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 [95].

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These findings suggested that HupA might be beneficial for VD therapy through its effects on the cholinergic system, the oxygen free radical system and energy metabolism.

A protective effect of HupA on hypoxic–ischemic (HI) brain injury has also been found in neonatal rats [62]. A unilateral HI brain injury was produced in 7-day-old rat pups by the ligation of the left CCA followed by 1 h hypoxia with 7.7% oxygen. After 5 weeks, the HI brain injury in these pups caused working memory impairments in water maze performance, as shown by an increased escape latency and a reduced time spent in the target quadrant. The combination of CCA ligation and exposure to a hypoxic environment also led to morphologic damage in the ipsilateral striatum, cortex, and also hippocampus, where it produced 12% neuronal loss in the CA1 region. Treatment with HupA at a dose of 0.1 mg/kg conferred significant protection against the behavioral and morphologic consequences of HI injury (fig. 7). The same treatment spared a significant fraction of the CA1 neurons relative...
Fig. 7. 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 the left hemisphere in huperzine A-treated HI rats (b, d) (n = 12).

to saline-treated HI group. These results raise the possibility that HupA have potential utility in treating HI encephalopathy in neonates.

Glutamate is the main excitatory neurotransmitter in the CNS, with important roles in neurotransmission and functional plasticity. Excitatory amino acid neurotransmitters are also involved in CNS pathology. The deleterious effects of overstimulation with excitatory amino acids have been implicated in a variety of acute and chronic neurodegenerative disorders such as ischemic brain damage, AD and neuronal cell death [11] [for reviews, see 13, 14, 26, 27, 35]. Glutamate-mediated overactivation of receptors induces excessive Ca$^{2+}$ influx, which results in elevated intracellular Ca$^{2+}$ concentrations [10, 12] with serious consequences such as necrosis and apoptosis [31]. Blockade of glutamate receptors prevents most of the Ca$^{2+}$ influx and neuronal cell death induced by glutamate exposure [50, 57].

It has been reported that HupA protects against glutamate-induced toxicity. HupA (100 $\mu$M) decreased neuronal cell death caused by a toxic level of glutamate (also 100 $\mu$M). 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 [58]. HupA dose-dependently inhibited the NMDA-induced toxicity in primary neuronal cells, most likely by blocking NMDA ion channels and the subsequent Ca$^{2+}$ mobilization at or near the PCP and MK-801 ligand sites [20]. Wang et al. [66] reported that HupA reversibly inhibited NMDA-induced current in acutely dissociated rat hippocampal pyramidal neurons and blocked specific $[^3]$HMK-801 binding in synaptic membranes from rat cerebral cortex. Of all the AChE inhibitors tested, HupA is the most powerful both in protecting mature neurons and in blocking the binding of $[^3]$HMK-801. Studies on the mechanism of receptor inhibition showed that HupA reversibly inhibited NMDA-induced currents. The effect was non-competitive, and showed neither ‘voltage-dependency’, nor ‘use-dependency’ [89]. Studies of $[^3]$HMK-801 binding in cortex membranes suggest that HupA acts as a non-competitive antagonist of the NMDA receptors, via a
competitive interaction with one of the polyamine binding sites [92]. Of interest, natural (−)-HupA and synthetic (+)-HupA reduced the binding of [3H]MK-801 with similar potencies [91] indicating that HupA inhibits NMDA receptors in rat cerebral cortex without stereoselectivity. This result is in dramatic contrast with the stereoselective inhibition of acetylcholinesterase.

NMDA-receptor activation also mediates the generation of long-term potentiation (LTP) – a cellular process that underlies learning and memory [3, 52]. There is evidence that the suppressive action of Aβ on LTP in both CA1 and dentate gyrus operates via a NMDA receptor-independent pathway that involves cholinergic terminals in the hippocampus. Of some interest, HupA (1.0 μM) was found to enhance LTP, while a much lower dose (0.1 μM) largely blocked the suppressive effects of Aβ on LTP induction [8, 80].

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 [20].

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