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Bis(12)-Hupyridone, a Promising Multi-Functional Anti-Alzheimer's Dimer Derived from Chinese Medicine

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1. Introduction

Alzheimer's disease (AD), clinically characterized by progressive impairments of memory, cognitive functions and behaviors, is a major form of dementia that mainly affects elderly individuals. Alzheimer's Disease International undertook a Delphi study that showed worldwide in 2001 there were 24.3 million people with dementia, most of whom with AD; and the figure will rise to 42.3 million and 81.1 million in 2020 and 2040, respectively (Ferri et al., 2005). It is estimated that dementia causes people over the age of 60 to spend 11.2% of their last years living with disability (Morris and Mucke, 2006).The rapid increase in the number of dementia patients, most of whom AD patients, imminently calls for effective therapeutic prevention and treatment, in particularly, for AD patients (van Marum, 2008).

The plaque of β-amyloid (Aβ) and the neurofibrillary tangle composed mainly by hyper-phosphorylated tau protein are two major pathological hallmarks of AD (Fu et al., 2009). Therefore, in developing anti-AD drugs, preventing the generations of abnormal Aβ and hyper-phosphorylated tau proteins is the major target. For example, bapineuzumab, the antibody of abnormal Aβ, semagacestat and tarenflubil, the modulators of γ-secretase, and tramiprosate, the blocker of Aβ aggregation, have been proposed to treat AD by targeting Aβ cascade (Aisen et al., 2007; Ballard et al., 2011; Green et al., 2009; Thakker et al., 2009). However, all these drugs have failed in randomized controlled trials (Ballard et al., 2011). Although several reasons might be provided to explain why these trials in AD failed, some

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scientists suggested the Aβ and tau hypotheses might be invalid (Smith, 2010). They proposed that the complexity of AD would require not one single drug, but multiple drugs or a multifunctional drug to modify the disease progress (Mangialasche et al., 2010; Smith, 2010).

The neuropathology of AD is characterized by a decreased cholinergic transmission caused by the loss of cholinergic neurons. Acetylcholinesterase (AChE) inhibitors, which enhance the function of cholinergic neurons by prolonging the duration in which acetylcholine stays in the synaptic clefts, have shown promising potential in the treatment of AD (Li et al., 2007b). The inhibitors of AChE could stabilize cognitive and behavior functions of AD patents at a steady level for at least 1 year in 50% and up to 2 years in about 24% of treated patients (Wang et al., 2006). Moreover, those AD patients who do not response to one AChE inhibitor could take another (Wang et al., 2006). So far, four AChE inhibitors, namely tacrine (Cognex), donepezil (Atricept), rivastigmine (Exelon) and galantamine (Reminyl), have been approved by the U.S. Food and Drug Administration (FDA) for the treatment of AD (Ellis, 2005; Francis et al., 2005).

Huperzine A, a *Lycopodium* alkaloid discovered from the traditional Chinese medicine *Huperzia serrata* (Qian Ceng Ta) (FIG. 1A), is also widely used in the treatment of AD in China (FIG. 1B). It is a selective AChE inhibitor with much higher potency and longer duration of AChE inhibition than those of tacrine, donepezil, rivastigmine and galantamine (Wang and Tang, 1998; Zhao and Tang, 2002). Double-blind, randomized clinical trials in China have demonstrated that huperzine A induces significant improvement in memory in elderly people and AD patients without any significant side effects (Wang et al., 2009). However, the lack of natural supply of *Huperzia serrata* and difficulty in its chemical synthesis have limited the clinical usage of huperzine A (Zhang and Tang, 2006).

Memantine (Namenda), which is an uncompetitive antagonist of N-methyl-D-aspartate (NMDA) receptors with a fast on-/off-rate, could reduce excessive glutamate-induced excitotoxicity. The success of memantine in clinical trials led to its being approved by FDA to treat moderate to severe AD in 2003 (Lipton, 2006). This encouraging news pointed to a new direction for the development of anti-AD drugs: by boosting the activities of healthy neurons and reducing abnormal brain functions (Gravitz, 2011). However, further studies have indicated that either AChE inhibitors or NMDA receptor antagonists have limited success in reversing AD progress as they are unable to stop neurodegeneration (Roberson and Mucke, 2006).

The effectiveness of multiple drug strategy has been proven. One of the examples is the HIV drug cocktail (Zhang, 2005). Combinations of drugs with different targets, are also widely used in cancer therapy (Hanahan and Weinberg, 2011). The involvement of multi-factorial etiopathogenesis in AD suggests that the treatment of AD may also require multiple drug therapy to target its different pathological aspects (Youdim and Buccafusco, 2005). However, there are some challenges in the use of drug cocktail strategy that works at different therapeutic targets. Different drugs have differences in bioavailability, pharmacokinetics and metabolisms. They may also cross-react with one another which in turn cause serious side-effects. Therefore, the one-compound-multiple-targets strategy, a novel drug development approach pioneered by Prof. Moussa Youdim, has emerged as a practical alterative to overcome these challenges (Youdim and Buccafusco, 2005). Designing a single molecule synergistically targeting two or more therapeutic pathways is reasonably

more proficient than the combination of one-compound-one-target drugs because of simple bioavailability and pharmacokinetics. Therefore, many neuroscience research institutes and pharmaceutical companies devote the majority of their resources to search for the effective one-compound-multi-functional agents for the treatment of AD.

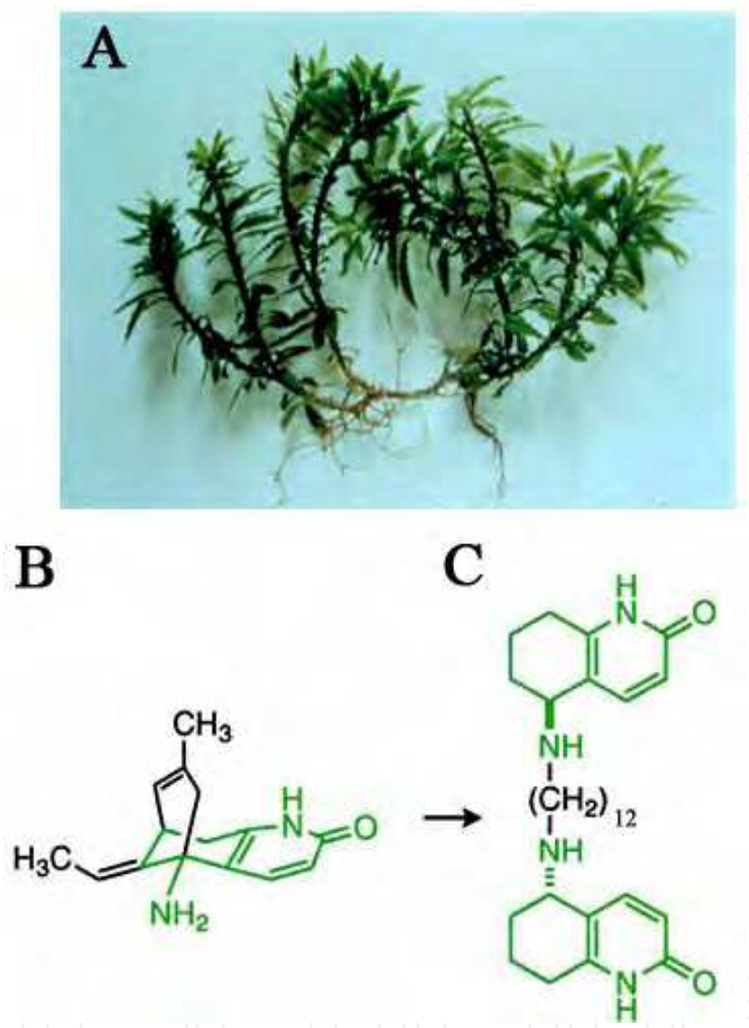


Fig. 1. The chemical structures of huperzine A and bis(12)-hupyridone.
(A) Chinese medicinal herb *Huperzia serrata* (Qian Ceng Ta); (B) The structure of huperzine A; (C) The structure of bis(12)-hupyridone.

Our group has devoted enormous efforts in developing drugs that are better efficacy than current AChE inhibitors the currently available AChE inhibitors over the past years. With the help of scientists from Israel, the USA and China, we have developed a series of novel bis(n)-hupyridones by the homo-dimerization of hupyridone, the ineffective fragments of huperzine A (Carlier et al., 2000; Wong et al., 2003). These dimeric compounds are easy to synthesis and have been shown to be more potent than huperzine A in the inhibition of AChE. In this article, we will review that bis(12)-hupyridone (FIG. 1C), one of our novel dimeric promising anti-AD candidates, possesses multiple functions that include the

enhancement of cognitive functions, the protection against neurotoxins, and the promoting of neuronal differentiation for the treatment of AD.

2. Design and synthesis of novel anti-AChE dimers derived from huperzine A

By studying the three-dimensional (3D) structure of AChE of *Torpedo californica* electric organ (*Tc*AChE), one active site of AChE, named the "catalytic anionic site", was found at the bottom of a deep narrow gorge (active-site gorge, 20 Å) (Axelsen et al., 1994), The quaternary amino group of acetylcholine interacts with the indole side chain of the conserved residue Trp84 in a cation-ð interaction at this "catalytic anionic site" (Ma and Dougherty, 1997). Moreover, another active site of AChE, named "peripheral anionic site", was also found near the top of the "active-site gorge", about 14 Å from the "catalytic anionic site" (Harel et al., 1993). The major element of the "peripheral anionic site" is the residue of Trp279. The bivalent ligand strategy is widely used in the synthesis of novel drugs in which identical or different pharmacophores are connected by a suitable linker (Haviv et al., 2005). The advantage of this strategy is the chelate effect, which creates a bifunctional ligand with enhanced affinity for its target. The molecular structure of AChE with one active site in the gorge ("catalytic anionic site") and another at the extremity ("peripheral anionic site") makes AChE a particularly attractive target to apply this strategy.

The crystal structure study of the complex of AChE with (-)-huperzine A has shown important hydrophobic interactions between (-)-huperzine A and Trp84 at the "catalytic anionic site" of AChE (Raves et al., 1997). We initially synthesized hupyridone (5-amino-2(1H)-quinolinones), a fragment which lacks the C6-C8 bridge of (-)-huperzine A. Although this fragment does not show any significant inhibition of AChE, it appears to possess much of the intrinsic functionality of (-)-huperzine A (FIG. 1). It retains the pyridine oxygen atom and NH group of (-)-huperzine A, which form hydrogen bonds to Tyr-130 and Gly-117, respectively (Raves et al., 1997). Most importantly, hupyridone also retains the 5-amino group of (-)-huperzine A, which is essential for the inhibitory activity of (-)-huperzine A by interacting with Try84. We speculated that it is possible to find high-affinity inhibitors of AChE with structure like that of hupyridone. Particularly, the loss of hydrophobic contact in the "catalytic anionic site" could be compensated by additional chelating interactions at the "peripheral anionic site" (Carlier et al., 2000; Wong et al., 2003).

A series of hupyridone dimer or bis(n)-hupyridones, with different alkylene chain lengths, has been synthesized from 7,8-dihydroquinoline-2,5(1H,6H)-dione by the condensation, the reduction and the dimerization (FIG. 2). Computational calculations showed that 12 methylene units were the most approximate chain length. The 3D study of *Tc*AChE-ligand complexes has shown that bis(12)-hupyridone binds more tightly to *Tc*AChE than (-)-huperzine A (Wong et al., 2003). Overlaying the structures of *Tc*AChE with those of bis(12)-hupyridone and (-)-huperzine A also reveals that the dimer makes cation-ð and hydrogen bonding interactions at the "peripheral anionic site" (Trp279), interactions that can contribute to bis(12)-hupyridone's higher affinity compared with (-)-huperzine A (FIG.3) (Wong et al., 2003). It is suggested that the tether of the hupyridone unit of bis(12)-hupyridone provides minimal entropy and substantially compensates for the weaker and/or missing interactions of (-)-huperzine A with AChE (Raves et al., 1997; Wong et al., 2003).

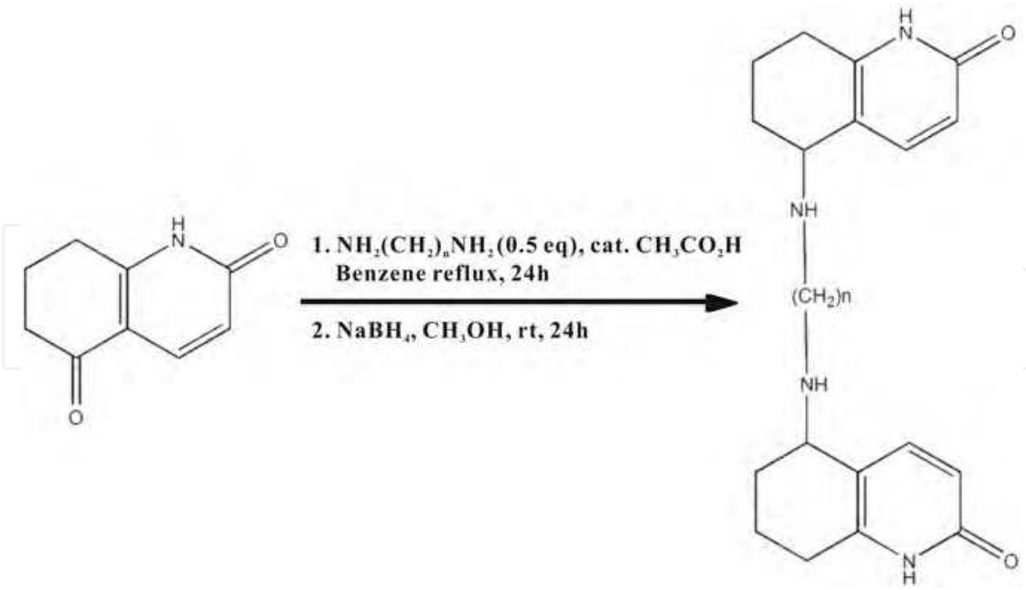


Fig. 2. Synthesis of bis(n)-hupyridones.

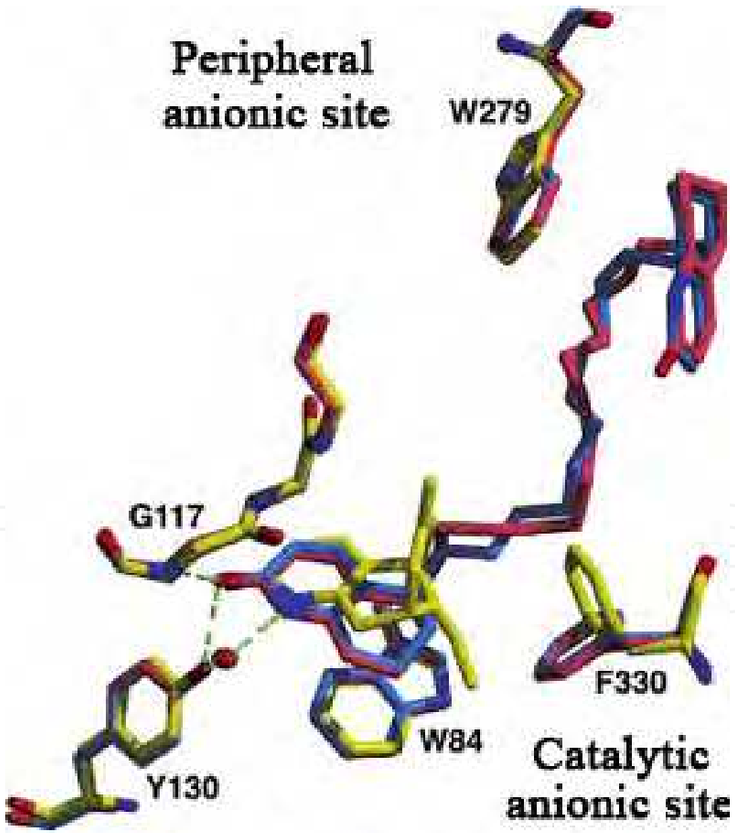


Fig. 3. Overlay of the refined structures of *Tc*AChE/(-)-bis(10)-hupyridone (sky blue), *Tc*AChE/(-)-bis(12)-hupyridone (pink), and *Tc*AChE/(-)huperzine A (yellow). Inhibitors and protein residues are rendered as sticks, and water molecules are shown as red spheres. The figure is modified from the reference (Wong et al., 2003).

3. Multifunctional potencies of bis(12)-hupyridone

3.1 Inhibition of AChE

The anti-cholinesterase activities of bis(n)-hupyridones were further tested *in vitro* and *in vivo*. It has been shown that bis(n)-hupyridones inhibit AChE in a tether-length-dependent manner. The 50% inhibitory concentration (IC_{50}) on AChE by bis(12)-hupyridone was about 52 nM, which was comparable to those of AChE inhibitors used for treating AD (Table 1) (Li et al., 2007b). Furthermore, kinetic analysis of bis(12)-hupyridone suggested that the inhibition pattern was mixed competitive with an apparent K_i value of 28.9 nM. *In vivo* study has also shown that single *p.o.* administration of bis(12)-hupyridone significantly inhibit AChE activity in various brain regions (cortex, hippocampus, striatum) in rats (Table 2) (Li et al., 2007b).

AChE inhibitors	IC_{50} (μM)		Ratio of IC_{50} (BuChE/AChE)	Inhibitory Pattern	K_i (μM)
	AChE	BuChE			
Bis(10)-hupyridone	0.151	1.82	12.1	N.D.	N.D.
Bis(11)-hupyridone	0.084	1.16	13.8	N.D.	N.D.
Bis(12)-hupyridone	**0.052**	**9.6**	**185.0**	**Mixed**	**28.9**
Bis(13)-hupyridone	0.052	16.7	321.0	N.D.	N.D.
Bis(14)-hupyridone	0.24	59.5	148.0	N.D.	N.D.
Huperzine A	**0.082**	**74.43**	**907.7**	**mixed**	**24.9**
Galantamine	**1.995**	**12.59**	**6.3**	**Competitive**	**210.0**
Donepezil	**0.010**	**5.01**	**501.0**	**Noncompetitive**	**12.5**
Tacrine	**0.093**	**0.074**	**0.8**	**Noncompetitive**	**105**

Table 1. Anti-AChE activities of bis(n)-hupyridones and other AChE inhibitors used in the treatment of AD.

The concentrators of inhibitors yield 50% inhibition of enzyme activity. The cortex homogenate was pre-incubated for 5 min with iso-OMPA 0.1 mM. The rate of color production was measured spetrophotometrically at 440 nM. N.D.: not determined, Data are from references (Cheng et al., 1996; Li et al., 2007b; Wang and Tang, 1998).

AChE inhibitor	Dose (μmol/kg)	AChE inhibition (%)			BuChE inhibition (%)
		cortex	hippocampus	striatum	serum
Bis(12)-hupyridone	**90**	**16 ± 5** **	**40 ± 3** **	**28 ± 6** **	**NA**
	45	**12 ± 3** **	**14 ± 4** **	**12 ± 5** *	**NA**
	22	**11 ± 3** **	**4 ± 3**	**5 ± 4**	**NA**

Table 2. Anti-cholinesterase activities of single *p.o.* administration of bis(12)-hupyridone in rats.

Values expressed as percentage of inhibition (*versus* saline control) were the means ± SD. *p < 0.05 and **p < 0.01 *versus* saline group (ANOVA and Dunnett's test). Basal saline control values of cortex, hippocampus and striatum are 1360 ± 70, 1540 ± 150 and 9390 ± 880 A values/g protein, respectively. Basal saline control value of serum is 23 ± 5 A values/g protein. Data are from the reference (Li et al., 2007b).

3.2 Blockade of NMDA receptors

There are increasing evidences that show that the overstimulation of glutamate receptors of the NMDA subtype may be involved in the neuronal loss of AD (Lipton, 2006; Parsons et al., 2007). With the disruption of the neuron-neuron and neuron-glial connections, glutamate might be not only improperly cleared, but also inappropriately released. Meanwhile, energetically compromised neurons become depolarized because they cannot maintain their ionic homeostasis in the absence of energy. The depolarization relieves the normal Mg^{2+} blockade of NMDA receptor-coupled channel, and then excessive stimulation of glutamate receptors occurs (Li et al., 2005; Lipton, 2004). Thus the NMDA receptor has been considered an attractive therapeutic target for the development of anti-stroke drugs. On the other hand, the NMDA receptor, as the major excitatory neurotransmitter receptor in the central nervous system, mediates many important physiological processes, such as synaptic plasticity, and learning and memory (Petrovic et al., 2005; Villmann and Becker, 2007). The therapeutic potential of many powerful NMDA receptor antagonists, such as MK-801, is limited and they fail in clinical trials because of the psychotropic side effects resulting from their interference with normal brain functions (Parsons et al., 2007). NMDA receptor blockers with moderate to low affinity, such as memantine, may inhibit NMDA receptor-mediated pathological but not NMDA receptor-mediated physiological functions. This kind of NMDA receptor antagonists has been at the center of interest in the search for the next generation of neuroprotective drugs for AD (Lipton, 2004; Lipton, 2007).

Using the receptor-ligand binding assay, bis(12)-hupyridone has been found to compete with [^{3}H]MK-801 with a K_i value of 7.7 μM. In the same testing system, memantine and MK-801 competed with [^{3}H]MK-801with a K_i value of 0.8 and 0.04 μM, respectively (Table 3) (Li et al., 2007a) (our unpublished data). These results suggested that bis(12)-hupyridone is a moderate NMDA receptor antagonist and thus might be useful in AD therapy.

NMDA receptor antagonists	[^{3}H]MK-801 binding K_i (μM)
Bis(12)-hupyridone	**7.7**
Memantine	**0.8**
MK-801	**0.04**

Table 3. Bis(12)-hupyridones moderator inhibits NMDA receptors at MK-801 site.

The membrane proteins from rat cerebellar cortex were incubated with 4 nM [^{3}H]MK-801.and treated with the serial concentrations of bis(12)-hupyridone/memantine/MK-801. The K_i values were calculated from the corresponding IC_{50} values, which were measured from the obtained data using at least eight concentrations of each chemical (in duplicates) based on the Cheng-Prusoff equation: $K_i = IC_{50}/(1+[\text{ligand}]/K_d)$. Data are either from our unpublished paper or from the reference (Li et al., 2007a).

3.3 Protection against excitotoxicity

It is well known that the overstimulation of NMDA receptors is essential to the neuronal apoptotic cell death induced by glutamate; and that the blockade of NMDA receptors may prevent neuronal cell death induced by excitotoxicity (Danysz and Parsons, 2003). We thus investigated the neuroprotective effects of bis(12)-hupyridone against excitotoxicity in the primary cerebellar granule neurons (CGNs). We have demonstrated that bis(12)-hupyridone inhibits glutamate-induced apoptosis in a concentration-dependent manner, and its preventive effect is significant even at the low dosage of 1 nM. Further study using fluorescein diacetate/propidium iodide double staining, Hoechst 33324 staining and DNA fragmentation gel assays have shown that bis(12)-hupyridone significantly reverses the glutamate-evoked nuclear condensation, apoptotic bodies and DNA fragmentation, indicating that this dimer is a powerful neuroprotectant against excitotoxicity *in vitro* (FIG. 4 and our unpublished data).

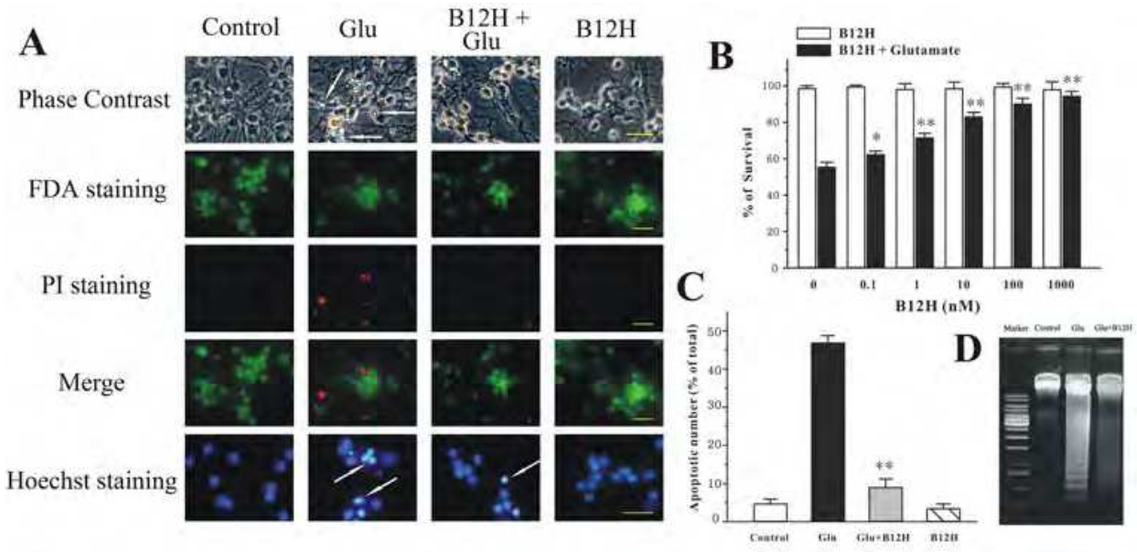


Fig. 4. Bis(12)-hupyridone prevents neuronal death induced by glutamate in primary CGNs. (A) CGNs were pre-incubated with or without 1 μM bis(12)-hupyridone and exposed to 75 μM glutamate 2 h later. At 24 h after glutamate challenge, CGNs were assayed with a phase contrast microscope, fluorescein diacetate/propidium iodide double staining and Hoechst 33324 staining. Apoptotic nuclei were indicated by white arrows. (B) CGNs were pre-incubated with bis(12)-hupyridone at different concentrations as indicated and exposed to 75 μM glutamate 2 h later. Cell viability was measured by MTT assay at 24 h after glutamate challenge. (C) The counts of apoptotic bodies by Hoechst staining. (D) Under the same treatment conditions as (B), DNA fragmentation was extracted from CGNs after 24 h of challenge, and then agarose gel electrophoresis and ethidium bromide staining were used to visualize the DNA extracted from the above samples. B12H: bis(12)-hupyridone; Glu: glutamate. All data, expressed as percentage of control, were the means ± SEM of three separate experiments; $*p < 0.05$, $**p < 0.01$ *versus* glutamate group (ANOVA and Dunnett's test).

3.4 Prevention of ROS-induced neuronal toxicity via regulating the VEGFR-2/Akt pathway

Oxidative stress plays an important role in the pathogenesis of AD as it is the main factor in the neuronal loss of this disease (Shibata and Kobayashi, 2008; Zhu et al., 2007). Although the detailed mechanisms underlying oxidative stress-induced neuronal death remain unknown, drugs with antioxidant properties have therapeutic significance in preventing AD (Pratico, 2008). H_2O_2 is widely used as a toxicant to establish *in vitro* models of oxidative stress-induced neuronal apoptosis as it is an uncharged and freely diffusible molecule (Lee et al., 2007).

Using primary CGNs as a cell model, we have demonstrated that bis(12)-hupyridone at a low concentration (3 nM) prevents H_2O_2-induced apoptosis(Cui et al., 2011c). We have also shown that this protection of bis(12)-hupyridone is a novel activity that is apart from its AChE inhibitory property. The decreased activation of glycogen synthase kinase (GSK) 3β was observed after H_2O_2 exposure, and bis(12)-hupyridone could reverse the altered activation of GSK3β, indicating that bis(12)-hupyridone may exert its neuroprotective effects via signaling molecule(s) upstream of GSK3β. Our further study using the antibody of phosphorylated vascular endothelial growth factor receptor-2 (VEGFR-2) and the inhibitor of VEGFR-2 has demonstrated that bis(12)-hupyridone prevents H_2O_2-induced neuronal apoptosis through regulating the VEGFR-2/Akt signaling pathway (FIG. 5) (Cui et al., 2011c; Liu et al., 2009). We speculated that bis(12)-hupyridone might either directly interact with VEGFR-2 as a potential agonist or indirectly facilitate the activation of VEGFR-2 such as by stabilizing the dimerization or increasing the endogenous VEGF from elevating its translation, transcription or post-transcription (Cui et al., 2011b). Further investigations on the exact role that bis(12)-hupyridone plays in the activation of VEGFR-2 are being undertaken in our laboratory.

3.5 Promoting neuronal differentiation via activating α7nAChR

Currently prescribed drugs that treat AD have shown only modest and symptomatic effects by reducing the degree of impairment without preventing or curing the disease. It is partially because that these drugs cannot induce neurogenesis to compensate for the neurons that have lost their functions (Maggini et al., 2006). Transplantation of stem cells is considered a potential strategy as it may provide neurons to replace those that have been lost in the brains of AD patients, and reverse the progress of neurodegeneration (Zhongling et al., 2009). However, there is one key problem with this strategy as grafted stem cells are not able to differentiate into fully mature neurons in the micro-environments of the brain of AD patients (Waldau and Shetty, 2008). The application of agents capable of promoting neuronal differentiation at the impaired site may be a valid alternative or adjunct to solve that problem.

With the help of rat hippocampus neural stem cells, we have evaluated the effects of bis(12)-hupyridone in promoting neural stem cell differentiation. The percentage of βIII-tubulin positively stained neurons gave evidence that the efficacy of 10 μM bis(12)-hupyridone was similar to that of 0.5 μM retinoic acid, a potent inducer of neuronal differentiation. Moreover, under the same condition, huperzine A was not able to induce differentiation (FIG. 6) (Cui et al., 2011a). Bis(12)-hupyridone therefore might be a promising anti-AD drug candidate to promote differentiation of neural stem cells.

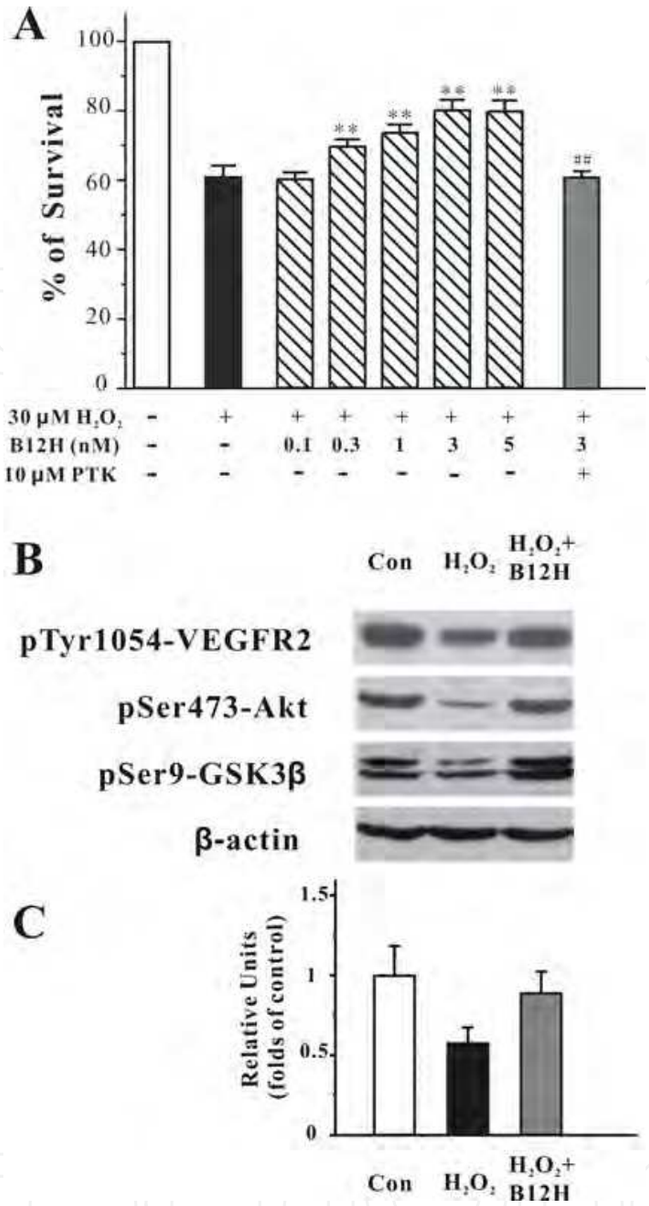


Fig. 5. Bis(12)-hupyridone inhibits H_2O_2-induced neuronal death from reversing VEGFR-2/Akt pathway. (A) The preventive functions of bis(12)-hupyridone against H_2O_2-induced cell death could be abolished by specific VEGFR-2 inhibitors in CGNs. CGNs with or without 30 min PTK787 (PTK, a specific VEGFR-2 inhibitor) pre-treatment were treated with bis(12)-hupyridone at the indicated concentrations for 2 h and then exposed to 30 μM H_2O_2. Cell viability was measured by the MTT assay at 6 h after H_2O_2 challenge. $^{**}p < 0.01$ *versus* H_2O_2 group, and $^{\#\#}p < 0.01$*versus* bis(12)-hupyridone plus H_2O_2 group (Tukey's test). (B) Bis(12)-hupyridone reversed H_2O_2-induced decreasing of pTyr1054-VEGFR-2, pSer473-Akt and pSer9-GSK3β. CGNs were pre-treated with 3 nM B12H for 2 h and then exposed to 30 μM H_2O_2 for 1 h, the total proteins were detected with using the specific antibodies. (C) The ratio to optical density (OD) values of pTry1054-VEGFR-2 over β-actin. The figure is modified from the reference (Cui et al., 2011c).

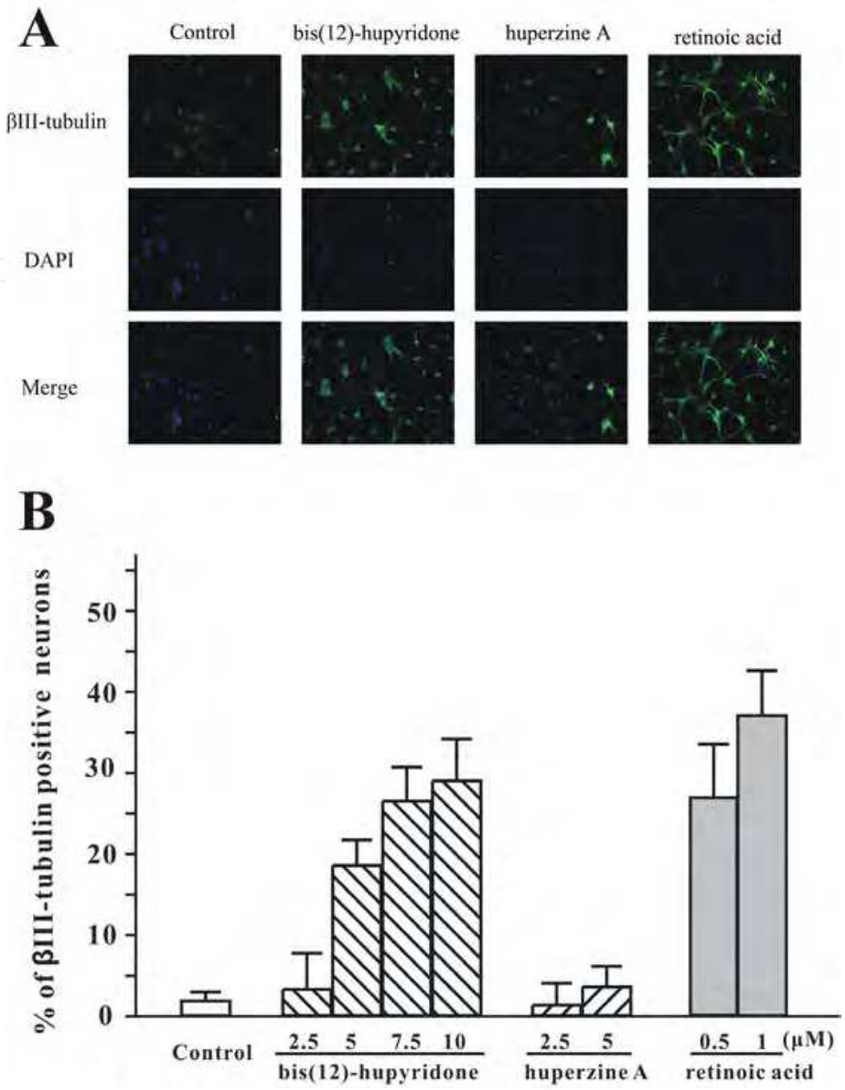


Fig. 6. Bis(12-hupyridone induces neuronal differentiation in adult rat hippocampus neural stem cells. (A) The expression of β-tubulin in neural stem cells was examined by fluorescence microscope. Neural stem cells were exposed to 10 μM bis(12)-hupyridone, 5 μM huperzine A or 1 μM retinoic acid for 48 h. The cells were then subjected to β-tubulin immunostaining and 4'-6-diamidino-2-phenylindole (DAPI) staining. (A) Bis(12)-hupyridone increased the percentage of β-tubulin positive neurons in a concentration-dependent manner. Neural stem cells were exposed to bis(12)-hupyridone, huperzine A or retinoic acid for 48 h, and the percentage of β-tubulin positive neurons was calculated. The figure is modified from the reference (Cui et al., 2011a).

We have also examined the neuronal differentiation promotion effects of bis(12)-hupyridone and its underlying mechanisms in the rat PC12 pheochromocytoma cell line, a well studied cell model of neuronal differentiation (Vaudry et al., 2002). Bis(12)-hupyridone (3 – 30 μM) has been demonstrated to induce neurite outgrowth in a concentration- and time-dependent manner with an efficacy that is three times higher than that of huperzine A in PC12 cells

(Cui et al., 2011a). Furthermore, mitogen-activated protein kinase kinase (MEK) inhibitor and alpha7-nicotinic acetylcholine receptor (α7nAChR) antagonist blocked the neurite outgrowth and the activation of extracellular signal-regulated kinase (ERK) induced by bis(12)-hupyridone, suggesting that bis(12)-hupyridone potently induces pro-neuronal cells into differentiated neurons by activating the ERK pathway via regulating α7nAChR (FIG. 7). As α7nAChR is essential for neuronal differentiation in the rat brain, and loss of α7nAChR impairs the maturation of dendritic neurons in adult hippocampus (Campbell et al., 2010; Le Magueresse et al., 2006), our results provide a novel insight into the possible therapeutic potential of bis(12)-hupyridone in treating AD. To date, as other clinically used anti-AD drugs such as huperzine A, donepezil and tacrine have also shown some activities in inducing neurite outgrowth in different neuronal cell lines *in vitro* (Table 4), it would be quite interesting to compare their effects on promoting neuronal differentiation in certain types of neurons, for example, neural stem cells with bis(12)-hupyridone (Cui et al., 2011a; De Ferrari et al., 1998; Oda et al., 2007; Sortino et al., 2004; Tang et al., 2005).

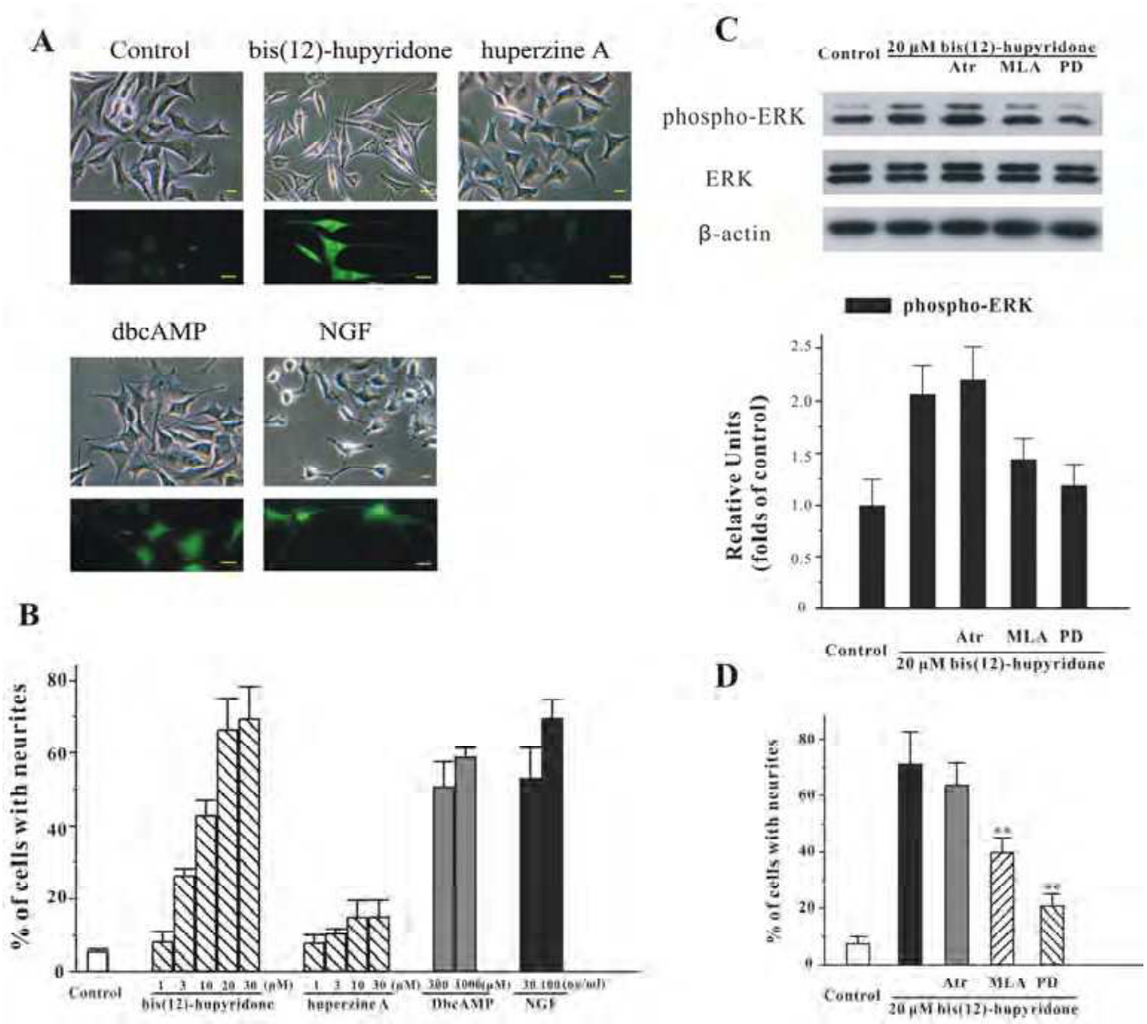


Fig. 7. Bis(12)-hupyridone induces neurite outgrowth from activating α7nAChR in PC12 cells. (A) The effects of bis(12)-hupyridone in promoting neurite outgrowth were evidenced by the

morphological changes and expression of GAP-43. PC12 cells were exposed to 20 μM bis(12)-hupyridone, 30 μM huperzine A, 3 mM dibutyryl cAMP (dbcAMP) or 100 ng/ml nerve growth factor (NGF) for 7 days. The morphological changes of neurites were examined by light microscope, and the expressions of GAP-43 were examined by fluorescence microscope. Scale bar 5 μm. (B) Induction of neurite outgrowth by bis(12)-hupyridone is in a concentration-dependent manner. PC12 cells were exposed to bis(12)-hupyridone, huperzine A, dbcAMP or NGF for 7 days, and the percentage of cells with neurites was measured. (C) The α7nAChR antagonist attenuates the activation of ERK induced by bis(12)-hupyridone. PC12 cells were treated with 0.3 μM methyllycaconitine (MLA, a specific α7nAChR antagonist), 10 μM atropine (Atr, a specific muscarinic acetylcholine receptor antagonist) or 30 μM PD98059 (PD, a specific MEK inhibitor) for 30 min before the administration of 20 μM bis(12)-hupyridone. The total proteins were extracted 30 min after the addition of bis(12)-hupyridone for Western blot analysis with specific antibodies. (D) The α7nAChR antagonist attenuates the neurite outgrowth induced by bis(12)-hupyridone. PC12 cells were incubated with 0.3 μM methyllycaconitine, 10 μM atropine or 30 μM PD98059 for 2 h and treated with 20 μM bis(12)-hupyridone. The percentage of cells with neurites was measured 7 days after treatment with bis(12)-hupyridone. The data, expressed as percentage of control, are the mean ± SEM of three separate experiments, with $^{**}p < 0.01$ *versus* the bis(12)-hupyridone group in employing ANOVA and Dunnett's test. The figure is modified from the reference (Cui et al., 2011a).

Drugs	Concentration (μM)	Neuronal Differentiation Models	Main Effects
Bis(12)-hupyridone	2.5 - 10	rat hippocampus neural stem cells	increased the percentage of βIII-tubulin positively stained neurons, induced neurite outgrowth
	3 - 30	PC12 cells	induced neurite outgrowth, and increased the expression of GAP-43
Huperzine A	10	PC12 cells	increased the number of neurite-bearing cells
Donepezil	1 -10	PC12 cells	potentiated the neurite outgrowth evoked by NGF
	0.1 - 10	SH-SY5Y cells	inhibited cell proliferation, and increased the expression of the neuronal marker MAP-2
Tacrine	10 - 50	Neuro 2A cells	induced neurite outgrowth

Table 4. Anti-AD drugs promote neuronal differentiation *in vitro*.
Data are from references (Cui et al., 2011a; De Ferrari et al., 1998; Oda et al., 2007; Sortino et al., 2004; Tang et al., 2005).

3.6 Enhancement of learning and memory

It is widely accepted that enhancement of learning and memory is beneficial for AD patients; and it has been proven that AChE inhibitors are the most effective agents in promoting cognitive functions in AD therapies. Our novel dimer bis(12)-hupyridone has demonstrated superior AChE inhibition *in vivo*. It is reasonable to expect that this dimer could remedy the impairments of learning and memory in AD patients. To prove this hypothesis, the model of scopolamine-induced performance deficits was used. We have demonstrated that *i.p.* injection of bis(12)-hupyridone (0.088 – 0.352 μmol/kg) significantly shortens the escape latency in Morris water maze after scopolamine administration in rats (Li et al., 2007b). Under the same condition, the relative potency of bis(12)-hupyridone (0.176 μmol/kg) to reverse the increased escape latency was higher than that of huperzine A (0.206 μmol/kg) (FIG. 8) (Li et al., 2007b).

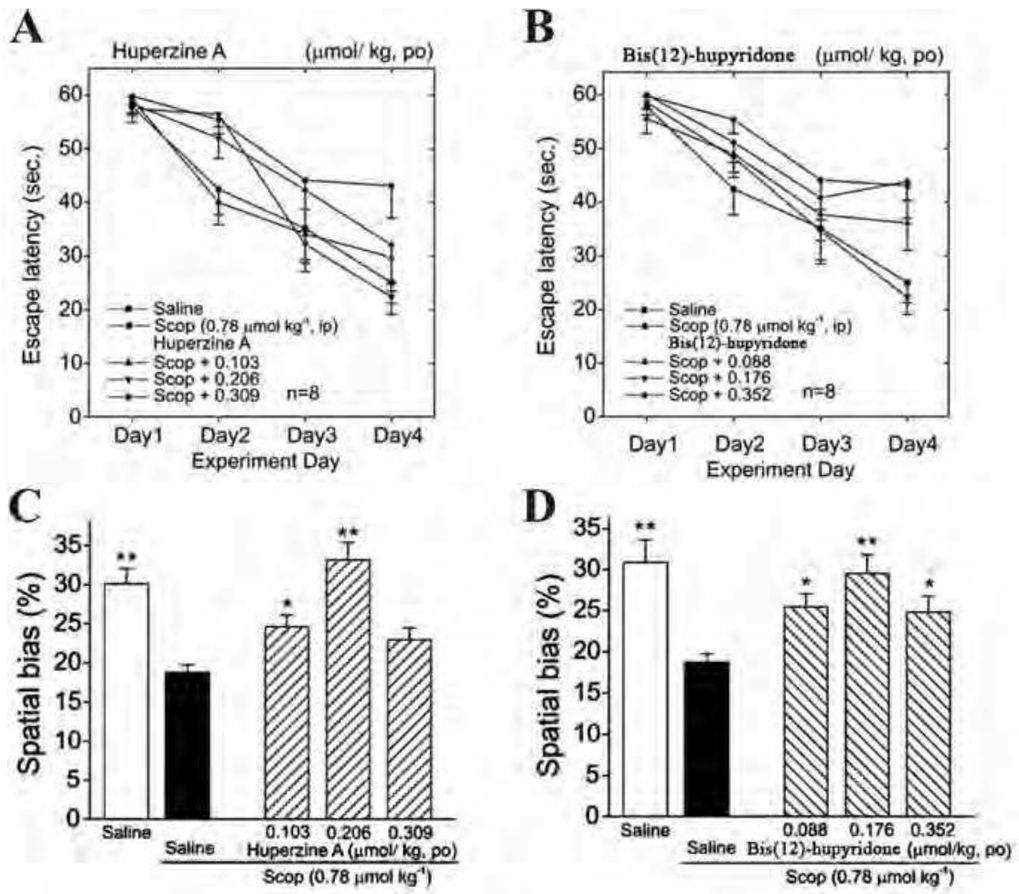


Fig. 8. Memory-enhancing effects of huperzine A and bis(12)-hupyridone on scopolamine-induced performance deficiency. Huperzine A (A) and bis(12)-hupyridone (B) reverse scopolamine-induced performance deficiency in rats. Huperzine A (C) and bis(12)-hupyridone (D) reverse scopolamine-induced decrease in spatial bias (% of total distances swum in the training quadrant during spatial probe trial) in rats. All data were expressed as means ± SD, $*p < 0.05$ and $**p < 0.01$ *versus* scopolamine group in (C) and (D) (ANOVA and Dunnett's test). The figure is modified from the reference (Li et al., 2007b).

3.7 Recovery of ischemic insult

Ischemia-induced insults result from the complex interplay of multiple pathways including excitotoxicity, oxidative stress and impairment of neurogenesis (Van der Schyf et al., 2006a). And some of these pathways are also underlying the impairments of AD progress. Therefore, agents targeting at multiple site for the treatment of stroke may also possess therapeutic effects for AD (Weinreb et al., 2009).

We have demonstrated in the 2-hour middle cerebral artery occlusion (MCAO) rat model, that bis(12)-hupyridone (0.70 - 1.41 μmol/kg, *i.p.*) could improve neurological behavior impairment, and reduce infarct volume as well as brain edema after ischemia. In addition, TUNEL staining assay has shown that bis(12)-hupyridone at quite a low concentration (0.70 μmol/kg, *i.p.*) could prevent cerebral ischemia-induced apoptosis in the penumbra region (FIG. 9, our unpublished paper). Compared with the currently used anti-AD drugs,

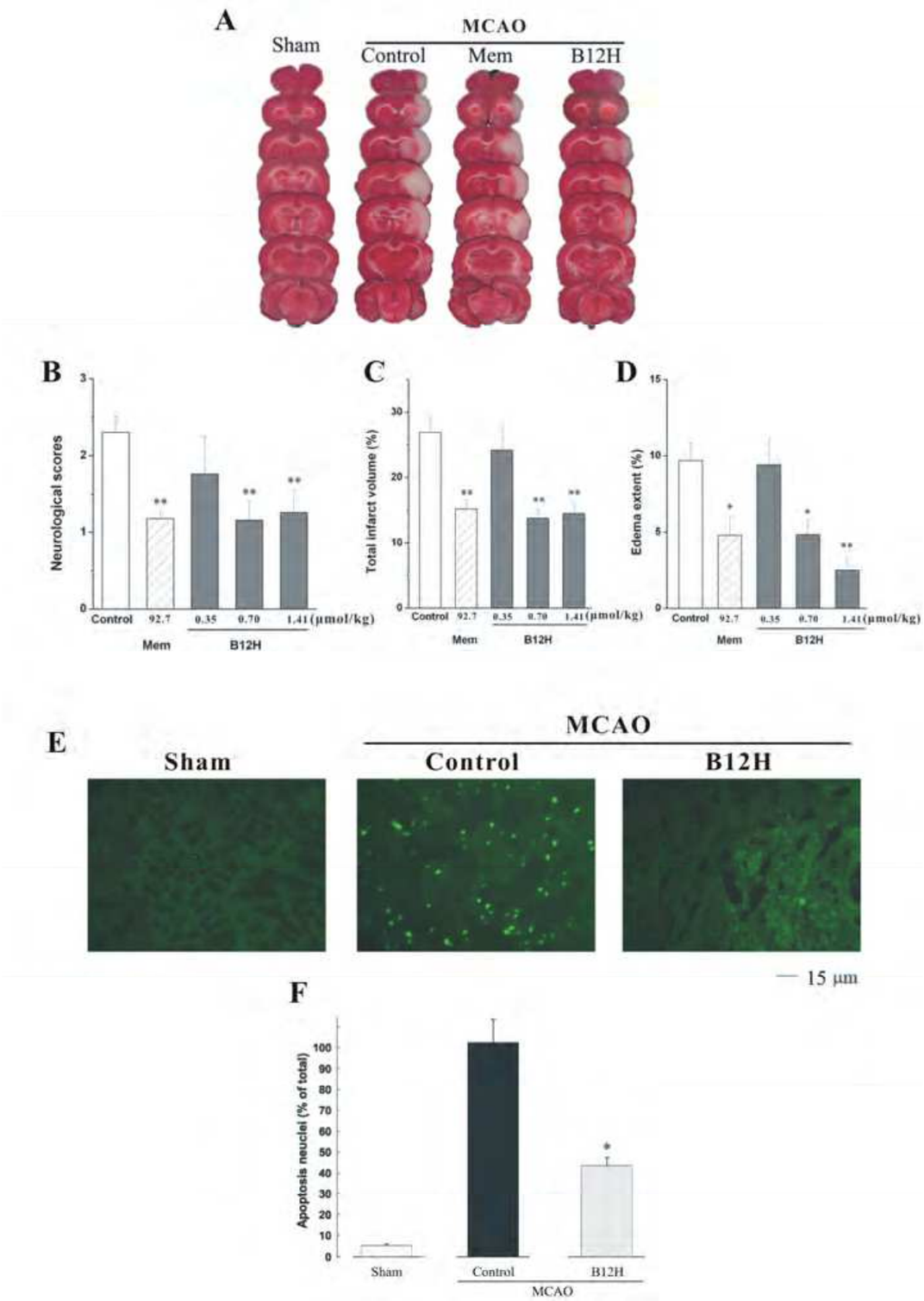


Fig. 9. Bis(12)-hupyridone rescues acute neurological impairments in rats after 2 h of MCAO followed by 24 h of reperfusion. Bis(12)-hupyridone was injected *i.p.* 30 min pre-ischemia and 15 min post-ischemia. (A) Representative photos of 2, 3, 5-triphenyltetrazolium

chloride (TTC)-stained brain slices showed that the enlarged infarct tissue area (pale unstained region) in the ischemic hemisphere of a control rat was reversed in animals treated with bis(12)-hupyridone (0.70 μmol/kg) or memantine (92.7 μmol/kg, 15 min post-ischemia *i.p.*). Bis(12)-hupyridone at both the concentrations of 0.70 and 1.41 μmol/kg reversed the decreases in neurological score (B), total infarction (C) and brain edema (D). (E) Bis(12)-hupyridone (0.70 μmol/kg) also rescued the apoptotic neurons in the penumbral region. Upper insets show representative photographs of terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) staining of the cerebral cortex penumbral zones of sham-treated rats, control animals, and bis(12)-hupyridone-treated rats. The lower panel shows the quantities of the TUNEL-positive cells. The number of TUNEL-positive neurons was randomly and double-blindly counted in three representative photomicrographs of each slice. B12H: bis(12)-hupyridone; Mem: memantine 92.7 μmol/kg. All data were expressed as means ± SEM, $*p < 0.05$, $**p < 0.01$ *versus* control group (ANOVA and Dunnett's test). The figure is adapted from our unpublished paper.

Drugs	Drug Dose (μmol/kg)	Transient Ischemia Model	Drug Treatment	Main Effects
Bis(12)-hupyridone	0.70 - 1.41	2 h middle cerebral artery occlusion followed by 24 h of reperfusion in rats	30 min pre- and 15 min post-ischemia, *i.p.*	attenuated ischemia-induced apoptosis in the penumbra region, improved neurological behavior impairment, and decreased cerebral infarct volume, cerebral edema
Memantine	46.3 - 92.7	3 h middle cerebral artery occlusion followed by 3 h of reperfusion in rat	15 min post-ischemia, *i.p.*	reversed ischemia-induced neurological deficit, reduced infract volumes, attenuated brain edema formation and blood-brain barrier permeability at the periphery
Huperzine A	0.41	45 min middle cerebral artery occlusion followed by 24 h of reperfusion in rats	at the onset and 6 h post-ischemia, *i.p.*	reversed ischemia-induced neurological deficit, reduced infract volumes, and decreased ROS production
Galantamine	7.0	20 min common carotid arteries occlusion followed by 24 h of reperfusion in rats	20 min post-ischemia, *i.p.*	reversed ischemia-induced learning impairment,
Tacrine	2.5 - 5.0	20 min common carotid arteries occlusion followed by 24 h of reperfusion in mice	1 h pre-ischemia, *p.o.*	prevented the reduction of step-down latency in the passive avoidance task, and shortened the escape latency in the Morris water maze task

Table 5. Anti-AD drugs protect transient ischemia induced impairments.
N.A.: not applicable. Data are either from our unpublished paper or from references (Gorgulu et al., 2000; Iliev et al., 2000; Wang et al., 2008; Xu et al., 2000; Zheng et al., 2008).

bis(12)-hupyridone has been shown to have high potency in preventing transient ischemia-induced neuronal impairments (Table 5) (Gorgulu et al., 2000; Iliev et al., 2000; Wang et al., 2008; Xu et al., 2000; Zheng et al., 2008). This high potency makes this dimer a promising drug candidate for the treatment of stroke and AD.

4. The physicochemical and pharmacokinetic properties of bis(12)-hupyridone

To predict the *in vivo* behaviors of bis(12)-hupyridone after dosing, its physicochemical properties have been studied and reported in our previous publication (Yu et al., 2008). As a

dihydrochloride salt, bis(12)-hupyridone presents a poor solubility (S_w: 11.16 mg/ml) with two ionization constants (pK_{a1}: 7.5 and pK_{a2}: 10.0) in water. Its solubility can be largely affected by the ionic strength (mainly the concentration of chloride ion) existed in the solution (*S*: 2.07 mg/ml in saline) and the pH value of the solution (*S*: 0.75 mg/ml in physiological phosphate buffer saline, pH 7.4). In addition, bis(12)-hupyridone has been determined to be highly lipophilic due to the symmetric chemical structure. The large difference of the oil-water partition coefficients between its neutral form (log P_N : 5.4) and ionized form (log $D_{pH\ 7.4}$: 1.1) suggests that bis(12)-hupyridone might be able to easily cross the biological barriers and reach to the site of effect (i.e. the central nerve system). Further investigated by an *in vivo* study, its maximum inhibition on AChE at mice brain could be reached in 15 min after an intraperitoneal injection (*i.p.*, 5.28 μmol/kg) and the effect could be lasted for more than 4 h (Yu et al., 2008).

Previously, bis(12)-hupyridone has been identified to be quite safe both *in vitro* and *in vivo*. No cytotoxicity was observed in the MTT assay after 3 h incubation of Caco-2 cells with bis(12)-hupyridone (264 μM) (Yu et al., 2011)) In addition, no side-effects were observed for bis(12)-hupyridone after an intravenous (*i.v.*) administration to rats even at a dosage as high as 8.8 μmol/kg which suggests the good compliance of bis(12)-hupyridone to rats (Yu et al., 2009).

The pharmacokinetic properties of bis(12)-hupyridone have been studied and reported (Yu et al., 2009). After the *i.v.* bolus injection (8.8 μmol/kg), bis(12)-hupyridone presents a two-compartmental elimination in rats with a first-order kinetic process. Comparing to huperzine A, bis(12)-hupyridone exhibits a relative faster distribution and elimination ($t_{1/2\alpha}$: 1.7 ± 0.4 min and $t_{1/2\beta}$: 92.9±7.9 min) *in vivo* than those of huperzine A ($t_{1/2\alpha}$: 6.6 ± 1.1min and $t_{1/2\beta}$: 149±96 min) (Wang et al., 2006). The greater distribution volume and mean blood clearance determined (V_d: 7.54 ± 0.88 L/(min kg) and *CL*: 0.067 ± 0.006 L/(min kg)) suggest the extensive tissue distribution and moderate blood elimination of bis(12)-hupyridone in rats. Furthermore, the previous pharmacokinetic study has revealed that bis(12)-hupyridone could be rapidly absorbed after *i.p.* administration to rats at a dose of 10 or 20mg/kg (t_{max} of 9.33 and 4.75 min, respectively), with an absolute bioavailability of >75% (Yu et al., 2008). It suggests that bis(12)-hupyridone could be well absorbed and most of the administrated drugs could enter into the systematic circulation after extra-vascular injection.

Although all the evidence from *in vitro* and *in vivo* studies suggest a reasonable permeation of bis(12)-hupyridone through the biological barrier after *i.p.* administration, it is somewhat surprising that bis(12)-hupyridone could not detectable in rat blood after oral administration (*p.o.*, 50 mg/kg), which suggesting its poor oral bioavailability. In order to assess its extent of absorption from the gastrointestinal (GI) tract, the mechanisms of bis(12)-hupyridone transport in intestine has been evaluated using Caco-2 cell model(Yu et al., 2011). As reported, bis(12)-hupyridone has been investigated to be a substrate to ATP-binding cassette (ABC) transporters and its directional transport could be regulated by the ABC-transporters mediated efflux. ABC-transporter inhibitors can significantly increase the absorptive transport of bis(12)-hupyridone thus facilitating its oral bioavailability. Since ABC-transporters are widely presented not only in the intestine but also at the blood-brain barrier (Dallas et al., 2006; Murakami and Takano, 2008), combined treatment of bis(12)-hupyridone with ABC-transporter inhibitors might to be developed as an effective approach

to improve its transport through the biological barriers and enhance its pharmacological effects at the central nerve system.

5. Conclusion

Based on the unique structure of the AChE enzyme and with the help of the bivalent ligand strategy, we have developed bis(n)-hupyridone, a novel series of dimers derived from the ineffective fragment of huperzine A. These dimers are proven to be potent and selective inhibitors of AChE both *in vitro* and *in vivo*. Bis(12)-hupyridone is a superior representative among these dimers. We have further shown that bis(12)-hupyridone, similar to memantine (an FDA approved anti-AD drug), moderately blocks NMDA receptors at the MK-801 site. Our studies have demonstrated that bis(12)-hupyridone could prevent excitotoxicity-induced neuronal loss and H_2O_2-induced neuronal apoptosis. Moreover, this dimer could promote neuronal differentiation with an efficacy similar to retinoic acid in neural stem cells. *In vivo* studies have shown that bis(12)-hupyridone possesses excellent efficacy in improving learning and memory deficits and protecting against neuronal loss *in vivo*. Our toxicological, physicochemical and pharmacokinetic studies have proved that bis(12)-hupyridone is promising for *in vivo* applications. Based on these novel findings, we conjecture that bis(12)-hupyridone could benefit AD patients by acting on multiple pathological targets concurrently (FIG. 10). As the synergism between anti-AChE, anti-NMDA receptors, anti-ROS, pro-neuronal differentiation might serve as the most effective

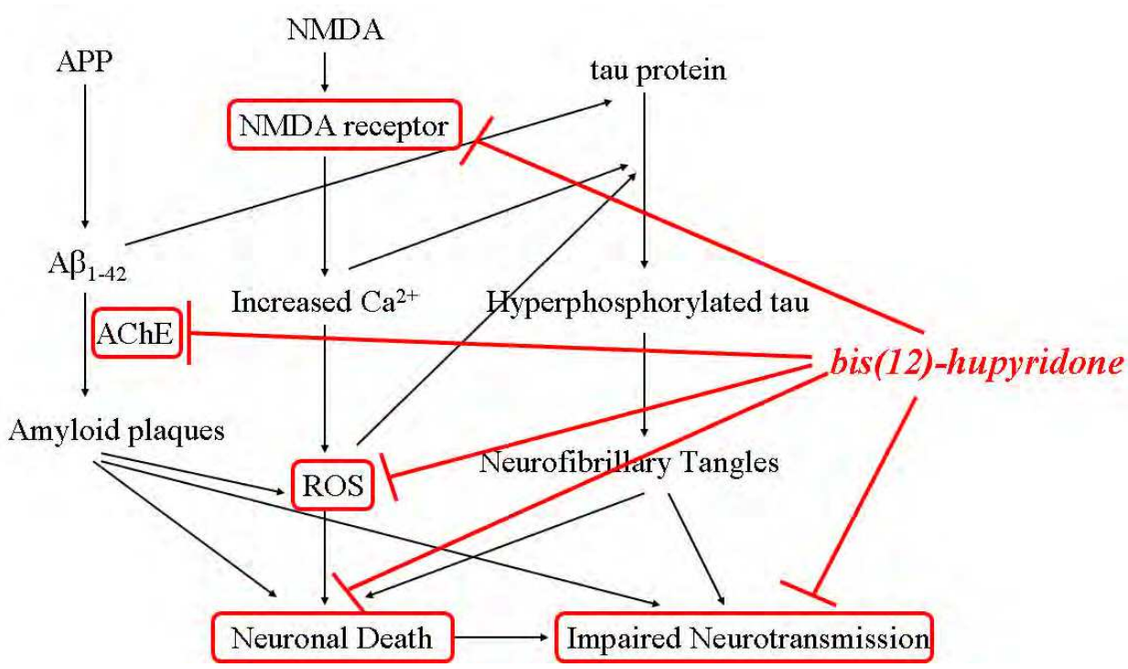


Fig. 10. Bis(12)-hupyridone acts as a multi-functional dimer for the treatment of AD.

therapeutic strategy to prevent and treat neurodegeneration AD, our findings not only provide a new direction for the design of effective compounds with multiple targets for the prevention and the treatment of AD, but also offer novel insights into the molecular basis for the development of potent therapeutic strategies for this disease.

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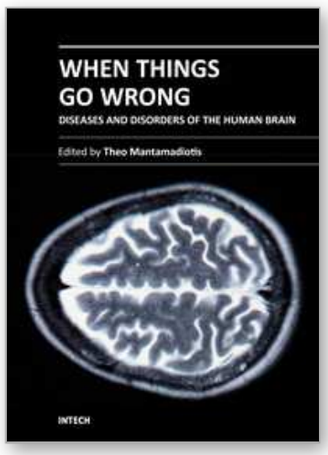
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When Things Go Wrong - Diseases and Disorders of the Human Brain

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In this book we have experts writing on various neuroscience topics ranging from mental illness, syndromes, compulsive disorders, brain cancer and advances in therapies and imaging techniques. Although diverse, the topics provide an overview of an array of diseases and their underlying causes, as well as advances in the treatment of these ailments. This book includes three chapters dedicated to neurodegenerative diseases, undoubtedly a group of diseases of huge socio-economic importance due to the number of people currently suffering from this type of disease but also the prediction of a huge increase in the number of people becoming afflicted. The book also includes a chapter on the molecular and cellular aspects of brain cancer, a disease which is still amongst the least treatable of cancers.

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