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Huperzine A from *Huperzia serrata:* a review of its sources, chemistry, pharmacology and toxicology

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Abstract The use and popularity of herbal medicines has been increasing worldwide. In fact, today, the traditional Chinese medicine offers a vast repertory for pharmaceutical research, as is the case of *Huperzia serrata, a* member of Huperziaceae family. This review reports the *Lycopodium* alkaloids that have been isolated from this plant. However, it was mainly focused on the huperzine A (HupA), a promising therapeutic option in several acute and chronic disorders. The major therapeutic interest described for HupA has been directed to the treatment of acetylcholine-deficit dementia, including Alzheimer's disease. However, HupA was also shown to be effective on cerebrovascular dementia and other neurodegenerative disorders with an ischemic component, as well as on other kind of cognitive impairments; the value of HupA on myasthenia gravis, organophosphate poisoning and schizophrenia has also been described. In addition, many other pharmacological properties have been ascribed to HupA, namely its anti-inflammatory, antinociceptive and anticonvulsant properties, which was recently identified, promoting a growing interest on HupA research. Furthermore, its particular chemical structure and the fact that HupA is well tolerated in humans, even at doses well above those clinically required, along with its favorable pharmacokinetics, also boosted an intense research in the pharmaceutical industry. Therefore, several HupA-related features are addressed in this review, including not only its therapeutic properties, but also its chemistry, biological and chemical sources, structure–activity relationship, pharmacokinetics and toxicology, which are discussed in detail covering the literature published from 1962 to 2014.

Keywords Huperziaceae · *Huperzia serrata* · *Lycopodium* alkaloids · Huperzine A · Alzheimer's disease

Abbreviations

ACh	Acetylcholine
AChE	Acetylcholinesterase
AD	Alzheimer's disease
bid	Twice-daily
BuChE	Butyrylcholinesterase
C_{max}	Peak concentration
CNS	Central nervous system

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CYP	Cytochrome P450
HupA	Huperzine A
im	Intramuscular
ip	Intraperitoneal
iv	Intravenous
LD_{50}	Median lethal dose
NMDA	*N*-Methyl-D-aspartate
po	*Per os*
sc	Subcutaneous

Introduction

The widespread use of herbs and plants as medicinal agents has dramatically increased in recent years (Tyagi and Delanty 2003; Zhu et al. 2004), estimating that 80 % of the world's population relies primarily on traditional medicines for their health care needs (Alves and Alves 2011). The herbal therapies are often the only available in many developing countries, contrary to what happens with pharmaceuticals, which is related to the economic and cultural factors underlying them (Schachter 2009). Although only less than 20 % of all plant species have been chemically or biologically evaluated, medicinal plants remain an important source of new chemical entities or new lead compounds, and they are frequently used as starting materials for semisynthetic analogs with improved pharmacological properties (Zhu et al. 2004; Bai 2007). Interestingly, about 30 % of the modern drugs have been developed based on phytochemicals that naturally exist in plants. The cardiac glycosides from foxglove (*Digitalis purpurea*), atropine from deadly nightshade (*Atropa belladonna*) and galantamine, an acetylcholinesterase (AChE) inhibitor naturally produced by *Galanthus nivalis* L., are some relevant examples of the current impact of herbal medicines (Tyagi and Delanty 2003).

In 1974, aiming at fulfill an unmet need through modern systems, the developing countries were encouraged to use traditional herbal medicines by the World Health Organization (Tyagi and Delanty 2003). Actually, although the traditional medicine has deep roots in societies of some countries, like Ayurveda in India, Kampo medicines in Japan, and Chinese herbal medicines in China, the popularity of herbal medicinal products has been increasing worldwide at a striking rate (Tyagi and Delanty 2003; Eisenberg et al. 2011). In this context, the traditional Chinese medicine offers a rich repertory for pharmaceutical research, among which the family Huperziaceae stands out (Zangara 2003). A single genus, the *Huperzia* Bernh, comprised this family in 1944 (Rothmaler 2008). However, the taxonomy of this family became complex and hotly debated among taxonomists due to the inclusion of the genus *Phlegmariurus* (Herter) Holub by Holub in 1964 (Holub 1985; Ji et al. 2008), being the family Huperziaceae divided into two separate genera—*Huperzia and Phlegmariurus* (Ma et al. 1998, 2006). The family Huperziaceae comprises 150–400 species (Luo et al. 2010; Szypuła et al. 2013). In China, 29 species, 2 varieties and 2 forma of *Huperzia* and 19 species of *Phlegmariurus* have been found until 2006 (Ma et al. 2006). Nevertheless, an important source of electronically available botanical systematic data, The Plant List, records 250 accepted species within *Huperzia* genus (The Plant List). In some classification systems a more broadly Lycopodiaceae family is defined including the genera of the Huperziaceae family, whereas in other classifications the genus *Huperzia*, as well as the genus *Phlegmariurus*, are treated in a separate Huperziaceae family (Ma and Gang 2004). In fact, the subdivision of the Lycopodiaceae has been matter of considerable disagreement. In some classification systems the separation of Lycopodiales into two separate families—Lycopodiaceae and Huperziaceae—was made on the basis of recognized differences in important characteristics (e.g., branching of the stem, spore-types, gametophyte-types), being the separation of *Huperzia* genus to the Huperziaceae family justified because its features differ substantially from those of other groups included in Lycopodiaceae family (Holub 1985). This taxonomic system is used in some countries and it is supported by chemotaxonomic analysis (Ma and Gang 2004; Ma et al. 2005). As a result, this classification system was followed in the present review. Huperziaceae is an ancient group of plants commonly known as the firmosses or fir clubmosses. They are mainly distributed in China, but also appear in America and Europe (Ji et al. 2008). The plants of this family grow very slowly and are usually required fifteen to 20 years from spore germination to maturity (Ma et al. 2007; Ma and Gang 2008; Luo et al. 2010). Among the species included in the family Huperziaceae, 33 species have been used for medicinal purposes (Ji et al. 2008), being, therefore, of great

interest for the scientific community, including pharmaceutical and medicinal research. Particularly the therapeutic potential of huperzine A (HupA), a naturally occurring alkaloid compound isolated from *Huperzia serrata,* has been increasingly recognized (Ma et al. 2007). In order to make clearer the information presented throughout this work, it should be mentioned that the term HupA is used whenever it is intended to refer to the racemic mixture [(±)-HupA]. On the other hand, the differentiation of the two stereoisomers of HupA as (−)-HupA and (+)-HupA (Fig. 1) will be properly performed (Haudrechy et al. 2000; Ding et al. 2012).

Therefore, this review extensively discusses the available knowledge about the medicinal properties of HupA, addressing also other relevant topics such as its biological sources, chemistry properties, chemical synthesis and structure–activity relationship, together with its pharmacokinetics and toxicology aspects.

Huperzia serrata

Huperzia serrata (Thunb. Ex Murray) Trev. (synonym *Lycopodium serratum* Thunb. ex Murray), also called Quian Ceng Ta, is today identified as a member of the family Huperziaceae (Kozikowski and Tückmantel 1999; Ma et al. 2007), which belongs to the *Huperzia* genus (Luo et al. 2010). Although with a higher incidence in the eastern, southern and southeastern areas of Asia, Oceania and Central America, *H. serrata* has a worldwide distribution (Ma et al. 2006; Huang and He 2010). *H. serrata* has been extensively used for diseases that affect the cardiovascular or neuromuscular systems, or those related to the cholinesterase activity including fever, contusions, strains, hematuria, and schizophrenia; it has also been used as an anti-inflammatory agent, as well as an antidote for organophosphate poisoning (Zangara 2003; Mukherjee et al. 2007; Ma et al. 2007; Sharma 2010; Wu et al. 2011; Zhang 2012). These medicinal properties ascribed to *H. serrata* are mainly due to its biologically active alkaloid compounds, named *Lycopodium* alkaloids (Ji et al. 2008).

Apart from *H. serrata,* other species of *Huperzia* have likewise been studied due to their diverse pharmacological properties. Studies conducted in adult male Wistar rats (Vallejo et al. 2007, 2009) revealed that the *Huperzia saururus* has effects on memory retention and learning process which were explained by the marked effects on the hippocampal synaptic plasticity (Ortega et al. 2006). Moreover, the species *Huperzia quadrifariata* and *Huperzia reflexa* appear to demonstrate an important anticholinesterase activity, both in in vitro and in vivo conditions (Konrath et al. 2012).

Biochemical constituents

Several *Lycopodium* alkaloids with diverse chemical structures have already been isolated from the *H. serrata* and they are still inspiring several research groups to design new alkaloid compounds; however, few reports have been published on their biological activities (Gao et al. 1999; Wu and Gu 2006; Jiang et al. 2010).

The majority of *Lycopodium* alkaloids are liposoluble (Jiang et al. 2010) and they are distinguished into four major structural classes, being the main representative compounds of each class shown in Fig. 2. The four classes of *Lycopodium* alkaloids found in *H. serrata* include fawcettimine-type (Fig. 3), lycodine-type (Fig. 4), lycopodine-type (Fig. 5), and a set of miscellaneous-type compounds (Fig. 6). The most

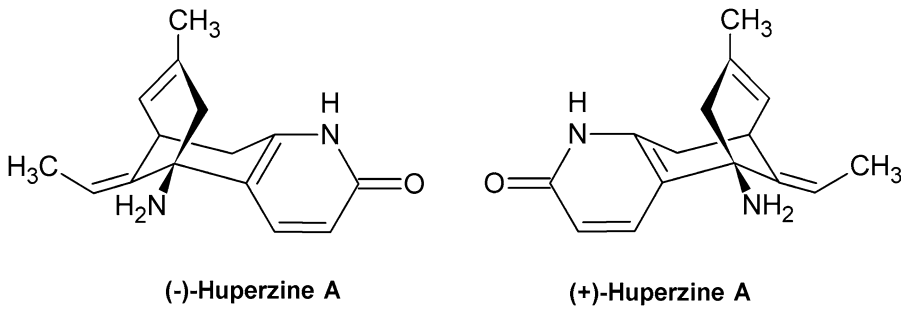


Fig. 1 Stereoisomers of huperzine A (Haudrechy et al. 2000; Ding et al. 2012)

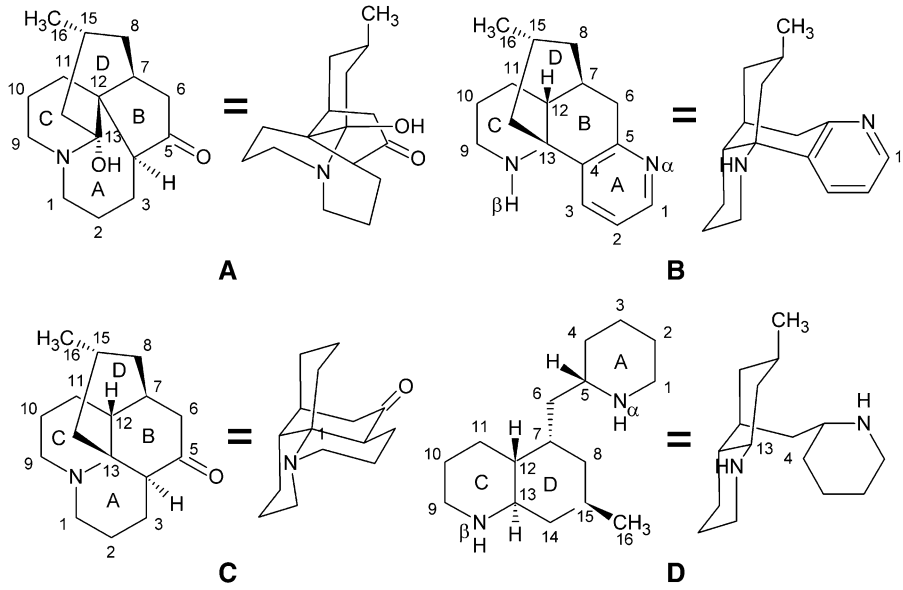


Fig. 2 Representative compounds of the four major classes of *Lycopodium alkaloids* from *H. serrata*: fawcettimine (**A**), lycodine (**B**), lycopodine (**C**), and phlegmarine (**D**) (Tan et al. 2003a; Ma and Gang 2004; Ma et al. 2007; Yuan et al. 2012)

part of alkaloid compounds that inhibit the AChE enzyme belong to the lycodine-type class and comprise HupA, 6β-hydroxyHupA, huperzine B, *N*-methylhuperzine B and huperzinine (Fig. 4) (Wu and Gu 2006; Ma et al. 2007; Wu et al. 2011). Among them, HupA is the foremost compound of interest since it revealed to be the most potent as AChE inhibitor (Wu et al. 2011). It is also important to highlight that Wang et al. (2002b) demonstrated that huperzine B can protect the neuron-like rat pheochromocytoma (PC12) cells against oxygen-glucose deprivation-induced injury, probably due to the alleviation of disturbances of oxidative and energy metabolism. Several *Lycopodium* alkaloids from the fawcettimine class have likewise been studied as AChE inhibitors. According to Tan et al. (2000a, 2002a), the huperzine P and huperzine R (Fig. 3) were also able to inhibit the AChE enzyme, but their inhibitory potency was less pronounced than that shown by HupA. In opposition, 11α-hydroperoxyphlegmariurine B, 7-hydroperoxyphlegmariurine B and phlegmariurine B, also from the fawcettimine class (Fig. 3) had no anticholinesterase activity (Tan et al. 2003b). On the other hand, the *Lycopodium* alkaloid 12-deoxyhuperzine O of lycopodine-type (Fig. 5) was recently reported as a naturally occurring alkaloid in *H. serrata*, and it was found to be an antagonist of the *N*-methyl-D-aspartate (NMDA) receptor, with an half maximal inhibitory concentration (IC_{50}) value of 0.92 μM (Yang et al. 2010).

Apart from *Lycopodium* alkaloid compounds, which have been the most extensively investigated, other naturally occurring bioactive compounds are commonly found in Huperziaceae plants (Luo et al. 2010). Particularly regarding the *H. serrata*, triterpenes like serrat-14-en-3β,21α,29-triol (Fig. 7A) (Zhou et al. 2004), flavones like 5,5′-dihydroxy-2′,4′-dimethoxyflavone-7-*O*-*β*-D-(6″-*O*-*Z*-*p*-coumaroyl)-glucopyranoside (Fig. 7B) (Yang et al. 2008), and also phenolic acids were identified (Ma et al. 2007; Luo et al. 2010).

Huperzine A

HupA was isolated from *H. serrata* for the first time in 1986 (Liu et al. 1986; Ma et al. 2006; Bai 2007; Ma et al. 2007). Over the years, HupA has been extensively studied by Chinese investigators, particularly due to the frequent use of firmoss *H. serrata* in traditional Chinese medicine for the treatment and prevention of dementia (Ma and Gang 2004; Ma et al. 2006). Up to date, it has been repeatedly proven that HupA is a potent AChE inhibitor relatively free of cholinergic toxicity (Rafii et al. 2011; Zhang 2012; Yu et al. 2013; Lunardi et al. 2013). Furthermore, HupA also showed to be an antagonist of cerebral NMDA receptors and, therefore, it is expected that HupA can be administered to subjects with epilepsy (Bialer et al. 2007, 2009, 2010; Yu et al. 2013).

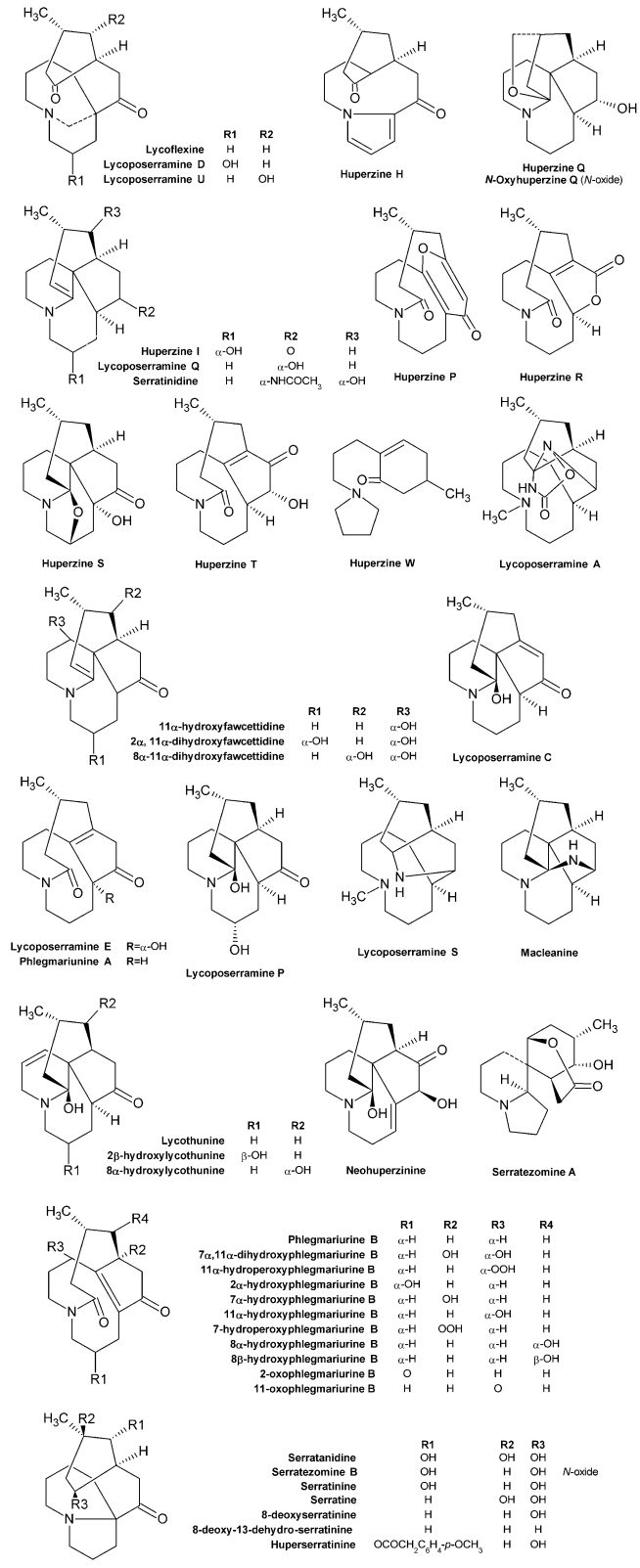


Fig. 3 Fawcettimine-type *Lycopodium* alkaloids from *Huperzia serrata* (Inubushi et al. 1967; Ayer et al. 1994; Zhu et al. 1994; Gao et al. 1999; Tan et al. 2000a, b, 2002a, b, c; d, 2003a, b Morita et al. 2000; Takayama et al. 2001; Ma and Gang 2004; Katakawa et al. 2007; Ma et al. 2007; Jiang et al. 2010; Gao et al. 2010; Yang et al. 2010; Yuan et al. 2012; Kitajima and Takayama 2012)

HupA seems to efficiently improve the age-associated learning and memory impairment in animals and humans, and it is also promising in the treatment of acetylcholine (ACh)-deficit dementia, including the Alzheimer's disease (AD) (Lallement et al. 2002; Zangara 2003; Ma and Gang 2004; Jiang et al. 2010). The drug "Shuangyiping", a tablet formulation of HupA produced from extracts of *H. serrata*, was developed in 1996 and it was approved as a new drug for the symptomatic treatment of AD in China (Ma et al. 2007). In 1997, HupA was classified by the Food and Drug Administration as a dietary supplement (Schachter 2009; Bialer et al. 2010; Wu et al. 2011), and it was marketed in the United States of America as powdered *H. serrata* in a twice-daily (bid) tablet or capsule formats (200–400 μg day^{-1}) for memory impairment (Xu et al. 1995; Chu et al. 2006, 2007; Ma and Gang 2008; Perry and Howes 2011). As a result, HupA rapidly became a cult supplement in the smart-drugs market and a best-selling product (Zangara 2003). HupA is widely available without prescription, in health food stores or via internet, labeled as a memory aid in its synthesized form (Zangara 2003; Bialer et al. 2007, 2009; Sharma 2010). Considering these aspects, some critical remarks related to the marketing of HupA as dietary supplement should be done. In fact, just because HupA is classified by the Food and Drug Administration as a dietary

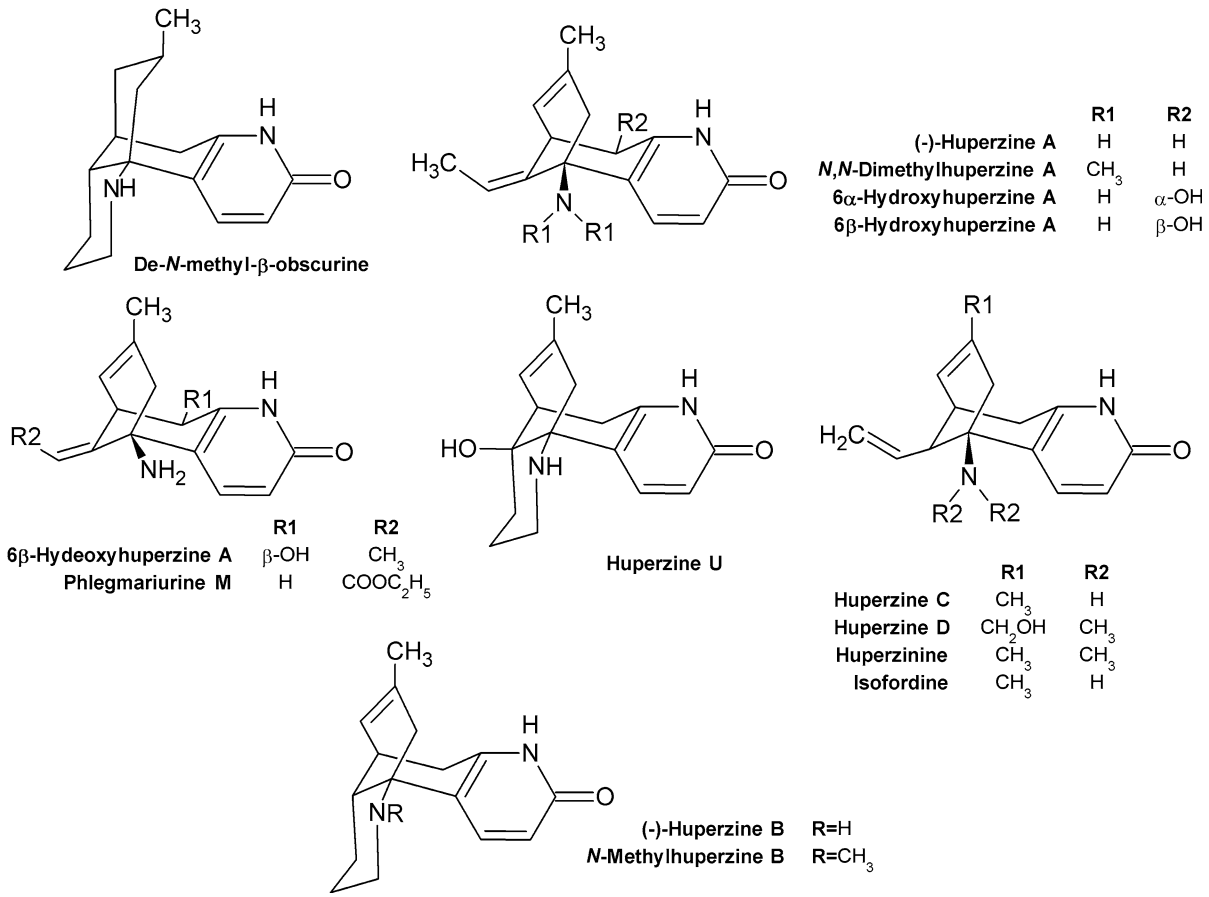
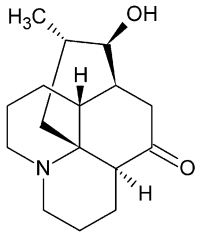
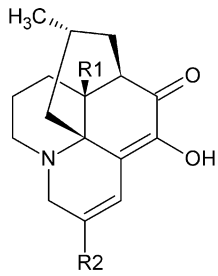


Fig. 4 Lycodine-type *Lycopodium* alkaloids from *Huperzia serrata* (Ayer et al. 1962; Hu et al. 1992; Dvir et al. 2002; Tan et al. 2003a; Zhu et al. 2004; Ma and Gang 2004; Toribio et al. 2007; Ma et al. 2007; Jiang et al. 2010; Yang et al. 2010; Yuan et al. 2012; Kitajima and Takayama 2012)

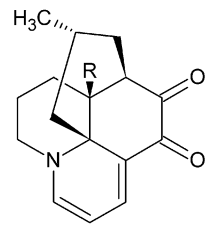
Fig. 5 Lycopodine-type *Lycopodium* alkaloids from *Huperzia serrata* (Inubushi et al. 1967; Lin et al. 1993; Wang et al. 1998, 2007; Morita et al. 2000; Tan et al. 2002e; Takayama et al. 2003; Tan and Zhu 2004; Ma and Gang 2004; Ma et al. 2007; Wang et al. 2009b; Jiang et al. 2010; Yang et al. 2010; Yuan et al. 2012; Kitajima and Takayama 2012)



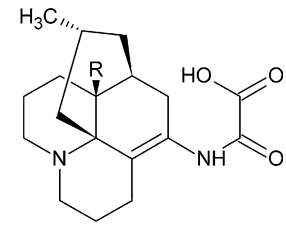
Clavolonine



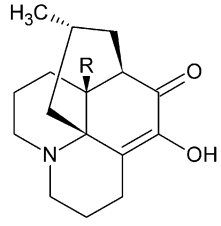
	R1	R2	
Huperzine E	H	H	
2-Chlorohuperzine E	H	Cl	
N-oxidehuperzine E	H	H	*N*-oxide
Huperzine F	OH	H	
N-oxidehuperzine F	OH	H	*N*-oxide



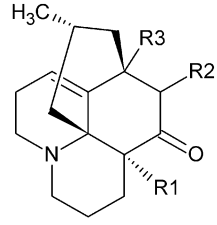
Huperzine E' R=H
Huperzine F' R=OH



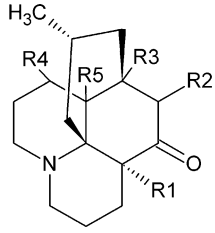
Huperzine G R=H
12-Hydroxyhuperzine R=OH



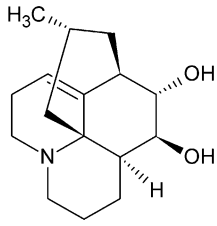
Huperzine O R=OH
12-Deoxyhuperzine O R=H



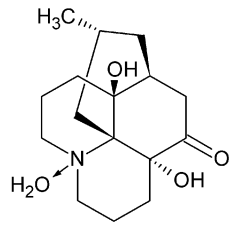
	R1	R2	R3
Serratidine	H	H	α-OH
4α-Hydroxyserratidine	OH	H	α-OH
4α,6α-Dihydroxyserratidine	OH	α-OH	α-OH
6α-Hydroxyserratidine	H	α-OH	α-OH
Lycoposerramine K	H	α-OH	H



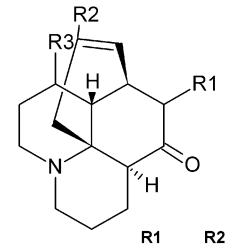
	R1	R2	R3	R4	R5	
Lycodoline	H	H	H	H	β-OH	
12-Epilycodoline *N*-oxide	H	H	H	H	α-OH	*N*-oxide
4α,6α-Dihydroxylycopodine	OH	α-OH	H	H	β-H	
6α-Hydroxylycopodine	H	α-OH	H	H	β-H	
7-Hydroxylycopodine	H	H	OH	H	β-H	
Lycoposseramine G	OH	H	H	H	β-OH	
Lycoposseramine L	H	β-OH	H	H	β-H	
Lycoposseramine M	H	H	H	α-OH	β-H	
Lycoposseramine N	H	H	H	α-$OCOCH_3$	β-OH	
Serratezomine C	H	α-OH	H	H	β-OH	



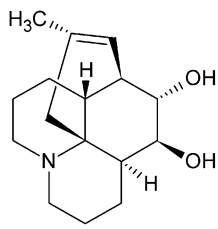
Lucidioline



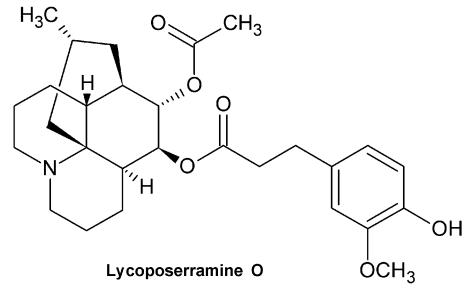
Lycoposerramine F



	R1	R2	R3
Lycoposerramine H	α-OH	H	H
Lycoposerramine H3c	α-OH	CH_3	H
Lycoposerramine I	H	H	α-OH



Lycoposerramine J



Lycoposerramine O

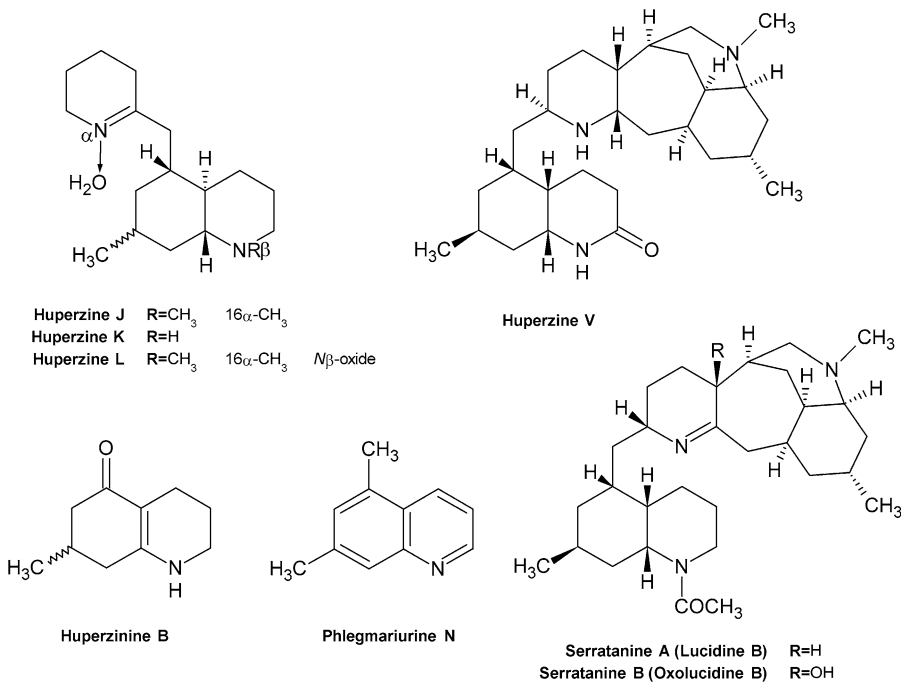


Fig. 6 Miscellaneous-type *Lycopodium* alkaloids from *Huperzia serrata* (Inubushi et al. 1967; Miao et al. 1989; Gao et al. 2000a; Liu et al. 2004; Ma and Gang 2004; Ma et al. 2007; Yuan et al. 2012)

supplement, it does not certify its safety and efficacy. Actually, few supplements have been submitted to rigorous studies to support their claims. Indeed, the regulation for this supplements is not as strict as to drugs, and in the case of HupA the therapeutic range does not seem to be as wide as one might think; indeed, side effects appear to occur at therapeutic doses, although with a mild intensity (Xu et al. 1995; Pepping 2000; Zangara 2003; Ma et al. 2007; Sharma 2010). In fact, herbal medicines are often not deeply investigated regarding to its mechanisms of action, toxicity, and clinical effects, and the studies conducted frequently fail in many requirements of the evidence-based medicine, being the efficacy of medicinal herbs mainly supported by empirical data and the traditional use (Chang 2000).

Nevertheless, due to its unique chemical structure (Ma and Gang 2008), good pharmacokinetic properties (Ha et al. 2011), and the fact that HupA is well tolerated in humans, even at doses well above those clinically required, an intense research on this compound has been performed by pharmaceutical industry (Tun et al. 2011). Indeed, multiple aspects about this phytochemical compound have been studied during the last years, including not only its therapeutic effects, but also its botanical sources, chemistry properties, chemical synthesis, structure–activity relationship, pharmacokinetics and toxicology, which will be described in next sections.

Natural sources and synthesis

Although *H. serrata* is the original herbal source of HupA, this compound is also produced in other species of Huperziaceae family that have close taxonomic relationships to *H. serrata*. Moreover, HupA equally occurs in other plant families including Lycopodiaceae and Selaginella. In *H. serrata* there is less than 0.02 % by weight content of HupA (Bialer et al. 2007, 2010) and the yields of the compound in dried herb range from 0.0047 to 0.025 %, depending on the collecting seasons, growing regions, process of collecting the herbs and extraction techniques (Bai 2007; Ha et al. 2011). Therefore, large quantities of *H. serrata* are required in order to yield practical usable quantities of HupA (Tang et al. 1994). In the *Huperzia* genus, *H. serrata, H. herteriana* and *H. ovatifolia* have higher contents of HupA than other species; while in

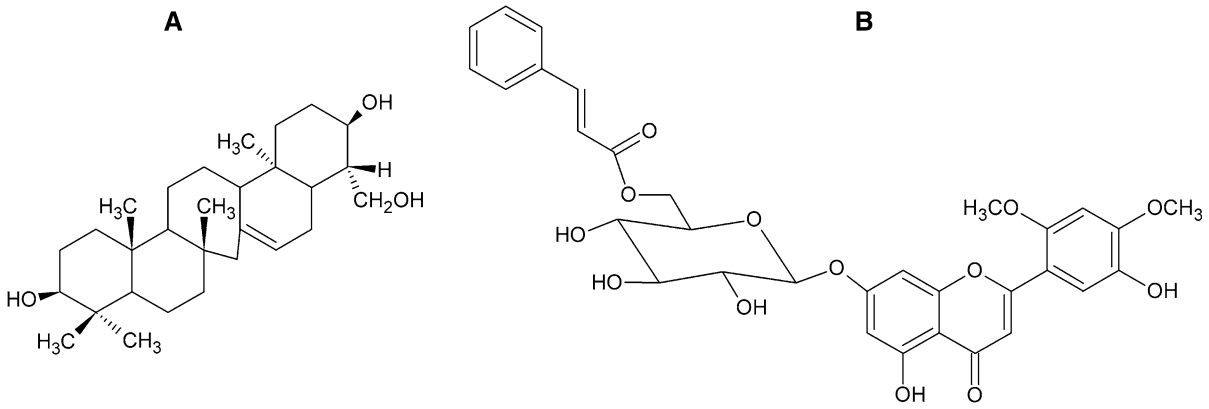


Fig. 7 Other compounds from *Huperzia serrata*: serrat-14-en-3β,21α,29-triol (**A**) (Zhou et al. 2004) and 5,5′-dihydroxy-2′,4′-dimethoxyflavone-7-*O*-*β*-D-(6″-*O*-*Z*-*p*-coumaroyl)-glucopyranoside (**B**) (Yang et al. 2008)

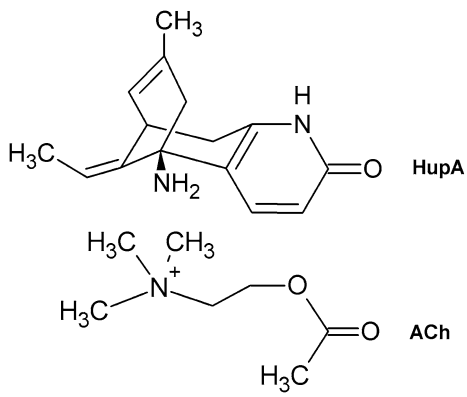


Fig. 8 Structural similarity between huperzine A (HupA) and acetylcholine (ACh) (Bai et al. 2000)

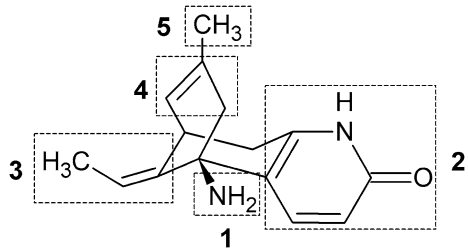


Fig. 9 Requeriments of huperzine A for its high anti-acetylcholinesterase activity: amine group (*1*); α-pyridone ring (*2*); exocyclic ethylydene residue (*3*); three-carbon bridge and its double bound (*4*); and methyl group (*5*) (Ashani et al. 1992; Ma and Gang 2004; Ma et al. 2007)

the *Phlegmariurus* genus, the highest content in HupA was found in *P. carinatus* and *P. mingcheensis* species (Ma et al. 2005; Bai 2007). However, the species of the *Phlegmariurus* genus still appear to possess higher levels of HupA than the species of *Huperzia* genus. Regardless, although some other species in Huperziaceae family produce larger amounts of HupA, they are less desirable candidates as natural sources of HupA, not only because they are more difficult to obtain, but also because they are scarcer than *H. serrata* (Ma and Gang 2008). It is also important to highlight that the plants of *H. serrata* that grow in humid forests are significantly more rich in HupA than those growing in less humid environments (Ma et al. 2005).

The fast growing demand and the high price of the raw material are increasing the pressure on the natural habitats and the *H. serrata* has become a threatened plant in China due to the over-exploitation and habitat fragmentation (Huang and He 2010). Furthermore, besides not being particularly abundant, these plants also grow extremely slowly (Ma et al. 2007; Ma and Gang 2008; Ding et al. 2012). Thus, owing to the unique bioactivity of HupA and its low yield from plants, several research groups have devoted intensive efforts in developing methods to synthesize HupA in high quantities. This chemical synthesis strategy initiated by Qian and Ji (1989) and Xia and Kozikowski (1989), was followed by Lucey et al. (2007) and, more recently, by Ding et al. (2014), who reported a more efficient total synthesis of HupA. The synthetic HupA resultant from these investigations is a racemic mixture. However, regarding to the in vitro inhibitory effects on AChE enzyme, the racemic mixture of HupA is three times less potent than (−)-HupA and, consequently, the formal synthesis of each pure

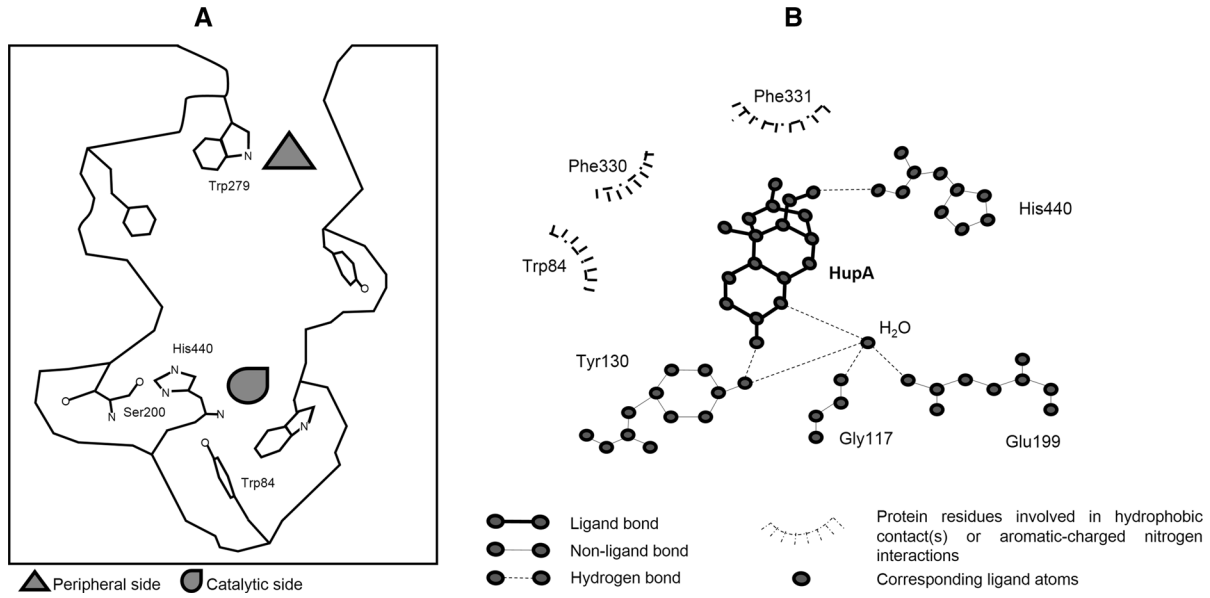


Fig. 10 **A** Representation of the gorge and the catalytic and peripheral sites of huperzine A (HupA) (Pang and Kozikowski 1994) and **B** a zoom-in look at the binding pocket of acetylcholinesterase showing the main interactions between the ligand (HupA) and the enzyme (*Glu* glutamic acid, *Gly* glycine, *His* histidine, *Phe* phenylalanine, *Ser* serine, *Trp* tryptophan, *Tyr* tyrosine) (Raves et al. 1997; Ha et al. 2011)

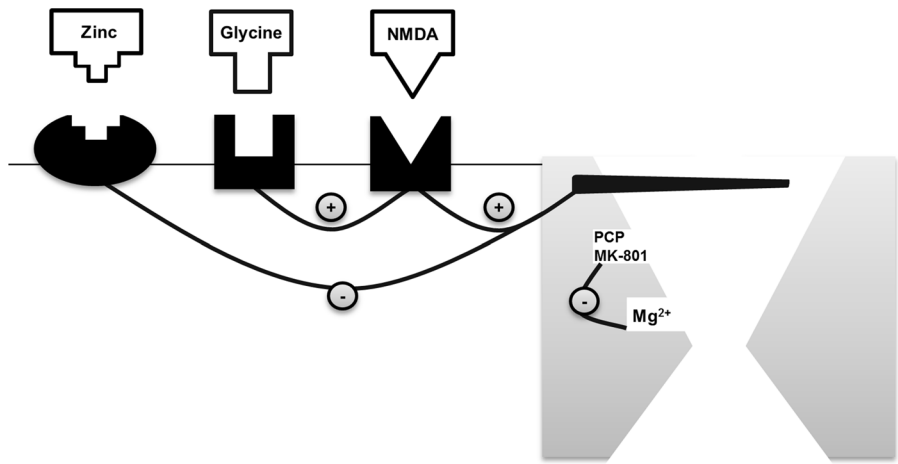


Fig. 11 Representation of the regulatory sites of *N*-methyl-D-aspartate (NMDA) receptor containing a recognition site for NMDA, a cation-selective ion channel, and binding sites for glycine, zinc and phencyclidine (PCP)-like compounds, where (+)-5-methyl-10,11-dihydro-5*H*-dibenzo[*a*,*d*]cyclohepten-5,10-imine maleate (MK-801) also binds. The channel can be blocked by magnesium (Mg^{2+}) (Reynolds and Miller 1988; Gordon et al. 2001)

enantiomer was attempted (Bai et al. 2000; Wu and Gu 2006). Indeed, today, the synthesis of (+)-HupA and the eutomer (−)-HupA is described in literature (Fig. 1), even though these processes are not yet industrialized (White et al. 2013).

Moreover, with the aim of surpass the low content of HupA in the raw plant material, Ma and Gang (2008) developed a method to propagate in vitro tissues of *Phlegmariurus squarrosus*, a member of Huperziaceae family that produces high levels of

Table 1 Analogs of huperzine A (Hup A) (Zhou and Zhu 2000; Camps et al. 2000; Ros et al. 2001; Alcalá et al. 2003; Ma and Gang 2004; Gemma et al. 2006; Jia et al. 2013)

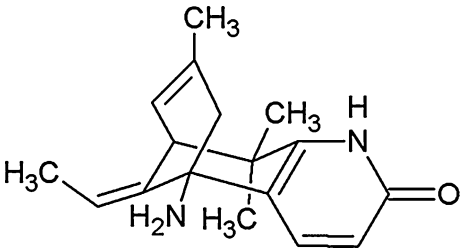
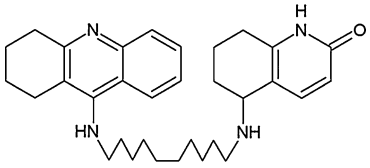
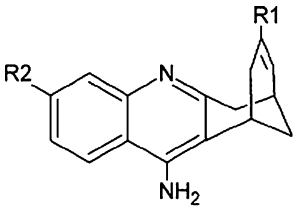
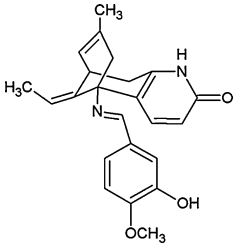
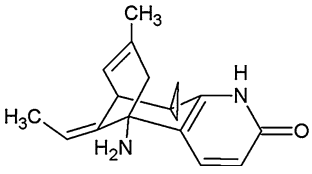
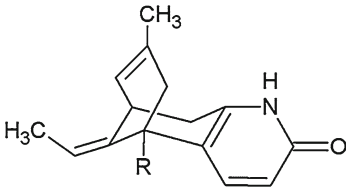
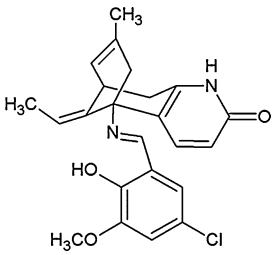
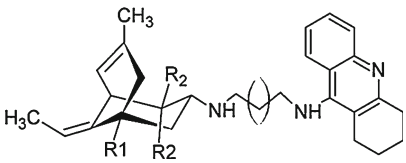
Analogs of HupA	Structural changes in relation to HupA	Consequences of the structural changes on compounds activity
(±)-10,10-Dimethyl-HupA 	Introduction of two methyl groups in the C-10 position	The axial methyl group into the C-10 position increased the inhibitory potency against AChE (8-fold increase) The corresponding equatorial isomer was about 1.5-fold less active than HupA
H-3 	Combination of tacrine with the possible effective structural essence of HupA (A and B rings) connected by an alkane tether	Good anti-AChE activity, but much less selectivity than HupA
Huprines  *(±)-Huprine X* R1 = C_2H_5 R2 = Cl *(±)-Huprine Y* R1 = CH_3 R2 = Cl *(±)-Huprine Z* R1 = CH_3 R2 = F	Combination of tacrine with the bridgehead ring of HupA	Higher anticholinesterase activity than HupA and tacrine Increase the levels of AChE in the synaptic cleft more effectively than tacrine Good selectivity in the ratio of AChE to BuChE activity The replacement of F of (±)-huprine Z by Cl in (±)-huprine Y probably improves the binding to AChE and explains its greater potency
IsovaniHupA 	Selected from the collection of Schiff bases at the HupA amino group	Activity close to that of HupA in some indexes Stability problems
(−)-10-Spiro-cyclopropyl-HupA 	Introduction of a cyclopropane group at C-10 position	In vitro activity similar to that of (−)-HupA

Table 1 continued

Analogs of HupA	Structural changes in relation to HupA	Consequences of the structural changes on compounds activity
5-Substituted analogs		
 *A* R = OH *B* R = F	Introduction of different substituents in the C-5 position.	The compounds exhibit 50 % of AChE inhibitory activity of HupA at the concentration of 35 mM (*A*) and 47 mM (*B*)
ZT-1		
	Pro-drug, rapidly absorbed and converted into HupA; Schiff base made by a condensation reaction between HupA and 5-Cl–*O*-vanillin.	Inhibition of AChE is more selective and the analogue is less toxic in mice than HupA Similar properties to HupA regarding the ability to cross the blood–brain barrier, oral bioavailability, and longevity of action A phase I study showed good tolerability in humans
Others		
 *A* R1 = NH_2 R2 = CH_3 *B* R1 = NH_2 R2 = H *C* R1 = CO_2CH_3 R2 = H	HupA-tacrine hybrids characterized by 3-methylbicyclo-[3.3.1]non-3-ene scaffolds	Biological profile markedly improved in relation to those of tacrine and HupA Potent cholinesterase multisite inhibitors of human cholinesterases, showing comparable inhibitory activities for AChE and BuChE

AChE acetylcholinesterase, *BuChE* butyrylcholinesterase

HupA. The authors referred that the in vitro propagated tissues may represent an excellent source for HupA due to the production of higher levels of this compound than the natural plant (Ma and Gang 2008).

Chemistry and physicochemical properties

HupA [(−)-HupA and (+)-HupA, Fig. 1], chemically designated as 9-amino-13-ethylidene-11-methyl-4-azatricyclo[7.3.1.0(3.8)]trideca-3(8),6,11-trien-5-one, is an unsaturated sesquiterpene *Lycopodium* alkaloid related to the quinolizidines (Howes and Houghton 2003; Mukherjee et al. 2007; Schachter 2009; Zhang et al. 2009). Structurally, HupA is a fairly unique molecule due to its compact and stringent skeleton that contains an ethylidene group and an aromatic pyridone moiety fused with a bicycle-ring system bearing a primary amino group (Raves et al. 1997; Patocka 1998; Zhao et al. 2007; Ha et al. 2011). Its empirical formula is $C_{15}H_{18}N_2O$ and its molecular weight is 242.32 g mol^{-1} (Zangara 2003; Zhao et al. 2007; Schachter 2009). HupA is optically active and it naturally exists as the pure isomer of (−)-HupA, also named L-HupA which is much more active than (+)-HupA, also called D-HupA (Fig. 1) (Haudrechy et al. 2000; Zangara 2003; Wu and Gu 2006; Ha et al. 2011; Ding et al. 2012). HupA is very stable, with a white-crystal appearance, and it is soluble in aqueous acids and chloroform (Raves et al. 1997; Patocka 1998; Zangara 2003). According to Ashani et al. (1992) no detectable changes in the chemical structure of HupA were detected in the long-term incubation at 24 °C

with AChE or butyrylcholinesterase (BuChE), or following 96 h of incubation with 0.1 N hydrochloric acid or sodium hydroxide, suggesting that the opening of the pyridone ring is not likely to occur. These data corroborate the high stability of HupA. At this point it is worthy to mention that the abbreviations BuChE and BChE have been randomly used in the literature for butyrylcholinesterase; however, in this case, to maintain the consistency the abbreviation BuChE was used throughout the article.

Structure–activity relationship

Studies of computer-generated superposition of HupA and ACh suggested that HupA possesses the basic structural features of ACh (Fig. 8). Actually, a reasonable structural similarity is found between the nitrogen, oxygen and carbonyl groups of ACh, and the corresponding amino-nitrogen, nitrogen and carbonyl groups of HupA. It seems that the amino-nitrogen atom of HupA is as distant of the carbonyl group of pyridine ring as the quaternary nitrogen atom is from the ester carbonyl in ACh and, therefore, the 5-aminomethyl-2(1*H*)-pyridone part of HupA is recognized as its pharmacophoric moiety (Bai et al. 2000). The simultaneous presence of the amine group, the α-pyridone ring, the exocyclic ethylidene residue, the three-carbon bridge with its double bound, and the methyl of the bridge properly aligned are required for HupA retain its high inhibitory effect on AChE enzyme (Fig. 9) (Ashani et al. 1992; Ma and Gang 2004; Ma et al. 2007). Thus, the elimination or substitution of at least one of these structural features is responsible by a dramatic reduction in HupA inhibitory activity on AChE (Ma and Gang 2004).

In fact, it is not surprising that small molecular differences determine notoriously the AChE inhibitory activity since the spatial configuration of HupA itself strongly determines the activity. Indeed, the (−)-HupA was found to be more potent than (+)-HupA relatively to the inhibition of the AChE enzyme (Fig. 1). Thus, according to Saxena et al. (1994), the

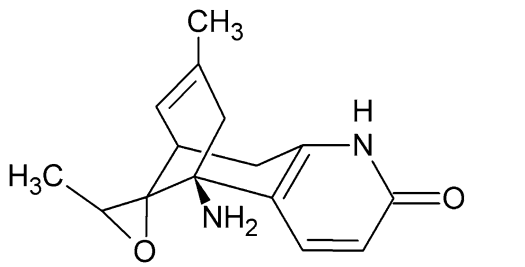


Fig. 12 Chemical structure of the major metabolite of huperzine A: the 13,14-epoxy-huperzine A (Garcia et al. 2004)

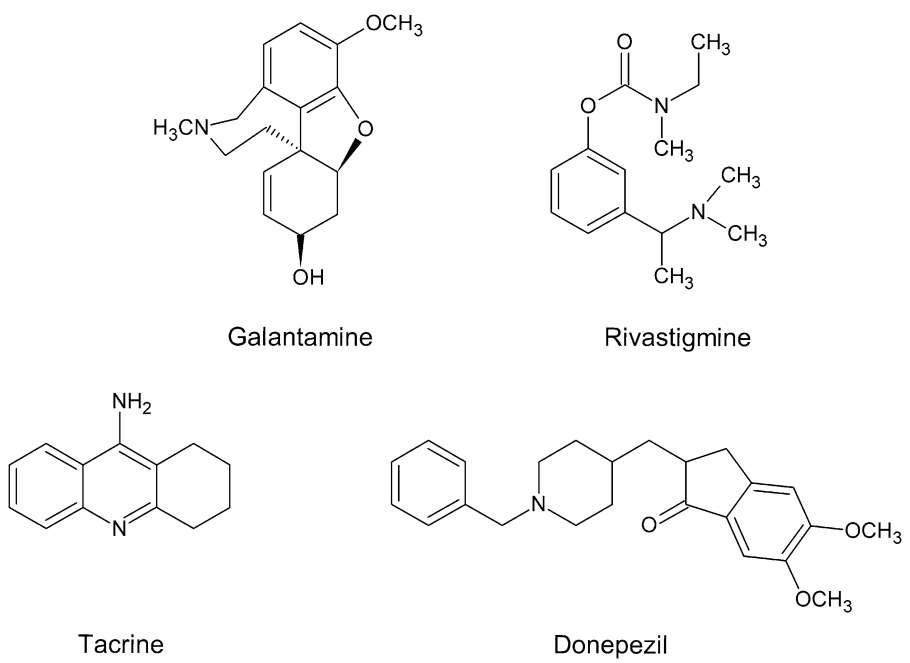


Fig. 13 Acetylcholinesterase inhibitors clinically used as therapeutic drugs for treatment of Alzheimer's disease (Ding et al. 2012)

molecular mechanics energy minimization of the complexes formed between each of the two stereoisomers of HupA and fetal bovine serum AChE, *Torpedo* AChE, or human BuChE revealed that (−)-HupA gave a better fit than (+)-HupA; importantly the tyrosine 337(330) is implicated in the stereoselectivity of the drug (Saxena et al. 1994). A similar biological action mechanism between the (±)-HupA and (−)-HupA was found on rat brain cholinergic function, both in vitro and in vivo (Hanin et al. 1993; Tang et al. 1994). However, the racemic mixture has a weaker biological activity than the natural product, probably due to the presence of (+)-HupA, which is the less potent enantiomer (Hanin et al. 1993; Tang et al. 1994; Ma and Gang 2004; Wang et al. 2011b). Actually, (+)-HupA showed to inhibited the AChE 38-fold less potently than (−)-HupA, with inhibition constant (*Ki*) values of 300 nM and 8 nM, respectively (Ma and Gang 2004). Despite these differences in the biological activity against the AChE, both enantiomers have much higher affinities for AChE than BuChE (Coleman et al. 2008).

According to Ashani et al. (1992), the number and type of aromatic amino acid residues in the catalytic pocket region of the cholinesterase enzymes (AChE and BuChE) may contribute to the thermodynamic stability of HupA-cholinesterase complex. Several studies were performed in order to understand the highly potent and selective AChE inhibition mediated by HupA. They include not only computer-aided docking studies (Pang and Kozikowski 1994; Dvir et al. 2002), but also X-ray crystallography studies (Raves et al. 1997; Kozikowski and Tückmantel 1999). It was recognized that HupA binds to the bottom of the gorge in *Torpedo* AChE above tryptophan 84 (catalytic domain, Fig. 10A) and to the opening of the gorge with its ammonium group partially interacting with the indole ring of tryptophan 279 (peripheral site, Fig. 10A). The serine 200 and histidine 440 residues were also recognized at the active site (Fig. 10A) (Sussman et al. 1991; Pang and Kozikowski 1994).

Effectively, the crystal structure of the optically pure (−)-HupA–AChE complex showed an unexpected orientation for HupA with surprisingly few strong direct interactions with protein residues, which explains its high affinity for the enzyme (Raves et al. 1997). Moreover, the importance of individual hydrophobic interactions between HupA and aromatic residues in the active site gorge of AChE has also been recognized (Raves et al. 1997; Patocka 1998). Thus, although only one strong hydrogen bond is established between the pyridone oxygen of the ligand and a protein residue, (−)-HupA has three potential hydrogen-bond donor and acceptor sites (Raves et al. 1997). Briefly, the principal interactions between (−)-HupA and AChE are the following: (1) direct and strong hydrogen bonds between the carbonyl group of HupA and the hydroxyl oxygen of tyrosine 130, located at the peripheral site of the enzyme, as well as between the ethylidene methyl group and the main-chain oxygen of histidine 440, a modality of the catalytic triad; (2) indirect hydrogen bonds mediated by one or two water molecules within the active site gorge which are, themselves, hydrogen-bonded to other water molecules or to side-chain and backbone atoms of the protein; (3) cation-π interactions of the primary amino group of HupA with the aromatic rings of tryptophan 84 and phenylalanine 330 at the choline site, as well as the ionic interactions with carboxyl groups of glutamic acid 199 and aspartic acid 72; and (4) several important hydrophobic interactions, particularly with the side chains and main-chain atoms of tryptophan 84, phenylalanine 331 and histidine 440 (Fig. 10B) (Pang and Kozikowski 1994; Raves et al. 1997; Kozikowski and Tückmantel 1999; Dvir et al. 2002). These structural biology investigations found that HupA directly binds to the opening of the active site in AChE, preventing the access of the endogenous substrate (Raves et al. 1997).

The NMDA receptor is constituted by a recognition site for NMDA, a cation-selective ion channel, and binding sites for glycine, zinc and phencyclidine-like compounds, where the (+)-5-methyl-10,11-dihydro-5*H*-dibenzo[*a,d*]cyclohepten-5,10-imine maleate also binds. Additionally, the channel can be blocked by magnesium (Fig. 11) (Reynolds and Miller 1988). It was demonstrated that HupA interacts with the NMDA ion channels inducing a dose-dependent inhibition of the binding of two other NMDA antagonists: [^{3}H](+)-5-methyl-10,11-dihydro-5*H*-dibenzo[*a,d*]cyclohepten-5,10-imine maleate and [^{3}H]thienylcyclohexylpiperidine. As result, HupA showed to interact with the NMDA receptor ion channel complex and it appeared to bind in the brain synaptosomal plasma membranes, but not to the glycine, polyamine or NMDA ligand-specific sites. The non-competitive binding results suggest that HupA binds

and blocks the NMDA receptor ion channel, with subsequent calcium mobilization, at or near the phencyclidine (the parent compound of thienylcyclohexylpiperidine) and (+)-5-methyl-10,11-dihydro-5*H*-dibenzo[*a,d*]cyclohepten-5,10-imine maleate ligand sites, without psychotomimetic side effects (Gordon et al. 2001).

Analogs of huperzine A

Following the interest of looking for more effective drugs against AD, several analogs of HupA have been prepared. However, due to the rigidity of HupA molecular structure, the majority of structurally simplified analogs were inactive or less active than HupA (Ma and Gang 2004). Indeed, only few of them demonstrated obvious AChE inhibitory activity. Table 1 summarizes the most relevant data about these HupA derivative compounds, which include the (±)-10,10-dimethyl-HupA, H-3, (±)-huprine X, (±)-huprine Y, (±)-huprine Z, isovaniHupA, 5-isosteres of HupA, (−)-10-spiro-cyclopropyl-HupA, and ZT-1 (Zhou and Zhu 2000; Ros et al. 2001; Alcalá et al. 2003; Ma and Gang 2004; Jia et al. 2013). Besides the H-3 and the huprines previously mentioned, other tacrine-HupA hybrids have also been developed as AChE inhibitors (Camps et al. 2000; Gemma et al. 2006). In fact, some of these new chemical entities were specifically designed to establish tight interactions, through different binding modes, with the midgorge recognition sites of human AChE and human BuChE, and also with the catalytic or peripheral sites. They showed a markedly improved biological profile in relation to those of tacrine and HupA (Table 1) (Gemma et al. 2006).

Pharmacokinetics

The pharmacokinetics of HupA has been studied not only in different animal species but also in healthy human volunteers (Tang and Han 1999; Little et al. 2008).

In mice, following intravenous (iv) or per os (*po*) administration of [^{3}H]HupA, the blood levels declined as a biphasic profile and the absolute bioavailability was considerably high (96.9 %) (Wang et al. 1988; Tang and Han 1999; Wang et al. 2006a). Moreover, HupA was mainly distributed for kidney and liver, moderately for spleen, lung and heart, and in a smaller extent to the brain (Wang et al. 1988). However, after iv injection of HupA to mouse, an autoradiographic study showed that the compound was present in all regions of the brain, but it was particularly concentrated in frontoparietal cortex, striatal cortex, hippocampus, and nucleus accumbens (Tang et al. 1994). It was also found that the extent of HupA bound to plasma proteins is only about 17 % (Wang et al. 1988). On the other hand, the majority of the compound was excreted in the urine in 24 h, with only 2.4 % being recovered in feces; indeed, a chromatographic analysis of urine revealed that [^{3}H]HupA was excreted as prototype and also as a metabolite. Interestingly, in pregnant mice, a small amount of radioactivity was shown in the fetus after iv administration of [^{3}H]HupA (Wang et al. 1988).

According to Ma et al. (2003a), the metabolism of HupA evaluated using rat liver microsomes is primarily mediated by the cytochrome P450 (CYP) 1A2 isoenzyme, with a probable secondary contribution of CYP3A1/2; in opposition, the CYP2C11 and CYP2E1 isoforms do not seem to be involved in the metabolism of HupA (Ma et al. 2003a). The major metabolite of HupA in the rat blood was demonstrated to be the 13,14-epoxy-HupA (Fig. 12) (Garcia et al. 2004). This compound was isolated from blood and liver samples, and subsequently analyzed by electrospray ionization mass spectrometry and proton nuclear magnetic resonance spectroscopy (Garcia et al. 2004).

Using a liquid chromatography–tandem mass spectrometric (LC–MS/MS) method, Wang et al. (2004) constructed the plasma concentration–time curve of HupA in dogs after the last intramuscular (im) injection of a sustained-release formulation (10 μg kg^{-1} per day for 15 days). The peak concentration (C_{max}) was 0.36 ng mL^{-1} and it was achieved at 48 h post-dosing; the elimination half-life was 54.8 h, and the area under the concentration–time curve (AUC) was 92.6 ng h mL^{-1} (Wang et al. 2004).

Until now, the data available on the pharmacokinetics of HupA in humans is limited, probably due to the difficulty of quantifying the compound in the systemic circulation following its administration at therapeutic doses (Li et al. 2007; Bialer et al. 2010). Qian et al. (1995) studied the pharmacokinetics of HupA in six Chinese volunteers after a single supratherapeutic oral dose of 0.99 mg (tablets). Taking into account the time course of HupA plasma

concentrations achieved, its pharmacokinetic behavior seems to fit to one-compartment open model assuming a first order absorption process. The values of C_{max} and time to reach C_{max} (T_{max}) were respectively 8.4 μg L^{-1} and 79.6 min post-dosing. The estimated value for the elimination half-life was 288.5 min, allowing a bid or three-times-daily dosing in humans. In summary, HupA is rapidly absorbed from the gastrointestinal tract and quickly distributed into the body, being eliminated at a moderate rate (Qian et al. 1995). The pharmacokinetics of HupA was also investigated in twelve healthy human volunteers (males and females, age ranged from 20 to 25 years) after its administration as a single dose of 0.4 mg (tablets). In this study, quantifiable levels of HupA were found in plasma after 5-10 min post-dosing and the overall results showed a pharmacokinetics of HupA fitted to a two-compartmental open model and a biphasic profile with a rapid distribution phase followed by a slower elimination phase (Li et al. 2007).

Routes of administration and formulations

As HupA can influence the cholinergic system which results in significant peripheral side effects, it is important to improve its brain-targeting efficiency. Thus, Zhao et al. (2007) showed that the intranasal administration of HupA as an in situ gel formulation significantly increased the distribution of the drug into the rat brain tissue, especially into the cerebrum and the hippocampus. Actually, the extent of systemic and brain exposure to HupA was found to be lower after oral administration than following intranasal administration. As result, an in situ gel system developed for intranasal delivery of HupA enabled the rapid absorption to the systemic circulation, avoiding the first-pass metabolism, and it may, hence, represent a viable and non-invasive strategy for delivering the drug into the brain (Zhao et al. 2007). Likewise, in order to evaluate the interest of the intranasal route to deliver HupA into the central nervous system (CNS), Yue et al. (2007) investigated the plasma and cerebrospinal fluid levels of HupA after its administration to Sprague–Dawley rats by three different routes (iv, intragastric and intranasal). The obtained data demonstrated that the intranasal administration provided high systemic and cerebrospinal levels of HupA which were similar to those achieved by iv administration. Thus, it was clearly demonstrated that the intranasal route is an attractive non-invasive alternative for CNS-delivery of HupA (Yue et al. 2007).

Other formulations for different administration routes have also been studied. Ye et al. (2008) compared the pharmacokinetics of HupA in beagle dogs after single and multiple dose regimens using controlled drug-release patches and conventional tablets. They showed that HupA patches were able to a sustained deliver or controlled drug release in vivo. Thus, the transdermal administration of HupA lowered C_{max} value, prolonged the time to reach C_{max} (T_{max}), and produced relatively constant serum concentrations up to 84 h after the administration of a single transdermal dose of 0.2 mg cm^{-2} HupA patches. Furthermore, following application of the patches, HupA concentrations in serum increased for approximately 12-24 h and the blood concentrations were maintained approximately at 2.1 ng mL^{-1} for up to 84 h. On the other hand, the serum concentrations were maintained within the range of 2.4–4.3 ng mL^{-1} during a 2-week wearing period after multiple dosing of HupA, and the degree of fluctuation at the steady-state of transdermal (0.51 ± 0.1) and *po* (1.99 ± 0.2) administration was significantly different (Ye et al. 2008).

The use of HupA loaded poly(D,L-lactic-*co*-glycolic acid) microspheres for controlled release of the drug was also studied in dogs and in mice (Chu et al. 2006, 2007). The increase of the molecular weight of poly($_{D,L}$-lactic-*co*-glycolic acid) in relation to HupA and the small particle size of microspheres resulted in the prolongation of the release period of HupA both in vitro and in vivo. Moreover, the release of HupA from microspheres after subcutaneous (sc) injection was faster than that after im injection (Chu et al. 2006). In addition, using the passive avoidance test, the therapeutic potential of HupA microspheres intragastrically administered to mice was improved in relation to that achieved with a suspension formulation (Chu et al. 2007).

On the other hand, a clinical study developed by Xu et al. (1999) compared the efficacy and safety of 200 μg of HupA administered *po*, bid, in capsules and tablets, to patients with AD. Accordingly, the formulation (capsules vs. tablets) did not influence the effects of HupA.

Drug interactions

The treatment of patients with HupA often needs a long course medication and the combination of drugs

is often required. As a result, this may originate pharmacokinetic-based drug interactions particularly by inhibiting or inducting CYP isoenzymes. Consequently, it is useful to identify the effects of HupA on CYP expression and activity as this may predict the consequences of co-administration of HupA with other drugs.

In this context, Ma et al. (2003b) examined the effects of HupA on the activity and expression of several CYP isoforms. The results indicated that the activity and expression of liver CYP1A2, 2C11, 2B1/2, 2E1 and 3A isoenzymes are not affected in rats treated with HupA at the dose of 0.1 mg kg^{-1}; however, at higher doses (1 and 2 mg kg^{-1}) HupA may elicit a slight inductive response in CYP1A2 (Ma et al. 2003b). Hence, as CYP1A2 is involved in the metabolism of several commonly used drugs, further studies should be performed in order to assess whether HupA causes clinically relevant interactions with other CYP1A2 substrates and/or inhibitors (Zhu et al. 2004). Nevertheless, to the best of our knowledge, no in vivo drug interactions involving HupA have been yet reported in the literature.

Although no pharmacokinetic interactions have been reported, it is noteworthy that taking into account the main mechanism of action ascribed to HupA, additive cholinergic effects are expected when the drug is co-administered with other medications that increase ACh levels in the central or peripheral tissues (Pepping 2000).

Therapeutic properties

HupA seems to be a valuable therapeutic option in a variety of acute and chronic disorders (Gordon et al. 2001). Although the use of HupA is primarily described for the treatment of AD, the drug may be also beneficial in cerebrovascular type dementia (Zhou et al. 2001b). Indeed, it is effective in the improvement of several cognitive impairments, such as multi-infarct dementia, brain trauma and benign senescent forgetfulness (Wu et al. 2011). Furthermore, other pharmacological properties have been ascribed to HupA, including anti-inflammatory, antinociceptive and anticonvulsant activities and its potential against organophosphate poisoning, which extends drug value for the treatment of multiple conditions (Pepping 2000; Bialer et al. 2007; Schachter 2009; Ruan et al. 2013). Moreover, HupA seems to be an interesting therapeutic choice for the treatment of myasthenia gravis and schizophrenia, being also possible that HupA can be used for the improvement of patient cognition ability (Sun et al. 1999; Pepping 2000; Ma et al. 2007).

Each one of these therapeutic properties will be described in detail in the following sections of the present review.

Alzheimer's disease

As previously mentioned, HupA showed to be promissory firstly in the treatment of AD, the most common form of dementia (Lallement et al. 2002; Ma et al. 2007). Pathologically, this disease is characterized by the excessive extracellular accumulation of β-amyloid peptide, in the form of senile plaques and intracellular neurofibrillary tangles (Xiao et al. 2000b; Zhang et al. 2004; Liang et al. 2008; Wang et al. 2011a). Due to the lack of symptomatic or preventive therapeutic strategies effective for an escalating dementia "epidemic" like AD, the research into ethnobotanicals for memory or cognition has increased over the last years (Perry and Howes 2011). A significant correlation has been reported between the cholinergic neurodegeneration in the CNS and the cognitive deficit found in patients with AD (Cheng et al. 1996; Bai et al. 2000; Ma and Gang 2004; Liang et al. 2008; Wang et al. 2009a). As a result, the enhancement of cholinergic neurotransmission has been the major strategy used to palliate the cognitive symptoms (Cheng et al. 1996; Bai et al. 2000; Ma and Gang 2004). The cholinergic neurons mainly affected are those localized in the basal forebrain and in brain regions that are involved in learning and memory (Liang et al. 2008; Wang et al. 2011a). Indeed, some drugs that target the cholinergic system have been approved by the Food and Drug Administration for the treatment of AD symptoms, including tacrine, donepezil, rivastigmine and galantamine (Fig. 13) (Zhang 2012; Ding et al. 2012). The latter, unlike the other three, is a natural alkaloid, original from *Galanthus nivalis* L. and related plants (Amaryllidaceae family) (Ma and Gang 2008). Although all of them are AChE inhibitors, and proved to be successful in alleviating some AD symptoms of mild to moderate intensity, none of these agents prevent disease progression (Ma and Gang 2004; Ma

et al. 2007; Konrath et al. 2012). Additionally, these AChE inhibitors produce excessive side effects related to the activation of the peripheral cholinergic systems including, for example, the stomach-related side-effects (nausea and vomiting) presented by rivastigmine and the liver toxicity produced by tacrine in more than 29 % of patients, which led to their withdrawal from the market (Tang and Han 1999; Ma et al. 2007).

Theoretically, as a selective AChE inhibitor, HupA may improve the symptoms of AD patients, not interfering with the pathogenesis process of the disease. However, due to the fact that the overstimulation of glutamate receptor, particularly the NMDA receptor, is involved in the pathogenesis of this disease, HupA, as potent NMDA receptor antagonist with few side effects, may be used as a preventive agent that slows down or block the pathogenesis process in the early stage of AD (Wang et al. 1999). It is also worthy to note that HupA has no presynaptic or postsynaptic activity, and therefore, it does not influence the synthesis or the release of ACh (Pepping 2000).

HupA has been found to reverse or attenuate cognitive deficits in several animal models. Additionally, some clinical trials have also demonstrated that HupA significantly relieves memory deficits in aged subjects, patients with benign senescent forgetfulness, AD and vascular dementia (Wang and Tang 2005). Moreover, several studies indicate that the drug may be effective against the reduced ACh levels in the brain and glutamate-induced neuronal death, which are two of the most common neuronal disorders observed in AD (Pepping 2000; Bai et al. 2000). Thus, the value of HupA as therapeutic agent for the treatment of AD would be enhanced by its pharmacologically dual actions (Bai et al. 2000). As a result, the AChE inhibition and, consequently, the improvement of cognitive ability, together with the neuroprotective activity of HupA are mainly responsible by the benefits showed by HupA in the treatment of AD. Furthermore, HupA exhibits other essential requirements to be therapeutically useful in AD and other memory disorder diseases (Tang and Han 1999; Gao et al. 2000b), including its high bioavailability after oral administration, ability to penetrate into the CNS, long duration of action and minimal side effects (Wang and Tang 1998b; Wang et al. 2002a; Toribio et al. 2007; Ma et al. 2007; Wu et al. 2011; Lunardi et al. 2013).

Acetylcholinesterase inhibition HupA is a potent, reversible, highly specific, centrally active and selective AChE inhibitor (Zhu and Giacobini 1995; Tang and Han 1999; Zhu et al. 2004; Ma et al. 2006, 2007). As, in general, the AChE inhibitors increase the availability of ACh in central cholinergic synapses, compounds with this kind of pharmacological properties are promising drug candidates for the treatment of AD (Zhao and Tang 2002). In this context, Tang et al. (1989) performed the first comprehensive study directed to assess the effects of HupA on AChE activity (evaluating the ACh levels and its release), and on the cholinergic receptors. They found that HupA could produce a long-term inhibition of AChE activity in rat brain (up to 360 min) and increase the ACh levels up to 40 % at 60 min. The degree of elevation of ACh brain levels after HupA was maximal in frontal (125 %) and parietal (105 %) cortex. In frontal cortex and also in whole brain, an inverse relationship was observed between ACh levels and AChE activities following the treatment with HupA (Tang et al. 1989). Importantly, the inhibitory potency of HupA against AChE was similar or superior to that of physostigmine, galantamine, donepezil and tacrine, which are already approved for AD (Fig. 13) (Xiao et al. 2002; Zangara 2003; Ma and Gang 2004; Park et al. 2010). Furthermore, in contrast to the drugs aforementioned, HupA is a poor inhibitor of human BuChE and highly selective for AChE, which may explain its tolerability profile clinically favorable in AD patients (Zhu 1991; Filliat et al. 2002; Zangara 2003; Zhu et al. 2004; Ma and Gang 2004; Bai 2007; Wang et al. 2009a). Compared to physostigmine, HupA was found to exhibit a threefold higher inhibitory effect against AChE (Zhu 1991), and it displayed, in rodents, a relative potency in inhibiting brain AChE activity 64-times higher than that of tacrine and eightfold superior to that shown by donepezil (Cheng et al. 1996; Wang and Tang 1998b; Cheng and Tang 1998). Additionally, in opposition to other AChE inhibitors (Hallak and Giacobini 1989), repeated doses of HupA do not increase the tolerance for AChE inhibition (Laganière et al. 1991), and the AChE inhibition seems to preferentially occur in the cortex and hippocampus areas, which are the cerebral regions where the presynaptic cholinergic markers are significantly reduced in AD (Cheng and Tang 1998; Pepping 2000). In fact, when compared with donepezil and rivastigmine, (−)-HupA showed the longest effects in the elevation of cortical ACh levels (Liang and Tang

2004). The results shown by Liang and Tang (2006) also indicated that (−)-HupA has a significant higher potency than donepezil (11-fold higher) and rivastigmine (twofold higher) on increasing medial prefrontal cortex ACh and dopamine levels.

In fact, regarding some studies performed in rats, (−)-HupA appears to preferentially inhibit the tetrameric AChE in multiple brain areas, being, along with *donepezil,* the most potent inhibitor of tetrameric AChE in the cortex and, along with physostigmine, the most potent inhibitor of tetrameric AChE in the hippocampus (Zhao and Tang 2002).

More recently, a screening study reported an inhibitory activity for (−)-HupA of 96.29 and 11.85 % against the human cholinesterases isoforms AChE and BuChE, respectively, using a concentration of 2 mg mL^{-1}. Therefore, the (−)-HupA stereoisomer showed high selectivity as AChE inhibitor (Brunhofer et al. 2012).

HupA can also produce a dose-dependent increase of the levels of several neurotransmitters (e.g., ACh, norepinephrine and dopamine). Thus, following intraperitoneal (ip) administration of (−)-HupA in the rat at the doses of 0.1, 0.3, and 0.5 mg kg^{-1}, the brain levels of AChE increased by 54, 129, and 220 %, respectively. In turn, norepinephrine and dopamine levels were respectively increased by 121 and 129 % above the baseline with 0.3 mg kg^{-1}, and 143 and 153 % with 0.5 mg kg^{-1}. These data suggest that HupA has both cortical and subcortical effects. In opposition, the levels of serotonin in the rat brain were not altered through HupA administration (Zhu and Giacobini 1995).

Hence, HupA fits closely with the established criteria for an ideal AChE inhibitor to be used in clinical studies (Cheng and Tang 1998). It has been observed in elderly people that HupA increases the ACh levels and it has a positive effect on the cerebral cholinergic system during the recovery from general anesthesia (Wang et al. 2006a).

Despite all these studies, some enzymes may significantly differ between species and it should be considered during the prediction of the most suitable properties of potential new compounds. In this context, it has been reported that cholinesterase activity in human blood is higher than in rodents. Actually, the human blood AChE is 4- and 6-fold more active than the corresponding enzyme in mouse and rat. On the other hand, for BuChE these proportions are of 2- and 11-fold, respectively (Rudakova et al. 2011). Moreover, another report refers that rat or rabbit plasma has lower BuChE than AChE activity, while mouse or guinea pig plasma has more BuChE than AChE activity (García-Ayllón et al. 2010). Relatively to BuChE, for example, it can be expressed in multiple molecular forms, being its catalytic activity also dependent on the tissue distribution and species involved. These inter-species differences are probably explained by subtle changes in the amino acid sequence as it can be seen by comparing the BuChE from human and rat, in which the amino acids of the active site region differ in eight residues (Pauliková et al. 2006). Thus, the differences between species need to be taken into account because they may greatly influence the results and final conclusions.

Cognitive decline The ability of HupA to improve the performance in neurobehavioral tasks that involve learning and memory, in which the central cholinergic system plays an important role (Xiong and Tang 1995), may be at least partly explained by its action as an AChE inhibitor, which leads to an increase of ACh levels in the synaptic cleft (Gao et al. 2000b). Moreover, compared with donepezil and tacrine, the improved effect of HupA in memory deficits is more potent on working memory than on reference memory. This is particularly important for AD patients because their severe cognitive deficits on the memory of recent events (Bai 2007). As a result, HupA is regarded as a promising clinical drug for the therapy of cognitive impairment observed in elderly people and patients with AD (Gao et al. 2000b). Recently, the findings of the study performed by Lunardi et al. (2013) reinforced the idea that (−)-HupA does not act exclusively on the ACh balance to improve the cognitive deficit observed in AD patients. In fact, the compound seems to act via nicotinic receptors when evaluating the astroglial S100B secretion, which is an astrocyte-derived protein that has been proposed to be a marker of brain injury (Lunardi et al. 2013).

Tang et al. (1986) and Lu et al. (1988) studied the effects of HupA on learning and retrieval discrimination processes performed by rats, and suggested that the effects occurred at the central cholinergic system. Thus, employing the in a Y-maze test to rats, HupA appeared to facilitate their learning and retrieval processes, being such effects antagonized by scopolamine or atropine

(Tang et al. 1989). The effects of HupA on learning and memory retention were superior to those of physostigmine and it was also found that HupA could reverse the scopolamine-induced memory deficits in rats more easily than donepezil and tacrine (Tang et al. 1989; Cheng et al. 1996). In this context, the treatment with HupA for eight consecutive days (0.25 mg kg^{-1}, *po*, once a day) was as potent as the acute treatment on attenuating the scopolamine-induced amnesia in rats (Xiong and Tang 1995). HupA (0.2 mg kg^{-1}, ip) has also been shown to ameliorate the nucleus basalis magnocellularis lesion-induced spatial working memory impairment in Sprague–Dawley rats (Xiong et al. 1998). Moreover, the daily oral administration of (−)-HupA (0.1 mg kg^{-1}) chronically to hypoperfused rats improved the cognitive dysfunction in the late phase. This effect derived not only from HupA actions in the cholinergic system, but also from the effects of the compound on the oxygen free radical system and energy metabolism. Thus, HupA showed potential for the treatment of dementia caused not only by cholinergic dysfunction, but also by decreasing the cerebral blood flow (Wang et al. 2000). The findings reported by Wang et al. (2001) revealed that the daily administration of (−)-HupA (ip) for twelve consecutive days to rats produced a significant reversal of the β-amyloid peptide-(1-40)-induced deficit in the water maze learning task. Consequently, the beneficial effects of HupA includes favorable changes in the expression of apoptosis-related proteins and in the extent of apoptosis in widespread regions of the brain (Wang et al. 2001). HupA has also been reported to improve the cognitive function of rats recovering from general anesthesia due to its inhibition of brain cholinesterases (Zhang et al. 2008).

Taking into account all the aforementioned works, it is undoubted that HupA exerts beneficial effects on memory deficits in various rodent models of amnesia. In order to investigate the antiamnesic action of HupA in non-human primates, Ye et al. (1999) evaluated the ability of HupA to reverse the deficits in spatial memory produced by scopolamine in young adult monkeys or those that naturally occur in aged monkeys using a delayed-response task. In both groups, the administration of HupA (0.01-0.1 mg kg^{-1}, im) improved the spatial working memory by a cholinergic mechanism (Ye et al. 1999). HupA also improved the memory impairments induced by the administration of reserpine or yohimbine to monkeys, probably through adrenergic mechanisms. Accordingly, these results also corroborate the potential application of HupA for clinical treatment of AD patients, since multiple neurotransmitters are decreased in these patients (Ou et al. 2001).

Resorting to the passive avoidance task test, the effects of HupA on disruption of spatial memory induced by scopolamine and muscimol [(a γ-aminobutyric acid A ($GABA_A$) agonist] in chick were also studied. By this means, HupA improved the process of memory formation, exhibiting a bell-shaped dose–response curve. These resulted not only from HupA action as a highly potent and selective inhibitor of AChE, but also from its antagonist effects mediated through the γ-aminobutyric acid A ($GABA_A$) receptor (Gao et al. 2000b).

A significant improvement of the memory deficiencies in aged and AD patients treated with HupA has been importantly demonstrated in several clinical trials (Ma and Gang 2004). Most of these clinical studies were performed in China, and their results indicated that HupA is an effective and safe drug that improves cognitive function, including in patients with AD (Ma et al. 2006; Ma et al. 2007). In a multicenter, prospective, double-bind, parallel, placebo controlled, randomized clinical trial, HupA was orally administrated at the dose of 0.2 mg to patients with AD and demonstrated to improve cognitive and behavioral functions in approximately 58 % of the patients (Xu et al. 1995). Other clinical trials demonstrated that HupA significantly improves memory deficits in elderly people with benign senescent forgetfulness and patients with AD (Zangara 2003; Ma et al. 2007). Zhang et al. (1991) conducted a randomized, double-blind trial to evaluate the effect of HupA on the treatment of senile memory disorders (multi-infarct dementia, senile and pre-senile simple memory disorders). Specifically, a dose of 0.05 mg of HupA (im, bid) or placebo was given to a first group of patients for four weeks, and a dose of 0.03 mg of HupA (im, bid) or placebo was administered to a second group for two weeks. In both conditions, the treatment with HupA showed significant improvements (Zhang et al. 1991; Zangara 2003; Ma et al. 2007). Additionally, another trial was conducted in 80 individuals, including patients diagnosed with vascular dementia ($n = 25$) and AD ($n = 55$). Using the popular Chinese memory quotient test, a better score for the group treated with HupA (0.1 mg four times

daily) was found in relation to the control group (Ha et al. 2011). These findings were similarly corroborated by Zhang et al. (2002). Accordingly, in this study, HupA showed to be a safe and effective medicine to AD, improving the cognition, behavior, daily life activities and mood of the treated patients (Zhang et al. 2002).

In United States of America, an open-label pilot study was performed to evaluate the administration of HupA to patients diagnosed with AD, and, once again, the results suggested that HupA improves the cognitive function measured by the mini-mental state examination. Importantly, HupA revealed to be very well tolerated at doses up to 200 μg bid (Little et al. 2008). More recently, based on phase II clinical study, Rafii et al. (2011) stated that the treatment with HupA (200 μg, bid, over 16 weeks) did not cause significant changes in mild to moderate patients with AD, but the higher dose of HupA 400 μg, bid, induced cognitive benefits.

Neuroprotection There are several evidences suggesting the interest of HupA as an useful neuroprotective agent (Kozikowski and Tückmantel 1999). These potential effects of HupA are related to its ability to regulate the expression of apoptotic proteins (Wang et al. 2001; Zhou et al. 2001b; Xiao et al. 2002; Zhou and Tang 2002), protect mitochondria (Gao and Tang 2006; Gao et al. 2009), attenuate oxidative stress (Shang et al. 1999; Xiao et al. 1999; Xiao et al. 2000a, b), and modulate the metabolism of β-amyloid precursor protein (Zhang et al. 2004; Liang et al. 2008; Wang et al. 2011a, 2012). HupA has also been found to exert effects on the nitric oxide-induced (Zhao and Li 1999; Zhao and Li 2002) and glutamate-mediated neurotoxicity (Raves et al. 1997; Ved et al. 1997; Wang et al. 1999; Pepping 2000; Bai et al. 2000; Zangara 2003). Interestingly, HupA appears to reduce the iron levels in the brain, which is a novel mechanism recognized to interfere in the pathologic process of AD (Huang et al. 2013). More recently, it was demonstrated that HupA also promotes the hippocampal neurogenesis in vitro and in vivo, enhancing significantly the proliferation of cultured hippocampal neural stem cells through a mechanism that involves the extracellular signal-regulated kinase activation. These findings suggest a new neurogenesis-related mechanism for HupA, showing again its interest for the prevention and treatment of a variety of neurological disorders (Ma et al. 2013). However, although HupA is a drug of interest to treat AD, the lack of efficacy of (−)-HupA in the prevention of colchicine-induced apoptosis in cerebellar granule neurons suggests that it cannot prevent neuronal loss due to cytoskeleton alterations (Jordá et al. 2004). Hence, the mechanisms suggested to be involved in the multiple neuroprotective effects induced by HupA include the activation of both muscarinic and nicotinic ACh receptors, the enhancement of the production of neurotrophic factors and the blocking of overstimulated NMDA receptors (Tang et al. 2005a, b; Wang et al. 2006c; Wu et al. 2011).

Protection against hypoxic-ischemic toxicity

HupA proved to be beneficial in cerebrovascular dementia and other neurodegenerative disorders with an underlying ischemic component (Zhou et al. 2001a, b). In fact, Zhou et al. (2001a) demonstrated the benefits of HupA in the neuron-like rat pheochromocytoma (PC12) cells against oxygen-glucose deprivation-induced toxicity, most likely by alleviating disturbances of the oxidative and energy metabolism. These results were confirmed and extended by Zhou et al. (2001b), who similarly investigated the protective effects of HupA on transient global ischemia in gerbils. The results of this study support that the oral treatment with HupA 0.1 mg kg^{-1}, bid, reduces the memory impairment and neuronal degeneration in the CA1 region of the gerbils, and partially restored the choline acetyltransferase activity in the hippocampus. Consequently, the ability of HupA to attenuate the memory deficits and neuronal damages after ischemia may be advantageous in cerebrovascular type dementia. The findings of this study also suggest that HupA has therapeutic and neurotrophic effects in cerebral ischemia stemming displayed by multiple mechanisms, which includes cholinergic function. Indeed, it was recognized that ACh can potentiate the protective actions of nerve growth factor against the ischemic insults (Zhou et al. 2001b). The mitochondrial dysfunction induced in a middle cerebral artery occlusion rat model was also ameliorated by treating the animals with HupA 0.1 mg kg^{-1}, which may partially contribute to HupA protective effects on brain damages after 24 h of reperfusion. Indeed, the mitochondrial dysfunction has been proved to

contribute to ischemia-induced brain damage (Zheng et al. 2008).

Moreover, HupA may also be beneficial in hypoxic–ischemic encephalopathy in neonatal rats, which is a major cause of acute mortality and chronic disability in survivors. Accordingly, this compound, administered at a dose of 0.1 mg kg^{-1} (ip) during 5 weeks, significantly attenuates the cognitive deficits and the brain injury in neonatal rats after hypoxic–ischemic brain insult (Wang et al. 2002a). These potential therapeutic properties of HupA were also confirmed latter by the same authors (Wang et al. 2003). More recently, a study performed in male Sprague–Dawley rats also suggested that the supplementation with oral HupA at 0.1 mg kg^{-1} improves the cognitive deficits, reduces the oxidative stress and inhibits the apoptotic cascade induced by acute hypobaric hypoxia in the hippocampus of the exposed rats (Shi et al. 2012).

Actually, associated with the properties previously described, it was recently suggested a cardioprotective potential for HupA in myocardial ischemic damage using a rat model. In this context, several mechanisms may contribute to this effect, including the anti-oxidative, anti-apoptotic and anti-inflammatory activities. Additionally, the infarct size was significantly reduced by HupA, which also inhibited the activity of the myocardial enzymes creatine kinase, MB isoenzyme of creatine kinase, lactate dehydrogenase and cardiac troponin T (Sui and Gao 2014).

Anti-inflammatory activity

Chronic cerebral hypoperfusion, which involves inflammatory processes and white matter lesions, is a common pathological feature that highly contributes to the progression of dementias (Wang et al. 2010). The inflammatory response is also involved in cerebral ischemia as a consequence of the activation of glial cells and resident macrophages, and of the infiltration of peripheral inflammatory cells into the brain (Wang and Tang 2007; Wang et al. 2008). Thereby, the process of inflammation contributes to the late stages of ischemic injury, reduces the neuronal survival and worsens the neurologic outcome. It was discovered that some AChE inhibitors, besides inhibiting the AChE, also suppress several inflammatory reactions such as T cell proliferation, cytokine production and CNS inflammation (Wang and Tang 2007). The oxidative stress plays a major role in the chronic inflammatory process named "inflamm-aging", which is characterized by the low-grade inflammation that occurs during aging and in age-associated diseases. Actually, AChE inhibitors demonstrated to enhance the cholinergic transmission and to act as anti-inflammatory agents under those circumstances (Ruan et al. 2013).

Aiming at investigating whether HupA has anti-inflammatory properties similar to those ascribed to other AChE inhibitors, Wang and Tang (2007) tested the anti-inflammatory effect of HupA in in vitro conditions applying an ischemia model based on oxygen–glucose deprivation in C6 rat glioma cells. The treatment with 1 μM HupA inhibited the activation of the nuclear translocation of nuclear factor-kappa B, attenuated the inducible nitric oxide synthase, cyclooxygenase-2 (COX-2) and nitric oxide overexpression, promoting the survival of the C6 cells subjected to oxygen–glucose deprivation (Wang and Tang 2007). These neuroprotective effects demonstrated by HupA against cerebral ischemia-induced brain injury may partly involve a cholinergic anti-inflammatory pathway in which α7 nicotinic ACh receptors play an essential role (Wang and Tang 2007; Wang et al. 2008; Wang et al. 2010). In this context, in the cell model of chronic hypoxia, HupA suppressed the inflammatory factor tumor necrosis factor-α and the overphosphorylation of c-Jun N-terminal kinases and p38 mitogen-activated protein kinases. As result, these effects of HupA could contribute to the amelioration of spatial cognitive impairment caused by chronic cerebral hypoperfusion, being a potential therapeutic strategy for the clinical treatment of long-term inflammation diseases (Wang et al. 2010).

The co-administration during 8 weeks of HupA (0.1 mg kg^{-1}, sc) and D-galactose (300 mg kg^{-1}, sc) not only significantly decreased hepatic function impairment, reactive oxygen species generation and oxidative damage, but also suppressed inflamm-aging by inhibiting hepatic replicative senescence, AChE activity, IκBα degradation, nuclear factor-kappa B p65 nuclear translocation and inflammatory responses. Furthermore, there was a decrease in the expression levels of pro-inflammatory cytokine messenger ribonucleic acid and tumor necrosis factor-α, *interleukin*-1β and *interleukin*-6, and an increase in the levels of the anti-inflammatory cytokine *interleukin*-10. Hence, the protective effects of HupA resulted from the

inhibition of AChE and from the activation of cholinergic anti-inflammatory pathway (Ruan et al. 2013). The treatment of Sprague–Dawley rats with HupA (0.1 mg kg^{-1} day^{-1}, ip) for 3 days also demonstrated to be beneficial in rat acetic acid-induced colitis model via inhibition of the release of reactive oxygen metabolites and pro-inflammatory cytokines, partly by neutrophils infiltrating the injured tissue. In this study, HupA reduced the extent of colonic lesions, increased colonic malondialdehyde level, high myeloperoxidase activity and nuclear factor-kappa B expression in the colitis group, attenuated the elevation of serum *interleukin*-1β level due to colitis, and was effective to reverse colitis-induced high lucigenin-enhanced chemiluminescence values and serum tumor necrosis factor-α levels (Kolgazi et al. 2013).

Antinociceptive activity

HupA displays antinociceptive activity which is primarily dependent of the stimulation of muscarinic cholinergic receptors, but independent of opioid and α2-adrenoceptors (Bialer et al. 2007; Park et al. 2010). The administration of HupA at 1 mg kg^{-1} (ip) to the mouse formalin pain model inhibited the pain behavior in all treated animals at all-time points. Moreover, a near complete inhibition of pain was also produced by HupA at the dose of 0.5 mg kg^{-1} (ip), which represents 60 % of the median toxic dose (TD_{50}) (Bialer et al. 2007; Bialer et al. 2010). Interestingly, in the sciatic ligature model of neuropathic pain, similar results were obtained with HupA 1 mg kg^{-1} (ip) (Bialer et al. 2010). Despite these findings it should be noted that the HupA doses that have demonstrated therapeutic potential as antinociceptive seem to be higher than the median toxic dose (TD_{50}) estimated for this compound. Thus, taking this information into account, it is not expected a favorable therapeutic index for the compound as antinociceptive agent. In order to better characterize the antinociceptive effects of HupA, Park et al. (2010) implanted intrathecal catheters in Holtzman rats to assess the thermal escape latency using Hargreaves thermal escape testing system and the flinching behavior elicited by formalin test. From these assays, it was observed that the intrathecal administration of HupA induced a dose-dependent increase in the thermal escape latency with a median effective dose (ED_{50}) of 0.57 μg and decreased in a dose-dependent manner the flinching behavior. Moreover, the intrathecal pre-treatment with atropine (15 μg), a nonspecific muscarinic antagonist, largely blocked the antinociceptive effects induced by HupA (10 μg) in both rat models, confirming the spinal muscarinic activity of HupA (Park et al. 2010). Recently, it was reported that (−)-HupA demonstrated significant analgesic properties when administrated ip or intrathecally to adult female Sprague–Dawley rats subjected to moderate static compression of T10 spinal cord. This pain-ameliorating effect of (−)-HupA is cholinergic dependent and the rats manifested no drug tolerance following repeated bolus ip. Moreover, (−)-HupA also appears to reduce neural inflammation, retain higher numbers of calcium-impermeable GluR2-containing AMPA (α-amino-3-hydroxy-5-methyl-4-isoxazolepropionate) receptors, and prevent Homer1a up-regulation in dorsal horn sensory neurons. This compound may, hence, provide a safe and effective option for chronic postneurotrauma pain by reestablishing homeostasis of sensory circuits (Yu et al. 2013).

Anticonvulsant activity

The anticonvulsant activity exhibited by HupA has been documented from several recent studies, making this compound of potential interest for controlling epileptic seizures (Bialer et al. 2007; Coleman et al. 2008; Bialer et al. 2009; Schachter 2009; Schneider et al. 2009; Bialer et al. 2010). Epilepsy is one of the most common serious chronic neurological disorders and, although a wide variety of pharmacological treatments is available to provide a better quality of life, more than 30 % of the patients remains not seizure free mainly due to the pharmacoresistance phenomena and, consequently, an extensive research for the ideal antiepileptic drug continues currently (Zhang et al. 2012). It is also true that patients with epilepsy have used a variety of herbal therapies over thousands of years. In fact, nowadays, herbal medicines are among the complementary and alternative medical therapies most commonly used in epilepsy (Schachter 2009).

Based on its proposed mechanism of action as a noncompetitive NMDA receptor antagonist, HupA has been evaluated as a potential anticonvulsant compound (Schachter 2009). Besides that, both (−)-HupA and (+)-HupA block the NMDA channel similarly (Fig. 1) (Coleman et al. 2008). Thus,

following oral administration of HupA (1 mg kg^{-1}) to Swiss-Webster mice, it was found that HupA protected the animals against pentylenetetrazole-induced seizures, reaching the peak anticonvulsant activity (62.5 % protection) at 1 h post-dosing; however, it was ineffective against the maximal electroshock-induced seizures in the same rodent species. The toxicity of HupA was investigated submitting treated mice to the rotarod test; a median toxic dose (TD_{50}) value of 0.83 mg kg^{-1} was achieved (Bialer et al. 2007, 2009; Schachter 2009; Bialer et al. 2010). In the 6-Hz model, the values found for the median effective dose (ED_{50}) after the administration of HupA (ip) were 0.28, 0.34 and 0.78 mg kg^{-1} for stimulation currents of 22, 32 and 44 mA, respectively. This data suggest a possible advantage over marketed anticonvulsant drugs such as phenytoin, carbamazepine, lamotrigine and topiramate, since they displayed limited efficacy in the 6-Hz model at doses devoid of behavioral toxicity. It is also suggested a further possible advantage of HupA over other active-drugs in this model, such as levetiracetam (Bialer et al. 2007, 2009; Schachter 2009; Bialer et al. 2010). A preliminary study was performed using the radiotelemetry rat seizure/*status epilepticus* model, and demonstrated that the animals pre-treatment with (+)-HupA did not exhibit the seizures usually induced by pilocarpine (a muscarinic agonist); in opposition, (−)-HupA did not exhibit the same protection effect (Fig. 1) (Coleman et al. 2008). The authors suggested that the protection induced by (+)-HupA was a result of its in vivo NMDA antagonism, without inhibiting AChE. Indeed, this hypothesis was proved because the pre- and post-exposure to (+)-HupA (3 mg kg^{-1}, im) protected animals against NMDA-induced seizures, increasing their survival (Coleman et al. 2008).

Furthermore, Schneider et al. (2009) described, for the first time, the use of HupA to treat partial behavioral seizures in dogs. Thus, when the compound was administered for more than 6 months as the only medication, it was successful in treatment of this type of seizure. In this case, the seizure activity improved considerably in both frequency and intensity. As a result, the authors stated that HupA might be an alternative to conventional anticonvulsant medications particularly in benign focal seizures (Schneider et al. 2009). Nevertheless, to better determine what types of seizures can be controlled with HupA and for how long the treatment is effective, appropriate clinical trials should be performed in order to evaluate the tolerability and efficacy of HupA in patients with epilepsy.

Organophosphate poisoning

Organophosphate compounds are very potent neurotoxic agents (Tonduli et al. 2001), recognized as potential threats in military and terrorism situations (Filliat et al. 2002; Lallement et al. 2002). These agents irreversibly inhibit the AChE enzyme in the peripheral nervous system and CNS (Tonduli et al. 2001; Lallement et al. 2002), leading to the accumulation of ACh and consequent release of excitatory amino acids, which appear to be responsible for the toxicity of organophosphate nerve agents (Filliat et al. 2002; Coleman et al. 2008). Among the excitatory amino acids released in organophosphate poisoning conditions, glutamate is one of the most potent one. Indeed, this excitatory amino acid over-stimulates NMDA receptors, contributing to uncontrolled seizures, development or evolution of *status epilepticus,* neuronal damage and neurobehavioral deficits (Coleman et al. 2008; Wang et al. 2013). It is worthy to note that the rapid "aging" of the inhibited AChE enzyme leads to a non-functional AChE and strongly limits the treatment options. Furthermore, organophosphate agents also inhibit the BuChE enzyme which contributes to their respiratory toxicity (Coleman et al. 2008).

HupA appears as one of the most effective agents for prophylaxis against the toxic effects of organophosphate nerve gases used in chemical warfare like sarin and soman. Compared with the already commonly available agents, such as pyridostigmine and physostigmine, HupA apparently has a better therapeutic index and longer half-life, protecting against not only ACh-related toxicity but also glutamate-related toxicity induced by poisons (Pepping 2000). Consequently, HupA may provide a safe and long-lasting prophylactic treatment against nerve agent poisoning in humans, particularly due to its remarkable selectivity for AChE enzyme and its chemical stability (Grunwald et al. 1994). In addition, in contrast to carbamates, such as physostigmine, HupA molecule is not modified upon the interaction with AChE enzyme and the HupA-AChE complex has a longer half-life time (Ashani et al. 1992; Gordon et al. 2001). Consequently, the pre-treatment with HupA may be promising against nerve agents toxicity by

protecting AChE from the irreversible organophosphate-induced phosphorylation (Gordon et al. 2001). On the other hand, the BuChE enzyme is poorly inhibited by HupA, hampering the respiratory toxicity induced by the organophosphate nerve agents (Filliat et al. 2002; Boudinot et al. 2005).

Grunwald et al. (1994) also studied the ability of HupA to protect against nerve agent poisoning. For that, HupA was intraperitoneally administered to mice, and the median lethal dose (LD_{50}) of the organophosphate nerve agent, soman (sc), was determined at various time points after HupA pretreatment. HupA exhibited a protective ratio (LD_{50} in protected animals divided by LD_{50} in untreated mice) of approximately 2, which was maintained for at least 6 h after a single injection, without requiring a post-challenge drug therapy. The authors reported that this long-lasting antidotal efficacy displayed by HupA was correlated with the time course of the blood-AChE inhibition, and suggested that the protective effect was a result from the temporary sequestration of the active site region of the AChE enzyme (Grunwald et al. 1994). In guinea pigs, HupA pretreatment with 0.5 mg kg^{-1} (ip) was found to totally prevent seizures and the subsequent hippocampal neuropathological changes, ensuring the survival of all animals for up to 24 h after intoxication with soman. In opposition, all animals pretreated with pyridostigmine exhibited epileptic seizures after soman poisoning, and five out of six animals died. This was the first evidence that HupA successfully protects against soman-induced convulsions and neuropathological changes in the hippocampus, as a consequence of its effect on peripheral and central AChE enzyme (Lallement et al. 1997). These findings were corroborated in a recent study in which (+)-HupA 40 mg kg^{-1} significantly reduced the behavioral signs of soman toxicity in guinea pigs and, importantly, preserved higher blood and brain AChE activity compared to pyridostigmine, demonstrating its less toxicity (Wang et al. 2011b). At this point it is important to highlight the pharmacotoxicological impact of the stereochemical properties of HupA; indeed, as referred by Wang et al. (2011b), (−)-HupA is toxic at higher doses due to the potent AChE inhibition which limits its use as neuroprotective, while (+)-HupA is a weak inhibitor of AChE and therefore is a non-toxic compound even at higher doses (e.g. 40 mg kg^{-1} in guinea pigs). Additionally, it was observed that the pre-treatment with HupA 500 μg kg^{-1} ip also prevented the epileptic activity induced by soman in male Sprague–Dawley rats. The AChE inhibition was reduced to 54 % and the ACh levels significantly increased in comparison to the baseline values. Accordingly, HupA acts at the enzymatic level protecting the AChE, reduces the hypercholinergic activity at the neurochemical level, and increases the gamma index at the electrophysiological level, which represent three parameters responsible for seizure occurrence in intoxicated animal (Tonduli et al. 2001). Consequently, HupA appears to be a promising antidote as a prophylactic drug against organophosphate compounds (Lallement et al. 1997).

Although each stereoisomer of HupA undoubtedly protect against NMDA-induced seizures, reduce glutamate-induced toxicity and prevent soman-induced toxicity, it is important to highlight that a unique combination of these two stereoisomers [40 mg kg^{-1} of (+)-HupA with 0.3 mg kg^{-1} of (−)-HupA, Fig. 1] offers a better protection than the single (+)-HupA isomer. Indeed, in comparison to (+)-HupA alone, this particular stereoisomer combination significantly increased the survival rate, reduced behavioral abnormalities and inhibited the development of high power of electroencephalogram in guinea pigs exposed to high doses of soman. The stronger protection effect observed may be result of the reversible AChE inhibition by (−)-HupA at low doses and the better neuroprotective effects of (+)-HupA at higher doses. Additionally, this combination may also reduce the toxicity of (−)-HupA for therapeutic application (Wang et al. 2013).

Other data demonstrated that the combination of HupA (50 μg kg^{-1} sc, 15 min before diisopropyl fluorophosphate) with imidazenil (2 mg kg^{-1} sc, 30 min before diisopropyl fluorophosphate), which is a partial agonist of benzodiazepine receptors, is a potent and safe prophylactic therapeutic strategy to overcome diisopropyl fluorophosphates toxicity in mice (Pibiri et al. 2008).

In mice, HupA also showed a protective effect on blood and brain AChE and inhibited the acute poisoning induced by isocarbophos, another organophosphate compound (Liu et al. 2006, 2013). However, it was also demonstrated that the administration of HupA has no effects on the neurotransmitter changes induced by the acute poisoning of phoxim (Liu et al. 2013).

In all these studies, as in others previously mentioned, the differences between species should be considered. Specifically, for organophosphate poisoning, the guinea pig is a commonly used animal model. In comparison with rat and mouse, guinea pig appears to be a more relevant species for studying the primate susceptibility to organophosphate poisoning. This fact is explain by its low relative concentration of serum carboxylesterase, which is an enzyme known to bind organophosphates in vitro, acting as an endogenous bioscavenger (Cadieux et al. 2010). However, Cadieux et al. (2010) reported some differences between human and guinea pig AChE (Cadieux et al. 2010). In fact, previous studies also reported some considerable differences between AChE enzymes from frog, chicken and rat brain regarding to the rate constants for AChE inhibition by some organophosphate compounds (Andersen et al. 1977). Consequently, the use of a specific animal model to evaluate some therapeutic properties should be careful selected in order to enable a suitable extrapolation of the results for humans.

Myasthenia gravis

The literature refers that HupA may improve the symptoms of myasthenia gravis, which is a rare but serious autoimmune neuromuscular disease (Pepping 2000; Ma and Gang 2004). To the best of our knowledge, there is only one study that investigated whether HupA would be therapeutically successful in the treatment of myasthenia gravis. In this open-label study, the clinical manifestations of myasthenia gravis were controlled in the treatment group ($n = 59$) administered with HupA 0.4 mg day^{-1} (im) for 10 days, while in the control group ($n = 69$) the patients were treated with neostigmine 0.5 mg day^{-1} (im) every other day and HupA 0.4 mg day^{-1} (im) on the intervening day. The results revealed that the administration of HupA improved muscle weakness in the 128 patients with myasthenia gravis. Overall, HupA exhibited a mean duration of action of 7 h whereas the neostigmine showed a duration of action of only 4 h (Pepping 2000).

Schizophrenia

In line with the effects of HupA on the cognitive impairment related with AD, the effect of HupA on memory disorders in schizophrenic patients have similarly been studied by some authors. In all those studies, the memory functions of patients were significantly improved after the treatment with HupA (Ma et al. 2007). The potential of HupA as add-on therapy in schizophrenic patients who did not obtain satisfactory response to antipsychotic treatments and had apparent cognitive impairments were likewise investigated in a small open-label clinical study ($n = 19$). This pilot clinical trial demonstrated the beneficial effects of HupA in treating cognitive and negative symptom clusters of schizophrenia over 12 weeks (Zhang et al. 2007). In this context, a clinical trial of phase II was performed in order to evaluate the potential of HupA in the cognitive and functional impairment in schizophrenia; although it was finished in the last year in the United States of America, the results are not yet available (Woods 2013).

Cognitive ability

Despite the recognized positive effects of HupA in patients with cognitive impairment and aged animals, permitting to restore or ameliorate a compromised system to a normal level of functioning, the compound may not be effective in enhancing cognitive function beyond normal levels. This idea is supported by the lack of a clear effect of (−)-HupA on the improvement cognitive function in normal young monkeys (Malkova et al. 2011). However, in a preliminary clinical study conducted in junior middle school students complained of memory inadequacy, HupA significantly improved the cognition ability. Accordingly, using a double-bind and matched pair method, it was also found that the oral administration of HupA (50 μg, bid) enhances the memory and learning performance of the students ($n = 34$) (Sun et al. 1999). In the same way, Filliat et al. (2002) also described a memory enhancing effect after the subchronic administration of HupA 1 μg h^{-1} to guinea pig, although such effect was limited only to the first day of the test.

Toxicity

The clinical use of HupA is becoming known on a widespread scale, not only because of its wide range of therapeutic applications, but also because it is a well-

tolerated drug with no serious adverse effects reported up to date (Pepping 2000; Ou et al. 2001; Wang et al. 2009a; Sharma 2010). Indeed, the side effects observed during HupA treatments are mild and only observed at high doses, with no adverse signs observed at doses lower than 0.3-0.5 mg kg^{-1} in rats, 0.1 mg kg^{-1} in monkeys and 0.5 mg kg^{-1} in humans (Filliat et al. 2002). Furthermore, no tolerance phenomena occur after multiple dosing treatments in rat (Little et al. 2008). However, due to the potent and strongly specific AChE inhibition, (−)-HupA has revealed some toxicity at doses greater than 0.5 mg kg^{-1} (Skolnick 1997; Wang et al. 2011b); whereas its synthetic stereoisomer is less toxic because of its weaker inhibitory activity on AChE (Wang et al. 2011b).

HupA adverse-effect profile seems to be favorable with those of the conventionally prescribed AChE inhibitors (Tang and Han 1999; Pepping 2000; Ma et al. 2007). Indeed, HupA has less severe undesirable side effects associated with cholinergic activation, as confirmed by several toxicological studies conducted in different animal species (Yan et al. 1987; Wang and Tang 1998a; Zangara 2003). Consequently, due to the higher selectivity of HupA for the AChE enzyme expressed in brain, its cholinergic adverse-effect profile is considerably less severe than those of tacrine or donepezil in rats (Wang and Tang 1998b; Tang and Han 1999; Pepping 2000). The adverse effects observed in guinea pigs consist mostly of fasciculation, which disappeared within 2–3 h after a dose of 0.5 mg kg^{-1} has been administered and 4 h after an administered dose of 2 mg kg^{-1} (Filliat et al. 2002). Nevertheless, a large number of studies conducted in other animal species (mice, rats, rabbits and dogs) showed that HupA has mild to no side effects or toxicity such as fasciculation or other cholinergic hyperactivity symptoms (Tang and Han 1999; Zhang et al. 2004). At this point, Wang and Tang (1998a) revealed that fasciculation and other cholinergic signs did not occur after administering HupA at the dose of 0.48 mg kg^{-1} to rats. The LD_{50} of HupA were 4.6 mg *po*, 3.0 mg sc, 1.8 mg ip and 0.63 mg iv, in mice (Tang and Han 1999; Zangara 2003; Wang et al. 2006b; Ma et al. 2007), whereas the value for (−)-HupA after its ip administration was 2.4 mg kg^{-1} in rats (Wang et al. 2011b). Moreover, the LD_{50} for HupA was found to be 2–4 mg kg^{-1} in female rats and greater than 4 mg kg^{-1} in male rats, when administered as a single oral dose (Little et al. 2008; Ha et al. 2011). Additionally, data included in a Memorandum from Food and Drug Administration identified a favourable treatment index [LD50/median effective dose (ED50)] of 23.1 for (−)-HupA following ip administration in mice, and of 72.9 following ip administration in rats. In fact, this treatment index appear to be more favourable for HupA than that reported for neostigmine (8.6 in mice and 34.0 in rats via ip testing) or for physostigmine (3.8 in mice and 7.2 in rats via ip testing) (FDA 1999).

Histopathological examinations following subacute toxicity studies showed no changes in liver, kidney, heart, lung or brain after administration of HupA for 180 days, neither in rats (1.5 mg kg^{-1} *po*) nor in dogs (0.6 mg kg^{-1}, im) (Tang and Han 1999; Zangara 2003; Zhang et al. 2004; Wang et al. 2006b; Ma et al. 2007). Additionally, although both HupA and tacrine increase the activity of serum aspartate aminotransferase and alanine aminotransferase enzymes in rats, histopathologic changes in liver were only induced by tacrine. Furthermore, in opposition to tacrine, atropine reverted the hepatic acute effects of HupA, suggesting that the effects of HupA on rat liver were not related to a direct hepatotoxicity (Ma et al. 2003c). In a recent study, where guinea pigs were exposed to (−)-HupA at doses in the range of 5-625 μg kg^{-1}, it was found an increase of the levels of antioxidants, glutathione reductase and oxidative stress markers in a dose-dependent manner in several brain areas and cerebellum; similar effects were observed in liver, kidney and spleen but with a milder intensity (Pohanka et al. 2012). On the other hand, after the administration of HupA, no mutagenicity was found in rats and no teratogenic effects was detected in mice (0.019-0.38 mg kg^{-1}, ip) or rabbits (0.02-0.2 mg kg^{-1}, im) (Tang and Han 1999; Zangara 2003; Wang et al. 2006b). In addition, HupA did not induce deleterious effects on spatial memory when administered subchronically to guinea pig (Filliat et al. 2002).

Regarding the respiratory function, (−)-HupA did not appear to perturb respiration at a dose that inhibits 40 % of AChE and, even at a lethal dose, HupA did not affect any important respiratory enzyme in mice (Boudinot et al. 2005). However, due to the cholinergic activity induced by HupA, the appearance of gastrointestinal-related side effects is expected. In fact, Zhang et al. (2013) found a significant inhibition of the AChE activity in the stomach and duodenum

and an increased gastrointestinal motility following a single dose of HupA; in contrast, no significant changes were identified in the AChE activity and gastrointestinal motility following multiple dose regimens administered to mice, suggesting that the gastrointestinal adverse effects of HupA are only transitory in mice. Therefore, after treatment of patients with HupA in multiple dose regimens only minimal gastrointestinal side effects are anticipated (Zhang et al. 2013).

Actually, in humans, similarly to the other AChE inhibitors, the adverse effects induced by therapeutic dosages of HupA are primarily related with the cholinergic system, but they tend to be manifested with a milder intensity (Xu et al. 1995; Pepping 2000; Zangara 2003; Ma et al. 2007; Sharma 2010), and particularly without the hepatotoxicity induced, for example, by tacrine (Zangara 2003; Ma et al. 2007). Those cholinergic adverse effects, which have been reported at a very low rate, include dizziness, nausea, vomiting, diarrhea, gastrointestinal discomfort, hyperactivity, anorexia, gastroenteric symptoms, headaches and depressed heart rate (Xu et al. 1995; Pepping 2000; Zangara 2003; Ha et al. 2011; Zhang et al. 2013). It is also important to highlight that, in a clinical study performed with HupA, relevant bradycardia abnormalities were reported and, therefore, the use of HupA in patients with cardiac diseases should be questionable (Pepping 2000).

Conclusion

Several years ago, herbs were the only source of medicines and, in spite of the wide variety of chemicals currently available in the modern medicine, the reemergence of its interest to treat various health problems is nowadays a reality. Thus, more and more people try to find answers on the knowledge and properties offered by the traditional and alternative medicines. Particularly the chemical, biological, pharmacological and therapeutic properties of Chinese Huperziaceae species have recently been a target of several research works, owing mainly to the strong relationship between its ethnopharmacological use and the medicinal properties of important constituents including HupA (Ma et al. 2007). This compound is the foremost *Lycopodium* alkaloid isolated from *H. serrata*, not only due to its therapeutic value in several pathological conditions, but also due to its unique chemical structure, with a compact and stringent skeleton, in addition to its favorable pharmacokinetic profile; notwithstanding, the data available in humans remains limited. It is also worthy to emphasize the fact that HupA is well tolerated in humans, even at doses well above those required clinically, being the adverse effects primarily related to the cholinergic system. As it was herein discussed, although only few compounds demonstrated obvious AChE inhibitory activity, several analogs of HupA have been prepared. In this context, the research in this field and the manipulation of appropriate structural parameters, alongside the investigation for new formulations and routes of administration, may allow further improvements in the therapeutic potency as well as a more ready penetration into the CNS.

The use of HupA in the treatment of AD is nowadays well supported by several in vitro studies, as well as in vivo non-clinical and clinical studies. Hence, HupA seems to offer benefits for patients with AD and age-associated memory decline. Moreover, the existing studies also suggest that HupA is potentially a more favorable AChE inhibitor than those conventionally used and already approved for the treatment of AD. HupA also seems to have an interesting potential as a pre-treatment agent against chemical weapons as nerve gases. On the other hand, the anticonvulsant properties of HupA were also recently identified, but a deeper and clinical characterization of its anticonvulsant profile needs to be further investigated in order to assess the potential HupA in the treatment of epilepsy. Additionally, the potential of HupA for the treatment of myasthenia gravis and schizophrenia needs likewise to be more explored, as well as its role in the improvement of cognitive ability. In fact, although the activity of HupA in the treatment of myasthenia gravis is one of the most described therapeutic properties for this compound, there is only one study performed in humans which, besides dating from 1986, was performed with standards somewhat dubious for the modern science. Furthermore, additional research is required to evaluate possible drug interactions involving HupA, which is a growing problem nowadays due to the frequent co-administration of various compounds; notwithstanding this issue may have potentially serious consequences for health, it is very often neglected.

While many compounds are starting to be used in the conventional medicine in the western world, HupA, which seems to have several promising medicinal properties particularly in AD and perhaps also in epilepsy, is only recognized as a dietary supplement by the Food and Drug Administration. As a result, the medicinal properties of HupA need to be heavily exploited by large clinical trials in the United States and/or in Europe, not only to confirm its efficacy for all the therapeutic actions that have been claimed to this compound, but also to effectively validate its safety. The data obtained from such clinical trials are certainly required to support a wider use of HupA in the Western world, following the steps of other phytochemicals. However, in these circumstances and bearing in mind that the Huperziaceae family is the main source of HupA, new methods to propagate HupA containing plants either in an agronomic context or in in vitro conditions, as well as new and improved ways for its chemical synthesis must be developed. Indeed, the development of new strategies to obtain HupA would be very valuable to protect the increasingly threatened group of Huperziaceae family species.

The rational discovery of multi-target drugs has been the status over the past years and this trend will possibly continue in the future. With the emerging scientific advances in the medicinal chemistry and pharmacology areas, the discovery of new drugs able to simultaneously modulate several therapeutic targets often interconnected in the pathological mechanisms of complex diseases will be an increasing goal (Lu et al. 2012; Prati et al. 2014). According to some researches, even if single-target drugs successfully inhibit or activate a specific target they cannot always induce the desired effect in the entire biological system. This may be explained by the development of compensatory ways in the organisms that affect the effectiveness of the drug (Lu et al. 2012). In fact, a complex disease condition cannot be fully corrected by many single-target drugs (Zimmermann et al. 2007). In the case of AD, for example, taking into account its multifactorial nature, a polypharmacology-based approach has been frequently reported to overcome some of the major limitations of the currently available drugs. In fact, a single compound able to interact with multiple targets responsible for disease mechanisms would have advantages over single-target drugs as well as over cotherapies (Capurro et al. 2013). Bearing in mind the several therapeutic properties described for HupA, this agent could represent a good example of multi-target compounds. In fact, many of the natural products are multi-target agents, being a great source for drug discovery due to their diverse and complex chemical structures (Lu et al. 2012). Actually, there are some efforts being made in this direction. For instance, Brunhofer et al. (2012) explored the natural compounds as sources of new bifunctional scaffolds targeting cholinesterases (AChE and BuChE) and β-amyloid aggregation, being (−)-HupA included in this screening. Thus, the study aimed to identify compounds that could target two mechanisms simultaneously associated with AD pathogenesis. This approach enabled to identify chelerythrine as an inhibitor of AChE and BuChE, with dual ability to inhibit Aβ aggregation as well as to disaggregate the preformed Aβ aggregates; hence, chelerythrine is potentially a starting point to the development of more successful anti-AD drugs (Brunhofer et al. 2012). Consequently, a multi-target approach appears to be a promising strategy to the drug discovery, especially for multifactorial diseases, being HupA an interesting compound within this line.

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