ABSTRACT The antinociceptive effect of huperzine A, a novel cholinesterase inhibitor, was investigated in the mouse hot-plate and abdominal constriction tests. Huperzine A induced a dose-dependent antinociception (70–110 μg kg⁻¹ i.p.) which was prevented by scopolamine (0.1 mg kg⁻¹ i.p.) and S(−)-ET 126 (0.01 μg per mouse i.c.v.), but not by naloxone (1 mg kg⁻¹ i.p.), mecamylamine (2 mg kg⁻¹ i.p.), α-methyl-p-tyrosine (100 mg kg⁻¹ i.p.), or CGP 35348 (100 mg kg⁻¹ i.p.). A dose-dependent inhibition of the antinociception induced by huperzine A (110 μg kg⁻¹ i.p.) was observed after inactivation of the M₁ gene by an antisense oligodeoxyribonucleotide (aODN). This effect was detected 24 h after the last intracerebroventricular injection of aODN. Time-course experiments revealed that, after the end of the aODN treatment, sensitivity to analgesic drugs progressively appeared, reaching the normal range at 96 h. Huperzine A, at the maximal effective doses, did not produce any alteration of mice motor coordination, as revealed by rota-rod experiments. These results indicate that huperzine A is endowed by muscarinic antinociceptive properties mediated by the activation of central M₁ muscarinic receptor subtype. Drug Dev. Res. 54:19–26, 2001. © 2001 Wiley-Liss, Inc.

Key words: huperzine A; cholinesterase inhibitor; analgesia; M₁ receptors; antisense oligonucleotides; central cholinergic system

INTRODUCTION It is widely accepted that organisms possess endogenous systems within the central nervous system that inhibit nociceptive transmission. Several reports have provided evidence for the critical involvement of the cholinergic system in such pain inhibitory pathways. The first observation that the cholinesterase inhibitor physostigmine increased the pain threshold in man was made more than 60 years ago [Pellandra, 1933]. Since then, a vast literature has appeared describing the antinociceptive action of both cholinesterase inhibitors and cholinomimetic drugs [Hartvig et al., 1989]. Bhargava and Way [1972] showed that elevation of acetylcholine (ACh) in brain, produced by cholinesterase inhibitors, enhanced morphine-induced analgesia. In addition, it has been reported that physostigmine is endowed with antinociceptive properties in rat cold-water swimming. The enhancement of pain threshold is prevented by the administration of the muscarinic antagonists scopolamine and benactyzine [Romano and Shih, 1983]. Intrathecal and systemic administration of acetylcholinesterase (AChE) inhibitors has been reported to produce antinociception in several animal species, including mice [Yaksh et al., 1985; Smith et al., 1989; Naguib and Yaksh, 1994; Ghelardini et al., 2000]. Human studies have confirmed the analgesic action of the anticholinesterase agent physostigmine given intravenously [Scott and Loh, 1984; Pettersson et al., 1986]. Furthermore, neostigmine has been recently shown to produce long-lasting postoperative analgesia.
It has been reported that muscarinic analgesia in mice and rats is mediated by postsynaptic M1 receptors. M1 selective agonists McN-A-343 and AF-102B are able to produce a significant enhancement of the pain threshold antagonized by the M1 antagonists dicyclomine and pirenzepine [Bartolini et al., 1992]. The analgesia induced by the cholinesterase inhibitors neostigmine and physostigmine was prevented by M1 antagonists [Bouaziz et al., 1995] and by anti-M1 ODN treatment [Ghelardini et al., 2000].

Huperzine A is an alkaloid isolated from a Chinese club-moss which is a potent and selective inhibitor of AChE [Tang et al., 1989]. Several pharmacological and clinical studies showed that huperzine A improves the mnesic capacity and cognitive functions [Ye et al., 1999]. Huperzine A was also found to be a neuroprotective agent [Ved et al., 1997]. This molecule which possesses a high pharmacological potential is under clinical evaluation for the palliative treatment of Alzheimer’s disease [Skolnick, 1997]. In addition, its use in the pretreatment of organophosphorous nerve agents could be another indication [Pilotaz and Masson, 1999].

On the basis of the observation that anticholinesterase inhibitors showed analgesic activity, the aim of the present study was to investigate the potential antinociceptive profile of huperzine A and its mechanism of action.

MATERIALS AND METHODS

Animals

Male Swiss albino mice (23–30 g) from Morini (San Polo d’Enza, Italy) were used. The mice were housed 15 per cage. The cages were placed in the experimental room 24 h before the test for adaptation. The animals were fed a standard laboratory diet and tap water ad libitum and kept at 23 ± 1°C with a 12-h light/dark cycle, lights on at 7 AM. Animals were used once. All experiments were carried out according to the guidelines of the European Community Council for experimental animal care.

Antisense Oligonucleotides

Low cell permeability and the high degradation of natural phosphodiester oligomers are considerable drawbacks in the application of antisense oligonucleotides (aODNs), both in vitro and in vivo. To overcome these drawbacks, phosphorothioate-capped phosphodiester oligonucleotides were used. The above-mentioned compounds are a class of ODN derivatives shown to maintain more stable and effective concentrations in the brain when compared with their unmodified counterpart [Whitesell et al., 1993]. Phosphodiester oligonucleotides (ODNs) protected by terminal phosphorothioate double substitution (capped ODNs) against possible exonuclease-mediated degradation were purchased from Genosys (Cambridge, UK) and purified by high-performance liquid chromatography (HPLC). The 18-mer antisense ODN (aODN) 5’-CAC TGA GGT GTT CAT TGC-3’ (phosphorothioate residues are underlined) complementary to the residues 112–129 of the published mouse M1 cDNA sequence [Shapiro et al., 1988] and the 18-mer fully degenerated ODN (dODN) 5’-NNN NNN NNN NNN NNN NNN NNN NNN NNN NNN NNN NNN NNN NNN NNN NNN NNN NNN-3’ (where N is G, or C, or A, or T and phosphorothioate residues are underlined) were vehiculated intracerebrally by an artificial cationic lipid (DOTAP; Boehringer-Mannheim, Germany) to enhance both uptake and stability, as described previously [Capaccioli et al., 1993; Quattrone et al., 1994], aODN or dODN (100–400 μM) were preincubated at 37°C for 30 min with 13 μM DOTAP, sterilized through a 0.2-μm filter, and supplied to mice by intracerebroventricular (i.c.v.) injection of a 5 μl solution as described in the next section.

GenBank Accession Numbers

The accession number of the cDNA sequence for the mouse muscarinic receptor subtype reported in this article (M1) is J04192.

Intracerebroventricular Injection of Oligonucleotides

Mice were randomly assigned to anti-M1 aODN, dODN, vehicle, saline, or naïve group. The antisense and dODNs were dissolved in a vehicle constituted of DOTAP. Each group received a single i.c.v. injection on days 1, 4, and 7, whereas naïve animals did not receive any treatment. i.c.v. administration was performed under ether anesthesia with isotonic saline as solvent, according to the method described by Haley and McCormick [1957]. During anesthesia mice were grasped firmly by the loose skin behind the head. A hypodermic needle (0.4 mm external diameter) attached to a 10 μl syringe was inserted perpendicularly through the skull and no more than 2 mm into the brain of the mouse, where 5 μl ODNs were then administered. The injection site was 1 mm to the right or left from the midpoint on a line drawn through to the anterior base of the ears. Injections were performed randomly into the right or left ventricle. To ascertain that ODNs were administered exactly into the cerebral ventricle, some mice (10%) were injected with 5 μl of diluted 1:10 India ink and their brains were examined macroscopically after sectioning. The accuracy of the injection technique was evaluated, with 95% of injections being correct.

Hot-Plate Test

The method adopted was described by O’Callaghan and Holtzman [1975]. Mice were placed inside a stainless steel container, which was set thermostatically at 52.5 ± 0.1°C in a precision water-bath from KW Mechanical...
Workshop (Siena, Italy). Reaction times(s) were measured with a stopwatch before and 15, 30, and 45 min after huperzine A administration. The endpoint used was the licking of the fore- or hindpaws. Those mice scoring less than 12 and more than 18 sec in the pretest were rejected (30%). To prevent tissue injury, an arbitrary cut-off time of 45 sec was adopted.

**Abdominal Constriction Test**

Mice were injected intraperitoneally (i.p.) with a 0.6% solution of acetic acid (10 ml kg\(^{-1}\)), according to Koster et al. [1959]. The number of stretching movements was counted for 10 min, starting 5 min after acetic acid injection.

**Rota-Rod Test**

The apparatus consists of a base platform and a rotating rod of 3 cm diameter with a nonskid surface. The rod was placed at a height of 15 cm from the base. The rod, 30 cm in length, was divided into five equal sections by six disks. Thus, up to five mice were tested simultaneously on the apparatus, with a rod-rotation speed of 16 rpm. The integrity of motor coordination was assessed on the basis of the number of falls from the rod in 30 sec, according to Vaught et al. [1985]. Performance time was measured before and 15, 30, and 45 min after i.p. administration of the investigated compounds.

**Reagents and Compounds**

The following drugs were used: huperzine A (gift of Prof. Nha, Medical School, University of Atlanta, Atlanta, GA), scopolamine hydrobromide, baclofen (β-p-chlorophenyl GABA), naloxone hydrochloride, DL-α-methyl-p-tyrosine methylester hydrochloride (α-MpT) (Sigma, St. Louis, MO), morphine hydrochloride (USL 10/D, Firenze, Italy); mecamylamine hydrochloride (RBI, Natick, MA); CGP 35348 (3-aminopropyl-diethoxy-methyl-phosphinic acid) (Ciba-Geigy, Summit, NJ); S-(–)-ET 126 (S-(–)-α-(hydroxymethyl)benzene-acetic acid 1-methyl-4-piperidinyl ester; prepared in the Department of Pharmaceutical Sciences of Florence, Italy, as described by Gualtieri et al. [1991]).

Drug concentrations were prepared in such a way that the necessary dose could be administered in a volume of 5 μl per mouse by i.c.v. injection and 10 ml kg\(^{-1}\) by i.p. injection. All drugs were dissolved in saline solution.

**Statistical Analysis**

All experimental results are given as the means ± SEM. An ANOVA followed by Fisher’s Protected Least Significant Difference (PLSD) procedure for post-hoc comparison was used to verify the significance of differences between two means. Data were analyzed with the StatView software for the Macintosh (1992).

**RESULTS**

**Antinociceptive Activity of Huperzine A**

The cholinesterase inhibitor huperzine A, as shown in Figure 1, produced a dose-dependent increase in the pain threshold in the mouse hot-plate test (70–110 μg kg\(^{-1}\) i.p.). The antinociceptive effect of huperzine A peaked 15 min after administration and then slowly diminished. Huperzine A was also able to produce antinociception in the mouse acetic acid abdominal constriction test. The cholinesterase inhibitor induced an increase in the pain threshold in a dose-dependent manner starting from the dose of 20 μg kg\(^{-1}\) i.p. (Fig. 2). In both tests the antinociception induced by huperzine A was compared with that exhibited by morphine, used as reference drug (Figs. 2, 3).

In the mouse hot-plate test, the antinociceptive effect of huperzine A (110 μg kg\(^{-1}\) i.p.) was not antagonized by naloxone (1 mg kg\(^{-1}\) i.p.), mecamylamine (2 mg kg\(^{-1}\) i.p.), α-MpT (100 mg kg\(^{-1}\) i.p.), or CGP-35348 (100 mg kg\(^{-1}\) i.p.), administered, respectively, 5, 15, 120, and 5 min before huperzine A (Table 1). Conversely, the unselective muscarinic antagonists scopolamine (0.1 mg kg\(^{-1}\) i.p.) and the selective M\(_1\) antagonist S-(–)-ET 126
aODN, at the dose of 0.3 nmol per i.c.v. injection, did not significantly affect huperzine A (Fig. 4A) analgesia, whereas at the dose of 1.0 and 2.0 nmol per i.c.v. injection, aODN dose-dependently prevented huperzine A (Fig. 4B,C) antinociception. This antagonistic effect was detected 24 h after the last i.c.v. injection.

The regression line which illustrates the dose-dependent reduction of huperzine A antinociception produced by increasing concentrations of aODN is shown in Figure 4D. The percentage of the maximum analgesic effect was evaluated in correspondence with the maximum effect of analgesic compounds that occurred 15 min after huperzine A administration. The hot-plate test was performed 24 h after the end of the ODN treatments.

A time-course study for aODN antagonistic effect showed that 48 h after the last i.c.v. injection of ODNs the antinociception produced by huperzine A was completely prevented (Fig. 5). At 72 h the aODN effect was still detectable against huperzine A antinociception, whereas at 96 h this compound was able to enhance the pain threshold at the same intensity in aODN-, dODN-, and vehicle-treated mice, indicating the loss of antagonistic activity by the anti-M1 aODN (Fig. 5).

The aODN pretreatment (2.0 nmol per i.c.v. injection) did not prevent the analgesia induced by morphine (7 mg kg\(^{-1}\) i.p.) and baclofen (4 mg kg\(^{-1}\) i.p.) 24 h after the end of ODN administration (data not shown).

The aODN pretreatment (2.0 nmol per i.c.v. injection) did not reduce the pain threshold in mice showing a lack of any hyperalgesic effect (Fig. 4). Furthermore, the pretreatment with the dODN never modified huperzine A-induced antinociception in comparison with mice injected with vehicle, as shown in Figure 4.

**Effect of Huperzine A on Mouse Behavior**

Mice pretreated with huperzine A were evaluated for motor coordination by use of the rota-rod test (Table 2). The number of falls, evaluated before and 15, 30, and 45 min after the beginning of the rota-rod test, showed the lack of any impairment in the motor coordination of animals pretreated with huperzine A at the dose of 110 µg kg\(^{-1}\) i.p. (Table 2). The number of falls by control animals progressively decreased at every measurement, since the mice learned how to balance on the rotating rod.

Conversely, a double dose of huperzine A produced an increase in the number of falls from the rotating rod, indicating the induction of motor side effects, as well as diphenhydramine 30 mg kg\(^{-1}\) i.p. (sedative reference drug) and scopolamine 5 mg kg\(^{-1}\) i.p. (amnesic reference drug) (Table 2).

**DISCUSSION**

Huperzine A was able to induce antinociception in mice that was elicited regardless of which noxious stimu-
lus was used: thermal (hot-plate) or chemical (abdominal constriction test). Huperzine A antinociception was obtained without producing any visible modification of animal gross behavior. Moreover, huperzine A-treated mice showed a complete integrity of motor coordination on the rota-rod test. Since animals learned how to balance on the rotating rod, the number of falls by control animals progressively decreased at every measurement. The increase or the lack of variation of the number of falls represents an index of the induction of motor incoordination that could lead to a misinterpretation of the results obtained in the hot-plate test. By contrast, a reduction of the number of falls after treatment indicates unimpaired mouse motor coordination. Mice, even if still sensitive to the thermal stimulus used in the hot-plate test, may have no reaction to pain (licking of paws) because of their impaired motility.

Huperzine A antinociception was found to be dependent on cholinergic activation since it was prevented by the nonselective muscarinic antagonist scopolamine at concentrations unable to produce analgesia induced by nonmuscarinic drugs such as morphine or baclofen administered at equiactive doses. The involvement of the M1 receptor subtype in huperzine A antinociception was postulated by the prevention exerted by the M1 selective antagonist S-(−)-ET 126 at a dose able to block the M1-induced analgesia [Ghelardini et al., 1996].

Furthermore, it has been demonstrated that cholinergic antinociception in mice is mediated by M1 receptor stimulation. The use of pharmacological agonists and antagonists evidenced the involvement of M1 receptors in muscarinic analgesia [Bartolini et al., 1992; Iwamoto and Marion, 1993; Ghelardini et al., 1996; Naguib and Yaksh, 1997]. The antinociception induced by huperzine A is prevented, in a dose-related manner, by i.c.v. administration of an antisense to the M1 gene coding for the mouse M1 receptor, further confirming that the enhancement of the pain threshold produced by this compound is of the cholinergic type. These results are in agreement with the prevention of the antinociception induced by physostigmine, a cholinesterase inhibitor, exhibited by anti-M1 ODN treatment [Ghelardini et al., 2000]. The specificity of the anti-M1 ODN employed was demonstrated by experiments that evidenced a selective reduction at both M1 mRNA and protein levels in mouse brain [Ghelardini et al., 1999, 2000].

The prevention by i.c.v. injection of the aODN also indicates that the antinociception induced by huperzine A is centrally mediated.

The aODN treatment induces a transient prevention of muscarinic antinociception since the inhibition of huperzine A antinociception disappeared 96 h after the last i.c.v. injection of the aODN. This return to normal sensitivity to analgesic treatments could imply both the total reversal of aODN-induced specific inhibition of M1 gene expression and a lack of damage or toxicity associated with aODN treatment.

In these experimental conditions, anti-M1 aODN did not modify the licking latency values of mice in comparison with control groups (dODN, saline, naive), excluding that the prevention of huperzine A antinociception is due to a hyperalgesic effect of the treatment used [Ghelardini et al., 2000]. Furthermore, the antinociception induced by activation of other neurotransmitter systems able to enhance the pain threshold, such as opioid and GABAergic, were not modified by anti-M1 aODN treatment [Ghelardini et al., 2000]. These data indicate not only the specificity of the aODN treatment, but also lead us to exclude a correlation between these two antinociceptive systems and muscarinic analgesia.

In comparison with the saline group, dODN and vehicle treatments did not modify the enhancement of

### TABLE 1. Lack of Effect of Naloxone, Mecamylamine, α-Methyl-p-tyrosine (α-MpT), and CGP-35348 on Antinociception Induced by Huperzine A (110 μg kg⁻¹ ip) in the Mouse Hot-Plate Test

<table>
<thead>
<tr>
<th>Pretreatment</th>
<th>Treatment</th>
<th>Before pretreatment</th>
<th>15 min</th>
<th>30 min</th>
<th>45 min</th>
</tr>
</thead>
<tbody>
<tr>
<td>Saline</td>
<td>Saline</td>
<td>13.6 ± 0.7</td>
<td>14.4 ± 1.0</td>
<td>13.9 ± 0.9</td>
<td>14.3 ± 0.8</td>
</tr>
<tr>
<td></td>
<td>Huperzine A</td>
<td>14.3 ± 0.9</td>
<td>33.1 ± 2.2*</td>
<td>26.2 ± 1.9*</td>
<td>19.0 ± 2.3</td>
</tr>
<tr>
<td>Naloxone</td>
<td>Saline</td>
<td>13.5 ± 0.8</td>
<td>14.0 ± 1.5</td>
<td>13.9 ± 1.6</td>
<td>14.3 ± 1.7</td>
</tr>
<tr>
<td>1 mg kg⁻¹ ip</td>
<td>Huperzine A</td>
<td>14.4 ± 0.7</td>
<td>31.5 ± 2.6*</td>
<td>24.6 ± 2.0*</td>
<td>18.7 ± 1.9</td>
</tr>
<tr>
<td>Mecamylamine</td>
<td>Saline</td>
<td>14.4 ± 0.9</td>
<td>15.2 ± 1.7</td>
<td>16.3 ± 1.3</td>
<td>14.4 ± 1.2</td>
</tr>
<tr>
<td>2 mg kg⁻¹ ip</td>
<td>Huperzine A</td>
<td>13.7 ± 1.0</td>
<td>36.7 ± 2.0*</td>
<td>24.5 ± 1.7*</td>
<td>18.4 ± 1.5</td>
</tr>
<tr>
<td>α-MpT</td>
<td>Saline</td>
<td>14.4 ± 0.9</td>
<td>13.8 ± 1.5</td>
<td>15.5 ± 1.3</td>
<td>15.4 ± 1.2</td>
</tr>
<tr>
<td>100 mg kg⁻¹ ip</td>
<td>Huperzine A</td>
<td>14.4 ± 1.1</td>
<td>34.0 ± 2.1*</td>
<td>25.9 ± 1.5*</td>
<td>17.3 ± 1.5*</td>
</tr>
<tr>
<td>CGP 35348</td>
<td>Saline</td>
<td>13.5 ± 0.7</td>
<td>11.4 ± 1.3**</td>
<td>12.5 ± 2.0</td>
<td>12.7 ± 1.5</td>
</tr>
<tr>
<td>100 mg kg⁻¹ ip</td>
<td>Huperzine A</td>
<td>14.5 ± 1.5</td>
<td>30.3 ± 2.2*</td>
<td>23.6 ± 2.6*</td>
<td>17.9 ± 1.7*</td>
</tr>
</tbody>
</table>

Each value is the mean of 8–12 mice. *P < 0.01; **P < 0.05 in comparison with saline. Naloxone, mecamylamine, α-MpT, and CGP-35348 were administered 15 min, 5 min, 2h, and 5 min before huperzine A, respectively.
the pain threshold produced huperzine A, ruling out the possibility that the antagonism exerted by aODN could be caused by sequence-independent effects on cerebral structures. This claim is supported by results obtained from the quantitative RT-PCR analysis of ODN effects on M1 gene expression. Quantitative results of M1 mRNA brain levels following aODN treatment confirmed that phenotypic effects of anti-M1 aODN on pain modulation were actually due to the specific inhibition of M1 gene expression, since dODN did not modify M1 mRNA brain levels [Ghelardini et al., 1999].

Considering the similarity between the structures of the muscarinic receptors, a specific anti-M1 aODN was employed in order to selectively inhibit the expression of this receptor subtype without interfering with the other subtypes. The sequence encompassing the translation start sites of mRNAs, which is considered particularly prone to aODN action [Goodchild, 1989; Stein and Cheng, 1993], was compared among the murine known muscarinic receptors gene family in order to design a specific antimouse M1 aODN with a very low sequence homology even with the nearest other members of the muscarinic receptor family. A homology search in the GenBank database confirmed the absolute specificity of the employed aODN. Furthermore, considering the de-

### Table 2. Effect of Huperzine in the Mouse Rota-Rod Test

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Dose ip</th>
<th>Number of falls in 30 s</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Before</td>
<td>15 min</td>
</tr>
<tr>
<td>Saline</td>
<td>10 ml kg⁻¹</td>
<td>3.3 ± 0.5</td>
</tr>
<tr>
<td>Huperzine A</td>
<td>110 µg kg⁻¹</td>
<td>3.5 ± 0.4</td>
</tr>
<tr>
<td>Huperzine A</td>
<td>220 µg kg⁻¹</td>
<td>3.4 ± 0.3</td>
</tr>
</tbody>
</table>

Each value represents the mean of 10–12 mice. *P < 0.01 in comparison with saline-treated mice.
scribed sequence-independent, nonantisense effects of ODNs [Storey et al., 1991; Gao et al., 1992; Blagosklonny and Neckers, 1994; Schick et al., 1995], a fully degenerated phosphorothioate capped phosphodiester ODN (dODN) was used as the most suitable control for these potentially confusing effects. The dODN used is a collection of about 3 × 10^14 different molecular species. At concentrations achieved in the nanomolar to micromolar range in in vitro antisense experiments, every species, i.e., every ODN of defined sequence, was present at the site of action at a concentration less than 10^{-18} M, which is totally insufficient to achieve any sense, or generally sequence-dependent, cellular effect. Therefore, if ODN i.c.v. administration per se had achieved any biological response, this would have been present in ODN-treated controls.

Other neurotransmitter systems are not involved in Huperzine A antinociception since the opioid antagonist naloxone, the GABA_A antagonist CGP-35348, the cathecolamine synthesis inhibitor α-methyl-p-tyrosine, and the nicotinic antagonist mecamylamine were all unable to prevent the effect of huperzine A. The doses and administration schedules of the above-mentioned drugs were ideal for preventing antinociceptions induced, respectively, by morphine [Ghelardini et al., 1992], the GABA_A agonist baclofen [Malcangio et al., 1991], nicotine [Ghelardini et al., 1997], and amphetamine [Bartolini et al., 1987].

In summary, our results have shown that huperzine A is able to produce dose-dependent antinociception in mice, without impairing motor coordination, by potentiating endogenous cholinergic activity. These data suggest a potential employment of huperzine A, as well as other cholinesterase inhibitors, as analgesics for the relief of painful human conditions.

REFERENCES


