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## Research Article

# Antinociceptive Profile of the Natural Cholinesterase Inhibitor Huperzine A

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Strategy, Management and Health Policy				
Venture Capital Enabling Technology	Preclinical Research	Preclinical Development Toxicology, Formulation Drug Delivery, Pharmacokinetics	Clinical Development Phases I-III Regulatory, Quality, Manufacturing	Postmarketing Phase IV

**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 ( $70\text{--}110\text{ }\mu\text{g kg}^{-1}$  i.p.) which was prevented by scopolamine ( $0.1\text{ mg kg}^{-1}$  i.p.) and S-(–)-ET 126 ( $0.01\text{ }\mu\text{g}$  per mouse i.c.v.), but not by naloxone ( $1\text{ mg kg}^{-1}$  i.p.), mecamylamine ( $2\text{ mg kg}^{-1}$  i.p.),  $\alpha$ -methyl-*p*-tyrosine ( $100\text{ mg kg}^{-1}$  i.p.), or CGP 35348 ( $100\text{ mg kg}^{-1}$  i.p.). A dose-dependent inhibition of the antinociception induced by huperzine A ( $110\text{ }\mu\text{g kg}^{-1}$  i.p.) was observed after inactivation of the  $M_1$  gene by an antisense oligodeoxynucleotide (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  $M_1$  muscarinic receptor subtype. *Drug Dev. Res.* 54:19–26, 2001. © 2001 Wiley-Liss, Inc.

**Key words:** huperzine A; cholinesterase inhibitor; analgesia;  $M_1$  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

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[Klamt et al., 1997] and to enhance opioid analgesia in human patients [Lauretti et al., 1996; Klamt et al., 1999; Nelson et al., 1999].

It has been reported that muscarinic analgesia in mice and rats is mediated by postsynaptic M<sub>1</sub> receptors. M<sub>1</sub> selective agonists McN-A-343 and AF-102B are able to produce a significant enhancement of the pain threshold antagonized by the M<sub>1</sub> antagonists dicyclomine and pirenzepine [Bartolini et al., 1992]. The analgesia induced by the cholinesterase inhibitors neostigmine and physostigmine was prevented by M<sub>1</sub> antagonists [Bouaziz et al., 1995] and by anti-M<sub>1</sub> 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 mnemonic 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 poisoning by 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 M<sub>1</sub> cDNA sequence [Shapiro et al., 1988] and the 18-mer fully degenerated ODN (dODN) 5'-NNN NNN NNN NNN NNN NNN-3' (where N is G, or C, or A, or T and phosphorothioate residues are underlined) were vehiculated intracellularly 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 (M<sub>1</sub>) is J04192.

### Intracerebroventricular Injection of Oligonucleotides

Mice were randomly assigned to anti-M<sub>1</sub> aODN, dODN, vehicle, saline, or naive 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 naive 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 ( $10 \text{ ml kg}^{-1}$ ), 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 ( $\beta$ -p-chlorophenyl GABA), naloxone hydrochloride, DL- $\alpha$ -methyl-*p*-tyrosine methylester hydrochloride ( $\alpha$ -MpT) (Sigma, St. Louis, MO), morphine hydrochloride (USL 10/D, Firenze, Italy); mecamlamine hydrochloride (RBI, Natick, MA); CGP 35348 (3-aminopropyl-diethoxymethyl-phosphinic acid) (Ciba-Geigy, Summit, NJ); S-(-)-ET 126 (S-(-)- $\alpha$ -(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  $5 \mu\text{l}$  per mouse by i.c.v. injection and  $10 \text{ ml kg}^{-1}$  by i.p. injection. All drugs were dissolved in saline solution.

### Statistical Analysis

All experimental results are given as the means  $\pm$  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 ( $70\text{--}110 \mu\text{g kg}^{-1}$  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  $20 \mu\text{g kg}^{-1}$  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 ( $110 \mu\text{g kg}^{-1}$  i.p.) was not antagonized by naloxone ( $1 \text{ mg kg}^{-1}$  i.p.), mecamlamine ( $2 \text{ mg kg}^{-1}$  i.p.),  $\alpha$ -MpT ( $100 \text{ mg kg}^{-1}$  i.p.), or CGP-35348 ( $100 \text{ mg kg}^{-1}$  i.p.), administered, respectively, 5, 15, 120, and 5 min before huperzine A (Table 1). Conversely, the unselective muscarinic antagonists scopolamine ( $0.1 \text{ mg kg}^{-1}$  i.p.) and the selective  $M_1$  antagonist S-(-)-ET 126

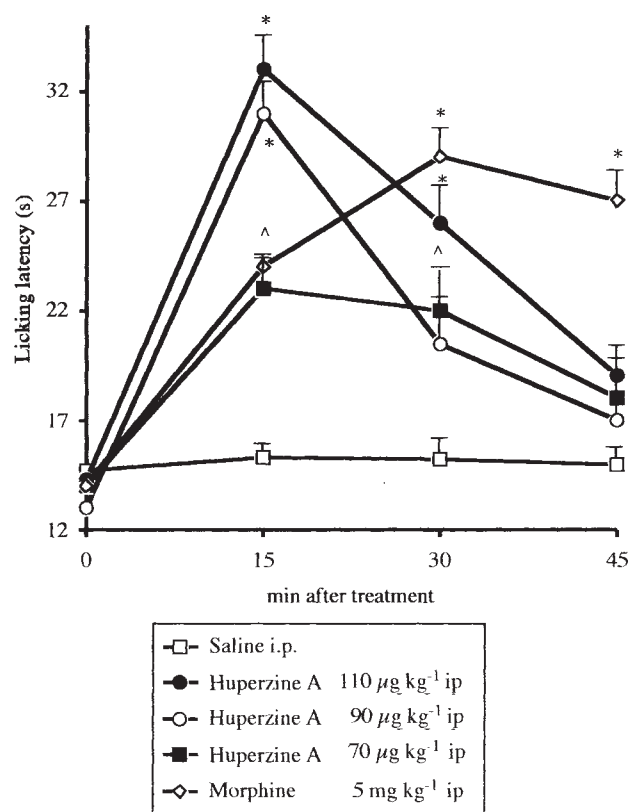
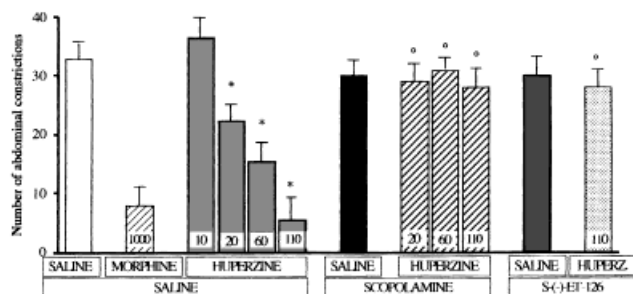


Fig. 1. Antinociceptive effect of huperzine A in comparison with morphine in mouse hot-plate test. Each point represents the mean of 10–22 mice. Vertical lines show SEM. \* $P < 0.01$ ; ^ $P < 0.05$  in comparison with saline-treated mice.



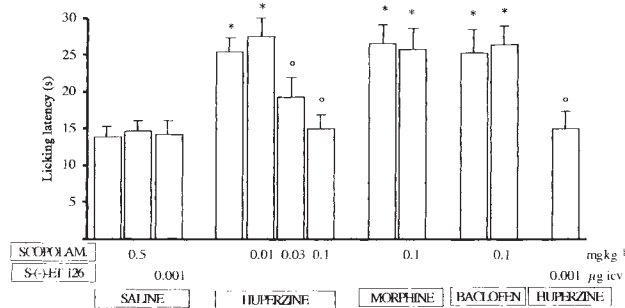


**Fig. 2.** Antagonism of scopolamine ( $0.1 \text{ mg kg}^{-1}$  i.p.) and S(-)-ET 126 ( $0.01 \text{ } \mu\text{g}$  per mouse i.c.v.) on the antinociceptive effect of huperzine A in mouse abdominal constrictions test. The nociceptive responses were recorded 15 and 30 min after huperzine A and morphine administration, respectively. Scopolamine and S(-)-ET 126 were injected 10 min before huperzine A. The doses of huperzine A and morphine expressed as  $\mu\text{g kg}^{-1}$  i.p. are inside the columns. Vertical lines show SEM. \* $P < 0.01$  in comparison with saline controls.  $^{\circ}P < 0.01$  in comparison with scopolamine/S(-)-ET 126-treated mice. Each column represents the mean of at least 10 mice.

( $0.01 \text{ } \mu\text{g}$  per mouse i.c.v.), administered 20 min before test, prevented huperzine A antinociception in the mouse abdominal constriction (Fig. 2) and hot-plate (Fig. 3) tests. Scopolamine, at the same concentration, did not prevent the analgesia induced by morphine ( $7 \text{ mg kg}^{-1}$  i.p.) and baclofen ( $4 \text{ mg kg}^{-1}$  i.p.) (Fig. 3).

### Effect of Anti- $M_1$ ODN on Huperzine A Antinociception

Mice were pretreated with a single i.c.v. injection of antisense ODN (aODN) to  $M_1$  gene, degenerate ODN (dODN), or vehicle on days 1, 4, and 7. The effect of aODN pretreatment on huperzine A ( $110 \text{ } \mu\text{g kg}^{-1}$  i.p.)-induced antinociception was then evaluated in the mouse hot-plate test.



**Fig. 3.** Effect of scopolamine (i.p.) and S(-)-ET 126 (i.c.v.) on huperzine A-, morphine-, and baclofen-induced antinociception in mouse hot-plate test. Scopolamine and S(-)-ET 126 were administered 10 min before the other drugs. Nociceptive response was recorded 15 min after huperzine A ( $110 \text{ } \mu\text{g kg}^{-1}$  i.p.) and 30 min after morphine ( $7 \text{ mg kg}^{-1}$  i.p.) and baclofen ( $4 \text{ mg kg}^{-1}$  i.p.) injection. Number of mice ranged between 8–18. Vertical lines show SEM. \* $P < 0.01$  vs. saline-treated mice.  $^{\circ}P < 0.01$  vs. huperzine A-treated mice.

aODN, at the dose of  $0.3 \text{ nmol}$  per i.c.v. injection, did not significantly affect huperzine A (Fig. 4A) analgesia, whereas at the dose of  $1.0$  and  $2.0 \text{ nmol}$  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- $M_1$  aODN (Fig. 5).

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

The aODN pretreatment ( $2.0 \text{ nmol}$  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  $110 \text{ } \mu\text{g kg}^{-1}$  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  $30 \text{ mg kg}^{-1}$  i.p. (sedative reference drug) and scopolamine  $5 \text{ mg kg}^{-1}$  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-

**TABLE 1. Lack of Effect of Naloxone, Mecamylamine,  $\alpha$ -Methyl-p-tyrosine ( $\alpha$ -MpT), and CGP-35348 on Antinociception Induced by Huperzine A (110  $\mu$ g kg<sup>-1</sup> ip) in the Mouse Hot-Plate Test**

Pretreatment	Treatment	Licking latency(s)			
		Before pretreatment	After treatment		
			15 min	30 min	45 min
Saline	Saline	13.6 $\pm$ 0.7	14.4 $\pm$ 1.0	13.9 $\pm$ 0.9	14.3 $\pm$ 0.8
	Huperzine A	14.3 $\pm$ 0.9	33.1 $\pm$ 2.2*	26.2 $\pm$ 1.9*	19.0 $\pm$ 2.3
Naloxone	Saline	13.5 $\pm$ 0.8	14.0 $\pm$ 1.5	13.9 $\pm$ 1.6	14.3 $\pm$ 1.7
1 mg kg <sup>-1</sup> ip	Huperzine A	14.4 $\pm$ 0.7	31.5 $\pm$ 2.6*	24.6 $\pm$ 2.0*	18.7 $\pm$ 1.9
Mecamylamine	Saline	14.4 $\pm$ 0.9	15.2 $\pm$ 1.7	16.3 $\pm$ 1.3	14.4 $\pm$ 1.2
2 mg kg <sup>-1</sup> ip	Huperzine A	13.7 $\pm$ 1.0	36.7 $\pm$ 2.0*	24.5 $\pm$ 1.7*	18.4 $\pm$ 1.5
$\alpha$ -MpT	Saline	14.4 $\pm$ 0.9	13.8 $\pm$ 1.5	15.5 $\pm$ 1.3	15.4 $\pm$ 1.2
100 mg kg <sup>-1</sup> ip	Huperzine A	14.4 $\pm$ 1.1	34.0 $\pm$ 2.1*	25.9 $\pm$ 1.5*	17.3 $\pm$ 1.5*
CGP 35348	Saline	13.5 $\pm$ 0.7	11.4 $\pm$ 1.3**	12.5 $\pm$ 2.0	12.7 $\pm$ 1.5
100 mg kg <sup>-1</sup> ip	Huperzine A	14.5 $\pm$ 1.5	30.3 $\pm$ 2.2*	23.6 $\pm$ 2.6*	17.9 $\pm$ 1.7

Each value is the mean of 8–12 mice. \* $P$  < 0.01; \*\* $P$  < 0.05 in comparison with saline. Naloxone, mecamylamine,  $\alpha$ -MpT, and CGP-35348 were administered 15 min, 5 min, 2h, and 5 min before huperzine A, respectively.

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 prevent analgesia induced by nonmuscarinic drugs such as morphine or baclofen administered at equiactive doses. The involvement of the M<sub>1</sub> receptor subtype in huperzine A antinociception was postulated by the prevention exerted by the M<sub>1</sub> selective antagonist S-(–)-ET 126 at a dose able to block the M<sub>1</sub>-induced analgesia [Ghelardini et al., 1996].

Furthermore, it has been demonstrated that cholinergic antinociception in mice is mediated by M<sub>1</sub> receptor stimulation. The use of pharmacological agonists and antagonists evidenced the involvement of M<sub>1</sub> 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 M<sub>1</sub> gene

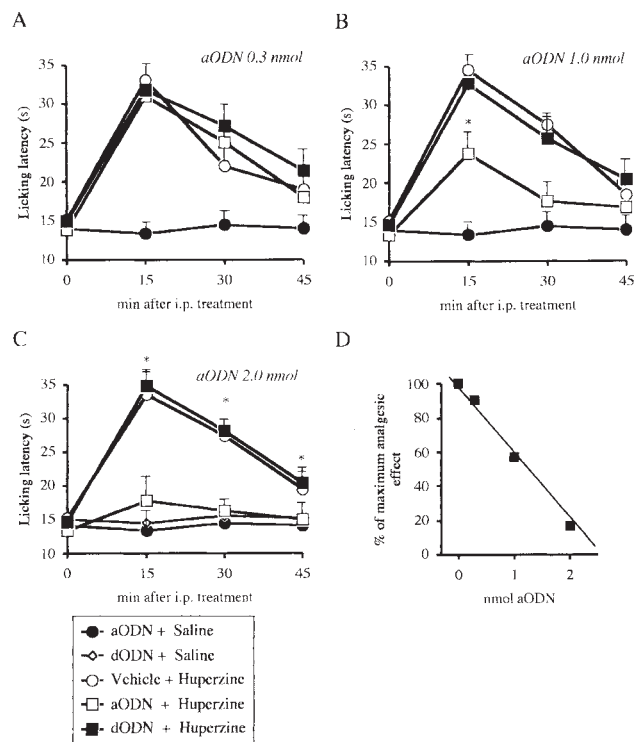
coding for the mouse M<sub>1</sub> receptor, further confirming that the enhancement of the pain threshold produced by this compound is of the cholinergic type. These results are in agreement with the prevention of the antinociception induced by physostigmine, a cholinesterase inhibitor, exhibited by anti-M<sub>1</sub> ODN treatment [Ghelardini et al., 2000]. The specificity of the anti-M<sub>1</sub> ODN employed was demonstrated by experiments that evidenced a selective reduction at both M<sub>1</sub> 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 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 M<sub>1</sub> gene expression and a lack of damage or toxicity associated with aODN treatment.

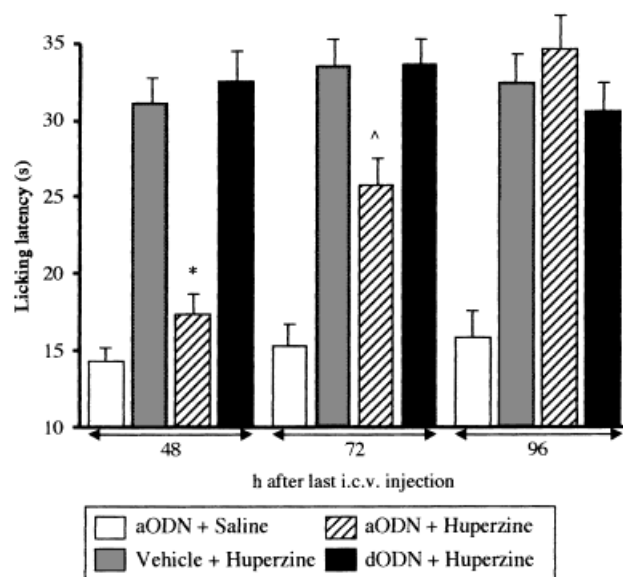
In these experimental conditions, anti-M<sub>1</sub> aODN did not modified the licking latency values of mice in comparison with control groups (dODN, saline, naive), excluding that the prevention of huperzine A antinociception is due to a hyperalgesic effect of the treatment used [Ghelardini et al., 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-M<sub>1</sub> 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



**Fig. 4.** Prevention of huperzine A ( $110 \mu\text{g kg}^{-1}$  i.p.)-induced antinociception by pretreatment with an antisense ODN (aODN) to  $M_1$  gene in the mouse hot-plate test (**A,B,C**) and effect of increasing concentrations of aODN to  $M_1$  gene on huperzine A ( $110 \mu\text{g kg}^{-1}$  i.p.)-induced antinociception in the same test (**D**). Mice were i.c.v. injected with vehicle, aODN, or degenerated ODN (dODN) at the dose of 0.3 (**A**), 1.0 (**B**), or 2.0 nmol (**C**) per single i.c.v. injection on days 1, 4, and 7. The hot-plate test was performed 24 h after the last i.c.v. injection. The evaluation of the analgesic effect was carried out 15 min after huperzine A administration. Vertical lines give SEM. Each point represents the mean of 10–14 mice. \* $P < 0.01$  in comparison with dODN + huperzine A-treated mice.

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  $M_1$  gene expression. Quantitative results of  $M_1$  mRNA brain levels following aODN treatment confirmed that phenotypic effects of anti- $M_1$  aODN on pain modulation were actually due to the specific inhibition of  $M_1$  gene



**Fig. 5.** Effect of antisense ODN (aODN) to  $M_1$  gene on huperzine A ( $110 \mu\text{g kg}^{-1}$  i.p.)-induced antinociception 48, 72, and 96 h after the end of the aODN treatment. Mice were i.c.v. injected with vehicle, aODN, or degenerated ODN (dODN) at a dose of 2.0 nmol per single i.c.v. injection on days 1, 4, and 7. Modification of pain threshold was evaluated by using the mouse hot-plate test. The licking latency was detected 15 min after huperzine A administration. Vertical lines give SEM. Each column represents the mean of 10–14 mice. ^ $P < 0.05$ ; \* $P < 0.01$  in comparison with vehicle-huperzine A-treated mice.

expression, since dODN did not modify  $M_1$  mRNA brain levels [Ghelardini et al., 1999].

Considering the similarity between the structures of the muscarinic receptors, a specific anti- $M_1$  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  $M_1$  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

Treatment	Dose ip	Number of falls in 30 s			
		Before pretreatment	15 min	30 min	45 min
Saline	10 ml $\text{kg}^{-1}$	3.3 $\pm$ 0.5	2.2 $\pm$ 0.4	1.5 $\pm$ 0.2	0.8 $\pm$ 0.2
Huperzine A	110 $\mu\text{g kg}^{-1}$	3.5 $\pm$ 0.4	1.8 $\pm$ 0.5	1.3 $\pm$ 0.2	1.0 $\pm$ 0.3
Huperzine A	220 $\mu\text{g kg}^{-1}$	3.4 $\pm$ 0.3	3.8 $\pm$ 0.4*	2.5 $\pm$ 0.4*	1.2 $\pm$ 0.3

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  $3 \times 10^{14}$  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 antisense, 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<sub>B</sub> antagonist CGP-35348, the catecholamine synthesis inhibitor  $\alpha$ -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<sub>B</sub> 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 analgesic for the relief of painful human conditions.

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