Development of Muscarinic Analgesics Derived from Epibatidine: Role of the M₄ Receptor Subtype

JAMES L. ELLIS, DEAN HARMAN, JAVIER GONZALEZ, MICHAEL L. SPERA, RONGGANG LIU, T. Y. SHEN, DONNA M. WYPJ, and FANGMING ZUO


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ABSTRACT

Epibatidine, a neurotoxin isolated from the skin of Epipedobates tricolor, is an efficacious antinociceptive agent with a potency 200 times that of morphine. The toxicity of epibatidine, because of its nonspecificity for both peripheral and central nicotinic receptors, precludes its development as an analgesic. During the synthesis of epibatidine analogs we developed potent antinociceptive agents, typified by CMI-936 and CMI-1145, whose antinociception, unlike that of epibatidine, is mediated via muscarinic receptors. Subsequently, we used specific muscarinic toxins and antagonists to delineate the muscarinic receptor subtype involved in the antinociception evoked by these agents. Thus, the antinociception produced by CMI-936 and CMI-1145 is inhibited substantially by 1) intrathecal injection of the specific muscarinic M₄ toxin, muscarinic toxin-3; 2) intrathecally administered pertussis toxin, which inhibits the G proteins coupled to M₂ and M₃ receptors; and 3) s.c. injection of the M₂/M₃ muscarinic antagonist himbacine. These results demonstrate that the antinociception elicited by these epibatidine analogs is mediated via muscarinic M₄ receptors located in the spinal cord. Compounds that specifically target the M₄ receptor therefore may be of substantial value as alternative analgesics to the opiates.

There has been considerable effort in the scientific/medical community to develop nonopiate painkillers that maintain the efficacy of opiates against severe and chronic pain but are devoid of the opiate liabilities of respiratory depression, constipation, and dependence. The discovery that epibatidine, isolated from the skin of the frog Epipedobates tricolor (Spande et al., 1992), is an extremely potent efficacious antinociceptive agent (Spande et al., 1992; Qian et al., 1993; Badio and Daly, 1994) and the subsequent observation by our group that its activity is mediated via nicotinic receptors (Qian et al., 1993) stimulated research into the discovery of epibatidine analogs without toxic side effects. This toxicity is due to its nonspecific activity at central and peripheral nicotinic receptors (Sullivan et al., 1994; Bonhaus et al., 1995).

We synthesized several hundred analogs using the 2.2.1 azaborinane bicycle of epibatidine as a template (Fig. 1). One of these series, containing a 1,2,4-oxadiazole ring attached to the bicycle in anexo configuration, was found to produce potent antinociception that, unlike that produced by epibatidine, is not blocked by the nicotinic antagonist mecamylamine. Two examples of this series are CMI-936 (2-exo[5-(3-methyl-1,2,4-oxadiazolyl)]-2.2.1.-7-azabicycloheptane) and CMI-1145 (2-exo[5-(3-amino-1,2,4-oxadiazolyl)]-2.2.1.-7-azabicycloheptane) (Fig. 1).

Having established the muscarinic nature of the antinociceptive response elicited by CMI-936 and CMI-1145, we set out to study which muscarinic subtype was involved in this antinociception. Despite five decades of research establishing a role for the muscarinic cholinergic system in antinociception (Chen, 1958; Herz, 1962; Pedigo et al., 1975; Widman et al., 1985), the subtype mediating this effect remains under debate. Dawson et al. (1991) reported that either M₁ or M₃ receptors are involved in the mouse tail-flick response to noxious stimuli. In rats and mice, Bartolini et al. (1992) suggested that an M₁ receptor is involved in this assay. In rats injected intrathecally with muscarinic agonists, it has been suggested that muscarinic antinociception is mediated via M₁ and/or M₃ receptors (Iwamoto and Marion, 1993) or else via M₁ and/or M₃ receptors (Naguib and Yaksh, 1997). Recent evidence using the highly M₁-selective agonist xanomeline strongly argues against a role for M₁ receptors in muscarinic antinociception (Sheardown et al., 1997). Further studies using agonists with selectivity for the various muscarinic subtypes also argue against a role of M₂ and M₃ receptors in antinociception (Sauerberg et al., 1995; Shannon et al., 1997). Also arguing against a role for M₁ receptors in

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ABBREVIATIONS: M, muscarinic; MT-3, muscarinic toxin-3; PTX, pertussis toxin; DMSO, dimethyl sulfoxide; CMI-936, 2-exo[5-(3-methyl-1,2,4-oxadiazolyl)]-2.2.1.-7-azabicycloheptane; CMI-1145, 2-exo[5-(3-amino-1,2,4-oxadiazolyl)]-2.2.1.-7-azabicycloheptane; MPE, maximum possible effect.
antinociception is the absence of M1 receptors from the rat spinal cord (Höglund et al., 1997). In addition, in the human spinal cord M4, but not M1, receptors have been shown to be the predominant receptor subtype (Borenstein et al., 1996).

The identification of muscarinic receptor subtypes involved in various physiological processes has been hampered severely by the lack of antagonists selective for the five individual subtypes (Caulfield, 1993; Eglen and Watson, 1996). We thus used a combination of an antagonist (himbacine) that has selectivity for the M2/M4 subtypes (Waelbroeck et al., 1990; Dorje et al., 1991; Miller et al., 1992) and pertussis toxin (PTX), which is also selective for the M2 and M4 subtypes (Hildebrandt et al., 1983; Sternweis and Robishaw, 1984; Hulme et al., 1990), and the muscarinic M4 toxin muscarinic toxin-3 (MT-3), which is selective for the M4 subtype (Max et al., 1993; Jolkkonen et al., 1994). PTX selectively inhibits G7 and G-coupled receptors (Hildebrandt et al., 1983; Sternweis and Robishaw, 1984). Thus, among the five muscarinic receptor subtypes, PTX inhibits M2 and M4 receptors that are coupled to these G proteins, but not M1, M3, and M5 receptors that are coupled to different G proteins (Hulme et al., 1990). MT-3 belongs to a family of muscarinic toxins that have been isolated from the venom of Dendroaspis angusticeps (Liang et al., 1996). MT-3 has a 40-fold selectivity for the M4 subtype over the M2 subtype and a greater than 500-fold selectivity for the M4 receptor over M2, M3, and M5 receptors (Jolkkonen et al., 1994).

Materials and Methods

Tail-Flick Test. Female CD-1 mice weighing 20 to 30 g were obtained from Charles River Laboratories (Wilmington, MA). A commercially available tail-flick analgesia meter was used (model TF-6 Analgesia Meter; Emdie Instrument Company, Maidens, VA). The radiant heat source was set so that control mice had a tail-flick latency of 2 to 4 s. A 10-s cutoff time was used as the maximum latency to avoid damage to the mice tails. The latency of each mouse (a mean of two separate test results for each time point) was obtained at 0- (immediately before dosing), 5-, 15-, 30-, and 60-min time points after injection of compounds, and the percent maximum possible effect (% MPE) was calculated by using the formula % MPE = [(postdrug latency – predrug latency) / (cutoff time – predrug latency)] × 100.

Body Temperature. At ambient temperature, a temperature probe (Type T Thermocouple Thermometer, BAT-10; Physitemp Inc., Clifton, NJ) was inserted 1.0 cm into the rectum of mice to measure their core temperature and recorded at 0 (before drug as a control baseline), 10, 25, and 55 min after injection of compounds.

Scoring of Salivation. The salivation was noted by close visual inspection of the animal’s mouth and was scored according to the following scale: 0, no sign of saliva within animal’s mouth; 1, evidence of saliva in animal’s mouth, but none on animal’s muzzle; and 2, evidence of saliva in animal’s mouth and on animal’s muzzle.

Each animal was scored at 5-, 15-, 30-, and 60-min time points after injection of compounds.

Intrathecal Injection. Intrathecal injections were done free-hand following the method of Hyliden and Wilcox (1980). Briefly, mice were held by the pelvic girdle in one hand, as the syringe was held in the other hand at an angle of about 20° above the vertebral column. The needle was inserted into the tissue to one side of the L5 or L6 spinal process so that it slipped into the groove between the spinous and transverse process. The needle was then moved carefully forward to the intervertebral space as the angle of the syringe was decreased to about 10°. The tip of needle was inserted approximately 0.5 cm within the vertebral column. The 5 μl of solution was injected in the needle was rotated on withdrawal. Hamilton 25-μl microsyringes and 30.5-gauge needles were used in this procedure.

Drugs. CMI-936, CMI-1145, and (-)-epibatidine were synthesized in the Chemistry Department of the University of Virginia. CMI-936 and CMI-1145 were prepared from the reaction of exo-2-carbomethoxy-7-azabicyclo[2.2.1]heptane with the appropriately substituted amidoxime (Carroll et al., 1993). The azanorbornane precursor was prepared as its racemic mixture either by the cycloaddition of an N-3,5-dimethylbenzaldehyde pyrrole complex of pentammineosmium (II) with methyl acrylate (Gonzalez et al., 1995) or by demethylation and ring-contraction sequence starting from tropine. (-)-Epibatidine was synthesized as previously described (Huang and Shen, 1993). Atropine, mecamylamine, and himbacine were purchased from Sigma Chemical Co. (St. Louis, MO). PTX was purchased from Research Biochemicals (Natick, MA), and MT-3 was purchased from Alexis Co. (San Diego, CA). Atropine and mecamylamine were dissolved in 0.9% saline. CMI-936, CMI-1145, (-)-epibatidine, and himbacine were dissolved in dimethyl sulfoxide (DMSO). Subsequent dilutions were made in 0.9% saline such that the final concentration of DMSO in the agonist studies was less than 0.1%. DMSO (0.1%) was found to have no antinociceptive, hypothermia, or salivary effect (data not shown). PTX (50 μg/vial) was reconstituted with 1000 μl of sterile 0.01 M sodium phosphate buffer (pH 7.0) containing 0.05 M sodium chloride solution. Five microliters of this solution was injected intrathecally into each mouse such that the final dose was 0.25 μg per mouse. MT-3 (10 μg/vial) was dissolved in 50 μl of sterile 0.9% saline. Five microliters of this solution was injected intrathecally such that the final dose was 1 μg per mouse. In studies using these toxins, control animals were injected intrathecally with 5 μl of the appropriate vehicle.

Fig. 1. The chemical structure of (-)-epibatidine, CMI-936 (2-exo{5-(3-methyl-1,2,4-oxadiazolyl)-[2.2.1]-7-azabicycloheptane}, and CMI-1145 (2-exo{5-(3-amino-1,2,4-oxadiazolyl)-[2.2.1]-7-azabicycloheptane}).

Fig. 2. Antinociceptive effect of CMI-936 ( ), CMI-1145 ( ), and (-)- epibatidine ( ) in the mouse tail-flick assay. The % MPE for CMI-936 and CMI-1145 is calculated at the 30-min time point after s.c. dosing, whereas the % MPE for (-)-epibatidine is calculated at the 5-min time point after s.c. dosing. Each point represents the mean ± S.E.M. of five mice.
Statistical Analysis. Results are given as the mean ± S.E.M. A general analysis of variance (multiway ANOVA) test using an SPSS computer program (Chicago, IL) was utilized to determine significance between control groups and antagonist/toxin-pretreated groups (multiple group comparison) for each time point in the antinociception and hypothermia data. A p value < .05 was considered significant in each case. Because the salivary data are nonparametric, a nonparametric test was used (Kruskal-Wallis H test; SPSS, Chicago, IL). A p value (corrected for ties) < .05 was considered statistically significant.

Results

Antinociception of CMI-936, CMI-1145, and (−)-Epibatidine. The ability of CMI-936, CMI-1145, and (−)-epibatidine to produce antinociception and hypothermia was evaluated in mice. The graphs show the percentage of maximum possible effect (% MPE) for antinociception and body temperature over time. The results were analyzed using multiway ANOVA, and significance was determined at p < .05.

Fig. 3. Effect of atropine or mecamylamine on the antinociception and hypothermia produced by 50 μg/kg s.c. CMI-936 (A and D), 30 μg/kg s.c. CMI-1145 (B and E), and 10 μg/kg s.c. (−)-epibatidine (C and F) in the mouse tail-flick assay. Results are shown as responses in the absence of antagonist (●), in the presence of 3 mg/kg s.c. atropine (△), and in the presence of 1 mg/kg i.p. mecamylamine (○). Each point represents the mean ± S.E.M. of five mice. *p < .05, **p < .01 compared with agonist responses in the absence of antagonists.
Effect of antagonists and toxins on the salivary response produced by CMI-936 and CMI-1145

<table>
<thead>
<tr>
<th>Compound</th>
<th>Dose (s.c.)</th>
<th>n</th>
<th>Antagonist, Dose</th>
<th>Score (mean ± S.E.M.) of salivation at</th>
</tr>
</thead>
<tbody>
<tr>
<td>CMI-936</td>
<td>50</td>
<td>5</td>
<td>Saline, 10 ml/kg s.c.</td>
<td>0.80 ± 0.20 1.00 ± 0.32 1.00 ± 0.00 0.40 ± 0.24</td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>5</td>
<td>Atropine, 3 mg/kg s.c.</td>
<td>0.00 ± 0.00* 0.00 ± 0.00* 0.00 ± 0.00* 0.00 ± 0.00</td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>5</td>
<td>Mecamylamine, 1 mg/kg i.p.</td>
<td>0.80 ± 0.20 1.00 ± 0.00 1.20 ± 0.20 0.20 ± 0.00</td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>5</td>
<td>DMSO/saline, 10 ml/kg s.c.</td>
<td>1.40 ± 0.24 2.00 ± 0.00 1.40 ± 0.24 0.60 ± 0.24</td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>5</td>
<td>Himbacine, 0.2 mg/kg s.c.</td>
<td>1.00 ± 0.00 1.80 ± 0.20 1.60 ± 0.24 0.60 ± 0.24</td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>5</td>
<td>Himbacine, 1.0 mg/kg s.c.</td>
<td>1.20 ± 0.20 1.80 ± 0.20 1.60 ± 0.24 0.40 ± 0.24</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>5</td>
<td>PBS, 5 μl/mouse i.t.</td>
<td>0.40 ± 0.24 0.90 ± 0.26 1.00 ± 0.17 0.40 ± 0.18</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>5</td>
<td>PTX, 0.25 μg/mouse i.t.</td>
<td>0.90 ± 0.18 1.20 ± 0.20 1.10 ± 0.18 0.60 ± 0.16</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>5</td>
<td>Saline, 5 μl/mouse i.t.</td>
<td>0.40 ± 0.15 1.00 ± 0.00 0.70 ± 0.14 0.40 ± 0.15</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>5</td>
<td>MT-3, 1.0 μg/mouse i.t.</td>
<td>0.50 ± 0.16 0.80 ± 0.13 1.00 ± 0.20 0.40 ± 0.15</td>
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<tr>
<td>CMI-1145</td>
<td>50</td>
<td>5</td>
<td>Saline, 10 ml/kg s.c.</td>
<td>0.80 ± 0.20 1.80 ± 0.20 1.20 ± 0.20 0.20 ± 0.20</td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>5</td>
<td>Atropine, 3 mg/kg s.c.</td>
<td>0.00 ± 0.00* 0.00 ± 0.00* 0.00 ± 0.00* 0.00 ± 0.00</td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>5</td>
<td>Mecamylamine, 1 mg/kg i.p.</td>
<td>0.80 ± 0.20 1.40 ± 0.24 0.40 ± 0.24 0.20 ± 0.20</td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>5</td>
<td>DMSO/saline, 10 ml/kg s.c.</td>
<td>0.80 ± 0.20 1.20 ± 0.20 0.80 ± 0.37 0.40 ± 0.24</td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>5</td>
<td>Himbacine, 0.2 mg/kg s.c.</td>
<td>0.40 ± 0.24 1.20 ± 0.20 1.20 ± 0.20 0.40 ± 0.24</td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>5</td>
<td>Himbacine, 1.0 mg/kg s.c.</td>
<td>0.00 ± 0.00* 0.20 ± 0.20* 0.80 ± 0.37 0.40 ± 0.40</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>5</td>
<td>PBS, 5 μl/mouse i.t.</td>
<td>0.90 ± 0.10 1.30 ± 0.15 1.10 ± 0.18 0.10 ± 0.10</td>
</tr>
<tr>
<td></td>
<td>9</td>
<td>5</td>
<td>PTX, 0.25 μg/mouse i.t.</td>
<td>0.67 ± 0.17 1.11 ± 0.11 0.89 ± 0.11 0.00 ± 0.00</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>5</td>
<td>Saline, 5 μl/mouse i.t.</td>
<td>0.80 ± 0.19 1.10 ± 0.15 0.90 ± 0.14 0.00 ± 0.00</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>5</td>
<td>MT-3, 1.0 μg/mouse i.t.</td>
<td>0.40 ± 0.23 0.70 ± 0.22 0.70 ± 0.22 0.00 ± 0.00</td>
</tr>
</tbody>
</table>

* p < .05 versus the vehicle control group.

Effect of Pertussis Toxin. The ability of PTX to inhibit receptors coupled with Gs and Gi, is dependent on the duration of PTX pretreatment. Several studies have shown that the effect of PTX is maximal after 7 to 12 days, at which time its effect lasts for several weeks (Hoehn et al., 1988; Galeotti et al., 1996). PTX (0.25 μg), therefore, was injected intrathecally 12 days before administration of CMI-936 and CMI-1145. The antinociception produced by CMI-936 (50 μg/kg s.c.) and CMI-1145 (30 μg/kg s.c.) is inhibited substantially by the PTX pretreatment (Fig. 5A and B). Neither the hyperthermia (Fig. 5, C and D) nor the salivation (Table 1) produced by CMI-936 and CMI-1145 is affected by the PTX treatment. The antinociception and hyperthermia produced by (−)-epibatidine (10 μg/kg s.c.) is also unaffected by the PTX pretreatment (data not shown).

Effect of MT-3 Toxin. Pretreatment with MT-3 (1 μg injected intrathecally) 20 min before the administration of CMI-936 (50 μg/kg s.c.) and CMI-1145 (30 μg/kg s.c.) significantly inhibits the antinociception produced by these agents (Fig. 6A and B). The salivation (Table 1) and hyperthermia (Fig. 6, C and D) produced by CMI-936 and CMI-1145 are unaffected by the MT-3 pretreatment. The antinociception and hyperthermia produced by (−)-epibatidine (10 μg/kg s.c.) is also unaffected by the MT-3 toxin (data not shown).

Discussion

Epibatidine has been shown to be an extremely potent antinociceptive agent (Spande et al., 1992; Qian et al., 1993;
Badio and Daly, 1994). The lack of separation of undesirable side effects from this antinociception (Sullivan et al., 1994; Bonhaus et al., 1995), however, preclude its development as a viable analgesic therapy. Therefore, we made several hundred analogs of epibatidine that we hoped would show separation of the antinociception from the side effects. As a result of this synthetic effort we made a series of epibatidine analogs that, in contrast to epibatidine’s nicotinic mode of action, elicited potent antinociception via an activation of muscarinic receptors.

As noted in the Introduction, which muscarinic subtype mediates antinociception remains under debate (Dawson et al., 1991; Bartolini et al., 1992; Iwamoto and Marion, 1993; Sauerberg et al., 1995; Naguib and Yaksh, 1997; Shannon et al., 1997) largely because of the lack of specific agonists and antagonists for the five muscarinic subtypes. Therefore, we used a combination of an antagonist that exhibits selectivity for the \( M_2 \) and \( M_4 \) subtypes (himbacine) (Waelbroeck et al., 1990; Dorje et al., 1991; Miller et al., 1992), a toxin that exhibits similar selectivity (pertussis toxin) (Hildebrandt et al., 1983; Sternweis and Robishaw, 1984; Hulme et al., 1990), and a toxin isolated from the venom of the green mamba \( D. \) angusticeps (Liang et al., 1996) (MT-3 toxin), which has a 40-fold selectivity for the \( M_4 \) receptor over the \( M_1 \) subtype and greater than 500-fold selectivity for the \( M_4 \) receptor over \( M_2, M_3, \) and \( M_5 \) receptors (Jolkkonen et al., 1994).

Himbacine was given s.c., and as it crosses the blood brain barrier it is a useful tool to examine the role of \( M_2 \) and \( M_4 \) receptors in both peripheral and central effects of the agents used in this study. By necessity, PTX and MT-3 were given intrathecally, which allows one to determine the site of the muscarinic antinociception elicited by CMI-936 and CMI-1145.

The results with himbacine indicate that the antinociception produced by CMI-936 and CMI-1145 is a result of acti-
vation of either M2 or M4 receptors. By contrast, neither the salivation nor hypothermia elicited by these agents appears to be mediated via M2 or M4 receptors.

Further supporting a role for either M2 or M4 receptors in the antinociception produced by CMI-936 and CMI-1145 are the data using PTX, which allow the differentiation of the M2 and M4 activity of a muscarinic agonist from activity at M1, M3, and M5 receptors (Hulme et al., 1990). After intrathecal injection the distribution of PTX is closely confined to the injection site (Chung et al., 1994). The data with PTX argue strongly that the muscarinic receptors mediating the antinociception are localized to the spinal cord. The lack of effect of PTX treatment on the hypothermia and salivation suggest that these effects are mediated by receptors other than spinal M2/M4 receptors.

To distinguish which of the M2 or M4 receptor subtypes is involved in the antinociception of CMI-936 and CMI-1145 it is necessary to have antagonists that show a high degree of selectivity for one subtype over the other. There are no reports to our knowledge describing compounds that exhibit this desired selectivity. Fortunately, a series of muscarinic toxins recently has been isolated from the venom of the green mamba, D. angusticeps (Liang et al., 1996). Among these is MT-3, which possesses greater than 500-fold selectivity for the M4 receptor over the M2 receptor (Jolkkonen et al., 1994). Because of its selectivity, this toxin has been used to show the localization of M4 receptors to various sites, including the pain-processing region of the human spinal cord (Borenstein et al., 1996). Here, we describe the first in vivo experiments with this toxin designed to probe which muscarinic subtype is eliciting a particular response. That MT-3 pretreatment inhibited the antinociception produced by CMI-936 and CMI-1145 provides the strongest evidence for a role of the M4 receptor in muscarinic antinociception. Because the MT-3

Fig. 5. Effect of pertussis toxin on the antinociception and hypothermia produced by 50 μg/kg s.c. CMI-936 (A, C and D), 30 μg/kg s.c. CMI-1145 (B and D) in the mouse tail-flick assay. Results are shown as responses in the absence of the toxin (●) and in the presence of 0.25 μg/mouse pertussis toxin (○). Each point represents the mean ± S.E.M. of 9 to 10 mice. *p < .05, **p < .01 compared with agonist responses in the absence of pertussis toxin.
toxin was administered intrathecally these data again argue that these M4 receptors are localized to the spinal cord.

Interestingly, it appears that the antinociceptive effect of CMI-1145 is more easily antagonized by himbacine and MT-3 than is the antinociceptive effect of CMI-936. One reason for this is that CMI-1145 may be mediating its antinociceptive effect solely through the M4 receptor subtype whereas the antinociceptive effect of CMI-936 is mediated through other muscarinic subtypes in addition to the M4 subtype.

In conclusion, we have demonstrated that certain oxadiazole analogs of epibatidine are potent antinociceptive agents in the mouse. In contrast to epibatidine, these analogs elicit their antinociception via muscarinic receptors. Furthermore, using a combination of selective toxins and antagonists we show that the M4 receptor subtype mediates this antinociception and that these receptors are spinally located. Our data also show that by selectively targeting the M4 receptor subtypes one should be able to elicit antinociception without eliciting the undesirable muscarinic side effects of salivation and hypothermia.

References
Chung KM, Song DK, Suh HW, Lee MH and Kim YK (1994) Effects of intrathecal or

Fig. 6. Effect of MT-3 toxin on the antinociception and hypothermia produced by 50 μg/kg s.c. CMI-936 (A, C and D), 30 μg/kg s.c. CMI-1145 (B and D), in the mouse tail-flick assay. Results are shown as responses in the absence of the toxin ( ● ) and in the presence of 1 μg/mouse muscarinic toxin-3 ( ○ ). Each point represents the mean ± S.E.M. of 10 mice. * p < .05, ** p < .01 compared with agonist responses in the absence of MT-3 toxin.
intracerebroventricular pretreatment with pertussis toxin on antinociception induced by b-endorphin or morphine administered intracerebroventricularly in mice. *Naunyn-Schmiedeberg's Arch Pharmacol* **349**:588–593.


Send reprint requests to: James L. Ellis, VCB Research Inc., 840 Memorial Drive, Cambridge, MA 02139. E-mail: james.ellis@vcb-group.com