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Antinociceptive effects of the novel neuronal nicotinic acetylcholine receptor agonist, ABT-594, in mice

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Abstract

ABT-594 [5-((2*R*)-azetidinylmethoxy)-2-chloropyridine], a novel neuronal nicotinic acetylcholine receptor agonist, produced significant antinociceptive effects in mice against both acute noxious thermal stimulation—the hot-plate and cold-plate tests—and persistent visceral irritation—the abdominal constriction (writhing) assay (maximally-effective dose in each test 0.62 μmol/kg, i.p.). This effect was not stereoselective since the *S*-enantiomer, A-98593 [5-((2*S*)-azetidinylmethoxy)-2-chloropyridine], produced similar antinociceptive effects in this dose range. The effect in the hot-plate test peaked at 30 min after i.p. administration and was still present 60 min, but not 120 min, after injection. ABT-594 was orally active, but 10-fold less potent by this route than after i.p. administration. The antinociceptive effect of ABT-594 was prevented, but not reversed, by the noncompetitive neuronal nicotinic acetylcholine receptor antagonist mecamylamine (5 μmol/kg, i.p.). In contrast, the antinociceptive effect of ABT-594 was not prevented by hexamethonium (10 μmol/kg, i.p.), a neuronal nicotinic acetylcholine receptor antagonist that does not readily enter the central nervous system, nor by naltrexone (0.8 μmol/kg), an opioid receptor antagonist. Thus, initiation of antinociception by ABT-594 involves activation of central nicotinic acetylcholine receptors, but does not require activation of naltrexone-sensitive opioid receptors. The antinociceptive effects of morphine and ABT-594 in the mouse hot-plate test appeared to be additive, but ABT-594 did not potentiate the respiratory depression produced by morphine when the two compounds were coadministered. ABT-594 reduced body temperature and spontaneous exploration in the antinociceptive dose range, but did not reliably impair motor coordination in the rotarod test. Thus, it is unlikely that the antinociceptive effects result simply from impaired motor function. The compound also produced an anxiolytic-like effect in the elevated plus maze (at 0.019 and 0.062 μmol/kg, i.p.). Preliminary safety testing revealed an ED_{50} for overt seizure production of 1.9 μmol/kg, i.p. and an LD_{50} of 19.1 μmol/kg i.p. in mice, values 10 and 100 times the minimum effective antinociceptive dose of the compound. ABT-594 increased the duration of ethanol-induced hypnotic effects, tended to increase pentobarbital-induced hypnotic effects ($P = 0.0502$), and had no effect on pentobarbital-induced lethality. These data indicate that ABT-594 is a centrally acting neuronal nicotinic acetylcholine receptor agonist with potent antinociceptive and anxiolytic-like effects in mice. © 1998 Elsevier Science B.V.

Keywords: Nicotinic acetylcholine receptor; Antinociception; Anxiety; Locomotor activity

1. Introduction

Analgesic agents are primarily from two general mechanistic classes; opioids and nonsteroidal antiinflammatory drugs. Opioids are fully efficacious in a variety of pain states, but side effects and dependence/tolerance issues limit their widespread use (Reisine and Pasternak, 1996). Side effects, primarily gastrointestinal, are also an issue with nonsteroidal antiinflammatory drugs (Insel, 1996). In addition, nonsteroidal antiinflammatory drugs have limited efficacy in the absence of prostaglandin-dependent hyperalgesia. Consequently, they are most often used to treat mild to moderate pain associated with inflammation and are ineffective in treating some types of nociceptive and neuropathic pain. Therefore, there is clearly a need for an additional class of compounds with the broad spectrum activity of opioids, but without the liabilities of opioids.

Compounds that act at nicotinic acetylcholine receptors also have antinociceptive activity, although this has not been exploited clinically. (−)-Nicotine, for example, pro-

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duces antinociception in rodents; however, this effect is typically relatively short-lasting and requires high doses (Aceto et al., 1983; Sahley and Berntson, 1979; Tripathi et al., 1982). Antinociceptive effects of (−)-nicotine have also been demonstrated in humans, but the magnitude of the effect may be related to smoking history (Pauli et al., 1993; Perkins et al., 1994; Pomerleau, 1986).

Interest in the potential analgesic activity of compounds acting at neuronal nicotinic acetylcholine receptors has recently been stimulated by the discovery that epibatidine, a potent, nonopioid antinociceptive compound isolated from frog skin (Spande et al., 1992), is a potent ligand at nicotinic acetylcholine receptors (Badio and Daly, 1994; Bonhaus et al., 1995; Qian et al., 1993; Sullivan et al., 1994). Epibatidine is greater than 100-fold more potent than either (−)-nicotine or morphine in rodent models of antinociception (Badio and Daly, 1994; Bannon et al., 1995a; Damaj et al., 1994; Sullivan et al., 1994). The antinociceptive effects of (±)-epibatidine are prevented by the noncompetitive nicotinic acetylcholine receptor antagonist mecamylamine but not by opioid receptor blockade (Bonhaus et al., 1995; Qian et al., 1993). Thus, (±)-epibatidine appears to be a potent antinociceptive agent that acts via neuronal nicotinic acetylcholine receptors and not through opioid receptors. Unfortunately, (±)-epibatidine is quite potent at all subtypes of the nicotinic acetylcholine receptor and is toxic at antinociceptive doses (Badio and Daly, 1994; Sullivan et al., 1994).

Given the evidence for neuronal nicotinic acetylcholine receptor diversity (for reviews, see Decker et al., 1995; Luetje et al., 1990; Sargent, 1993), it is possible that nicotinic acetylcholine receptor ligands with greater subtype selectivity might have enhanced separation between antinociception and toxicity. We have previously demonstrated the utility of this strategy with the identification of ABT-418 and ABT-089, nicotinic acetylcholine receptor ligands that maintain the cognition-enhancing activities of (−)-nicotine in preclinical models, but have improved safety profiles relative to (−)-nicotine (Arneric et al., 1994; Decker et al., 1994a, 1997; Lin et al., 1997). Although ABT-418 has modest antinociceptive activity at high doses (Damaj et al., 1995; Decker et al., 1995), ABT-418 and ABT-089 are not potent as antinociceptive agents. These compounds, however, illustrate the general principle that desirable and undesirable effects of neuronal nicotinic acetylcholine receptor activation can be dissociated by more selective agents.

ABT-594 (5-((2*R*)-azetidinylmethoxy)-2-chloropyridine) is a potent ligand at neuronal nicotinic acetylcholine receptors that has a profile in in vitro functional assays that is distinct both from (±)-epibatidine and from ABT-418 and ABT-089 (Arneric et al., 1994; Donnelly-Roberts et al., 1998; Sullivan et al., 1997). The experiments described in this report were conducted for the purpose of determining the analgesic potential of ABT-594 using mouse models of antinociception and preliminary acute safety assessment in mice. Effects of ABT-594 on acute thermal pain were assessed using the hot-plate and cold-plate tests. Effects of ABT-594 on more persistent, visceral pain were assessed using the abdominal constriction assay.

2. Materials and methods

2.1. Subjects

Subjects were male, CD-1 mice obtained from Charles River Laboratories (Portage, MI), ranging in size from 30–40 g at the time of testing. The mice were allowed free access to food and water except during test procedures and were group-housed (12–14 mice per cage) in a temperature-regulated environment with lights on between 7:00 and 20:00 h. The mice were tested during the light phase. All animals were acclimated to the animal colony for at least 72 h prior to use. Except where otherwise noted, animals were used for only one test. All testing was conducted according to protocols approved by Abbott's Institutional Animal Care and Use Committee.

2.2. Hot-plate testing

For the hot-plate test (Woolfe and MacDonald, 1944), an automated hot-plate device (Model #AHP16AN, Omnitech Electronics, Columbus, OH) was used. Each mouse was placed in one of 16 test chambers ($15 \times 7 \times 10$ cm) on a heated (55°C) copper plate and the latency to jump 10 times was recorded as an index of nociception. Mice who did not respond within 180 s (approximately 2–3 times control latency) were removed from the plate and a latency of 180 s was assigned. Jumps were recorded automatically using photocell detectors located 12.5 cm above the surface of the hot-plate. Test compounds were administered 30 min prior to conducting the hot-plate test.

2.3. Cold-plate testing

A 'cold-plate' test was used to assess the antinociception in mice subjected to noxious cold stimulation. For this test, a 4000 ml glass beaker was placed on crushed ice until the temperature of the beaker bottom had equilibrated (at approximately 1°C). The mouse was then placed into the beaker and observed. When placed on the cold surface, mice exhibited a stereotypical shaking/rubbing of their forepaws and/or attempted to jump out of the beaker. These behaviors were not observed when mice were placed in the beaker maintained at room temperature. The latency to engage in either the shaking/rubbing behavior or to jump from the container was recorded. A cut-off latency of 180 s was employed. Compounds were administered 30 min prior to testing.

2.4. Abdominal constriction assay

Persistent pain was assessed using an abdominal constriction assay (also known as the writhing test) (Collier et al., 1968). For this test, the mouse was given an i.p. injection of the irritant phenyl-*p*-quinone (68 μmol/kg dissolved in 5% ethanol). The presence of characteristic stretching or writhing responses was noted during a 10-min period beginning 5 min after the injection of phenyl-*p*-quinone. Mice displaying one or more of these 'nociceptive' responses were categorized as responders and mice who did not display these behaviors were regarded as nonresponders.

2.5. Locomotor activity, motor coordination, and body temperature

Locomotor activity was monitored under dim light in a 41×41 cm. open field using a photobeam activity system (San Diego Instruments, San Diego, CA). Motor coordination was assessed using an accelerating rotarod apparatus (Omnitech Electronics, Columbus, OH). The mouse was placed on a 3.5 cm diameter rod which increased in speed from 0 to 40 rpm over 120 s. The time required for the mouse to fall from the rod was recorded with a maximum score of 120 s. Body temperature was assessed using a probe inserted 3 cm into the rectum (YSI Tele-Thermometer, Yellow Springs Instrument, Yellow Springs, OH). All three measures were made in the same animals. Twenty-five minutes after receiving an i.p. injection, the mice were placed in the open field for 5 min. After removal from the open field (i.e., 30 min after injection), they were immediately tested on the rotarod, followed by determination of rectal temperature (approximately 35 min after injection). Diazepam (10.5 μmol/kg, i.p.) was used as a positive control.

2.6. Preliminary safety tests

Lethality was assessed using both an approximate lethal dose procedure and by determining an LD_{50} value. To determine the approximate lethal dose, a range of doses was given to a group of mice (each mouse receiving a single i.p. dose) and the lowest dose producing death within 24 h was recorded as the approximate lethal dose. When multiple groups were used, the approximate lethal doses for the groups were averaged to obtain a mean. For determination of an LD_{50}, doses of compound were administered i.p. (10 mice per dose), and deaths were recorded out to 7 days.

Seizure thresholds were determined by administering a range of doses (i.p.), and observing mice for overt signs of seizures ('running fit,' myoclonus, or tonic–clonic) for a period of at least 15 min. An ED_{50} and an ED_{16} were then calculated for the production of seizures.

Respiratory depression was assessed by determining the pCO_2 in trunk blood obtained after sacrifice. Mice were killed by cervical dislocation and trunk blood was obtained immediately after death. A sample was collected in a heparinized capillary tube and pCO_2 determined using a blood gas analyzer (ABL5, Radiometer, Copenhagen, Denmark).

2.7. Ethanol- and pentobarbital-induced hypnotic effects

Effects of ABT-594 on ethanol- and pentobarbital-induced hypnotic effects were assessed by determining the time required for animals to regain the righting reflex. ABT-594 or saline was injected i.p. followed 15 min later by an i.p. injection of ethanol (87 mmol/kg using a 20% solution) or pentobarbital (160 μmol/kg). At these doses, ethanol and pentobarbital cause a loss of the righting reflex, and the time required to regain the righting reflex was recorded as the 'sleep time' (with the time of injection being regarded as time zero). Animals were placed on their backs and observed for spontaneous righting. Following a spontaneous righting response, the mouse was again placed on its back to determine if it would right itself. When the animal was able to right itself three times in this manner, the time was recorded. Observation was terminated at 90 min and mice who had not displayed a righting response by this time were assigned 90 min as their sleep time.

2.8. Elevated plus-maze

Because some nicotinic acetylcholine receptor agonists produce anxiolytic-like effects in animal models (Brioni et al., 1993), the effects of ABT-594 were tested for anxiolytic-like activity using the elevated plus-maze procedure (Pellow et al., 1985). The apparatus used in this test was made of grey Plexiglas and consisted of two open arms (17×8.0 cm) and two enclosed arms ($17 \times 8 \times 15$ cm) extending from a central platform (8×8 cm) at 90° angles, with the enclosed arms opposite each other. The maze was mounted on a Plexiglas base and elevated 39 cm from the bench surface. Light levels on the open and enclosed arms were balanced. Thirty minutes after an i.p. injection of ABT-594 (0.019, 0.062, or 0.19 μmol/kg) or saline, the mouse was placed in the center of the maze and allowed to explore the maze for 5 min. During this period, an automated video tracking system (Videomex, Columbus Instruments, Columbus, OH) recorded the time spent on the open arms and the total distance traveled. Diazepam (10.5 μmol/kg, i.p.) was used as a positive control compound.

2.9. Compounds

ABT-594 [5-((2*R*)-azetidinylmethoxy)-2-chloropyridine] and its *S*- enantiomer, A-98593 [5-((2*S*)-azetidinylmethoxy)-2-chloropyridine] were synthesized by Abbott Laboratories (Abbott Park, IL), as described by Holladay et al. (1998). Monohydrochloride, dihydrochloride, benzoate, and tosylate salts were used; comparable behavioral results were observed with all salt forms. (±)-Epibatidine

dihydrochloride was obtained from Research Biochemicals International (Natick, MA); and morphine sulfate, mecamylamine hydrochloride, hexamethonium chloride, and naltrexone hydrochloride were obtained from Sigma (St. Louis, MO). These compounds were dissolved in sterile 0.9% saline. Pentobarbital sodium solution (Nembutal) was obtained from Abbott Laboratories (North Chicago, IL) and diluted as necessary with sterile 0.9% saline. Diazepam was obtained from Sigma (St. Louis, MO) and was suspended in 0.9% saline to which a drop of Tween-80 was added. Doses are expressed in μmol/kg of free base or free acid, and compounds were administered in a volume of 10 ml/kg.

2.10. Statistics

In most cases, statistical analysis was by analysis of variance (ANOVA) with post-hoc analysis using Fisher's protected least significant difference test. These analyses were conducted using SuperANOVA and StatView software programs (Abacus Concepts, Berkeley, CA). Determination and comparisons of ED_{16}, ED_{50}, and LD_{50} values were by the method of Litchfield and Wilcoxon using the PHARM/PCS program (MCS MicroComputer Specialists, Philadelphia, PA). The results obtained with the abdominal constriction assay were nominal data and required analysis with nonparametric statistics. The Chi-Square statistic was used to evaluate statistical significance in this assay (StatView).

3. Results

3.1. Hot-plate testing

As shown in Fig. 1, ABT-594 increased jump latencies in the hot-plate test when administered i.p. ($F(3,28) = 17.936$, $P < 0.0001$) or p.o. ($F(4,35) = 4.474$, $P = 0.005$), although the compound was about 10-fold more potent after i.p. administration than after p.o. administration. As can also be seen in Fig. 1, ABT-594 did not exhibit stereoselectivity in this test, as its *S*-enantiomer, A-98593, produced comparable effects ($F(3,25) = 16.419$, $P < 0.0001$). (−)-Nicotine also produced a significant increase in jump latencies on this test ($F(4,45) = 3.586$, $P = 0.013$), but (−)-nicotine was markedly less potent than either ABT-594 or A-98593. Significant elevation of jump latencies was only observed with 19 μmol/kg, i.p., with (−)-nicotine and at 0.62 μmol/kg, i.p., for both ABT-594 and A-98593.

The duration of action of ABT-594 in the hot-plate test was assessed by testing mice given either ABT-594 (0.62 μmol/kg, i.p.) or saline 15, 30, 60, 120, or 180 min after injection. Separate groups of mice were used for each time point. As seen in Fig. 2, significant effects of ABT-594 ($F(1,129) = 32.090$, $P < 0.0001$) and time ($F(4,129) = 7.970$, $P < 0.0001$) and a significant ABT-594 by time interaction ($F(4,129) = 4.872$, $P = 0.0011$) were obtained. The antinociceptive effect of ABT-594 peaked at 30 min and was statistically significant at 60 min, but not at 120 or 180 min.

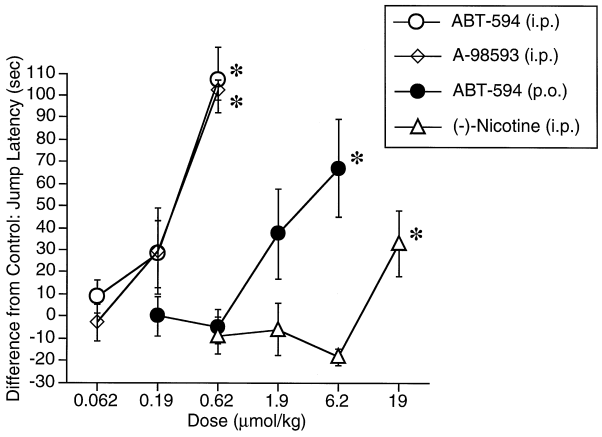

Fig. 1. The effects of i.p. administration of ABT-594, its *S*-enantiomer (A-98593), and (−)-nicotine and of p.o. administration of ABT-594 on jump latencies in the hot plate test. Data are from separate experiments and normalized by presentation as the difference from the saline control group. Shown are means ± S.E.M. $n = 6$–10 mice per group. * Significantly different from saline control group, $P < 0.05$.

The antinociceptive effects of ABT-594 in the hot-plate test were prevented by pretreatment with the noncompetitive neuronal nicotinic acetylcholine receptor antagonist mecamylamine. In this experiment mecamylamine (5 μmol/kg) or saline was administered i.p. 15 min before an i.p. injection of ABT-594 (0.62 μmol/kg) or saline. Analysis of hot plate latencies measured 30 min after the second injection (see Fig. 3A) revealed a marginal main effect of ABT-594 ($F(1,27) = 4.017$, $P = 0.055$) and no significant main effect of mecamylamine ($F(1,27) = 2.024$, $P = 0.166$), but a significant ABT-594 by mecamylamine interaction ($F(1,27) = 20.120$, $P < 0.0001$). Mecamylamine modestly increased jump latencies by itself ($P <$

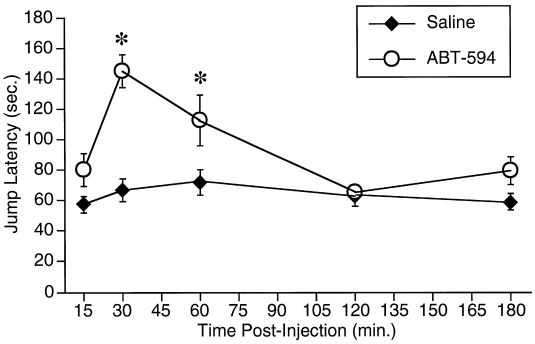

Fig. 2. Duration of the antinociceptive effects of ABT-594 (0.62 μmol/kg, i.p.) in the hot plate test. Shown are the jump latencies (mean ± S.E.M.) for animals treated with ABT-594 or saline and tested the indicated time after injection. Separate groups ($n = 14$ mice/group) were used for each time point. * Significantly different from saline control group, $P < 0.05$.

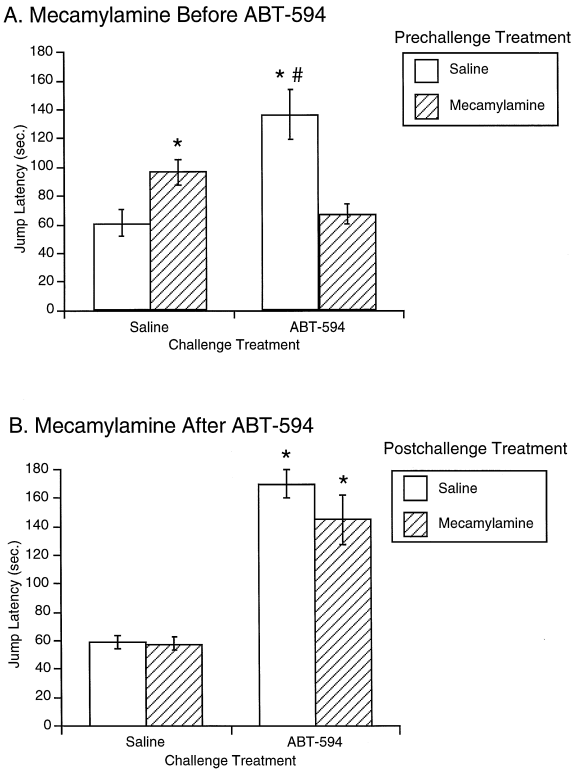

Fig. 3. Effects of the noncompetitive neuronal nicotinic acetylcholine receptor antagonist, mecamylamine (5 μmol/kg, i.p.) administered either 15 min before (A) or 15 min after (B) ABT-594 (0.62 μmol/kg, i.p.) on the antinociceptive effect of ABT-594 in the hot-plate test. Shown are jump latencies (mean + S.E.M.). Mice were tested 30 min after ABT-594. $n = 7–8$ per group. # Significantly different from mecamylamine/ABT-594 group, $P < 0.001$. * Significantly different from saline/saline control group, $P < 0.05$.

0.05) in this experiment, but still prevented the ABT-594-induced increase in jump latencies. ABT-594 produced significantly higher jump latencies in saline-pretreated than in mecamylamine-pretreated mice ($P < 0.001$).

In contrast to its effects when given before ABT-594, mecamylamine did not substantially reduce the antinociceptive effect of ABT-594 in the hot-plate test when administered after ABT-594 (Fig. 3B). In this experiment, mecamylamine (5 μmol/kg, i.p.) or saline was administered 15 min after ABT-594 (0.62 μmol/kg, i.p.) or saline, and hot plate jump latencies were determined 30 min after the first injection. The main effect of ABT-594 was significant ($F(1,27) = 94.424$, $P < 0.0001$), but the main effect of mecamylamine ($F(1,27) = 1.625$, $P = 0.213$) and the ABT-594 by mecamylamine interaction ($F(1,27) = 1.385$, $P = 0.250$) were not. In this experiment, mecamylamine by itself did not affect jump latencies and did not alter the effect of previously administered ABT-594.

The antinociceptive effect of ABT-594 was not prevented by hexamethonium, a neuronal nicotinic acetylcholine receptor antagonist that does not readily enter the central nervous system. In this experiment, saline or hexamethonium (10 μmol/kg, i.p.) was injected 15 min prior to an injection of saline or ABT-594 (0.62 μmol/kg, i.p.). The mice were tested in the hot-plate test 30 min after the second injection. These results (Table 1) show a significant effect of ABT-594 ($F(1,28) = 13.365$, $P = 0.001$). However, the effect of hexamethonium ($F(1,28) = 0.001$, $P = 0.975$) and the hexamethonium by ABT-594 interaction ($F(1,28) = 0.587$, $P = 0.450$) were not statistically significant. ABT-594 produced antinociceptive effects in saline-pretreated and in hexamethonium pretreated mice. Thus, the peripherally-active neuronal nicotinic acetylcholine receptor antagonist was ineffective at this dose.

Table 1
Effects of hexamethonium (10 μmol/kg, i.p.) given 15 min before ABT-594 (0.62 μmol/kg, i.p.) on the antinociceptive effects of ABT-594 in the hot-plate test in mice

Treatment	n	Latency to the 10th jump (s) (mean ± S.E.M.)
Saline/Saline	8	73.6 ± 11.3
Hexamethonium/Saline	8	85.2 ± 10.7
Saline/ABT-594	8	143.6 ± 20.1[a]
Hexamethonium/ABT-594	8	131.0 ± 18.8[a]

[a] Significantly different from all other groups ($P < 0.05$).

The possible role of opioid receptor activation in the antinociceptive effects of ABT-594 was assessed by attempting to prevent ABT-594-induced nociception in the hot plate test with the opioid receptor antagonist, naltrexone (0.8 μmol/kg, i.p.). The effect of naltrexone on morphine (21 μmol/kg) antinociception was assessed in the same experiment, but the effects of naltrexone on morphine and ABT-594 were analyzed separately by 2-way ANOVA. These results are presented in Table 2. In the ABT-594 analysis, an ABT-594-induced increase in jump latencies was noted ($F(1,28) = 14.029$, $P = 0.0008$), but neither the naltrexone main effect ($F(1,28) = 0.972$, $P = 0.333$) nor the naltrexone by ABT-594 interaction effect ($F(1,28) = 0.046$, $P = 0.832$) were significant. Thus, there was no evidence that naltrexone altered the antinociceptive effect of ABT-594 in this experiment. In contrast, the morphine analysis revealed significant morphine ($F(1,28)$

Table 2
Effects of naltrexone (0.8 μmol/kg, i.p.) given 15 min before ABT-594 (0.62 μmol/kg, i.p.) or morphine (21 μmol/kg, i.p.) on the antinociceptive effects in the hot-plate test in mice

Treatment	n	Latency to the 10th jump (s) (mean ± S.E.M.)
Saline/Saline	8	100.6 ± 14.6
Naltrexone/Saline	8	82.4 ± 9.6
Saline/ABT-594	8	154.4 ± 19.1[a]
Naltrexone/ABT-594	8	142.6 ± 16.0[a]
Saline/Morphine	8	157.5 ± 7.2[a]
Naltrexone/Morphine	8	81.9 ± 14.0[b]

[a] Significantly different from saline/saline ($P < 0.05$).
[b] Significantly different from saline/morphine ($P < 0.05$).

$= 5.755$, $P = 0.023$) and naltrexone ($F(1,28) = 15.957$, $P = 0.0004$) main effects, as well as a significant naltrexone by morphine interaction ($F(1,28) = 5.961$, $P = 0.021$). Post-hoc analysis confirmed that naltrexone blocked the effect of morphine in this test.

3.2. Cold-plate testing

Since noxious thermal stimulation is not limited to high temperature stimuli, effects of noxious cold stimulation were also evaluated. In the cold-plate test, the effects of doses of ABT-594 and morphine that produced maximal antinociceptive effects in the hot plate test (0.62 μmol/kg, i.p. and 21 μmol/kg, i.p., respectively) were compared. Control mice received saline injections. The results (mean latencies in s ± S.E.M.: saline group, 29.0 ± 3.7; morphine group, 84.9 ± 18.5; and ABT-594 group, 92.9 ± 19.8; $N = 8$ per group) revealed a significant treatment effect ($F(2,21) = 4.854$, $P = 0.018$), with both ABT-594 and morphine significantly increasing response latencies ($P < 0.05$). The effects of ABT-594 and morphine were comparable, although morphine was far less potent than ABT-594. Preliminary tests had established that lower doses of morphine were not effective in this model (data not shown).

3.3. Abdominal constriction assay

To determine if the antinociceptive effects of ABT-594 observed in a test of acute thermal pain were also seen in a test of persistent chemical pain, ABT-594 was tested in the abdominal constriction assay. When injected i.p. 30 min before administration of the chemical irritant phenyl-*p*-quinone, ABT-594 significantly reduced the number of animals responding to the chemical irritant at 0.19 and 0.62 μmol/kg ($P < 0.05$). As in the hot-plate test, the effect of ABT-594 was not stereoselective since A-98593 was also effective at these doses. (−)-Nicotine, however, was ineffective in this test at doses as high as 19 μmol/kg. Higher doses of (−)-nicotine could not be used because of overt toxicity. These results are shown in Fig. 4.

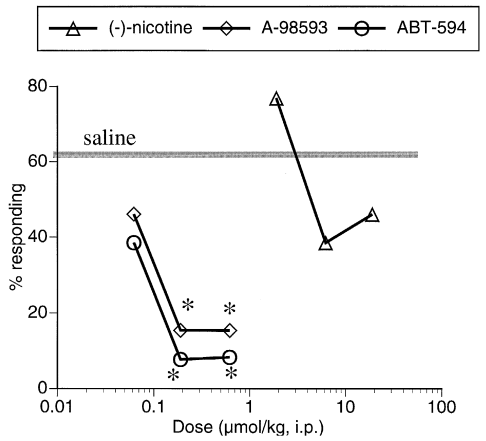

Fig. 4. The effects of i.p. administration of ABT-594, its *S*-enantiomer (A-98593), and (−)-nicotine on nociceptive responses to i.p. injection of the irritant phenyl-*p*-quinone. Shown are the percentages of mice responding to the injection of phenyl-*p*-quinone. $N = 13$ mice per group. * Significantly different from saline control group (shown as the shaded line), $P < 0.05$.

3.4. Locomotor activity, motor coordination, and body temperature

Because both models of antinociception used here assess pain intensity by measuring behavioral responses to painful stimuli, it is possible that compounds that disrupt an animal's ability to make the required behavioral responses could inaccurately be identified as potential analgesics. To assess the effects of ABT-594 on motor performance, the effects of the compound (0.62 and 1.9 μmol/kg, i.p.) on locomotor activity in an open field and on rotarod performance were determined. In addition, the ability of the compound to lower body temperature was assessed since compounds acting at nicotinic acetylcholine receptors can decrease body temperature. As can be seen in Table 3, significant reductions in activity and body temperature were observed at 1.9 μmol/kg of ABT-594. At 0.62 μmol/kg, the dose at which maximal antinociceptive effects were observed, ABT-594 decreased vertical activity and body temperature but not horizontal activity. No overall treatment effect was noted in the rotarod experiment ($F(3,25) = 1.700$, $P = 0.193$). However, given the high degree of variance that resulted from the inability of 4 of the 7 diazepam-treated mice to stay on the rod for more than a second, a separate comparison was conducted using a nonparametric test. This evaluation revealed a significant reduction in rotarod performance by diazepam (2-tailed Mann–Whitney's *U*-test, $z = 2.003$, $P = 0.045$) but not by either dose of ABT-594 ($z = 1.504$, $P = 0.1325$ for 0.62 μmol/kg and $z = 1.153$, $P = 0.249$ for 1.9 μmol/kg).

An additional test over a wider dose range (0.019, 0.19, and 1.9 μmol/kg) revealed similar effects. Significant treatment effects were observed on vertical activity ($F(4,33) = 6.196$, $P = 0.0008$), horizontal activity ($F(4,33) = 7.013$, $P = 0.0003$), rotarod ($F(4,33) = 6.317$, $P = 0.0007$), and body temperature ($F(4,33) = 35{,}514$, $P < 0.0001$). ABT-594, at a dose of 1.9 μmol/kg, significantly reduced all measures. Lower doses of ABT-594 were less effective, with 0.19 μmol/kg reducing all measures except the rotarod and 0.019 μmol/kg reducing only vertical activity. Diazepam (10.5 μmol/kg, i.p.) significantly reduced all measures except horizontal activity.

3.5. Interaction with morphine antinociception

Possible interactions between the antinociceptive effects of ABT-594 and morphine were evaluated using the hot-plate test (Table 4). In this experiment, morphine (0, 2.6, 5.25, 10.5, and 21 μmol/kg) and ABT-594 (0, 0.1, 0.2, 0.4, and 0.8 μmol/kg) were co-administered i.p., as a

Table 3
Effects of ABT-594 and diazepam administered 25–35 min before testing for locomotor activity, rotarod performance and body temperature in mice

ABT-594 (μmol/kg, i.p.)	n	Vertical activity counts (mean ± S.E.M.)	Horizontal activity counts (mean ± S.E.M.)	Latency to fall (s) (mean ± S.E.M.)	Rectal temperature (°C) (mean ± S.E.M.)
Experiment 1					
Saline	7	116.7 ± 9.5	583.0 ± 33.8	67.1 ± 13.6	38.51 ± 0.11
0.62	8	17.8 ± 12.4[a]	375.1 ± 107.1	36.8 ± 10.9	32.88 ± 0.49[a]
1.9	7	10.7 ± 3.3[a]	232.4 ± 52.5[a]	41.7 ± 12.9	32.43 ± 0.62[a]
Diazepam (10.5 μmol/kg)	7	112.1 ± 27.2	604.7 ± 88.5	26.4 ± 15.2[b]	35.27 ± 0.60[a]
Experiment 2					
Saline	7	216.1 ± 9.5	665.6 ± 35.4	86.1 ± 11.8	39.10 ± 0.04
0.019	7	98.6 ± 15.1[a]	484.7 ± 64.4	82.1 ± 14.4	38.13 ± 0.17
0.19	8	59.0 ± 57.7[a]	289.2 ± 105.9[a]	79.5 ± 11.2	35.44 ± 0.81[a]
1.9	8	5.4 ± 5.1[a]	246.1 ± 70.4[a]	46.1 ± 12.6[a]	32.71 ± 0.29[a]
Diazepam (10.5 μmol/kg)	8	112.1 ± 26.0[a]	712.9 ± 96.1	19.9 ± 7.2[a]	35.01 ± 0.28[a]

[a]Significantly different from saline ($P < 0.05$).
[b]Significantly different from saline ($P < 0.05$) using a Mann–Whitney's U-test but no significant F-value for experiment from ANOVA.

cocktail, 30 min before testing. All combinations of the compounds were used, resulting in a total of 25 treatment groups. ANOVA of the jump latencies revealed main effects of both morphine ($F(4,170) = 27.544$, $P < 0.0001$) and ABT-594 ($F(4,170) = 14.165$, $P < 0.0001$). Evaluation of these main effects by collapsing across all animals receiving a given dose of morphine or ABT-594 revealed significant differences from control for 10.5 and 21 μmol/kg of morphine and for 0.2, 0.4, and 0.8 μmol/kg ABT-594. No significant interaction was noted between morphine and ABT-594 in this experiment [$F(16,170) = 0.821$, $P = 0.66$], suggesting that their effects were additive.

3.6. Preliminary safety testing

The LD_{50} value for ABT-594 was determined in mice with i.p. dosing and a 7 day duration of observation. In this experiment, doses of 5, 10, 15, and 20 μmol/kg were administered to separate groups of 10 mice each. No deaths were observed after either of the lower doses. Three deaths and seven deaths were noted at the 15 μmol/kg and 20 μmol/kg doses respectively. Deaths were preceded by tonic/clonic seizures, although seizures were also noted in animals that did not die. From these results, an LD_{50} value of 19.1 μmol/kg (95% C.I. 14.8–24.7) was calculated. Animals who did not die during the first few hours after the injection appeared normal when checked 24 h later, and no additional mortality was noted after the first day.

The ED_{50} value for seizure activity was determined in a separate experiment. In this experiment, the effects of (±)-epibatidine were also assessed. For ABT-594 and A-98593, 10 mice were used for each dose (1, 1.5, 2, 2.5, 3, 3.5, and 4 μmol/kg, i.p.). Seizures were assessed by behavioral observation and those that were noted occurred within a few min of injection. The ED_{50} and ED_{16} values for ABT-594 were 1.9 μmol/kg (95% C.I. 1.5–2.5) and

Table 4
Antinociceptive effects of co-administration (30 min. before testing) of morphine and ABT-594 in the hot-plate test in mice

Morphine (μmol/kg)	ABT-594 (μmol/kg)				
	Saline	0.1	0.2[b]	0.4[b]	0.8[b]
Saline	66.6 ± 6.8	62.3 ± 7.2	61.1 ± 4.6	70.7 ± 8.4	102.9 ± 15.4
2.6	63.3 ± 4.8	71.7 ± 13.0	81.6 ± 13.1	93.9 ± 22.4	116.5 ± 16.6[a]
5.2	59.3 ± 3.0	73.0 ± 4.6	92.9 ± 16.6	98.2 ± 14.9	109.1 ± 19.1[a]
10.5[b]	98.3 ± 11.2	70.2 ± 11.6	120.7 ± 18.6[a]	122.1 ± 19.8[a]	159.8 ± 13.1[a]
21[b]	112.0 ± 17.6[a]	126.6 ± 13.0[a]	165.3 ± 13.8[a]	170.5 ± 5.2[a]	178.4 ± 1.6[a]

[a]Significantly different from saline ($P < 0.05$) by post-hoc pairwise comparison.
[b]Significant main effect (relative to saline) at this dose.
Shown are means (±S.E.M.) for jump latencies.
$n = 7$–8 per group.

Table 5
Effects of 15-min. pretreatment with ABT-594 (0.62 μmol/kg, i.p.) on ethanol- and pentobarbital-induced hypnotic effects in mice

Group	n	Min to recover righting reflex (mean ± S.E.M.)
Saline/Ethanol	7	28.0 ± 3.0
ABT-594/Ethanol	7	85.7 ± 4.3[a]
Saline/Pentobarbital	7	31.4 ± 8.9
ABT-594/Pentobarbital	7	49.4 ± 8.3

[a] Significantly different from saline pretreatment ($P < 0.05$).

1.1 μmol/kg (95% C.I. 1.0–1.2), respectively. Comparable potency was observed with A-98593 which had ED_{50} and ED_{16} values of 2.0 μmol/kg (95% C.I. 1.7–2.4) and 1.3 μmol/kg (95% C.I. 1.3–1.4), respectively. (±)-Epibatidine was 10-fold more potent ($P < 0.05$) than ABT-594 and A-98593 in producing seizures with ED_{50} and ED_{16} values of 0.16 μmol/kg (95% C.I. 0.13–0.21) and 0.11 μmol/kg (95% C.I. 0.10–0.12), respectively.

Potential interactions with ethanol and barbiturates were evaluated by assessing the effect of ABT-594 on ethanol and pentobarbital-induced hypnotic effects. In addition, the effect of ABT-594 on the approximate lethal dose of pentobarbital was determined. A dose of 0.62 μmol/kg (i.p.) of ABT-594 was selected for use in these experiments given its consistent antinociceptive effects in the hot-plate and abdominal constriction assays. The compound was administered 15 min prior to ethanol (87 mmol/kg, i.p.) or pentobarbital (160 μmol/kg, i.p. in the hypnotic effect experiment and 320, 400, 480, 560, 640, 720, or 800 μmol/kg in the approximate lethal dose experiment). Saline was administered before ethanol or pentobarbital to mice in the control groups. ABT-594 significantly potentiated ethanol-induced hypnotic effects (Table 5), increasing 'sleep time' by 3-fold ($F(1,12) = 121.501$, $P < 0.0001$). There was a tendency for ABT-594 to increase pentobarbital-induced sleep time (Table 5) that was nearly statistically significant ($F(1,19) = 4.368$, $P = 0.0503$), but ABT-594 did not affect the lethality of pentobarbital (in each case the approximate lethal dose for pentobarbital was 520 μmol/kg, based on the mean of 4 determinations).

To determine if ABT-594 altered the effects of morphine on blood pCO_2, ABT-594 (0 or 0.4 μmol/kg) and morphine (0, 10.5, 21, or 42 μmol/kg, i.p.) were administered alone or in combination, and pCO_2 levels were determined in trunk blood obtained 30 min after injection. As seen in Table 6, there was a significant main effect of morphine ($F(3,61) = 3.381$, $P = 0.024$). This effect was the result of a significant increase in pCO_2 (hypercapnia) at the highest dose of morphine. ABT-594 had no significant effect on its own ($F(1,61) = 0.654$, $P = 0.422$) and did not alter the effect of morphine (morphine by ABT-594 interaction, $F(3,61) = 0.087$, $P = 0.967$). In a separate experiment, the effect of morphine (21.0 μmol/kg, i.p.) or a higher dose of ABT-594 (1.24 μmol/kg, i.p., twice the maximally effective antinociceptive dose) on pCO_2 was determined. This experiment (Table 6) revealed a significant treatment effect ($F(2,19) = 11.917$, $P = 0.0004$), which was the result of a morphine induced increase in pCO_2. In contrast, no evidence of respiratory depression with ABT-594 was observed in this experiment.

3.7. Effects of ABT-594 on the elevated plus-maze test

The anxiolytic-like effects of ABT-594 and the anxiolytic positive control, diazepam, in the elevated plus-maze were assessed using one-way ANOVA. This was determined at 30 min after injection, the time of peak antinociceptive activity on the hot-plate. As can be seen in Table 7, significant treatment effects were noted on the time spent on the open arms ($F(4,54) = 3.293$, $P = 0.017$) and on the total distance traveled ($F(4,54) = 4.622$, $P = 0.003$). Post-hoc comparisons with saline-injected controls, revealed that diazepam (10.5 μmol/kg, i.p.) significantly increased both measures ($P < 0.05$), whereas ABT-594

Table 6
Effects of co-administration (30 min before testing) of morphine and ABT-594 on blood pCO_2 in mice

Treatment (μmol/kg, i.p.)	n	pCO_2 (mean ± S.E.M.)	% difference from saline
(A) Combined treatment			
Saline	13	42.8 ± 1.2	
Morphine (10.5)	8	44.8 ± 1.9	+4.7
Morphine (21.0)	9	44.8 ± 1.4	+4.7
Morphine (42.0)	9	47.4 ± 1.3[a]	+10.7
ABT-594 (0.4)	9	44.0 ± 1.7	+2.8
ABT-594 (0.4)/Morphine (10.5)	7	45.1 ± 2.1	+5.4
ABT-594 (0.4)/Morphine (21.0)	7	45.1 ± 1.6	+5.4
ABT-594 (0.4)/Morphine (42.0)	7	49.3 ± 2.1[a]	+15.2
(B) Separate treatment			
Saline	7	37.7 ± 1.3	
ABT-594 (1.24)	7	36.1 ± 1.5	−4.2
Morphine (21.0)	8	44.9 ± 1.3[a]	+19.1

[a] Significantly different from saline ($P < 0.05$).

Table 7
Effects of ABT-594 and diazepam administered 30 min before testing in the elevated plus-maze test

ABT-594 (μmol/kg, i.p.)	n	Time spent on open arms (s) (mean ± S.E.M.)	Distance traveled (cm) (mean ± S.E.M.)
Saline	12	37.9 ± 5.8	741 ± 104
0.019	11	63.4 ± 4.9[a]	840 ± 40
0.062	12	66.4 ± 7.1[a]	812 ± 26
0.19	12	54.5 ± 7.5	646 ± 104
Diazepam (10.5 μmol/kg)	12	79.6 ± 13.9[a]	1230 ± 172[a]

[a] Significantly different from saline ($P < 0.05$).

selectively increased the time spent on the open arms at 0.019 and 0.062 μmol/kg ($P < 0.05$). Control animals spent less than 13% of the time on the two open arms. Increases in time spent on the open arms can be interpreted as an anxiolytic-like effect. In the case of ABT-594, this increase cannot be attributed to a non-specific increase in motor activity since the compound did not alter the total distance traveled during the test.

4. Discussion

These data demonstrate that ABT-594 exhibits antinociceptive effects in mice subjected to either acute thermal or persistent chemical pain stimuli. Moreover, antinociception observed in thermal threshold tests was present when either noxious cold or noxious hot stimuli were used. ABT-594 was about 30-fold more potent than (−)-nicotine in the hot-plate test and was somewhat more potent in the abdominal constriction assay than in the hot-plate. In contrast to previous findings that (−)-nicotine is active in the abdominal constriction assay when administered 5–10 min before evaluation (Aceto et al., 1983), (−)-nicotine was inactive in this assay at in the current study at doses as high as 19 μmol/kg administered 35–45 min before evaluation. This is consistent with the short duration of action observed with (−)-nicotine. Higher doses of (−)-nicotine were not tested since we have previously observed an ED_{50} for seizures of 41 μmol/kg and an approximate lethal dose of 70 μmol/kg with (−)-nicotine (Decker et al., 1994a).

The antinociceptive effects of ABT-594 were not stereoselective, as its *S*-enantiomer, A-98593, produced effects similar to those of ABT-594 on the hot-plate and abdominal constriction tests. The lack of stereoselectivity we observed with ABT-594 is distinct from the stereoselectivity previously found with nicotine (Rao et al., 1996) but is consistent with in vitro nicotinic acetylcholine receptor binding data for ABT-594, since ABT-594 and A-98593 have similar potencies in displacing [^{3}H]cytisine from nicotinic acetylcholine receptors in rat brain (Donnelly-Roberts et al., 1998). Moreover, the present results with ABT-594 resemble the lack of stereoselectivity found for the antinociceptive effects of epibatidine (Bonhaus et al., 1995; Damaj et al., 1994; Li et al., 1993).

Antinociceptive effects of ABT-594 were observed at doses that decrease spontaneous exploratory activity and body temperature. Both (−)-nicotine and (±)-epibatidine also reduce spontaneous exploratory activity and body temperature at antinociceptive doses (Bannon et al., 1995a; Collins et al., 1986; Decker et al., 1994b). Motor effects of compounds could confound the interpretation of antinociceptive tests, since the tests utilized require a motor response. However, there is evidence that the motor and antinociceptive effects of (±)-epibatidine can be dissociated (Bannon et al., 1995a). Similarly, the motor effects of ABT-594 do not appear to explain the effects on the antinociceptive tests in the current study. Reliable impairment of motor coordination, as measured by the rotarod test, was not observed at antinociceptive doses of ABT-594. Since doses beyond the antinociceptive dose range of ABT-594 were required for consistent disruption of rotarod performance, motor impairment cannot account for all of the effects of this compound in the hot-plate and abdominal constriction assays. Moreover, behavioral observations suggested that even when ABT-594 reduced spontaneous exploration, animals displayed normal responses to tactile stimulation. Dissociation of the antinociceptive and motor effects of ABT-594 can also be demonstrated in rats, where repeated dosing produces tolerance to the motoric effects but not to the antinociceptive effects of the compound (Bannon et al., 1998b).

Further characterization of the antinociceptive effects of ABT-594 using the hot-plate test revealed that the antinociceptive effect peaked at 30 min and was still present at 60 min after i.p. administration. The compound was also active after oral administration but appeared to be about 10-fold less potent after p.o. than after i.p. administration, which probably reflects differences in bioavailability with these two routes of administration.

Administration of the neuronal nicotinic acetylcholine receptor antagonist mecamylamine prior to ABT-594 prevented the antinociceptive effect of ABT-594 in the hot-plate test. However, administration of mecamylamine after ABT-594 did not reverse the antinociceptive effects of ABT-594. A modest increase in jump latencies was observed with mecamylamine alone in the first of these two experiments. The nature of mecamylamine-induced increase in jump latencies is not clear, and this particular

effect of mecamylamine alone was not observed in the second experiment. Moreover, we have not previously observed a significant effect of mecamylamine on jump latencies in the hot plate under similar conditions (Bannon et al., 1995b; Sullivan et al., 1994).

Although mecamylamine pretreatment prevented the antinociceptive effect of ABT-594, the neuronal nicotinic acetylcholine receptor antagonist hexamethonium, a compound that does not readily enter the central nervous system after parenteral administration, did not prevent this effect. This finding suggests that the antinociceptive effect of ABT-594 is not mediated solely by activation of peripheral neuronal nicotinic acetylcholine receptors. A central site of action is also suggested by findings from rats that intracerebroventricular administration of chlorisondamine, which produces a long-lasting blockade of central, but not peripheral, nicotinic acetylcholine receptors, prevents the antinociceptive effects of systemically-administered ABT-594 (Bannon et al., 1998b) and by findings that direct injection of ABT-594 into the nucleus raphe magnus produces antinociception (Bannon et al., 1998a).

In contrast to the involvement of nicotinic acetylcholine receptors in the antinociceptive effect of ABT-594, activation of opioid receptors is not required for the effect of ABT-594 in the mouse hot-plate test since the opioid receptor antagonist naltrexone did not prevent the effect. This dose of naltrexone was sufficient to prevent the antinociceptive effect of morphine, suggesting that the dose was sufficient to block μ opioid receptors.

An additive interaction with the antinociceptive effects of morphine was noted in hot-plate testing, but ABT-594 did not alter the hypercapnia produced by morphine as reflected by blood pCO_2. A potential interaction with alcohol was noted with ABT-594, as an antinociceptive dose significantly increased the duration of ethanol-induced hypnotic effects. This interaction is similar to that we have observed with (±)-epibatidine, which markedly enhances ethanol-induced hypnotic effects at doses as low as 0.002 μmol/kg (unpublished observations). A similar trend toward ABT-594 potentiation of pentobarbital-induced hypnotic effects was noted, but this same dose of ABT-594 did not alter the acute lethality of pentobarbital.

Preliminary safety testing revealed an ED_{50} value for seizures of 1.9 μmol/kg, which is 3 times the dose required to obtain the maximum effect in the antinociceptive tests and more than 10 times the seizure ED_{50} for (±)-epibatidine (ED_{50} value of 0.16 μmol/kg). As for the antinociceptive effects, the ability of ABT-594 to produce seizures at high doses was not stereoselective since its *S*-enantiomer, A-98593, displayed comparable potency. An LD_{50} of 19 μmol/kg was determined for ABT-594, which is 30 times the dose eliciting maximal antinociception in the hot-plate test and 100 times the dose at which significant antinociception was observed on the abdominal constriction assay. In contrast, we have previously found that (±)-epibatidine is lethal at a dose only 6 times that eliciting maximal antinociception in the hot-plate test (Sullivan et al., 1994). Thus, ABT-594 has a larger safety index than (±)-epibatidine.

Interestingly, ABT-594 had anxiolytic-like activity in the elevated plus-maze test. Anxiolytic-like effects have also been observed with other nicotinic acetylcholine receptor agonists, including (−)-nicotine and ABT-418 (Brioni et al., 1994; Brioni et al., 1993; Decker et al., 1994a). However, not all nicotinic acetylcholine receptor agonists are active in this test. For example, neither cytisine (Brioni et al., 1993) nor (±)-epibatidine (Sullivan et al., 1994) have anxiolytic-like activity in this test. The apparent difference between ABT-594 and (±)-epibatidine on this measure is particularly interesting given the similar antinociceptive effects obtained with these two compounds and further serves to distinguish between these two potent neuronal nicotinic acetylcholine receptor agonists. Moreover, a compound with both anxiolytic and antinociceptive activity might be particularly useful in pain management. It should be noted, however, that the anxiolytic-like effects of ABT-594 could be obtained at slightly lower doses than its antinoceptive effects.

In summary, data obtained in this study indicate that the novel nicotinic acetylcholine receptor agonist, ABT-594, had antinociceptive effects in mouse models of acute and persistent pain, which is a broader spectrum of activity than displayed by (−)-nicotine. ABT-594 has an improved safety profile relative to both (−)-nicotine and the potent antinociceptive nicotinic acetylcholine receptor agonist, (±)-epibatidine. This suggests that it is possible to increase separation between antinociceptive effects and undesirable side effects of nicotinic acetylcholine receptor stimulation. Thus, the findings with ABT-594 suggest that nicotinic acetylcholine receptor agonists may have potential for development as a new class of analgesics.

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