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European Journal of Pharmacology 521 (2005) 43–48
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In vivo characterization of a novel inhibitor of CNS nicotinic receptors

M. Imad Damaj [a,*], Jenny L. Wiley [a], Billy R. Martin [a], Roger L. Papke [b]

[a] Department Pharmacology and Toxicology, Medical College Virginia Campus, Virginia Commonwealth University, Box 980613, Richmond VA 23298-0613, United States
[b] Department of Pharmacology and Therapeutics, University of Florida, College of Medicine, Gainesville, FL 32610, United States

Received 24 March 2005; received in revised form 27 June 2005; accepted 28 June 2005
Available online 21 September 2005

Abstract

There are multiple types of nicotine acetylcholine receptors (nAChR) in the brain associated with synaptic function, signal processing, or cell survival. The therapeutic targeting of nicotinic receptors in the brain will benefit from the identification of drugs, which may be selective for their ability to activate or inhibit a limited range of these receptor subtypes. We previously identified a family of bis-tetramethylpiperidine compounds as selective inhibitors of neuronal-type nicotinic receptors. In the present study we describe the in vivo effects and properties of 2,2,6,6-tetramethylpiperidin-4-yl heptanoate (TMPH), a novel inhibitor of neuronal nicotinic receptors. Delivered systemically, this drug can block central nervous system effects of nicotine, indicating that this drug is able to cross the blood–brain barrier and access sites in the brain. Unlike the prototype CNS-active nicotinic inhibitor, mecamylamine, TMPH blocked some but not all of the CNS effects of nicotine, indicating that it has a unique selectivity for specific receptor subtypes in the brain. The nAChR subtypes that mediate the locomotor effects and hypothermic effects of nicotine appear to be less sensitive to TMPH than those which mediate analgetic effects and discriminative stimuli. These results indicate that TMPH may possess unique selectivity for specific nicotinic receptor subtypes.
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Keywords: Nicotine; Nicotinic antagonists; Analgesia; Drug discrimination

1. Introduction

During the past several years, considerable efforts have been directed towards the development of ligands for nicotinic acetylcholine receptors (nAChRs) in the brain. These compounds are of interest because of their potential therapeutic utility in the treatment of central nervous system (CNS) disorders including Alzheimer's and Parkinson's disease, pain, schizophrenia, anxiety, depression, Tourette's syndrome, and smoking cessation. Most of the efforts have been directed towards nAChR agonists. However, interest in nAChR antagonists has increased since studies have shown that bupropion (Zyban®), the antidepressant that has proven useful in treatment for smoking cessation, is a noncompetitive nAChR antagonist (Slemmer et al., 2000; Fryer and Lukas, 1999). In addition, mecamylamine, a ganglionic blocker developed many years ago as an antihypertensive, was recently shown to be useful alone and in combination of nicotine as a component in the pharmacotherapy for Tourette's syndrome (Sanberg et al., 1998) and smoking cessation (Rose et al., 1994). Therefore, the therapeutic targeting of nicotinic receptors in the brain will benefit from the identification of drugs, which may be selective for their ability to activate or inhibit a limited range of nicotinic receptor subtypes. However, electrophysiological characterization of mecamylamine has shown it to be relatively nonselective (Papke et al., 2001), consistent with the observation that it effectively blocks all of the peripheral and central nervous system (CNS) effects of nicotine (Martin et al., 1993). We previously identified a family of bis-tetramethylpiperidine compounds as inhibitors of neuronal type nicotinic receptors (Francis et al., 1998). The prototype compound in this series is BTMPS (bis-(2,2,6,6-tetramethyl-4-piperidinyl)-sebacate), which produces a readily reversible block of muscle-type nAChR and a nearly irreversible use-dependent, voltage-independent block of neuronal nAChR. The tetramethyl-

Abbreviations: TMPH, 2,2,6,6-tetramethylpiperidin-4-yl heptanoate; BTMPS, bis-(2,2,6,6-tetramethyl-4-piperidinyl)-sebacate.

* Corresponding author. Tel.: +1 804 828 1676; fax: +1 804 828 2117.
E-mail address: mdamaj@hsc.vcu.edu (M.I. Damaj).

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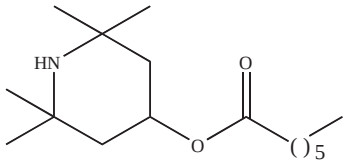
doi:10.1016/j.ejphar.2005.06.056



2,2,6,6-tetramethylpiperidin-4-yl heptanoate

Fig. 1. Structure of TMPH.

piperidine groups of BTMPS are sufficient to produce block of nAChR, and the conjugation of two such groups with a long aliphatic chain accounts for both the selectivity and slow reversibility of BTMPS inhibition of neuronal nAChR (Francis et al., 1998). In the present study we describe the in vivo effects of 2,2,6,6-tetramethyl-4-hydroxy piperidinyl octenoate (TMPH), a novel compound that has a single tetramethyl-piperidine group and an aliphatic chain similar to that of BTMPS (Fig. 1). The modulatory action of systemically injected TMPH on nicotine's centrally mediated behavioral effects was studied in comparison with those of mecamylamine. The data show TMPH can block CNS effects of nicotine, indicating that this drug is able to cross the blood–brain barrier and access sites in the brain. Notably however, unlike the prototype CNS-active nicotinic inhibitor, mecamylamine, TMPH blocked only some of the CNS effects of nicotine, indicating that it may have unique selectivity for specific nicotinic receptor subtypes in the brain.

2. Methods

2.1. Animals

Male ICR mice (20–25 g) obtained from Harlan Laboratories (Indianapolis, IN) were used throughout the study. Animals were housed in groups of six and had free access to food and water. Adult, male Long–Evans rats (350–460 g), obtained from Harlan (Dublin, VA), were individually housed in a temperature-controlled (20–22 °C) environment with a 12 h light–dark cycle (lights on at 7 a.m.). Rats were maintained within the indicated weight range by restricted post-session feeding and had ad libitum water in their home cages. Rats were drug-naive at the beginning of the study. Animals were housed in an AALAC approved facility and the study was approved by the Institutional Animal Care and Use Committee of Virginia Commonwealth University.

2.2. Drugs

TMPH was synthesized as reported elsewhere (Papke et al., 2005) and supplied by Dr. Nicole Horenstein (University of Florida). Mecamylamine hydrochloride was supplied as a gift from Merck, Sharp and Dohme & Co. (West Point, PA). (−)-Nicotine was obtained from Aldrich Chemical Company, Inc. (Milwaukee, WI) and converted to the ditartrate salt as described by Aceto et al. (1979). All drugs were dissolved in physiological saline (0.9% sodium chloride). All doses are expressed as the free base of the drug.

2.3. Behavioral assays

2.3.1. Locomotor activity

Mice were placed into individual Omnitech photocell activity cages (28 × 16.5 cm) immediately after s.c. administration of either 0.9% saline or nicotine (6.2 μmol/kg or 1 mg/kg) and were allowed to acclimate for 10 min. Interruptions of the photocell beams (two banks of eight cells each) were then recorded for the next 10 min. Data were expressed as number of counts per 10 min session. Mice were pretreated s.c. with either saline or TMPH 10 min before nicotine.

2.3.2. Antinociception

1. Tail-flick test. Antinociception was assessed by the tail-flick method as modified by Dewey et al. (1970). Briefly, mice were lightly restrained while a radiant heat source was shone onto the upper portion of the tail. Latency to remove the tail from the heat source was recorded for each animal. A control response (2–4 s) was determined for each mouse before treatment, and a test latency was determined after drug administration. In order to minimize tissue damage, a maximum latency of 10 s was imposed. Antinociceptive response was calculated as percent maximum possible effect (% MPE), where % MPE = [(test − control)/(10 − control)] × 100.
2. Hot-plate test. Mice were placed into a 10 cm wide glass cylinder on a hot plate (Thermojust Apparatus) maintained at 55.0 °C. Two control latencies at least 10 min apart were determined for each mouse. The normal latency (reaction time) was 8 to 12 s. Antinociceptive response was calculated as percent maximum possible effect (% MPE), where % MPE = [(test − control)/(40 − control) × 100]. The reaction time was scored when the animal jumped or licked its paws. In order to minimize tissue damage, a maximum latency of 40 s was imposed. Antagonism studies were carried out by pretreating the mice with either saline or TMPH 10 min before nicotine. The animals were tested 5 min after administration of nicotine.

2.3.3. Body temperature

Rectal temperature was determined by a thermistor probe (inserted 24 mm) and digital thermometer (Yellow Springs Instrument Co., Yellow Springs, OH). Readings were taken just before and 30 min after the s.c. injection of nicotine at a dose of 12.3 μmol/kg (2 mg/kg). Mice were pretreated with either saline or TMPH (s.c.) 10 min before nicotine. The difference in rectal temperature before and after treatment was calculated for each mouse. The ambient temperature of the laboratory varied from 21 to 24 °C from day to day.

The doses of nicotine used in the different tests represent approximately an ED_{84} (effective dose 84%) which were determined from previous works (Damaj et al., 1995). Eight to twelve mice were tested in each treatment group and each animal was tested only once.

2.3.4. Drug discrimination in rats

1. Apparatus: Rats were trained and tested in standard operant conditioning chambers (Lafayette Instruments Co., Lafayette, IN) housed in sound-attenuated cubicles. Each chamber had three retractable levers, only two of which were used for this study. Pellet dispensers delivered 45-mg BIO SERV (Frenchtown, NJ) food pellets to a food cup on the front wall of the chamber between the two response levers and over the third (retracted) lever. Fan motors provided ventilation and masking noise for each chamber. House lights located above the food cup were illuminated during training and testing sessions. A micro-computer with Logic '1' interface (MED Associates, Georgia, Vermont) and MED-PC software (MED Associates) was used to control schedule contingencies and to record data.
2. Procedure: Rats were trained to press one lever following administration of 0.4 mg/kg nicotine and to press another lever after injection with saline, each according to a fixed ratio 10 schedule of food reinforcement. Completion of 10 consecutive responses on the injection-appropriate lever resulted in delivery of a food reinforcer. Each response on the incorrect lever reset the ratio requirement on the correct lever. The position of the drug lever was varied among the group of rats. The daily injections for each rat were administered in a double alternation sequence of 0.4 mg/kg nicotine and saline. Rats were injected and returned to their home cages until the start of the experimental session 5 min later. Training occurred during sessions conducted five days a week (Monday–Friday) until the rats had met three criteria during eight of ten consecutive sessions: (1) first completed fixed ratio 10 on the correct lever; (2) percentage of correct-lever responding >80% for the entire session; and (3) response rate >0.4 responses/s.

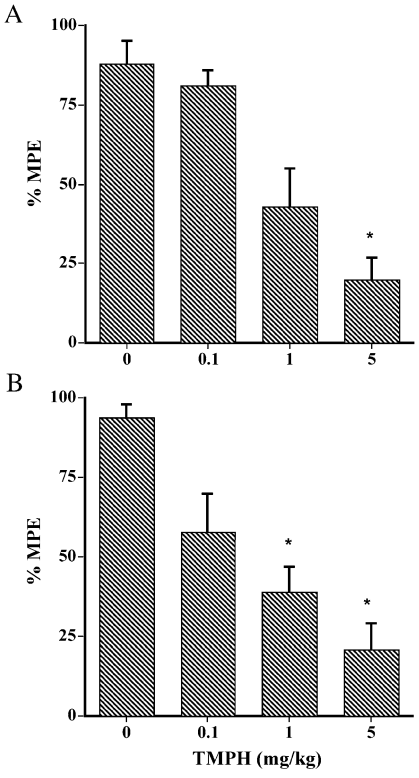


Fig. 2. Dose–response blockade of nicotine-induced antinociception in the tail-flick (Panel A) and the hot-plate test (Panel B) by TMPH after s.c. injection in mice. TMPH at different doses was administered s.c. 10 min before nicotine (2.5 mg/kg, s.c.) and mice were tested 5 min later. Each point represents the mean±SE of 8 to 12 mice.

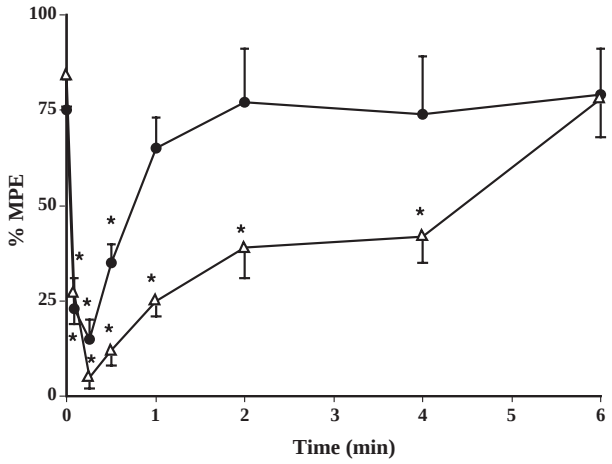


Fig. 3. Time-course of TMPH effect on nicotine-induced antinociception (2.5 mg/kg) in (—△—) the tail-flick and (—●—) the hot-plate tests after s.c. administration of 5 mg/kg in mice. Each point represents the mean±SE of 8 to 12 mice. *$p<0.05$ compared to correspondent zero time point.

Following successful acquisition of the discrimination, stimulus substitution tests with test compounds were conducted on Tuesdays and Fridays during 15-min test sessions. Training continued on Mondays, Wednesdays, and Thursdays. During test sessions, responses on either lever delivered reinforcement according to a fixed ratio 10 schedule. In order to be tested, rats must have completed the first FR and made at least 80% of all responses on the injection-appropriate lever on the preceding day's training session. In addition, the rat must have met these same criteria during at least one of the training sessions with the alternate training compound (nicotine or saline) earlier in the week.

A nicotine dose–effect determination [0.1, 0.2, 0.4, 0.8, and 1.2 mg/kg] was performed first in each rat. Then, combination

Table 1
Effect of TMPH on nicotine-induced hypomotility and hypothermia after s.c. administration

Treatment (mg/kg)	Locomotor activity # Interrupts	Body temperature Δ °C
	(Mean±SEM)	(Mean±SEM)
Saline/saline	1931±120	−0.3±0.1
TMPH (20)/saline	2031±160	−1.0±0.2
Saline/nicotine (1.5)	358±92*	−5.0±0.3*
TMPH (20)/nicotine (1.5)	397±170*	−5.2±0.4*

Each point represents the mean±SE of 6 to 8 mice.
* $p<0.05$ from saline/saline.

tests with nicotine, and TMPH followed (see figures for specific doses). Doses of each compound were administered in ascending order. Throughout the study, control tests with saline and 0.4 mg/kg nicotine were conducted during the week before the start of each dose–effect curve determination.

2.4. Statistical analysis

Statistical analysis of all analgesic and in vivo studies was performed using either *t*-test or analysis of variance (ANOVA) with Tukey's test post hoc test when appropriate. All differences were considered significant at $p<0.05$. AD_{50} values with 95% CL for behavioral data were calculated by unweighted least-squares linear regression as described by Tallarida and Murray (1987).

3. Results

3.1. Antinociception

Nicotine-induced antinociception in the tail-flick and hot-plate tests after systemic administration in mice (2.5 mg/kg) was blocked by TMPH in a dose-dependent manner (Fig. 2A and B). Calculation of the AD_{50} 15 min after the antagonist administration showed that TMPH is 1.7 times more potent in blocking the antinociceptive effect of nicotine in the hot-plate than in tail-flick test (0.7 versus 1.2 mg/kg). By itself, TMPH after s.c. injection did not cause antinociception at the indicated doses and times.

3.2. Time-course of TMPH effects

The duration of action of TMPH in the tail-flick test was time-dependent with maximum blockade occurring between 15 and 30 min after a dose of 5 mg/kg dose. The effect of TMPH lasted for at least 4 h after its administration. Indeed, as illustrated in Fig. 3, nicotine's effect started to recover within 60 min after pretreatment with a dose of 5 mg/kg of TMPH, but was still significantly different from control 4 h after. Similar to the tail-flick test, TMPH time-dependently blocked nicotine-induced antinociception as measured by the hot-plate test with however, a shorter duration of action. As shown in Fig. 3, nicotine's effects in the hot-plate test recovered entirely 60 min after pretreatment with a dose of 5 mg/kg of TMPH.

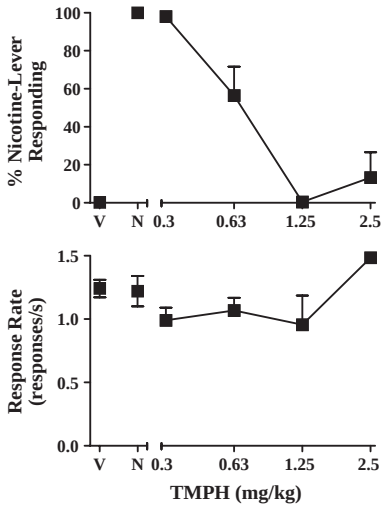


Fig. 4. Effects of TMPH in combination with 0.4 mg/kg nicotine on percentage of nicotine-lever responding (upper panel) and response rates (lower panel) in rats trained to discriminate 0.4 mg/kg nicotine from vehicle. Points above V and N represent the results of control tests with two saline injections and saline plus 0.4 mg/kg nicotine, respectively, conducted before the dose–effect curve determination. Each value represents the mean (+SEM) of 4–6 rats.

Table 2
Comparison of the blockade potency of TMPH and mecamylamine on nicotine′s pharmacological and behavior effects after systemic and administration in mice and rats

Test	TMPH[a]	Mecamylamine[b]
	(AD_{50} mg/kg±CL)	(AD_{50} mg/kg±CL)
Tail-flick	1.2 (0.6–1.8)	0.045 (0.03–0.1)
Hot-plate	0.7 (0.3–1.7)	0.8 (0.5–1.1)
Drug discrimination	0.74 (0.56–0.98)	0.91 (0.63–1.32)
Hypothermia	0% blockade at 20	1.2 (0.9–1.8)
Hypomotility	0% blockade at 20	1.95 (1.1–2.5)

[a] AD_{50} values (±CL) were calculated from the dose–response and expressed as mg/kg. Each dose group included 6 to 8 animals.
[b] AD_{50} values (±CL) were taken from Damaj et al. (1995) and Wiley et al. (2002).

3.3. Locomotor activity and body temperature

TMPH at 20 mg/kg administered s.c. 15 min prior to the injection of nicotine (1.5 mg/kg) failed to significantly reduce the hypomotility induced by nicotine (Table 1). In addition, nicotine-induced hypothermia after systemic administration in mice (2.5 mg/kg) was also not blocked by both TMPH given at 20 mg/kg. By itself, TMPH after s.c. injection did not have a significant effect on the body temperature or the locomotor activity at the indicated doses and times.

3.4. Nicotine discriminative stimulus in rats

Fig. 4 shows the results of combination tests with the training dose of nicotine and various doses of TMPH. TMPH dose-dependently antagonized the discriminative stimulus effects of 0.4 mg/kg nicotine (Fig. 4 top right panel) with an AD_{50} value of 0.74 mg/kg (0.56–0.98) (Table 2). The TMPH–nicotine combination did not alter response rates (compared to vehicle) at any of the dose combinations tested ($p>0.05$; Fig. 4, bottom panel). TMPH alone also did not produce nicotine-lever responding at the doses at which antagonism was observed (data not shown).

4. Discussion

The main goal of this study was to investigate the antagonistic effect of TMPH on central behavioral effects of nicotine, since this antagonist had not been investigated previously for its blocking effects in vivo. Our data show that

TMPH represents a prototype for new CNS-active nAChR antagonists with selectivity for specific CNS effects of nicotine, based on its differential potency for the blockade of nicotine's effects in vivo.

We tested its antagonistic effects on four different nicotinic responses: antinociception, discriminative cue, locomotor activity and body temperature. It has been shown that only the first two responses are antagonized by TMPH in a dose-related manner. The failure of TMPH to block nicotine-induced motor decrease and hypothermia suggests that it may inhibit neuronal nicotinic receptors in a selective manner.

TMPH was equipotent in blocking the effects of nicotine in the mouse hot-plate test and the rat drug discrimination. In contrast, mecamylamine was much more potent in blocking the other effects of nicotine (Table 2). Nicotine-induced antinociception in the hot-plate test and the nicotine discriminative stimulus were recently reported to be largely mediated by $\alpha_4\beta_2^*$ subtypes (Marubio et al., 2003; Shoaib et al., 2002). The effects on the tail-flick test seem to involve both $\alpha_4\beta_2^*$ and non-$\alpha_4\beta_2^*$ receptor subtypes (Marubio et al., 2003). Indeed, contrary to the hot-plate test where a nearly complete loss of the effect was observed, nicotine-induced antinociception in the tail-flick test showed a significant rightward shift in α_4 or β_2 knock-out mice (Marubio et al., 2003). Compared to mecamylamine, the effects of TMPH on nicotine-induced antinociception in the tail-flick test suggest a lower blockade potency of TMPH on the non-$\alpha_4\beta_2^*$ receptor subtypes. At this point, it is difficult to predict which nicotinic receptor subtypes are involved in this non-$\alpha_4\beta_2^*$ component, but it seems that mecamylamine possesses much higher affinity than TMPH to these subtypes. One possible candidate is α_7 nAChRs subtype. However, recent results (Damaj et al., 1998; Rao et al., 1996) indicate little involvement of α_7 subtypes in the antinociceptive effects of nicotinic agonists in the tail-flick test.

The lack of TMPH effect on nicotine-induced hypomotility and hypothermia is very interesting and points out further to the in vivo selectivity of TMPH in blocking different nicotinic receptors. The nAChR subtypes that mediate the locomotor effects and hypothermic effects of nicotine appear to be less sensitive to TMPH than those that mediate analgesic effects. The depressing effect of nicotine on locomotor activity in mice involves α_5 (Salas et al., 2003) and β_2 subunits but not α_4 (Marubio et al., 2003), α_7 and β_4 subunits as reported in recent studies using knock-out mice of these various subunits. These results suggest that the higher blockade activity of mecamylamine on nicotine-induced antinociception may involve α_5-containing receptor subtypes. Although little data are available on nicotine-induced hypothermia, the lack of TMPH's effects may possibly involve similar receptor mechanisms. Since the systemic administration of TMPH can inhibit selective effects of nicotine in the CNS, TMPH can apparently pass the blood–brain barrier. The selectivity of TMPH effects in vivo suggests that it may inhibit the effects of nicotine at some nAChR subtypes but not others.

In conclusion, the results we report suggest that drug therapies for the inhibition of CNS nicotinic receptors may be developed with greater selectivity than previously appreciated. While more selective antagonists such as MLA are known, these generally work poorly with systemic administration. Mecamylamine has previously been proposed for adjunct therapy for Tourette's syndrome (Sanberg et al., 1998) and smoking cessation (Rose et al., 1994). The characterization of selective antagonists such as TMPH may lead the way to the development of better therapies for these, and potentially other, neuropsychiatric indications based on a more limited profile of side effects. For example, in regard to smoking cessation, it is particularly interesting to note that TMPH blocks nicotine discrimination with a potency equal to or greater than that of mecamylamine. However, while the concentrations of mecamylamine required to block drug discrimination would profoundly block potentially desirable antinociception mechanisms (as measured by tail-flick, Table 2), concentrations of TMPH effective at blocking drug discrimination leave the effects of nicotinic receptors on tail-flick responses largely intact. In addition to the potential therapeutic significance of TMPH, this drug may also prove to be a valuable tool to combine with selective agonists and knock-out animals to further unravel the mystery of how neuronal nicotinic receptors play a role in brain function.

Acknowledgements

This work was supported by National Institute on Drug Abuse grant # DA-05274, NIH grants NS 32888 and MH 11258 and the University of Florida College Incentive fund. We thank Tie Han for his technical assistance.

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