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Functional diversity among 5-substituted nicotine analogs; in vitro and in vivo investigations

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Abstract

Two 5-substituted derivatives of nicotine (nicotinic acetylcholine receptor: $K_i = 2.4$ nM) were synthesized and evaluated: 5-bromonicotine ($K_i = 6.9$ nM) and 5-methoxynicotine ($K_i = 14.3$ nM). Despite their high affinity, neither 5-bromonicotine nor 5-methoxynicotine mimicked nicotine in producing antinociceptive (tail-flick, hotplate), hypolocomotor, or hypothermic effects in mice. Neither agent antagonized the hypolocomotor actions of nicotine, whereas 5-methoxynicotine, but not 5-bromonicotine, antagonized the antinociceptive (tail-flick) activity of nicotine in a dose-related manner. In tests of stimulus generalization using rats trained to discriminate 0.6 mg/kg of (−)-nicotine from vehicle, 5-bromonicotine substituted for nicotine. Further evaluation of 5-bromonicotine indicated that it might be a partial agonist at $\alpha4\beta2$ receptors (stimulation of Rb^+ efflux; $\alpha4\beta2$ receptors expressed in oocytes) and at $\alpha3$-containing nicotinic acetylcholine receptors (synaptosomal dopamine release). Thus, 5-bromonicotine might be acting as a partial agonist at $\alpha4\beta2$ receptors and/or some of its effects might be related to interactions with non-$\alpha4\beta2$ receptors. Clearly, the effects of 5-bromonicotine and 5-methoxynicotine are different from those of nicotine, and from one another. These actions demonstrate that substitution at the 5-position of nicotine exerts a profound influence on the pharmacological profile as well as agonist/antagonist properties of nicotine.

Keywords: Nicotinic receptor; (−)-Nicotine; 5-Bromonicotine; 5-Methoxynicotine; In vitro model; In vivo model

1. Introduction

Rapid advances in molecular biology, and discovery of the potent nicotinic acetylcholine receptor ligand epibatidine, have evoked renewed interest in nicotinic acetylcholine receptors in the past decade (Holladay et al., 1997; Arneric and Brioni, 1998; Corringer et al., 2000; Glennon and Dukat, 2000; Lloyd and Williams, 2000; Schmitt, 2000). Cloning of new nicotinic acetylcholine receptor subtypes called for a reformulation or at least a reevaluation of existing structure–activity relationships and a reexamination of nicotinic receptor pharmacophores. Although nicotine itself has served as a template in the formulation of structure–activity relationships and structure–affinity relationships, little is known about the 5-position of the nicotine nucleus and the pharmacology associated with modification at this position. The first mention of 5-substituted nicotine derivatives was by Leete et al. (1971); while investigating the biosynthesis of nicotine they prepared 5-fluoronicotine. No pharmacological data accompanied this study. Several years later, Rondahl (1977) reported the synthesis and biological activity of 5-halo (i.e., -Cl, -Br, -I, -F) nicotine analogs. The biological activity of the halogenated nicotines was examined using a peripheral assay—isolated guinea pig vas deferens—with *S*(−)-nicotine as standard. The most active analog in this assay was 5-fluoronicotine, which was about 40% as active as nicotine. Nothing was reported about the action of 5-substituted nicotine analogs on neuronal nicotinic acetylcholine receptors.

Having studied the structure–activity relationships and pharmacology of 6-substituted nicotine analogs (Dukat et al., 1996, 1999) we turned our attention to an investigation of the 5-position. Cosford et al. (1996) recently reported the synthesis of 5-ethynyl- (i.e., SIB-1765F), (*S*)-5-ethynyl- (i.e., SIB-1508Y), (*R*)-5-ethynyl- and 5-ethylnicotine, their binding at nicotinic and muscarinic acetylcholine receptors, and their actions in several functional assays. The displacement of

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[^{3}H]nicotine in rat cortical membranes by the above analogs resulted in IC_{50} values of 4.6, 3, 75, and 11 nM, respectively. SIB-1508Y was promoted as a nicotinic acetylcholine receptor agonist to treat the symptoms of Parkinson's disease. Subsequent communications (Menzaghi et al., 1997; Sacaan et al., 1997) characterized the nicotinic receptor agonist actions of SIB-1765F. The 5-bromo compound, synthesized as a synthetic intermediate in the preparation of SIB-1765F, was found to bind at nicotinic receptors (IC_{50} = 19 nM) and, at the single concentration examined (300 μM), was found to release dopamine from superfused striatal slices (Cosford et al., 1996). Recently, Vernier et al. (1999) revealed that replacement of the 5-ethynyl moiety in SIB-1508Y with a phenyl group results in an analog that retains high affinity for displacing [^{3}H]nicotine from rat cortical membranes (IC_{50} = 37 nM), but shifts subtype selectivity ($\beta2 \rightarrow \beta4$) in the calcium flux assay using human recombinant nicotinic acetylcholine receptors.

The above findings prompted us to investigate what influence an electron withdrawing (i.e., 5-bromo) and an electron donating (i.e., 5-methoxy) group would have on the pharmacological profile of nicotine. Our aim was to obtain binding data on these two analogs and to evaluate them in several in vitro and in vivo models that have been used to describe the actions of nicotine. The analogs were evaluated for their ability to produce antinociception (tail-flick and hot-plate tests), hypothermia, and to decrease spontaneous activity in mice. In addition, the 5-bromo analog was evaluated in drug discrimination, a model which has greater selectivity in identifying nicotine-like activity (Stolerman, 1988; Rosecrans, 1989).

A biochemical assay that measures nicotinic receptor agonist-stimulated $^{86}Rb^+$ efflux in synaptosomes isolated from mouse brain was also used to evaluate potential nicotinic responses of 5-bromonicotine. Indeed, several observations suggest that the $^{86}Rb^+$ efflux assay is measuring a response that corresponds to the receptor that is labeled with [^{3}H]nicotine in binding assays (Marks et al., 1993, 1994). Additional mechanistic evidence was obtained by assessing the activity of 5-bromonicotine at $\alpha4\beta2$ nicotinic acetylcholine receptors expressed in oocytes. We finally tested the potency of 5-bromonicotine in inducing dopamine release from striatal synaptosomes, an effect thought to be mediated by a nicotinic acetylcholine receptor that contains an $\alpha3$ subunit.

2. Materials and methods

2.1. Animals

Male ICR mice (20–25 g) and male Sprague–Dawley rats (175–225 g) obtained from Harlan Laboratories (Indianapolis, IN) were used throughout the study. Mice were housed in groups of six, and rats were housed individually; all animals had free access to food and water. 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

(−)-Nicotine was obtained from Aldrich (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) and administered in a total volume of 1 ml/100 g body weight for s.c. injections in rat, or 10 ml/100 g in mice. All doses are expressed as the free base of the drug, except in the drug discrimination study.

The free base of (±)5-bromonicotine was prepared according to literature procedures by Rondahl (1977) and Jacob (1982). The ethereal solution of (±)5-bromonicotine was treated with an ethereal solution of maleic acid and the resulting solid was recrystallized from a mixture of isopropanol:anhydrous ether to afford the desired product, 5-bromonicotine maleate, in 44% overall yield as a white crystalline solid; mp 85–88 °C.

(±)5-Methoxynicotine was prepared by methylation of (±)5-hydroxynicotinic acid (Ueno and Imoto, 1967) with diazomethane followed by condensation with *N*-vinylpyrrolidinone followed by sodium borohydride reduction under acidic condition in a manner similar to that reported for the synthesis of (±)5-bromonicotine (Jacob, 1982). The resulting (±)5-methoxynornicotine was methylated with sodium cyanoborohydride according to the procedure of Borch and Hassid (1972). The crude product was converted to its maleate salt and recrystallized from a mixture of isopropanol:anhydrous ether to afford the desired product, 5-methoxynicotine maleate, in 61% yield as a white crystalline solid; mp 128–130 °C.

Both 5-bromonicotine and 5-methoxynicotine were homogeneous as evidenced by thin-layer chromatography; proton magnetic resonance spectral data were consistent with the assigned structures, and both derivatives analyzed correctly for C, H, and N to within 0.4% of theory (Atlantic Microloab, Norcross, GA).

2.3. [^{3}H](−)-nicotine binding in vitro

The receptor binding assay was conducted as previously reported in greater detail (Scimeca and Martin, 1988; Dukat et al., 1996). In brief, rat brain without cerebellum was homogenized in 10 volumes of ice-cold 0.05 M sodium potassium phosphate buffer (pH 7.4) and centrifuged at 17 500 × *g* (4 °C) for 30 min. The pellet was resuspended in 20 volumes of ice-cold glass-distilled water and allowed to incubate on ice for 60 min prior to centrifugation as described above. The final pellet was resuspended (40 mg/ml) in buffer; [^{3}H](−)-nicotine was incubated with 0.5 ml of tissue homogenate (final volume 1 ml) for 2 h at 4 °C, and samples were rapidly filtered through Whatman GF/C filters. Specific binding was defined as the difference in the amount of

binding in the presence and absence of 100 μM (−)-nicotine tartrate. Following buffer wash, the filters were air-dried, placed in scintillation vials, and radioactivity was quantified. Following transformation of the data by the Scatchard method, the K_d and B_{max} values were determined using the program LIGAND (Munson and Rodbard, 1980). Displacement of [^{3}H](−)-nicotine binding at 1 nM was determined in the presence of increasing concentrations of test compound and converted to percent displacement of specific binding. IC_{50} values were determined from a plot of the log concentration vs. percent displacement and converted to K_i values by the method of Cheng and Prusoff (1973). K_i values were determined at least in triplicate.

2.4. Intrathecal injections

Intrathecal injections were performed free-hand between the L5 and L6 lumbar space in unanesthetized male mice according to the method of Hylden and Wilcox (1980). The injection was performed using a 30-gauge needle attached to a glass microsyringe. The injection volume in all cases was 5 μl. The accurate placement of the needle was evidenced by a quick "flick" of the mouse's tail. In protocols where two sequential injections were required in an animal, the flicking motion of the tail could be elicited with the subsequent injection.

2.5. Behavioral and pharmacological assays in mice and rats

2.5.1. Spontaneous activity

Mice were placed into individual Omnitech photocell activity cages (28 × 16.5 cm) 5 min after s.c. administration of either 0.9% saline or the nicotine analogs. 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 photocell interruptions. Each dose of each agent was examined in a minimum of six animals.

2.5.2. Body temperature

Rectal temperature was measured by a thermistor probe (inserted 24 mm) and digital thermometer (Yellow Springs Instrument, Yellow Springs, OH). Readings were taken just before and at 30 min after the s.c. injection of either saline or the nicotine analogs. 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. Each dose of each agent was examined in a minimum of six animals.

2.5.3. Antinociception

2.5.3.1. Tail-flick test. Antinociception was assessed by the tail-flick method of D'Amour and Smith (1941) as modified by Dewey et al. (1970). 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. Groups of 8 to 12 animals were used for each dose and for each treatment. The mice were tested 5 min after either s.c. or i.t. injections of the nicotine analogs. Antagonism studies were carried out by pretreating the mice with either saline or drug at different times before (−)-nicotine. The animals were tested 5 min after administration of drug.

2.5.3.2. Hot-plate test. The method is a modification of that described by Eddy and Leimbach (1953) and Atwell and Jacobson (1978). 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 6 to 10 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. Eight mice per dose were injected s.c. with (−)-nicotine or the nicotine analogs and tested 5 min after injection. Antagonism studies were carried out by pretreating the mice with either saline or drug at different times before nicotine or its analogs. The animals were tested 5 min after administration of (−)-nicotine.

2.5.4. Drug discrimination

2.5.4.1. Subjects. Six male Sprague–Dawley rats (Charles River Laboratories; Wilmington, MA) weighing 350–400 g at the beginning of the study were used. Rats were housed individually and, prior to the start of the study, their body weights were reduced to approximately 80% of their free-feeding weight. During the entire course of the study, the animals' body weights were maintained at this reduced level by partial food deprivation. In their home cages, the animals were allowed drinking water without restriction.

2.5.4.2. Apparatus. Standard two-lever operant conditioning chambers (Model E10-10, Coulbourn Instruments, Lehigh Valley, PA) in sound- and light-attenuating outer chambers were used. The apparatus has been previously described (Young and Glennon, 1998).

2.5.4.3. Training and testing. The training procedure was essentially the same as that recently reported for another training drug (Young and Glennon, 1998). In brief, the rats were trained to respond under a fixed ratio 1 (FR1) schedule of reinforcement. The FR1 schedule of reinforcement was gradually increased until all animals were reliably responding under a variable interval 15-s schedule of reinforcement. After 15 weeks, all animals successfully discriminated i.p. injections (15-min presession injection interval) of 0.6 mg/kg

of (−)-nicotine hydrogen tartrate from vehicle (sterile 0.9% saline) under a variable-interval 15-s schedule of sweetened milk reinforcement using a 15-min training session. Daily training sessions were conducted with (−)-nicotine or saline. On every fifth day, learning was assessed during an initial 2.5-min non-reinforced (extinction) session followed by a 12.5-min training session. For three of the animals, the left lever was designated the drug-appropriate lever, whereas the situation was reversed for the remaining animals. Data collected during the extinction session included responses per second (i.e., response rate) and number of responses on the drug-appropriate lever (expressed as a percent of total responses).

Once the animals had been trained to discriminate (−)-nicotine from vehicle (i.e., after the animals made >80% of their responses on the drug appropriate lever after administration of (−)-nicotine, and <20% of their responses on this same lever after administration of saline, for 3 consecutive weeks), tests of stimulus generalization or substitution were conducted in order to determine if 5-bromonicotine would substitute for (−)-nicotine. During this phase of the study, maintenance of the (−)-nicotine/saline discrimination was insured by continuation of the training sessions on a daily basis (except on a substitution test day; see below). On one of the 2 days before a substitution test, half of the animals would receive (−)-nicotine and half would receive saline; after a 2.5-min extinction session, training was continued for 12.5 min. Animals not meeting the original criteria (i.e., >80% of total responses on the drug-appropriate lever after administration of training drug, and <20% of total responses on the same lever after administration of saline) during the extinction session were excluded from the immediately subsequent substitution test session. During the investigations of substitution, test sessions were interposed among the training sessions. The animals were allowed 2.5 min to respond under non-reinforcement conditions; the animals were then removed from the operant chambers and returned to their home cages. An odd number of training sessions (usually five) separated any two substitution test sessions. Doses of the test drug were administered in a random order, using a 5-min presession injection interval to the group of six rats. Stimulus generalization or substitution was considered to have occurred when the animals, after a given dose of drug, made ≥80% of their responses on the nicotine-appropriate lever. ED_{50} values were calculated by the method of Finney (1952). The ED_{50} doses are doses at which the animals would be expected to make 50% of their responses on the drug-appropriate lever. Insufficient quantities of 5-methoxynicotine precluded evaluation in the drug discrimination assay.

Tests of antagonism were conducted with mecamylamine. These tests were conducted in approximately the same manner as the substitution tests. The animals were administered 1 mg/kg of mecamylamine along with either the training dose of (−)-nicotine or the generalization dose of 5-bromonicotine. Mecamylamine was administered 15 min prior to (−)-nicotine or 5-bromonicotine and, 5 min later, the animals were placed in the operant chambers for a 2.5-min extinction session.

2.6. Oocyte expresion system

2.6.1. Oocyte preparation

Oocytes preparation was performed according to the method of Mirshahi and Woodward (1995) with minor modifications. Briefly, oocytes were isolated from female adult oocyte-positive *Xenopus laevis* frogs. Frogs were anesthetized in a 0.2% 3-aminobenzoic acid ethyl ester solution (Sigma, St. Louis, MO) for 30 min and a fraction of the ovarian lobes were removed. The eggs were rinsed in Ca^{2+}-free ND96 solution, treated with Collagenase type IA (Sigma) for 1 h to remove the follicle layer, and then rinsed again. Healthy stage V–VI oocytes were selected and maintained for up to 14 days after surgery in 0.5 × L-15 media.

2.6.2. mRNA preparation and microinjection

α4 and β2 rat subunit cDNA's contained within a pcDNAIneo vector were kindly supplied by Dr. James Patrick (Baylor College of Medicine, Houston, TX). The template was linearized downstream of coding sequence and mRNA was synthesized using an in vitro transcription kit from Ambion (Austin, TX). The quantity and quality of message were determined via optical density (spectrophotometer Beckman Instruments, Chaumburg, IL) and denaturing formaldehyde gel analysis. Oocytes were injected with 51 ng (41 nl) of α4 and β2 mixed in a 1:1 ratio using a Variable Nanoject (Drummond Scientific, Broomall, PA). Oocytes were incubated in 0.5 × L-15 media IA (Sigma) supplemented with penicillin, streptomycin, and gentamicin for 4–6 days at 19 °C before recording.

2.6.3. Electrophysiological recordings

Oocytes were placed within a plexiglass chamber (total volume 0.2 ml) and continually perfused (10 ml/min) with buffer consisting of 115 mM NaCl, 1.8 mM $CaCl_2$, 2.5 mM KCl, 1.0 μM atropine and 10.0 mM HEPES at pH 7.2. Oocytes were impaled with two microelectrodes containing 3M KCl (0.3–3 MΩ) and voltage-clamped at −70 mV using an Axon Geneclamp amplifier (Axon Instruments, Foster City, CA). Oocytes were stimulated for 10 s with various concentrations of nicotine analogs using a 6-port injection valve. Except where noted, applications were separated by 5-min periods of washout. Currents were filtered at 10 Hz and collected by a Macintosh Centris 650 with a 16-bit analog digital interface board, and data were analyzed using Pulse Control voltage-clamp software running under the Igor Pro graphic platform (Wavemetrics, Lake Oswego, Foster City, CA). Oocytes were stimulated for 10 s with various concentrations of nicotine analogs using a six-port injection valve. Except where noted, applications were separated by 5-min periods of washout. Currents were filtered at 10 Hz and collected by a Macintosh Centris 650 with a 16-bit analog digital interface board, and data were analyzed using Pulse

Control voltage-clamp software running under the Igor Pro graphic platform (Wavemetrics, Lake Oswego, OR). Nicotinic analogs were applied at different concentrations and concentration–response curves were normalized to the current induced by 1 μM acetylcholine. The normalizing concentration of acetylcholine was applied before and after drug application to each oocyte to check for desensitization. Data were rejected if responses to the normalizing dose fell below 75% of the original response. Dose–response curves were analysed by nonlinear regression analysis using the Allfit analysis program (DeLean et al., 1978). EC_{50} and EC_{max} values (with S.E.M.) were then determined for normalized responses.

2.7. $^{86}Rb^{+}$ efflux

The stimulation of $^{86}Rb^{+}$ efflux from crude mouse thalamic synaptosomes by (−)-nicotine and 5-bromonicotine was measured using the method of Marks et al. (1993). Crude synaptosomes were prepared by homogenizing the mouse thalamus in 0.32 M sucrose, 5 mM HEPES 1/2Na (pH = 7.5), centrifuging the homogenate for 10 min at $1000 \times g$ and centrifuging the resulting supernatant at $10\,000 \times g$ for 20 min. The resulting pellet was resuspended in 150 μl incubation buffer per thalamus (incubation buffer: NaCl, 140 mM; KCl, 1.5 mM; $CaCl_2$, 2 mM; $MgSO_4$, 1 mM; glucose, 20 mM; HEPES 1/2Na, 25 mM; pH = 7.5). Loading with $^{86}Rb^{+}$ was achieved by incubating a 25 μl aliquot of the synaptosomes with 10 μl of incubation buffer containing 4 μCi of isotope for 30 min. Following the incubation with $^{86}Rb^{+}$, the synaptosomes were harvested by filtration onto a 7-mm Gelman A/E glass fiber filter under gentle vacuum (−100 Torr). Following two washes with 0.5 ml of incubation buffer, the filter containing the synaptosomes was transferred to the apparatus and perfused with 1.5 ml/min experimental buffer (NaCl, 135 mM; CsCl, 5 mM; KC1, 1.5 mM; $CaCl_2$, 2 mM; $MgSO_4$, 1 mM; glucose, 20 mM; tetrodotoxin, 50 nM; bovine serum albumin, 0.1%; HEPES 1/2Na, 25 mM; pH = 7.5). Following a 6-min wash period, sample collection was begun. Fractions were collected every 30 s for 6 min. Stimulation of $^{86}Rb^{+}$ efflux by (−)-nicotine and 5-bromonicotine was tested by exposing the thalamic synaptosomes to known concentrations of the test compounds for 1 min.

The EC_{50} values and maximal efflux rates were calculated using the Michaelis–Menten equation: $V = V_{max}D/(D + K_d)$. All curve fits were achieved using the nonlinear least squares algorithm in Sigma Plot 5.0 (Jandel Scientific, San Rafael, CA).

2.8. Striatal dopamine release

The release of [^{3}H]dopamine from a striatal P2 preparation was measured according to the method of Grady et al. (1992). Tissues were incubated with 0.1 μM [^{3}H]dopamine for 5 min at 37 °C. After the incubation, the samples were collected by filtration and the filter transferred to a platform that allows continuous superfusion of the sample. Buffer was applied by a peristaltic pump at room temperature. After exposure to test solutions, fractions were collected in 6-ml scintillation vials each minute. Radioactivity was determined in a scintillation counter, and the data were plotted as radioactivity in each fraction vs. time elapsed. The peaks were identified, and peak size was determined by summing the radioactivity released above baseline. Basal release was determined by averaging the cpm in the fractions immediately preceding and after the peak.

2.9. Statistical analysis

Data was analyzed statistically by an analysis of variance (ANOVA) followed by the Fisher's protected least significant difference (PLSD) multiple comparison test. The null hypothesis was rejected at the 0.05 level. For the time-course studies, each animal was used once. Data were analyzed by a two-factor ANOVA. ED_{50} values with 95% Confidence Limits for behavioral data were calculated by unweighted least-squares linear regression as described by Tallarida and Murray (1987).

3. Results

3.1. Radioligand binding

(−)-Nicotine binds with high affinity ($K_i = 2.4 \pm 0.4$ nM). 5-Bromonicotine ($K_i = 6.9 \pm 2.6$ nM) and 5-methoxynicotine ($K_i = 14.3 \pm 1.5$ nM) were also found to bind with high affinity, but with approximately 3- and 6-fold lower affinity, respectively, than (−)-nicotine itself. The affinity of the 5-bromo analog is consistent with that reported earlier by Cosford et al. (1996): $IC_{50} = 19$ nM.

3.2. Spontaneous activity

Administration (s.c.) of 1.0 mg/kg of (−)-nicotine decreased mouse locomotor activity by >90%, from 1635 photocell interruptions in 10 min (i.e., saline baseline) to 171 interruptions (Table 1). 5-Bromonicotine, at doses of 5 and 10 mg/kg, and 5-methoxynicotine at 10 mg/kg, produced saline-like effects. Examined in combination, 10 mg/kg of 5-bromonicotine or 5-methoxynicotine failed to significantly antagonize the locomotor actions of (−)-nicotine (Table 1).

3.3. Antinociceptive activity

The antinociceptive properties of 5-bromonicotine and 5-methoxynicotine were compared with those of (−)-nicotine. In the tail-flick assay 2.5 mg/kg of (−)-nicotine produced 77% of the maximal possible effect (MPE) (Table 1) when administered via the s.c. route. 5-Bromonicotine produced 2–5% MPE at doses of 10 and 30 mg/kg, and 5-methoxynicotine produced 7% MPE at 10 mg/kg. Even

Table 1
Effect of nicotine analogs on mouse tail-flick, hotplate, spontaneous activity, and body temperature following s.c. administration

Treatment	Tail-flick % MPE (± S.E.M.)	Hotplate % MPE (± S.E.M.)	Spontaneous activity (interruptions ± S.E.M.)	Body temperature Δ °C (± S.E.M.)
Nicotine (1.0 mg/kg)	–	–	171 (± 37)	
Nicotine (2.5 mg/kg)	77 (± 15)	93 (± 8)	–	− 3.9 (± 1.2)
Saline	3 (± 2)	3 (± 2)	1635 (± 164)	+ 0.3 (± 0.2)
5-Br nicotine				
5 mg/kg	–	–	1749 (± 152)	–
10 mg/kg	5 (± 3)	5 (± 3)	1548 (± 174)	–
15 mg/kg[a]	–	–	–	0
30 mg/kg	2 (± 1)	16 (± 8)	–	–
5-Br nicotine (10 mg/kg) + nicotine (2.5 mg/kg)	–	69 (± 12)	175[b] (± 57)	− 5.5 (± 1.4)
5-Br nicotine (15 mg/kg) + nicotine (2.5 mg/kg)	69 (± 14)	–	–	–
5-Br nicotine (30 mg/kg) + nicotine (2.5 mg/kg)	82 (± 12)	95 (± 5)	–	–
5-OMe nicotine (10 mg/kg)	7 (± 2)	–	1857 (± 316)	–
5-OMe nicotine (10 mg/kg) + nicotine (1.0 mg/kg)	–	–	182 (± 31)	–
5-OMe nicotine (10 mg/kg) + nicotine (2.5 mg/kg)	25[c] (± 10)	–	–	–

[a] 5-Bromonicotine was administered 30 min prior to testing; when administered 15 and 60 min prior to testing, the results were not different, i.e., − 0.3 (± 0.1) and + 0.1 (± 0.1) °C, respectively.
[b] In the spontaneous activity assay, the dose of (−)-nicotine was 1 mg/kg.
[c] 5-Methoxynicotine dose-dependently antagonized the tail-flick response induced by (−)-nicotine (see Fig. 1).

when administered by the i.t. route, 5-bromonicotine failed to produce a significant antinociceptive effect (Table 2). Similar results were observed in the hotplate test; (−)-nicotine produced 93% MPE, whereas 5-bromonicotine produced 5% and 16% MPE at 10 and 30 mg/kg (Table 1).

Both 5-bromonicotine and 5-methoxynicotine were examined in the tail-flick assay as possible nicotine antagonists. Table 1 shows that s.c. doses of 15 and 30 mg/kg of 5-bromonicotine had essentially no effect on the antinociceptive actions of 2.5 mg/kg of (−)-nicotine. In contrast, 5-methoxynicotine, with an AD_{50} value of 1.1 (95% Confidence Limits = 0.4–2.5) mg/kg, dose-dependently antagonized the effect of (−)-nicotine (Fig. 1).

3.4. Body temperature

(−)-Nicotine (2.5 mg/kg) reduced mouse body temperature by 3.9 °C (Table 1). 5-Bromonicotine had little effect on mouse body temperature when administered at a dose of 15 mg/kg under the same conditions; variation of the time-to-test interval from 15 to 60 min was without effect. Administered in combination, 2.5 mg/kg of (−)-nicotine plus 10 mg/kg of 5-bromonicotine produced a 5.5 °C decrease in body temperature.

Table 2
Effect of 5-bromonicotine alone and in combination with nicotine on tail-flick activity following i.t. administration

Treatment	Tail-flick % MPE (± S.E.M.)
5-Br nicotine	
20 μg/mouse	3 (± 1)
40 μg/mouse	2 (± 1)
Nicotine (20 μg/mouse)	83 (± 10)
Nicotine (20 μg/mouse) + 5-Br nicotine (40 μg/mouse)	96 (± 4)
Saline	3 (± 2)

3.5. Drug discrimination studies

Administered to animals trained to discriminate (−)-nicotine from vehicle, 0.9% saline produced 3% drug-appropriate responding (response rate = 1.2 ± 0.4 responses/s). The training dose of (−)-nicotine elicited 93% drug-appropriate responding (response rate = 1.1 ± 0.4 responses/s). Several lower doses of (−)-nicotine were examined to generate a dose–response curve (Fig. 2); ED_{50} = 0.2 (95% Confidence Limits = 0.1–0.3) mg/kg. Eight doses of 5-bromonicotine were examined in tests of stimulus generaliza-

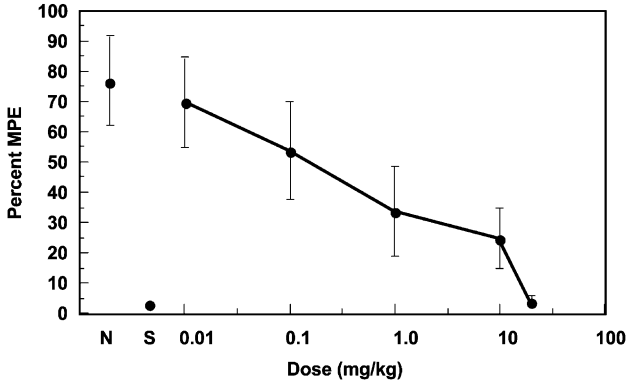

Fig. 1. Antinociceptive actions (± S.E.M.) of nicotine (2.5 mg/kg) administered alone and in combination with various doses of 5-methoxynicotine in the tail-flick assay, plotted as drug dose vs. percent maximal possible effect (% MPE). S = effect of saline; N = effect of 2.5 mg/kg of (−)-nicotine.

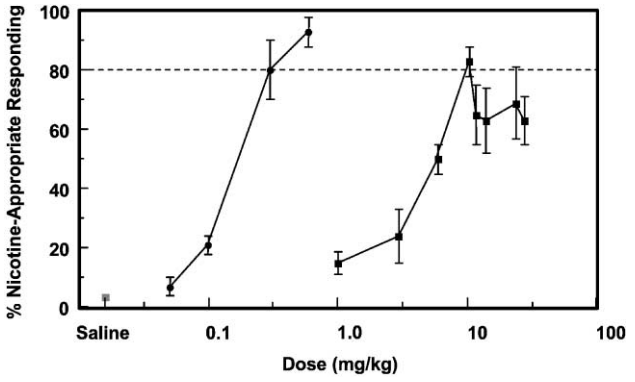

Fig. 2. Results (mean ± S.E.M.) of stimulus generalization studies with (−)-nicotine and 5-bromonicotine in rats trained to discriminate 0.6 mg/kg of (−)-nicotine from saline vehicle.

tion. At a dose of 10.5 mg/kg of 5-bromonicotine, the animals made 83 (± 5)% of their responses on the drug-appropriate lever but response rates were depressed (response rate = 0.3 ± 0.2 responses/s). Administration of higher drug doses resulted in a decrease in (−)-nicotine-appropriate responding and at the highest dose tested (28 mg/kg) the animals made 63% drug-correct responding (response rate = 0.6 ± 0.2 responses/s); higher doses were not evaluated. The calculated ED_{50} dose for 5-bromonicotine is 6.0 (95% Confidence Limits = 2.4–14.9) mg/kg; doses higher than 10.5 mg/kg were not used in calculating the ED_{50} dose. Limited supplies of 5-methoxynicotine precluded its examination in the drug discrimination assay.

Tests of stimulus antagonism were conducted with a single dose of the nicotine antagonist mecamylamine. Administered in combination, 1 mg/kg of mecamylamine and the training dose of (−)-nicotine elicited 13 (± 7)% drug-appropriate responding, whereas a combination of mecamylamine and the generalization dose of 5-bromonicotine elicited 25 (± 12)% drug-appropriate responding (data not shown).

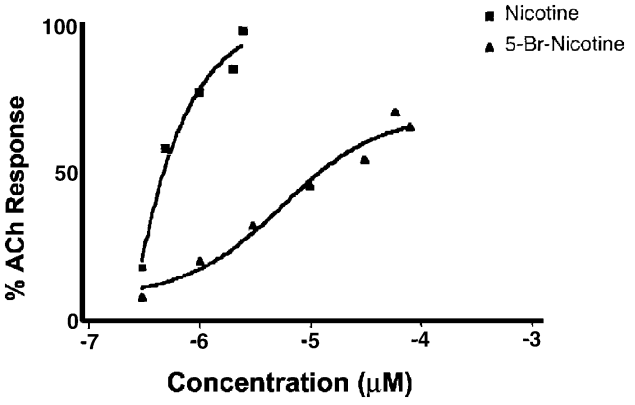

Fig. 3. Concentration–response relationship of (−)-nicotine and 5-bromonicotine on α4β2 receptors expressed in *Xenopus* oocytes. Responses are presented after normalization to the response of the same oocyte to 1 μM acetylcholine (ACh). Normalized curves are plotted against concentration. Each point represents the normalized mean of the responses of four to six separate oocytes held at −70 mV.

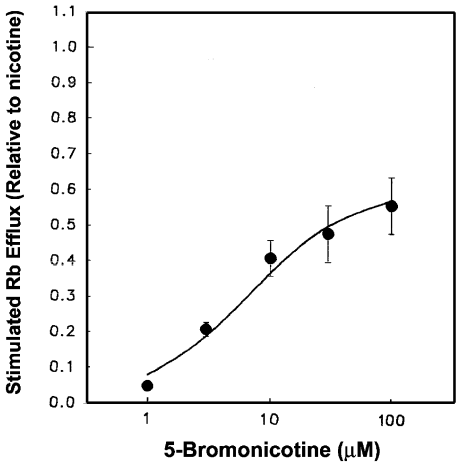

Fig. 4. Concentration–effect curve for stimulation of $^{86}Rb^+$ efflux by 5-bromonicotine. Results were normalized to the response of 1 μM (−)-nicotine. Points represent the mean ± S.E.M. of values obtained for six separate stimulations at each concentration of agonist. Curves are theoretical nonlinear least-squares fits of the data to the Michaelis–Menton equation.

3.6. Effect of α4β2 receptors expessed in oocytes

(−)-Nicotine produced a concentration-dependent inward current in voltage-clamped oocytes expressing α4β2 subunit combinations and held at −70 mV. The (−)-nicotine effect was concentration-dependent (data not shown) with an EC_{50} of 0.35 μM. In contrast, partial responses were generated by 5-bromonicotine with a maximal response of 70% of that observed for (−)-nicotine (Fig. 3). In addition, 5-bromonicotine was 57 times less potent (EC_{50} = 20 μM) as an agonist than (−)-nicotine.

3.7. Rb^+ efflux and dopamine release studies

A concentration–effect curve for 5-bromonicotine for the stimulation of $^{86}Rb^+$ efflux from mouse thalamic synapto-

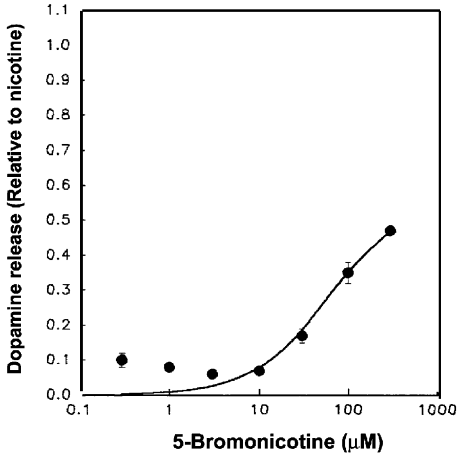

Fig. 5. 5-Bromonicotine-induced [^{3}H]dopamine release from striatal synaptosomes. Each point is the mean ± S.E.M. of two to six determinations.

somes is shown in Fig. 4. 5-Bromonicotine evoked concentration-dependent efflux of $^{86}Rb^+$ with an EC_{50} value of 6.6 μM, whereas (−)-nicotine had an EC_{50} of 0.46 μM (data not shown). In addition, the maximal $^{86}Rb^+$ efflux elicited by 5-bromonicotine was only 60% (at 100 μM) of that observed for (−)-nicotine.

Similarly, 5-bromonicotine induced a concentration-dependent release of striatal dopamine with an EC_{50} value of 60 μM, whereas (−)-nicotine had an EC_{50} of 0.48 μM (data not shown). However, the efficacy of 5-bromonicotine in releasing synaptosomal dopamine was low with a maximum of 45% (at 500 μM) of that observed for (−)-nicotine (Fig. 5).

4. Discussion

(−)-Nicotine has a diverse pharmacological profile and acts at multiple nicotinic acetylcholine receptor types. For example, in vivo (−)-nicotine produces hypolocomotion, antinociception, and hypothermia in mice, and serves as a discriminative stimulus in rats. Many simple analogs of nicotine can mimic these actions, with potencies greater than or, more typically, less than that of (−)-nicotine. This has served as the basis for structure–activity studies and has led to formulation of pharmacophores for nicotinic action (reviewed: Glennon and Dukat, 2000). One region of the nicotine molecule that has received relatively little attention is the 5-position. What little is known about 5-substituted nicotine analogs suggests that such agents can bind at α4β2 receptors but that some of their actions might involve other populations of nicotinic acetylcholine receptors or the differential activation of multiple nicotinic acetylcholine receptors (Menzaghi et al., 1997; Sacaan et al., 1997; Vernier et al., 1999). The potential therapeutic utility of 5-substituted nicotine analogs as cognition enhancers (Vernier et al., 1999) or for the treatment of certain neurological disorders (Menzaghi et al., 1997) also makes them attractive targets for drug development.

The three agents used in the present investigation varied only with respect to the substituent at the pyridine-ring 5-position of nicotine. 5-Bromonicotine and 5-methoxynicotine were found to bind at nicotinic acetylcholine receptors with affinities roughly comparable to that of (−)-nicotine, but displayed different pharmacological profiles relative to the parent agent. For example, (−)-nicotine produces a dose-related decrease in spontaneous motor activity in mice; 5-bromonicotine and 5-methoxynicotine failed to produce (or antagonize) these effects (Table 1). It is unlikely that distributional factors are involved because the corresponding 6-substituted analogs of nicotine have been demonstrated to display nicotine-like agonist actions (Dukat et al., 1999).

(−)-Nicotine produces an antinociceptive effect in mice as measured both by the tail-flick assay and the hotplate assay. Administered by the s.c. route, 5-bromonicotine and 5-methoxynicotine failed to produce this effect at the doses evaluated (Table 1). 5-Bromonicotine also failed to produce an effect when administered via the i.t. route (Table 2). However, when administered in combination with (−)-nicotine, 5-methoxynicotine antagonized the antinociceptive actions of nicotine in the tail-flick assay in a potent and dose-related manner (Fig. 1). In contrast, 5-bromonicotine lacked antagonist character at the doses evaluated.

(−)-Nicotine produces hypothermia in mice. 5-Bromonicotine failed to produce a hypothermic effect (Table 1). When administered in combination with (−)-nicotine, 5-bromonicotine failed to antagonize (−)-nicotine's effect on body temperature.

(−)-Nicotine has been previously shown to serve as a discriminative stimulus in rats. In the present investigation, tests of stimulus generalization (substitution) were conducted with 5-bromonicotine using rats trained to discriminate (−)-nicotine (ED_{50} = 0.2 mg/kg) from vehicle. Fig. 2 shows that the (−)-nicotine stimulus generalized to 5-bromonicotine (ED_{50} = 6.0 mg/kg). At the dose where generalization occurred (i.e., 10.5 mg/kg), the animals' response rates were severely depressed; however, higher doses (up to 28 mg/kg) failed to disrupt the animals' behavior and resulted in 60–69% drug-appropriate responding. Although 5-bromonicotine is about 30 times less potent than nicotine, the results suggest that there is a similarity in the stimulus effects produced by these two agents. This similarity of effect is supported by the ability of mecamylamine to attenuate the effects of both agents. However, due to the disruptive nature of 5-bromonicotine, it is likely that the effects of the two agents are also somewhat dissimilar. This was the only in vivo assay in which 5-bromonicotine displayed agonist actions; it is also the only in vivo assay that employed rats rather than mice. Hence, the possibility exists that the observed effect could be species-dependent.

All three agents bind with high affinity at nicotinic acetylcholine receptors with K_i values spanning less than a six-fold range. However, unlike (−)-nicotine, 5-bromonicotine and 5-methoxynicotine failed to produce hypolocomotor effects in mice, and 5-bromonicotine had little effect on body temperature. Likewise, both 5-bromonicotine and 5-methoxynicotine failed to produce nicotine-like antinociceptive effects in mice. Indeed, 5-methoxynicotine dose-dependently antagonized the antinociceptive effects of s.c. administered (−)-nicotine as measured in the tail-flick assay. Interestingly, however, 5-bromonicotine failed to produce this effect even at nearly 30 times the AD_{50} dose of 5-methoxynicotine. In contrast, 5-bromonicotine seemingly behaves as an agonist in the drug discrimination assay in that it produced (−)-nicotine-like stimulus effects in rats. It would seem, then, that although 5-bromonicotine and (−)-nicotine bind at nicotinic acetylcholine receptors with similar affinity, 5-bromonicotine is unable to either mimic or antagonize any of the nicotinic effects examined, except one. Curiously, although 5-bromonicotine produced (−)-nicotine-like stimulus effects in rats, it was found to be much less potent than (−)-nicotine in this regard.

Stolerman et al. (1995) have reported an excellent correlation between binding at α4β2 receptors and drug discrimination potency. Given its affinity for these receptors, 5-bromonicotine might have been expected to substitute for (−)-nicotine; however, the relative potencies of these two agents cannot be explained solely on the basis of their affinities. Furthermore, the inability of 5-bromonicotine to either mimic or antagonize the actions of nicotine in some of the other assays is also inconsistent with its high affinity for α4β2 receptors. To gain further insight into the actions of 5-bromonicotine, the agent was examined in several additional in vitro functional assay systems. At α4β2 receptors expressed in oocytes, 5-bromonicotine demonstrated partial agonist action (Fig. 3) producing only about 70% of the effect produced by (−)-nicotine. Likewise, 5-bromonicotine seemed to behave as a partial agonist relative to (−)-nicotine in the agonist-mediated Rb^+ efflux assay where it produced only about 60% of the effect of nicotine (Fig. 4). The partial agonist nature of 5-bromonicotine at α4β2 receptors could explain its substitution in the stimulus generalization studies. 5-Bromonicotine also behaved as a partial agonist in inducing dopamine release from striatal synaptosomes (Fig. 5). Because this action is thought to be mediated by nicotinic acetylcholine receptors that possess an α3 subunit, 5-bromonicotine could be producing some of its effects through non-α4β2 nicotinic acetylcholine receptors. But its weak partial agonist effect in releasing dopamine suggests that activation of an α3-containing nicotinic acetylcholine receptor subtype does not play a major role in the action of 5-bromonicotine.

In summary, the results of this investigation show that 5-bromonicotine and 5-methoxynicotine bind at nicotinic acetylcholine receptors with affinities similar to (−)-nicotine, but that the action of these two 5-substituted nicotine analogs not only differ from those of nicotine, they differ from one another. Neither 5-bromonicotine nor 5-methoxynicotine was able to mimic the antinociceptive, hypolocomotor, or hypothermic effects of (−)-nicotine. Interestingly, 5-methoxynicotine, but not 5-bromonicotine, was able to antagonize the antinociceptive actions of (−)-nicotine in the tail-flick assay. Given that 5-methoxynicotine and 5-bromonicotine bind at nicotinic acetylcholine receptors with similar affinity, it is apparent that binding data alone are not sufficient to explain the observed pharmacology of these agents. 5-Bromonicotine produced nicotine-like effects in a stimulus generalization study and in the Rb^+ efflux assay. 5-Bromonicotine also produced agonist actions at expressed α4β2 receptors. However, in the latter two assays, 5-bromonicotine was clearly a partial agonist. Taken together, the results suggest that substituents at the 5-position of nicotine significantly influence functional activity. That is, nicotine behaved as an agonist in all assays, 5-bromonicotine seemed to act as a partial agonist in certain assays but lacked agonist character in other assays, and 5-methoxynicotine was without agonist activity in any assay examined. Indeed 5-methoxynicotine antagonized the effect of nicotine in the tail-flick assay, but lacked antagonist activity in the mouse locomotor assay. The role of 5-position substituents is not inconsistent with what has been recently reported by others for a series of A-84543 analogs (Lin et al., 1998, 2001). Finally, evidence from the dopamine release assay suggests that 5-bromonicotine might act as a partial agonist at non-α4β2 receptors. Further investigation of substituted nicotine analogs might result in the development of novel probes with which to investigate nicotine cholinergic mechanisms and functional activity; specifically, the profound differences observed between (−)-nicotine, 5-bromonicotine, and 5-methoxynicotine emphasize the continued need for investigation of the nicotine 5-position.

Acknowledgements

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