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Synthesis and nicotinic activity of epiboxidine: an isoxazole analogue of epibatidine

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Abstract

Synthetic (±)-epiboxidine (exo-2-(3-methyl-5-isoxazolyl)-7-azabicyclo[2.2.1]heptane) is a methylisoxazole analog of the alkaloid epibatidine, itself a potent nicotinic receptor agonist with antinociceptive activity. Epiboxidine contains a methylisoxazolyl ring replacing the chloropyridinyl ring of epibatidine. Thus, it is also an analog of another nicotinic receptor agonist, ABT 418 ((*S*)-3-methyl-5-(1-methyl-2-pyrrolidinyl)isoxazole), in which the pyridinyl ring of nicotine has been replaced by the methylisoxazolyl ring. Epiboxidine was about 10-fold less potent than epibatidine and about 17-fold more potent than ABT 418 in inhibiting [^{3}H]nicotine binding to $\alpha_4\beta_2$ nicotinic receptors in rat cerebral cortical membranes. In cultured cells with functional ion flux assays, epiboxidine was nearly equipotent to epibatidine and 200-fold more potent than ABT 418 at $\alpha_3\beta_{4(5)}$ nicotinic receptors in PC12 cells. Epiboxidine was about 5-fold less potent than epibatidine and about 30-fold more potent than ABT 418 in TE671 cells with $\alpha_1\beta_1\gamma\delta$ nicotinic receptors. In a hot-plate antinociceptive assay with mice, epiboxidine was about 10-fold less potent than epibatidine. However, epiboxidine was also much less toxic than epibatidine in mice.

Keywords: Epibatidine; Epiboxidine; ABT 418; Nicotinic receptor; Analgesia

1. Introduction

The alkaloid (−)-epibatidine, isolated from skin of an Ecuadoran frog (Spande et al., 1992), has potent antinociceptive activity due to activation of central nicotinic receptors (Badio and Daly, 1994). The novel structure of epibatidine and its marked antinociceptive activity stimulated synthetic efforts in many laboratories (see Szántay et al., 1996, for a review of synthesis and pharmacological studies). Both (+)- and (−)-epibatidine show selectivity for central neuronal $\alpha_4\beta_2$ and ganglionic $\alpha_3\beta_{4(5)}$ nicotinic receptors compared to neuromuscular $\alpha_1\beta_1\gamma\delta$ and central neuronal α_7 receptors (Badio and Daly, 1994; Sullivan et al., 1994; Alkondon and Albuquerque, 1995; Decker et al., 1995; Gerzanich et al., 1995). Toxicity is a major consideration for any therapeutic development of epibatidine and it was thought that alterations in the ring systems might provide compounds with better ratios of pharmacological to toxicological activity. The replacement of the pyridinyl ring in nicotine by a methylisoxazolyl ring, as in the nicotinic receptor agonist ABT 418, (*S*)-3-methyl-5-(1-methyl-2-pyrrolidinyl)isoxazole (Elliott et al., 1995; Garvey et al., 1994), represents a relatively accessible alteration that could be also applied to epibatidine. ABT 418 has been reported to have cognitive enhancing, anxiolytic and antinociceptive effects in mice (Briggs et al., 1995; Brioni et al., 1995; Damaj et al., 1995; Holladay et al., 1995). The methylisoxazole analog of epibatidine, therefore, was synthesized and the nicotinic receptor agonist and antinociceptive activities of the resultant racemate, which has been named epiboxidine (exo-2-(3-methyl-5-isoxazolyl)-7-azabicyclo[2.2.1]heptane), were compared to the activities of (+)- and (−)-epibatidine and of ABT 418, itself the *S*-(+)-enantioner. (±)-Epiboxidine proved even more selective than epibatidine for ganglionic nicotinic receptors, less potent as an antinociceptive agent, and much less toxic.

2. Materials and methods

2.1. Materials

Synthetic ABT 418, the *S*-(+)-enantiomer, was generously provided by Dr. M. Damaj (Medical College Vir-

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ginia, Richmond, VA, USA). Synthetic (+)- and (−)-epibatidine were initially supplied by Drs. Tech-Peng Loh and E.J. Corey (Harvard University, Cambridge, MA, USA). Subsequently, (+)-epibatidine tartrate (levorotatory as free base) was from Research Biochemicals International (Natick, MA, USA) as were (−)-nicotine ditartrate and mecamylamine. [^{3}H]Nicotine (75.7 Ci/mmol) was from New England Nuclear (Boston, MA, USA); ^{22}NaCl (0.2 mCi/ml) from Amersham Life Science (Arlington Heights, IL, USA); *d*-tubocurarine from Boehringer-Mannheim (Mannheim, Germany); carbamylcholine from Sigma (St. Louis, MO, USA). Other compounds were from standard commercial sources.

2.2. Synthesis of epiboxidine

The synthetic route to (±)-epiboxidine (**5**) originates from the commercially available tropinone (Aldrich, Milwaukee, WI, USA) and includes a Favorskii rearrangement to contract the tropane ring to the 'nortropane' ring found in epibatidine, followed by reaction of the resultant ester with the dianion of acetone oxime which after deblocking yields (±)-epiboxidine (Fig. 1). The Favorskii reaction was recently reported in a route to (±)-epibatidine (Xu et al., 1996) and the acetone oxime reaction was employed in the synthesis of the nicotine analog ABT 418 (Elliott et al., 1995). The details of the synthesis are as follows: Tropinone (**1**, 1 g) was dissolved in tetrahydrofuran (10 ml) and ethyl chloroformate (5 ml) was added. After stirring at room temperature overnight, aqueous $NaHCO_3$ (sat. soln., 30 ml) was added and the neutral mixture was extracted with ethyl acetate (3 × 10 ml). The organic layer was dried (anhydrous Na_2SO_4) and the solvent evaporated to yield a residue of tropinone carbamate (**2**, 1.01 g, 71%) homogeneous by gas chromatography-mass spectrometry (gc-ms). Tropinone carbamate (**2**, 169 mg) was dissolved in CH_2Cl_2 (600 μl) and a solution of bromine (50 μl) in 30% HBr in acetic acid (550 μl) was added with stirring. After 45 min, the solution was neutralized with aqueous $NaHCO_3$ (sat. soln., 10 ml) and the mixture extracted with ethyl acetate (3 × 5 ml). The organic layer was dried (anhydrous Na_2SO_4) and the solvent evaporated to yield a residue (198 mg) consisting of two monobromo derivatives (60%), a mixture of dibromo derivatives (20%) and starting material (20%). A sodium ethoxide solution was prepared by reaction of sodium (55 mg) with ethanol (2 ml) and this solution was added to an ethanol (6 ml) solution of the crude monobromo derivatives. The reaction mixture was stirred for 45 min and then H_2O and ethyl acetate were added. The organic layer was dried (anhydrous Na_2SO_4), the solvent evaporated and a residue (115 mg, estimated yield 24% from tropinone) was obtained. Part of the residue (54 mg) was purified by reverse phase high-performance liquid chromatography (hplc), yielding the ester **4** (12 mg), homogeneous by gc-ms. Acetone oxime (3.3 mg) was dissolved in tetrahydrofuran (145 μl) and butyllithium (1.6 M in hexanes, 200 μl) was added. This solution was warmed to 60°C for 5 min in a capped tube and then, a solution of **4** (10 mg) in tetrahydrofuran (60 μl) was injected. After stirring at 60°C for 45 min, the solvent was evaporated with N_2 and concentrated HCl (200 μl) was added. This solution was heated in a capped tube at 100°C for 3 h. After neutralization with aqueous $NaHCO_3$ (sat. soln., 2 ml) extraction with ethyl acetate (3 × 2 ml), drying of the organic layer (anhydrous Na_2SO_4) and evaporation of the solvent, a residue of (±)-epiboxidine **5** was obtained (3.5 mg, 47% from **4**) homogeneous by gc-ms. Ion-trap ms of **5**: m/z 179 (M + 1, 11), 110 (5), 82 (9), 70 (32), 69 (100), 68 (78), 55 (6). ^{1}H-nmr ($CDCl_3$): δ 5.85 (s, 1H, H-12), 3.80 (t, 1H, H-5), 3.75 (d, 1H, H-2), 3.00 (m, 1H), 2.24 (s, 3H, CH_3-13). Ftir (vapor phase): 2979 (100), 2884 (24), 1599 (46), 1422 (42), 1368 (12), 1221 (9), 1048 (9), 1009 (12), 912 (34), 790 (34) cm^{-1}.

2.3. Ion flux assays with cultured cells

Rat pheochromocytoma PC12 cells were provided by Dr. G. Guroff (NIH, Bethesda, MD, USA). Human medul-

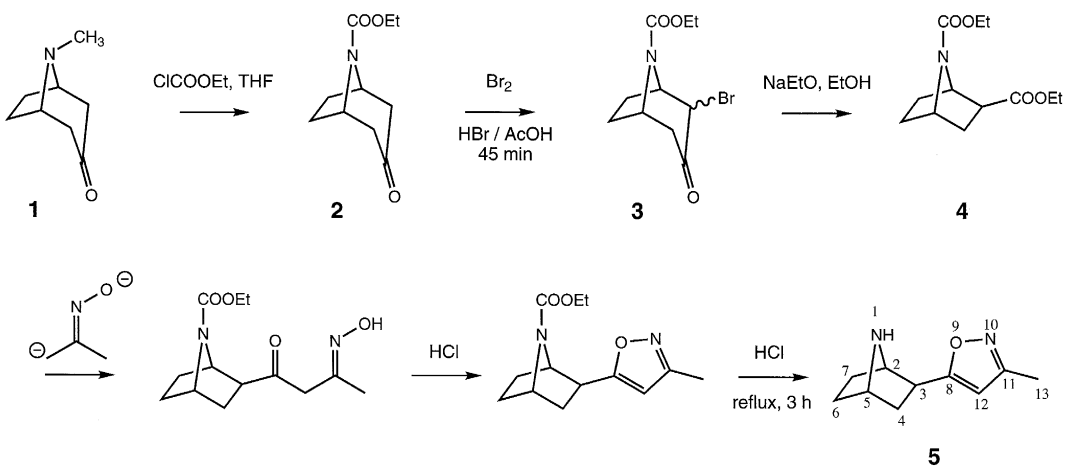

Fig. 1. Synthetic route to (±)-epiboxidine.

loblastoma TE671 cells were from the American Type Culture Collection (Rockville, MD, USA). The PC12 cells were grown in Dulbecco's modified Eagle medium with 6% fetal calf serum, 6% horse serum and penicillin (100 U/ml) and streptomycin (100 mg/ml). The TE671 cells were grown in Dulbecco's modified Eagle medium with 10% fetal calf serum and penicillin (100 U/ml) and streptomycin (100 mg/ml). Cells were grown at 37°C in an atmosphere enriched in CO_2.

Stimulation of sodium-22 influx was assayed in cultured cells as described previously (Badio and Daly, 1994). Cells were plated in six-well culture plates (poly-D-lysine coated) and cultured with [^{3}H]leucine-containing medium for 48 h (PC12 cells) and 24 h (TE671 cells). Medium was removed by aspiration and 0.5 ml of preincubation buffer (concentrations in mM: NaCl 150; KCl 5.4; $CaCl_2$ 2; HEPES/Tris buffer, pH 7.4, 50 (HEPES = 4-(2-hydroxyethyl)-1-piperazinethane sulfonic acid, Tris = 2-amino-2-hydroxymethyl-1,3-propanediol); glucose 5) was added at 22°C. After 10 min, the preincubation buffer was replaced with influx buffer (concentration in mM: NaCl 5; KCl 5.4; $CaCl_2$ 2; HEPES/Tris, pH 7.4, 50; glucose 5; sucrose 224; ouabain 1) containing ^{22}NaCl (0.7 μCi) and (±)-epiboxidine or ABT 418. The antagonist mecamylamine or *d*-tubocurarine was present in both preincubation and influx buffer. After 2 min at 22°C, the influx buffer was removed by aspiration and the cells washed three times with wash buffer (same composition as for preincubation buffer). Cells were solubilized with 0.5 ml 1% sodium dodecylsulfate in 0.5 M NaOH for 30–60 min, pipetted into counting vials with 5 ml Hydrofluor and 0.25 ml 1 M HCl and radioactivity (^{3}H, $^{22}Na^+$) determined in a scintillation counter.

2.4. Nicotinic binding assay with rat brain cerebral cortical membrane

Rat brains, obtained from Pel Freez Biological (Rogers, AK, USA), were thawed in ice-cold 50 mM Tris-HCl buffer (pH 7.4). Rat cerebral cortical tissue was removed and homogenized using a Polytron (setting 6, 10 s). The homogenate was centrifuged for 15 min at $35\,000 \times g$ at 4°C. The pellet was washed once by suspension and recentrifugation in ice-cold Tris buffer. The final pellet was resuspended in ice-cold Tris buffer and stored at −70°C until needed. Aliquots were diluted to a concentration of 1–5 mg/ml for binding assays. Protein concentrations were determined by the bicinchoninic acid protein assay (Pierce Chemical Co., Rockford, IL, USA), using bovine albumin as a standard. [^{3}H]Nicotine receptor binding was assayed as described previously (Badio and Daly, 1994) in 20 mM HEPES buffer (pH 7.4), containing 1 mM $MgCl_2$, 120 mM NaCl, 5 mM KCl and 2 mM $CaCl_2$. Each assay contained the test agent, a suspension of the rat cerebral cortical membranes (100 μl containing 200–300 μg protein), 200 μM diisopropyl fluorophosphate and 2 nM [^{3}H]nicotine in a final volume of 0.5 ml. Each assay was for 120 min at 0–4°C and was performed in triplicate. Nonspecific binding was determined with 10 μM nicotine. Binding reactions were terminated by filtration through Whatman GF/B filters using a Brandel M24R Cell Harvester (Brandel, Gaithersburg, MD, USA). Filters were washed twice with 5 ml ice-cold buffer and placed in scintillation vials with 5 ml of Hydrofluor scintillation fluid, followed by counting for tritium. The filters were presoaked in 0.3% polyethylenimine to reduce nonspecific binding.

2.5. Hot-plate assay for antinociceptive activity

Adult male NIH Swiss strain mice, weighing 25–30 g were used. All drugs were dissolved in a 20:80 v/v mixture of Emulphor EL-620 (Rhône-Poulenc, Cranbury, NJ, USA) and 0.9% saline solution and administered intraperitoneally in a volume corresponding to 5 ml/kg body weight. The hot-plate antinociceptive assay was based on a method previously described (Eddy and Leimbach, 1953). Briefly, mice were placed on a metal plate heated to 55–56°C, enclosed by a glass cylinder. Time to appearance of the first sign of pain (licking or shaking of hind paw, jumping or climbing the sides of the cylinder) was measured. The reaction time for each mouse without drug was determined twice. Each mouse was then injected intraperitoneally with test agent and the reaction time of each mouse was again determined. Mice were used only once for each test agent.

Table 1
Inhibition of [^{3}H]nicotine binding in rat cerebral cortical membranes and stimulation of sodium-22 influx in cultured cells

Agonist	K_i (nM) binding	EC_{50} (μM) sodium-22 influx	
	Rat cerebral membrane	PC12 cells	TE671 cells
(±)-Epiboxidine	0.6 ± 0.1	0.18 ± 0.06	2.6 ± 0.2
(−)-Epibatidine	0.058 ± 0.007	0.11 ± 0.02	0.52 ± 0.02
(+)-Epibatidine	0.045 ± 0.004	0.07 ± 0.007	0.67 ± 0.1
ABT 418	10 ± 3	40 ± 7.0	101 ± 6
(−)-Nicotine	1.01 ± 0.09	20 ± 1.8	60 ± 3.2

Assays were as described in Section 2. Values are mean ± S.E.M. ($n = 3$). Hill coefficients were greater than 0.9, values for epibatidines and (−)-nicotine are from prior publications (Badio and Daly, 1994; Badio et al., 1995) using the same protocols.

3. Results

(±)-Epiboxidine and ABT 418 inhibited the binding of [^{3}H]nicotine to rat cerebral cortical membranes with (±)-epiboxidine being much more potent than ABT 418 (Table 1). The K_i values for (−)- and (+)-epibatidine and (−)-nicotine are from a prior study with an identical protocol (Badio and Daly, 1994; Badio et al., 1995). Neither (±)-epiboxidine nor ABT 418 at 100 μM inhibited binding of [^{3}H]quinuclidinylbenzilate to muscarinic receptors of rat cerebral cortical membranes (data not shown). Epibatidine was previously reported to cause no inhibition of binding at muscarinic receptors (Badio and Daly, 1994).

(±)-Epiboxidine and ABT 418 were full agonists in cells expressing ganglionic-type (PC12 cells) and neuromuscular-type (TE671 cells) nicotinic receptors (Fig. 2A,B). The maximal stimulation of sodium-22 influx by both agents was blocked by mecamylamine in PC12 cells and by *d*-tubocurarine in TE671 cells (data not shown). (±)-Epiboxidine at 50 and 100 μg/kg caused marked antinociception as measured in the hot-plate assay, while mice given 20 μg/kg showed no effect (Fig. 3). (−)-Epibatidine at 5 μg/kg caused a similar degree of antinociception to that evinced with 50 μg/kg (±)-epiboxidine. (±)-Epiboxidine at 50 and 100 μg/kg caused a dose-related Straub tail, hypomotility, hypoventilation and piloerection in mice, similar to the effect of 5 μg/kg (−)-epibatidine. The antinociceptive effect of 100 μg/kg (±)-epiboxidine was completely blocked by 2 mg/kg mecamylamine (Fig. 4). Mice given the nicotinic receptor antagonist mecamylamine showed little or no Straub tail, hypomotility, hypoventilation, or piloerection. There was only one death (convulsions) in the group of mice ($n = 10$) given 100 μg/kg (±)-epiboxidine alone. (−)-Epibatidine at 10 μg/kg caused death in 6 of 6 mice. Mice recovered fully 1 h after 100 μg/kg of (±)-epiboxidine or 5 μg/kg of (−)-epibatidine.

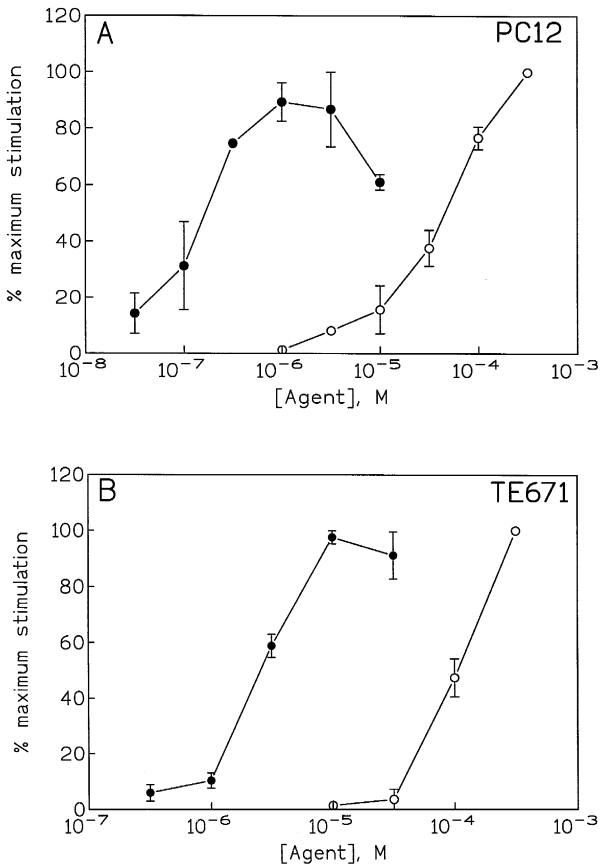

Fig. 2. Stimulation of influx of sodium-22 by (±)-epiboxidine and ABT 418 in (A) rat pheochromocytoma PC12 cells and (B) human medulloblastoma TE671 cells. Influx assays were as described in Section 2 with (±)-epiboxidine (●) or ABT 418 (○). Each value is the percentage of maximal influx obtained with (−)-nicotine and is the mean ± S.E.M. ($n = 3$).

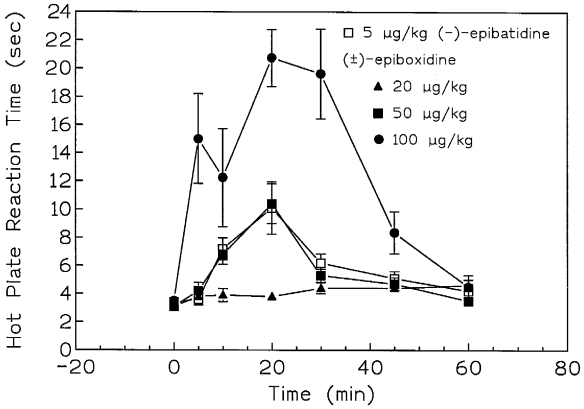

Fig. 3. Antinociceptive activity of (±)-epiboxidine and (−)-epibatidine in mice. Hot-plate assays were as described in Section 2. Each value is the mean of the reaction time in seconds ± S.E.M. ($n = 6$–10).

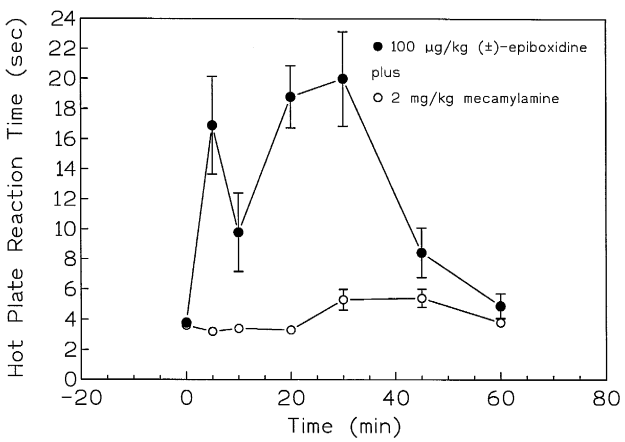

Fig. 4. Blockade of (±)-epiboxidine-elicited antinociception by the nicotinic antagonist mecamylamine. (±)-Epiboxidine at 100 μg/kg was administered 5 min after 2 mg/kg mecamylamine. Hot plate assays were as described in Section 2. Each value is the mean ± S.E.M. ($n = 10$) and is from a different set of mice than those reported in Fig. 3.

4. Discussion

(±)-Epiboxidine proved to be a potent nicotinic receptor agonist, particularly at the ganglionic-type receptor of PC12 cells, where it was not significantly less potent than (−)-epibatidine (Table 1). Activity at the neuromuscular-type receptor of TE671 cells and affinity versus nicotinic-binding to neuronal-type receptors in brain membranes was, in contrast, markedly reduced by replacement of the chloropyridinyl moiety in epibatidine with the methylisoxazolyl moiety in epiboxidine. Replacement of the pyridinyl moiety of (−)-nicotine (*S*-enantiomer), with the methylisoxazolyl moiety in ABT 418 (*S*-enantiomer), resulted in about a 2-fold reduction in activity at both the ganglionic-type receptor and the neuromuscular-type receptor, while a 10-fold reduction occurred for the central neuronal-type receptor (Table 1). Thus, it appears that the nortropane ring of epiboxidine and epibatidine is the primary determinant of high nicotinic activity, while substitution of the pyridinyl moiety in either nicotine or epibatidine by a methylisoxazolyl moiety markedly decreases affinity/potency at central neuronal-type receptors. In the case of epibatidine the replacement of the pyridinyl moiety with a methylisoxazolyl moiety also markedly reduces potency at the neuromuscular-type receptors.

The altered profile of selectivity of (±)-epiboxidine and ABT 418 for nicotinic receptor subtypes would be expected to change the in vivo profile of pharmacologic and toxicologic activity. (±)-Epiboxidine was about 10-fold less potent than (−)-epibatidine as an antinociceptive agent (Fig. 3), but appeared about 20-fold less toxic in a comparison with (−)-epibatidine (see Section 3). Comparisons of in vivo effects of ABT 418 and (−)-nicotine have been previously reported (Briggs et al., 1995; Brioni et al., 1995; Damaj et al., 1995; Holladay et al., 1995). ABT 418, because of our limited supplies, could not be evaluated in vivo in the present study. The (+)- and (−)-epibatidine show little enantioselectivity in a variety of nicotinic assays including in vivo antinociception (see Szántay et al., 1996 and ref. therein). There are, however, differences with regard to time course and tolerance (Badio and Daly, 1994; Damaj et al., 1994; Badio et al., 1995 Damaj and Martin, 1996). (−)-Epibatidine in our hot-plate assay with NIH Swiss strain male mice always showed a biphasic time course for antinociceptive effects (Badio and Daly, 1994). An apparent biphasic antinociceptive time course also occurs with (±)-epiboxidine at least in two sets of experiments (Figs. 3 and 4). Tolerance occurs with (−)- but not (+)-epibatidine (Damaj and Martin, 1996). Further studies with enantiomers of epiboxidine and of ABT 418 are warranted. It would be predicted that the epiboxidines, like the epibatidines, would show little enantioselectivity, while the (−)- and (+)-enantiomers of ABT 418, like the nicotines, should show enantioselectivity, except at neuromuscular-type receptors, where the two enantiomers of nicotine also show little enantioselectivity (Badio et al., 1995). ABT 8295, the *R*-(−)-enantiomer of ABT 418, was stated to be 2-fold less active than ABT 418 (Holladay et al., 1995). Both in vitro and in vivo, the effects of (±)-epiboxidine were blocked by nicotinic antagonists (see Section 3) as was previously reported for the epibatidines (Badio and Daly, 1994).

In summary, (±)-epiboxidine represents a potent antinociceptive agent with a better activity/toxicity ratio than epibatidine, the natural alkaloid that served as a molecular model. Tentatively, it might be concluded that central $\alpha_4\beta_2$ nicotinic receptors are primarily involved in the antinociceptive activity of (−)-epibatidine, since a 10-fold reduction of affinity of (±)-epiboxidine at such receptors compared to (−)-epibatidine seems paralleled by about a 10-fold reduction in antinociceptive activity of (±)-epiboxidine compared to (−)-epibatidine. Ganglionic receptors seem less likely to be involved since (±)-epiboxidine is not significantly less potent than (−)-epibatidine at such receptors. (±)-Epiboxidine provides a novel new agonist for investigation of the receptor subtypes involved in the central pharmacological activities of nicotine.

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