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Homoepiboxidines: further potent agonists for nicotinic receptors

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Abstract—Homoepiboxidine (**3**) and the corresponding *N*-methyl (**4**) and *N*-benzyl (**5**) derivatives were prepared from a 6β-carbomethoxynortropane (**8**). Affinities and functional activities at neuromuscular, central neuronal and ganglionic-type nicotinic receptors were compared to those of epibatidine **1**, and epiboxidine **2**. Homoepiboxidine had equivalent affinity/activity to epiboxidine at neuromuscular, neuronal α4β2, and most α3-containing ganglionic-type nicotinic receptors. The *N*-substituted derivatives showed reduced affinity/activity at most receptor subtypes. Replacement of the methylisoxazole moiety of **3** and **4** with a methyloxadiazole moiety provided analogues **6** and **7**, which had greatly reduced affinity/activity in virtually all assays at nicotinic receptors. Marked analgetic activity in mice occurred at the following ip doses: epibatidine 10 μg/kg; epiboxidine 25 μg/kg; homoepiboxidine 100 μg/kg; *N*-methylhomoepiboxidine 100 μg/kg; the methyloxadiazole (**6**) 100 μg/kg. The time course at such ip doses was significantly longer for homoepiboxidine **3** with marked analgesia still manifest at 30 min post-injection. Epiboxidine and the homoepiboxidines were less toxic than epibatidine.
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1. Introduction

The discovery of the alkaloid epibatidine (**1**)[1] as a potent analgetic and agonist for neuronal and ganglionic nicotinic receptors[2] has led to the synthesis of a wide range of structural analogues with the objective of establishing a potential potent analgetic with greatly reduced toxicity. Analogues have been described with alterations to the azabicycle[3–14] and the heteroaromatic ring[15–20] (Fig. 1). A methylisoxazole analogue, epiboxidine (**2**), showed a greater separation of analgetic potency and toxicity than epibatidine.[15] We now report the synthesis of homoepiboxidine (**3**), the corresponding *N*-methyl (**4**) and *N*-benzyl (**5**) derivatives, and two oxadiazole analogues **6** and **7**. The nicotinic and analgetic activities, as well as toxicities of these compounds were compared with those of epibatidine and epiboxidine.

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2. Results

2.1. Chemistry

Starting material *N*-benzyl-2-oxo-6β-carbomethoxynortropane (**8**)[21] was deoxygenated by conversion to the dithiolane (**9**) and subsequent desulfurization with Raney nickel to give nortropane **10**. This was converted to *N*-benzylhomoepiboxidine **5** in 40% yield, following a literature method for formation of the isoxazole[22] (Scheme 1). Two minor by-products, β-ketooxime **11** and dimethylpyridyl *N*-oxide **12** were obtained in 9 and 6% yield, respectively. Oxime **11** could be converted to **5** in 68% yield by treatment with methanesulfonyl chloride/Et_3N in CH_2Cl_2. Catalytic hydrogenation of **5** failed to produce homoepiboxidine **3**. Rather, products of isoxazole cleavage were obtained. Subsequently, **3** was prepared by an alternate route. Nortropane **13** was *N*-protected as urethane **14** by treatment with Boc_2O. This was then treated with the dianion of acetone oxime to afford methylisoxazole **15** in 42% yield. Subsequent removal of the urethane with trifluoroacetic acid (TFA) gave **3**, which was then *N*-methylated using formalin/formic acid to give **4**. Urethane **14** was treated as

described[23] with acetamide oxime and sodium hydride to give the methyloxadiazole **16** in 80% yield. Deprotection of **16** with TFA gave the nortropane **6**, which was *N*-methylated to give **7**.

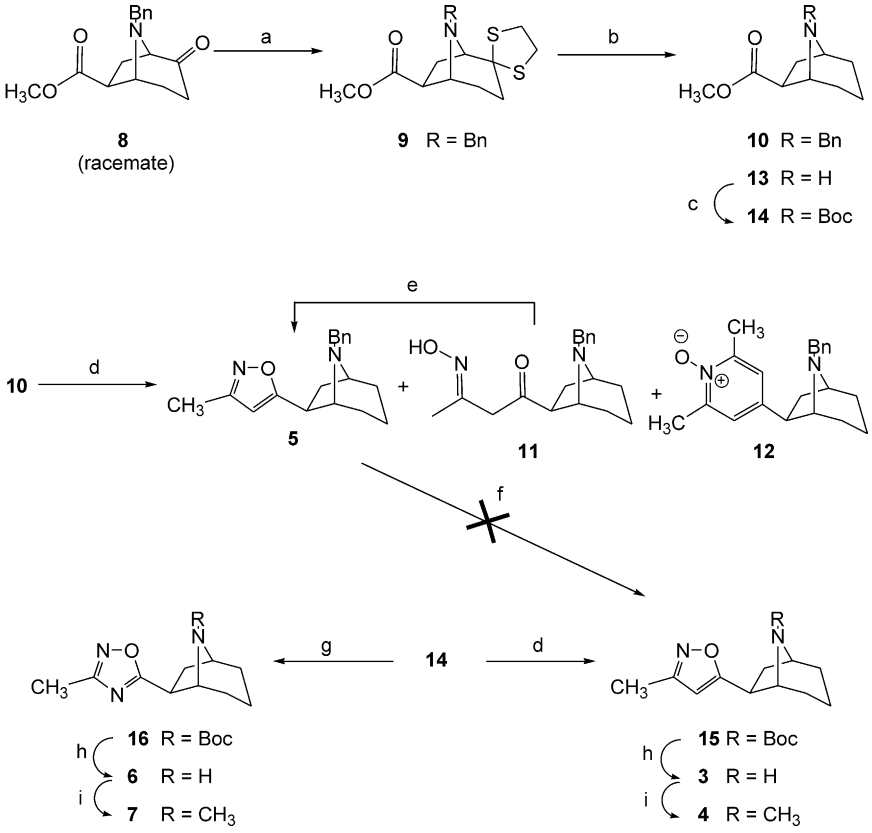
2.2. Biology

Both binding affinity and functional activity studies (see Experimental) were conducted with cultured cells expressing human α1β1γδ receptors (TE-671),[25] human neuronal α4β2 receptors (K-177 cells),[26] and rat ganglionic α3β4 receptors (KXα3β4R2 cells)[27] or in cells expressing either rat or human ganglionic-type α3-containing receptors (PC-12,[28,29] IMR-32,[30–34] SH-SY5Y[32–36] cells), where the subunit composition is not fully defined. The rat ganglionic-type receptors of PC-12 cells contain α3 and β4 subunits, but mRNA for the α5 subunit is also highly expressed.[28,29] No mRNA for β2 subunits was detected. The human ganglionic-type α3β4*-receptors of IMR-32 cells might also contain α5 or β2 subunits.[30,31] However, a recent study[32] suggests that the primary functional responses of IMR-32 cells are those of an α3β4 receptor. The human ganglionic α3β2* receptors of SH-SY5Y cells probably also contain β4 subunits.[33]

2.3. Binding assays

The affinities for the homoepiboxidines and related nicotinic agonists were determined using the standard [^{3}H]nicotine binding assay in rat cerebral cortical membranes (Table 1).[24] [^{3}H]Nicotine, a commonly used nicotinic radioligand was used in rat cerebral cortical membranes and this data is presented for comparison with the existing literature. [^{3}H]Epibatidine[37] was used instead of [^{3}H]nicotine for binding studies with cultured cell membranes (see below) because of higher affinity and low nonspecific binding as well as to compensate for the low density of receptors in cell membranes, compared with brain membranes. K_i values were determined using the Cheng–Prusoff equation, thus enabling rough comparisons between binding data from the two radioligands. As binding in cortical membranes reflect primarily the α4β2 subtype, the binding data for K-177 cells represent a point of comparison for brain and cell membranes. The low density of the human α1β1γδ neuromuscular receptors in TE671 and SH-SY5Y cell membranes proved difficult to assess using either [^{3}H]nicotine or [^{3}H]epibatidine as the radioligand.

(±)-Homoepiboxidine (**3**, K_i 0.74 nM) had comparable affinity to epiboxidine (**2**, K_i 0.46 nM) with both having several-fold lower affinity than epibatidine (**1**, K_i 0.093 nM). The *N*-methyl group in **4** reduced the affinity relative to **3** by about 6-fold, while the *N*-benzyl group in **5** reduced affinity by 40-fold. The methyloxadiazole analogues **6** and **7** had 15-fold and 5-fold lower affinities, respectively, than the corresponding homoepiboxidines **3** and **4**, being many-fold less potent than (−)-nicotine (K_i 1.2 nM).



Scheme 1. (a) $HSCH_2CH_2SH$, $BF_3{\cdot}OEt_2$; (b) H_2, Raney Ni, THF; (c) Boc_2O, THF, $NaHCO_3$; (d) (i) $(CH_3)_2C{=}NOH$, *n*BuLi; (ii) 10% aq HCl; (e) MsCl, Et_3N, CH_2Cl_2; (f) H_2, $Pd(OH)_2/C$, THF; (g) (i) $CH_3C(=NOH)NH_2$, NaH/Δ; (ii) 10% aq HCl, Δ; (h) CF_3CO_2H, CH_2Cl_2; (i) 37% aq CH_2O, HCO_2H.

Homoepiboxidines (**3**, **4**, **5**) had lower affinities for the ganglionic α3-receptors of the cultured cells compared to the neuronal α4β2 receptors, as was the case for the epibatidines and epiboxidine (Table 1). Oxadiazole **6** had low affinity for the neuronal receptors and very low affinity for ganglionic-type receptors. The corresponding *N*-methyl derivative **7** had still lower affinities for the various subtypes.

2.4. Functional assays

Functional agonist activity of homoepiboxidines and the related nicotinic agents were determined based on either stimulation of ^{22}Na influx[38] (Table 2) or fluorometric assessment of changes in intracellular calcium[39] (Table 3) or membrane potential[40] (Table 4). Influx of $^{22}Na^+$ was measured either with the six-well paradigm of earlier studies[15,34] or with a 96-well assay using phosphorimaging detection and quantitation (see Experimental). Both assays gave similar results when compared for TE-671 cells (Table 2). Fluorometric measurements of calcium were performed using a fluorescence plate reader modified to allow addition of reagents manually while reading (see Experimental), while fluorescence measurements of membrane potential were performed using a fully automated plate reader.[40] The potencies of nicotinic agonists in functional assays are typically orders of magnitude less than measured inhibition constants in the same tissues. This has been ascribed to a so-called high affinity desensitized state of nicotinic receptors[24] as

Table 1. Affinity of epibatidine, epiboxidine, and related analogues for subtypes of nicotinic receptors in membranes from brain and cultured cells

Nicotinic agent	$K_i \pm$SEM (nM)			
	Neuronal		Ganglionic	
	Rat Brain[a] Rat α4β2	K-177[b] Human α4β2	Kxa3b4R2[b] Rat α3β4	IMR-32[b] Rat α3β4*
(±)-Epibatidine **1**	0.093±0.004	0.044±0.002	0.51±0.04	0.22±0.01
(1*R*,2*R*,4*S*)-(−)-Epibatidine	0.058±0.007[c]	—	—	—
(1*S*,2*S*,4*R*)-(+)-Epibatidine	0.045±0.004[c]	—	—	—
N-Methyl-(1*R*,2*R*,4*S*)-(−)-epibatidine	0.10±0.03[d]	0.17±0.01	2.3±0.1	0.7±0.2
N-Methyl-(1*S*,2*S*,4*R*)-(+)-epibatidine	0.26±0.05[d]	0.26±0.05	0.95±0.07	1.6±0.2
(±)-Epiboxidine **2**	0.46±0.12	1.2±0.4	15±4	19±1
(±)-Homoepiboxidine **3**	0.74±0.04	7.8±3.6	16±2	41±12
(±)-*N*-Methylhomoepiboxidine **4**	4.7±0.02	25±2	320±120	160±50
(±)-*N*-Benzylhomoepiboxidine **5**	30±5	20±2	57±4	110±20
(±)-Oxadiazole analogue **6**	11±0.4	28±3	890±50	460±50
(±)-*N*-Methyloxadiazole analogue **7**	24±3	70±4	3800±300	1900±300
S-(−)-Nicotine	1.2±0.1	7.2±0.6	390±30	320±40

Affinities determined as described in Experimental. Values represent the average±SEM of three experiments performed in triplicate.
[a] K_i verses 5 nM [^{3}H]-*S*-nicotine.
[b] K_i verses 0.5 nM [^{3}H]-(±)-epibatidine.
[c] Taken from ref 2.
[d] Taken from ref 42.

Table 2. Agonist activity of epibatidine, epiboxidine, and related compounds in cultured cells: ^{22}Na influx[c]

Nicotinic agent	$EC_{50} \pm$SEM (μM)						
	Neuromuscular		Neuronal	Ganglionic			
	TE-671 Human α1β1γδ		K-177 Human α4β2	KXα3β4R2 Rat α3β4	IMR-32 Human α3β4*	SH-SY5Y Human α3*	PC-12 Rat α3*
	six-well	96-well	six-well	96-well	96-well	96-well	six-well
(±)-Epibatidine **1**	—	0.35±0.17	—	0.049±0.005	0.012±0.002	0.0036±0.0020	—
(1*R*,2*R*,4*S*)-(−)-Epibatidine	0.53±0.02[a]	0.62±0.23	0.038±0.002	0.034±0.010	0.014±0.002	0.0044±0.0015	0.11±0.02[a]
(1*S*,2*S*,4*R*)-(+)-Epibatidine	0.67±0.10[a]	0.24±0.30	0.040±0.009	0.037±0.011	0.014±0.009	0.0054±0.0031	0.072±0.009[a]
N-Methyl-(1*R*,2*R*,4*S*)-(−)-epibatidine	0.092±0.010[a]	0.097±0.052	—	0.057±0.019	0.025±0.003	0.0039±0.0007	0.032±0.004[a]
N-Methyl-(1*S*,2*S*,4*R*)-(+)-epibatidine	0.6±0.09[a]	1.0±0.2	—	0.18±0.05	0.051±0.010	0.014±0.008	0.26±0.07[a]
(±)-Epiboxidine **2**	2.6±0.2[b]	1.1±0.27	0.39±0.10	0.14±0.02	0.069±0.021	0.93±0.83	0.18±0.06[b]
(±)-Homoepiboxidine **3**	—	1.5±0.8	0.49±0.05 (74)	0.51±0.10	0.44±0.25	0.59±0.47	1.0±0.1 (76)
(±)-*N*-Methylhomoepiboxidine **4**	—	2.5±1.3	1.8±0.3	2.4±0.4	0.86±0.56	0.53±0.19	1.8±0.1
(±)-*N*-Benzylhomoepiboxidine **5**	—	>10 μM	>10 μM	>10 μM	3.5±3.4 (33)	0.88±0.43 (52)	>10 μM
(±)-Oxadiazole analogue **6**	—	—	—	52±15	—	—	—
(±)-*N*-Methyloxadiazole analogue **7**	—	—	—	190±20 (61)	—	—	—
S-(−)-Nicotine	41±4	110±60	3.8±0.7	16±4	16±5	13±9	20±2

Pharmacological parameters were determined as described in the Experimental. Values represent the average±SEM of three experiments performed in triplicate for six-well data and 3–5 experiments perfomed in duplicate for 96-well data. Efficacies relative to (±)-epibatidine were 80% or greater except where indicated in parentheses.
[a] Data taken from ref 42.
[b] Data taken from ref 15.
[c] Efficacy at 1 mM.

Table 3. Agonist activity of epibatidine, epiboxidine and related compounds in cultured cells: calcium fluorescence assay

Nicotinic agent	EC_{50} ± SEM (μM)		
	Neuronal	Ganglionic	
	K-177 Human α4β2	KXα3β4R2 Rat α3β4	IMR-32 Human α3β4*
(±)-Epibatidine **1**	0.027 ± 0.005	0.072 ± 0.004	0.027 ± 0.009
(1*R*,2*R*,4*S*)-(−)-Epibatidine	0.027[b]	0.033 ± 0.008	0.015, 0.051[a]
(1*S*,2*S*,4*R*)-(+)-Epibatidine	0.0073[b]	0.016 ± 0.005	0.013, 0.022[a]
N-Methyl-(1*R*,2*R*,4*S*)-(−)-epibatidine	0.0029[b]	0.18 ± 0.10	0.0021, 0.0094[a]
N-Methyl-(1*S*,2*S*,4*R*)-(+)-epibatidine	0.059[b]	1.2 ± 0.1	0.033, 0.12[a]
(±)-Epiboxidine **2**	0.35 ± 0.05	0.32 ± 0.02[c]	0.47 ± 0.19
(±)-Homoepiboxidine **3**	9.3 ± 2.2 (74)	0.72 ± 0.12 (75)	5.9 ± 1.3 (54)
(±)-*N*-Methylhomoepiboxidine **4**	18 ± 8	10 ± 1	7.1 ± 2.6 (68)
(±)-*N*-Benzylhomoepiboxidine **5**	> 100	9.6 ± 2.0 (12)	> 100
(±)-Oxadiazole analogue **6**	81 ± 59 (70)	26 ± 4 (54)	18 ± 8 (58)
(±)-*N*-Methyloxadiazole analogue **7**	95 ± 45 (42)[a]	> 100	23 (12)[b]
S-(−)-Nicotine	5.0 ± 1.1 (79)	14 ± 2 (72)	28 ± 3 (74)

Pharmacological parameters were determined as described in the Experimental. Except where otherwise indicated, values represent the average ± SEM of 3–5 experiments performed duplicate. Efficacies relative to (±)-epibatidine were 80% or greater except where indicated in parentheses.
[a] Average of two experiments.
[b] Single experiment.
[c] The value for (±)-epiboxidine in Table 2 of ref 40 was reported in

Table 4. Nicotinic agonist activity of epibatidine, epiboxidine and related compounds in cultured cells: membrane potential fluorescence assay

Nicotinic agent	EC_{50} ± SEM (μM)				
	Neuromuscular	Neuronal	Ganglionic		
	TE-671 Human α1β1γδ	K-177 Human α4β2	KXα3β4R2 Rat α3β4	SH-SY5Y Human α3*	IMR-32 Human α3β4*
(±)-Epibatidine **1**	0.27 ± 0.03	0.0046 ± 0.0022	0.018 ± 0.011	0.0080 ± 0.0005	0.021 ± 0.010
(1*R*,2*R*,4*S*)-(−)-Epibatidine	0.26 ± 0.07	0.018 ± 0.011	—	0.0022 ± 0.0010	0.013 ± 0.003
(1*S*,2*S*,4*R*)-(+)-Epibatidine	0.099 ± 0.024	0.0057 ± 0.0016	—	0.0026 ± 0.0007	0.0041 ± 0.0007
N-Methyl-(*1R*,2*R*,4*S*)-(−)-epibatidine	0.12 ± 0.02	0.0043 ± 0.0021	—	0.0022 ± 0.0017	0.014 ± 0.012
N-Methyl-(1*S*,2*S*,4*R*)-(+)-epibatidine	0.76 ± 0.10	0.064 ± 0.051	—	0.0096 ± 0.0019	0.091 ± 0.071
(±)-Epiboxidine **2**	0.51 ± 0.07	0.20 ± 0.08	0.18 ± 0.11	0.065 ± 0.013	0.59 ± 0.32
(±)-Homoepiboxidine **3**	1.0 ± 0.3	9.6 ± 4.2 (75)	0.52 ± 0.41	0.14 ± 0.07	22 ± 21 (76)
(±)-*N*-Methylhomoepiboxidine **4**	4.7 ± 0.9	2.1 ± 1.0	3.8 ± 3.5	2.9 ± 0.4	11 ± 8
(±)-*N*-Benzylhomoepiboxidine **5**	16 ± 4 (62)	30 ± 23 (54)	3.0 ± 0.8	9.2 ± 5.5 (24)	300 ± 290 (15)
(±)-Oxadiazole analogue **6**	8.3 ± 2.1 (69)	25 ± 10 (36)	29 ± 25	0.55 ± 0.08 (66)	0.98 ± 0.10 (72)
(±)-*N*-Methyloxadiazole analogue **7**	28 ± 5	7.0 ± 1.4 (56)	3.7 ± 3.6	3.0 ± 2.6	39 ± 2 (64)
S-(−)-Nicotine	27 ± 3 (76)	0.54 ± 0.18	8.0 ± 4.6	6.2 ± 0.2	21 ± 9 (63)

Pharmacological parameters were determined as described in the Experimental. Values represent the average ± SEM of 3–5 experiments performed in duplicate. Estimated efficacies relative to (±)-epibatidine were 80% or greater except where efficacy is indicated in parentheses.

assessed in binding assays, which are performed over the course of many minutes to hours as compared to functional assays which are on the order of subsecond to a few minute time scales.

In the functional assays, (±)-homoepiboxidine **3** was similar in potency to (±)-epiboxidine **2** in TE671 cells expressing the neuromuscular α1β1γδ nicotinic receptor (Tables 2–4). An *N*-methyl group (**4**) or an *N*-benzyl group (**5**) reduced functional activity 2-fold and 5-fold, respectively. In functional assays with K-177 cells expressing the human neuronal α4β2 receptor, homoepiboxidine **3** was equipotent with epiboxidine **2** and both were about 10-fold less potent than epibatidine **1**, consonant with binding data (Table 1) on rat neuronal α4β2 receptors. The solubility of 5 appeared limited to < 100 μM. Efficacy values for **5** were less than 50% in nearly all cases and less than 20% in most. Thus, EC_{50} values with this compound could not be reliably estimated for four of the cell lines.

In functional assays with cells expressing ganglionic α3 nicotinic receptors (PC12, KXα3β4R2, IMR-32, SH-SY5Y), homoepiboxidine **3** was either equipotent or less potent than epiboxidine **2**. The *N*-methyl group of **4** reduced potency at ganglionic receptors in most cell lines. The *N*-benzyl group of **5** reduced potency even further and also reduced apparent efficacy. The results for cells expressing various ganglionic-type nicotinic receptors differed considerably, suggesting that such receptors may have markedly different interactions with the agonists. The oxadiazole analogues **6** and **7** were very weak agonists at the ganglionic receptors of KXα3β4R2 cells as compared to (−)-nicotine. Limited supplies prevented detailed evaluation of **6** and **7** in other cells.

2.5. In vivo assays

Analgesia of compounds **1**–**4** and **6**, and toxicity of compounds **1**–**5** were compared in NIH Swiss strain

male mice on ip and sc injection, respectively. The homoepiboxidines were about 10-fold less potent as analgetics, but fully as efficacious as epibatidine using the hot-plate assay[41,42] (Fig. 2A–D). However, the analgetic effects of homoepiboxidine had a longer duration (Fig. 2B). Oxadiazole **6** was about 10-fold less potent than epibatidine (Fig. 2E).

The homoepiboxidines appeared much less toxic than epibatidine as follows: (±)-Epibatidine (**1**) at 10 μg/kg caused death in one of three mice, while at 20 μg/kg all three mice died. (±)-Epiboxidine (**2**) at 200 μg/kg caused death in one of three mice. (±)-Homoepiboxidine (**3**) at 200 μg/kg caused prostration, labored breathing, but no deaths in three mice. (±)-*N*-Methylhomoepiboxidine (**4**) at 200 μg/kg caused prostration, labored breathing, but no deaths in three mice. (±)-*N*-Benzyhomoepiboxidine (**5**) at 1600 μg/kg caused mild prostration and no deaths in three mice.

3. Discussion

Homoepiboxidines (**3–5**) represent another structural class of azabicyclics developed as analogues of the potent nicotinic analgetic epibatidine (**1**). In these analogues the chloropyridyl moiety of epibatidine is replaced by a methylisoxazole moiety as first reported for epiboxidine (**2**).[15] Previously, the pyridyl moiety of *S*-(−)-nicotine had been replaced with a methylisoxazole moiety to afford ABT-418.[43] In contrast to epibatidine and epiboxidine, an additional methylene is present in the azabicyclic ring system of homoepiboxidine, a compound analogous to homoepibatidine.[3,4] Such structural alterations affect both potency and selectivity of homoepiboxidine **3** as compared to **1** and **2** (Tables 1–4).

3.1. Epibatidine/epiboxidine comparison

Epibatidine (**1**) had subnanomolar affinity for neuronal α4β2 nicotinic receptors and only a few-fold lower affinity for ganglionic-type α3-containing nicotinic receptors (Table 1) and commensurate submicromolar functional activity at such receptors (Tables 2–4). Epibatidine had much lower affinity and functional activity at neuromuscular receptors. Epiboxidine (**2**) proved to have significantly lower affinity and functional activity than epibatidine at central neuronal α4β2 receptors (ref 15 and Tables 2–4), but both had similar activity at ganglionic-type α3β4*-nicotinic receptors of PC12 cells.[15] The activity of epiboxidine was also reduced compared to epibatidine at α3-containing nicotinic receptors of other cell types (Tables 2–4). The 10-fold reduction in the activity of epiboxidine relative to epibatidine at α4β2 receptors may account for the 5- to 10-fold reduction in analgetic potency of epiboxidine.[15] The toxicity of epiboxidine was also markedly reduced perhaps due to a reduction in activity at neuromuscular receptors[15] and/or some classes of ganglionic-type α3-containing nicotinic receptors (Tables 2–4). In the present study, epiboxidine was about 5-fold less potent than epibatidine as an analgetic, while the homoepiboxidines (**3**, **4**) were about 10-fold less potent (Fig. 2A–D). The homoepiboxidines also were significantly less toxic that epibatidine (see Results). A comparison of the functional activities of epiboxidine and homoepiboxidine is presented below.

Another synthesis of epiboxidine (**2**) was recently reported, along with synthesis of an isoxazole analogue and a phenylisoxazole analogue.[17] In that study, the methylisoxazole (epiboxidine) had about 2-fold higher affinity for rat α4β2 receptors than the unsubstituted isoxazole analogue, while the phenylisoxazole had 100-fold reduced affinity. The reported LD_{50} values of epiboxidine and the unsubstituted isoxazole analogue were about 10-fold higher (2 mg/kg) than the LD_{50} for epibatidine (0.2 mg/kg).

3.2. Homoepiboxidines and homoepibatidines

Homoepiboxidine (**3**) proved to have similar functional activity to epiboxidine (**2**) at the neuromuscular and neuronal α4β2 receptors and to be less active at ganglionic-type α3-containing receptors (Table 2). Homoepiboxidine was also less potent as an analgetic (Fig. 2). The synthesis of the structurally related (±)-homoepibatidine was reported for the first time in 1996.[3ab,4a] (±)-Homoepibatidine, and the *N*-methyl derivative were about 4-fold less potent as analgetics than (±)-epibatidine.[3a] *N*-Benzyl derivative was less potent still in both affinity and functional assays. Both enantiomers of homoepibatidine have been synthesized.[4d] The (−)-enantiomer had only slightly more affinity than the (+)-enantiomer, and both had about 3-fold less affinity than the corresponding enantiomers of epibatidine in binding assays at rat brain α4β2 receptors.[4d] Recently, (+)-homoepibatidine was reported to be equipotent with (−)-homoepibatidine in activating α4β2 and α3β4 receptors expressed in *Xenopus* oocytes, while being much less potent at α7 receptors.[19] The (−)-homoepibatidine was many-fold less potent than the (+)-enantiomer at all three receptor subtypes. An isomeric homoepibatidine (isohomoepibatidine) has also been described in which the chloropyridyl substituent is on the six-membered ring.[6] The enantiomer, which was synthesized from (−)-cocaine, had 27-fold lower affinity than (±)-epibatidine at neuromuscular-type nicotinic receptors in membranes of *Torpedo* electroplax. A mesosymmetric homoepibatidine[14] and the corresponding (±)-dehydro- precursor[14,20] have also been synthesized as well as UB-165,[5] an epibatidine/anatoxin hybrid (see Fig. 1).

The effect of absolute stereochemistry and *N*-substitution on activity of epibatidines have been reported.[2,42] Both enantiomers of epibatidine had similar affinities and functional activities at nicotinic receptors (Tables 1–4). *N*-Methylation of the natural (−)-(RRS) enantiomer tended to enhance functional activity, while having little effect or reducing activity of the unnatural (+)-(SSR) enantiomer. Enantioselectivity for epiboxidine (**2**) and homoepiboxidines (**3–5**) are unknown. The (±)-*N*-methylhomoepiboxidine (**4**) tended to be somewhat less potent than (±)-homoepiboxidine (**3**).

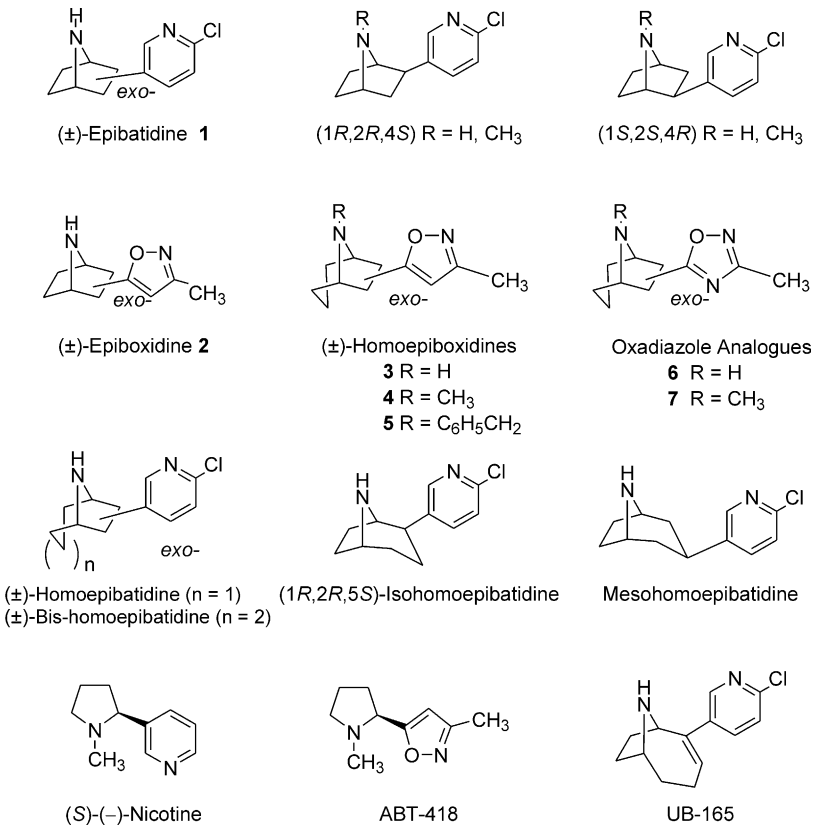


Figure 1. Nicotinic agonists.

3.3. Oxadiazole analogues

The methyloxadiazole analogue (**6**) of (±) homoepiboxidine (**2**) was considerably less potent at nicotinic receptors (Tables 1–4), as was the *N*-methyl analogue (**7**). The methyloxadiazole analogue of (±)-epibatidine has been reported.[19] Only in vivo effects were reported, but it was clear that, in contrast to epibatidine[2] and epiboxidine,[15] the potent analgetic activity of the methyloxadiazole analogue of epibatidine was mediated by activation of muscarinic rather than nicotinic receptors. The analgetic activity of methyloxadiazole **6** (Fig. 1E) also might be mediated by muscarinic receptors, but this was not investigated.

3.4. Functional assay comparisons

The three functional assay methods (^{22}Na influx, calcium dynamics, and membrane potential) gave similar EC_{50} values (2-fold difference or less) for most compounds in the three cell lines (K-177, KXα3β4R2, and IMR-32) where all three methods were used (Tables 2–4). However, certain compounds appeared more potent in the ^{22}Na influx assay as compared to calcium dynamics. These included epiboxidine in IMR-32, the *N*-methylepibatidines in KXα3β4R2, homoepiboxidine (**3**) in K-177, and *N*-methylhomoepiboxidine (**4**) in all three cell lines. We have previously observed a greater (2- to 30-fold) potency for nicotinic agonists in the membrane potential assay as compared to calcium dynamics for KXα3β4R2 cells.[40] In the present study, most compounds were only 2- to 3-fold more potent in causing membrane depolarization in these cells. However, in the IMR-32 cells, oxadiazole **6** appeared significantly less potent in the membrane potential assay than in calcium dynamics. In the K-177 cells, all but two of the compounds showed more potency in the membrane potential assay as compared to calcium dynamics. While most were only modestly more potent (2- to 3-fold), four appear to be significantly more potent (6- to 14-fold). In SH-SY5Y cells, where only membrane potential and ^{22}Na influx data were obtained, nearly all compounds had similar potencies, with the single exception of *N*-methylhomoepiboxidine (**4**), which appeared significantly (5-fold) more potent in the membrane potential assay. In the calcium and membrane potential assays, desensitization/blockade data for the compounds was also obtained by subsequent addition of nicotine as a reference agonist.[40] Although inhibition data for agonists should be viewed with caution, none of the compounds presented herein were found to be more potent as antagonists than as agonists as might be expected from weak partial agonists.

4. Summary

The present study introduces one more class of potent azabicyclic nicotinic agonists, as documented by both binding affinity and functional activity at neuromuscular, neuronal and ganglionic-type receptors. The results clearly indicate that alterations in the structure of nicotinic agonists, such as epibatidine, can have diverse effects on affinity and functional activity at various subtypes of nicotinic receptors and that the full

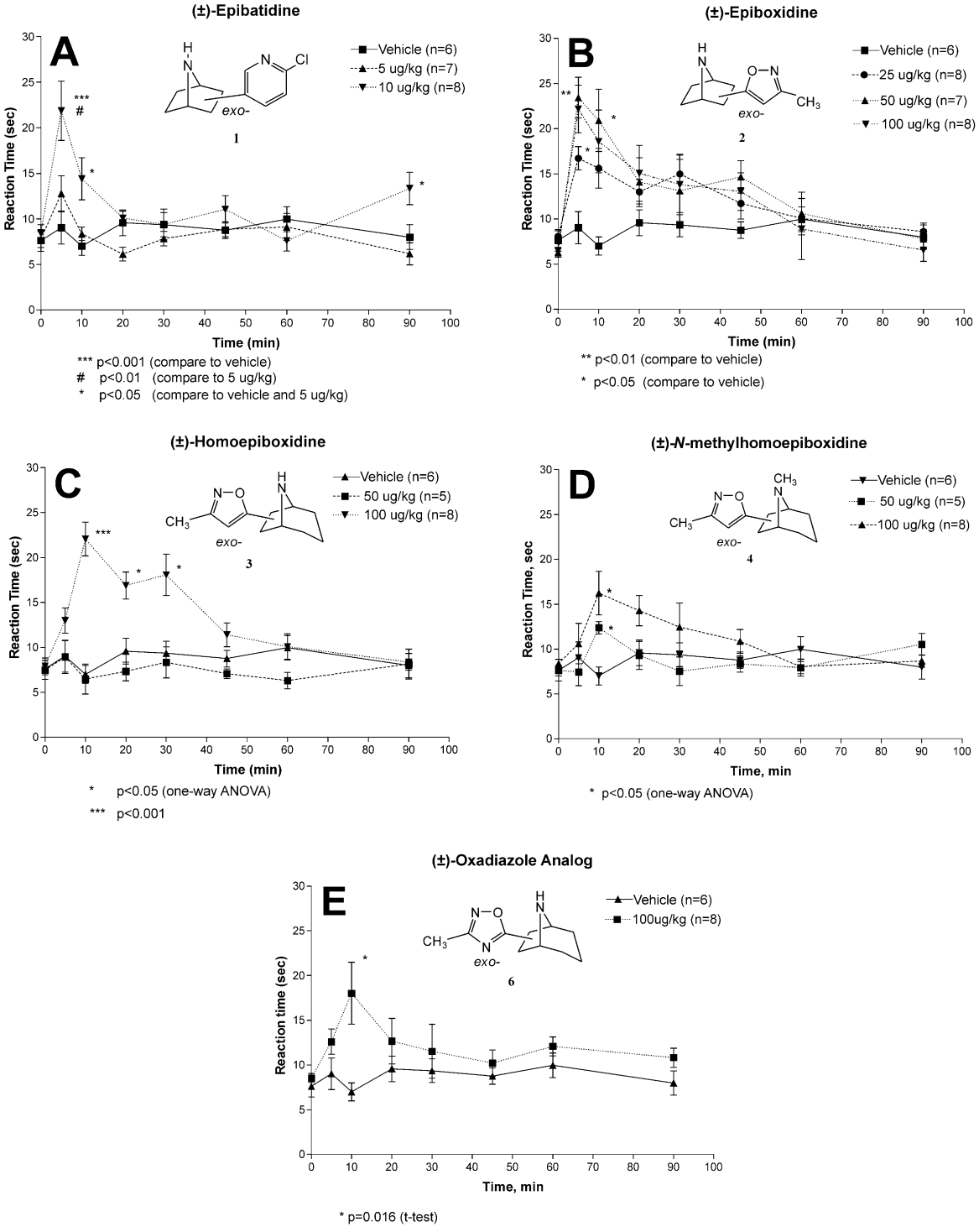


Figure 2. Analgesia studies. Analgetic activity was determined using the hot-plate assay as described in the Experimental. Error bars represent the SEM of 5–9 mice. Significance levels of data are shown below graphs.

potential for developing selective, even specific, nicotinic agonists for such receptors can only be realized through detailed studies of structure–activity correlations at the many often co-existing nicotinic subtypes. The homoepiboxidines were less potent than epibatidine as analgetics, and also were less toxic. Thus, little dissociation of analgetic and toxic properties occurred with these analogues.

5. Experimental

5.1. Chemistry

Melting points were measured with a Thomas-Hoover capillary melting point apparatus and are uncorrected. ^{1}H NMR were recorded on a Varian Gemini XL-300 MHz spectrometer. Chemical shifts are expressed as δ ppm relative to TMS, referenced to the appropriate

residual proton signal of the deuterated solvent used. Chemical ionization (CI) mass spectra were recorded on a Finnigan-1015D mass spectrometer. Elemental analyses were performed by Galbraith Laboratories Inc., Knoxville, TN, USA. Unless otherwise indicated all column chromatographic separations were carried out on silica gel (E. Merck 7734, 230–400 mesh). All reactions involving nonaqueous solutions were performed under an inert atmosphere and with anhydrous solvents unless otherwise noted. Acetamide oxime was prepared essentially as described previously.[44] All new compounds are racemates.

5.1.1. 6β-(3-Methylisoxazol-5-yl)-8-azabicyclo[3.2.1]octane (3). To a solution of compound **15** (69 mg, 0.24 mmol) in CH_2Cl_2 (0.5 mL) at 0 °C was added trifluoroacetic acid (0.5 mL). After stirring for 1 h at 0 °C, H_2O (4 mL), was added, then neutralized with $NaHCO_3$, and extracted with CH_2Cl_2 (4×5 mL). The solvent was removed by rotary evaporation, and the residue was flash chromatographed (9:1 CH_2Cl_2/MeOH) to give **3** (44 mg, 95%) as a colorless oil. It was converted to an oxalate. **3** (oxalate): mp 141–142 °C. **3**. Free base: CI-MS *m*/*z* 193 (MH^+); ^1H NMR ($CDCl_3$) δ 5.86 (s, 1H, 4′-CH), 3.70 (t, 1H, *J* = 3.9, 3.0 Hz, 1-CH), 3.56 (br.s, 1H, 5-CH), 3.38 (dd, 1H, *J* = 9.2, 5.4 Hz, 6α-CH), 2.34 (br.s, 1H, NH), 2.26 (s, 3H, CH_3), 2.05–1.49 (m, 8H, 2,3,4,7-CH_2). Anal. calcd for ($C_{11}H_{16}N_2O \cdot C_2H_2O_4$): C, 55.31; H, 6.43; N, 9.92. Found: C, 55.27; H, 6.40; N, 9.94.

5.1.2. 8-Methyl-6β-(3-methylisoxazol-5-yl)-8-azabicyclo[3.2.1]octane (4). Compound **3** (21 mg, 0.11 mmol) was refluxed with CH_2O (37%, 0.5 mL) and HCO_2H (89%, 0.5 mL) for 1 h. After cooled to room temperature, H_2O (3 mL) was added, then neutralized with $NaHCO_3$, and extracted with CH_2Cl_2 (3×5 mL). The solvent was removed by rotary evaporation, and the residue was flash chromatographed (9:1 CH_2Cl_2/MeOH) to give **4** (22 mg, 98%) as a colorless oil. **4**: CI-MS *m*/*z* 193 (MH^+); ^1H NMR ($CDCl_3$) δ 5.87 (s, 1H, 4′-CH), 3.45 (br.s., 1H, 1-CH), 3.32 (br.s, 1H, 5-CH), 3.31 (dd, 1H, *J* = 9.3, 5.4 Hz, 6α-CH), 2.36 (s, 3H, 3′-CH_3), 2.35–2.25 (m, 1H, 7β-CH), 2.25 (s, 3H, N–CH_3), 2.17 (dd, 1H, *J* = 12.7, 9.8 Hz, 7α-CH), 1.65–1.42 (m, 6H, 2,3,4-CH_2). Anal. calcd for ($C_{16}H_{24}N_2O_3$): C, 65.72; H, 8.27; N, 9.58. Found: C, 65.80; H, 8.25; N, 9.51.

5.1.3. 8-Benzyl-6β-(3-methylisoxazol-5-yl)-8-azabicyclo[3.2.1]octane (5). To a solution of *n*-BuLi (4.0 mL, 1.6 M in hexane) under argon at 0 °C was added dropwise in 10 min a solution of acetone oxime (215 mmol, 2.94 mmol) in THF (2.0 mL). After stirring for 2 h at 0 °C, a solution of compound **10** (380 mg, 1.47 mmol) in THF (2 mL) was added over 10 min. After stirring at 0 °C for 20 h, the reaction mixture was slowly transferred into a vigorously stirred solution of 10% HCl (50 mL, cooled to 0 °C) over 40 min. The layers were separated and the aqueous layer was washed with ether (20 mL), then neutralized with $NaHCO_3$, and extracted with CH_2Cl_2 (2×20 mL). The solvent was removed by rotary evaporation, and the residue was flash chromatographed (20:1 CH_2Cl_2/MeOH) to give **5** (164 mg, 40%), the intermediate β-keto oxime **11** (40 mg, 9%), and dimethylpyridyl-*N*-oxide **12** (30 mg, 6%) as colorless oils. Ketooxime **11** could be converted into **5** according to following procedure. Ketooxime **11** (24 mg, 0.08 mmol) was dissolved in CH_2Cl_2 (1 mL) and cooled to 0 °C. Under argon, Et_3N (14.5 μL, 10.5 mg, 0.10 mmol) was added followed by MsCl (7.4 μL, 11.0 mg, 0.10 mmol). The mixture was stirred at room temperature for 18 h, then poured into H_2O (5 mL), and extracted with CH_2Cl_2 (2×5 mL). After removal of solvent by rotary evaporation, the residue was flash chromatographed (20:1 CH_2Cl_2/MeOH) to give **5** (16 mg, 68%). 5: CI-MS *m*/*z* 283 (MH^+); ^1H NMR ($CDCl_3$) δ 7.33–7.20 (m, 5H, Ph), 5.85 (s, 1H, 4′-CH), 3.74 (s, 2H, $PhCH_2$), 3.40 (br.s, 1H, 1-CH), 3.33 (dd, 2H, *J* = 8.8, 5.9 Hz, 6α-CH, 5-CH), 2.27 (s, 3H, CH_3), 2.28–1.26 (m, 8H, 2,3,4,7-CH_2): **11**: CI-MS *m*/*z* 301 (MH^+); ^1H NMR ($CDCl_3$) δ 7.34–7.23 (m, 5H, Ph), 3.98 (s, 2H, $PhCH_2$), 3.67 (br.s, 1H, 1-CH), 3.21 (br.s, 1H, 5-CH), 2.78 (d, 2H, *J* = 0.9 Hz, COCH_2CNOH), 2.38 (dd, 1H, *J* = 11.6, 5.7 Hz, 6α-CH), 1.99 (s, 3H, CH_3), 2.09–1.16 (m, 9H, OH, 2,3,4,7-CH_2): 12: CI-MS *m*/*z* 323 (MH^+); ^1H NMR ($CDCl_3$) δ 7.38–7.23 (m, 5H, Ph), 7.20 (s, 2H, 3′,5′-CH), 3.95 (s, 2H, $PhCH_2$), 3.41 (br.d, 1H, *J* = 5.8 Hz, 1-CH), 3.03–2.99 (m, 2H, 6α-CH, 5-CH), 2.49 (s, 6H, 2×CH_3), 2.24–1.16 (m, 8H, 2,3,4,7-CH_2).

5.1.4. 6β-(5-Methyl-1,3,4-oxadiazol-5-yl)-8-azabicyclo[3.2.1]octane (6). In a similar way to the preparation of **3**, compound **6** was obtained from **16** as a colorless oil (yield 93%). It was converted to oxalate. **6**·Oxalate: mp 163–165 °C. Free base: CI-MS *m*/*z* 194 (MH^+); ^1H NMR ($CDCl_3$) δ 3.73 (br.s, 1H, 1-CH), 3.64 (br.s, 1H, 5-CH), 3.50 (dd, 1H, *J* = 8.8, 5.9 Hz, 6α-CH), 2.37 (s, 3H, CH_3), 2.30-2.16 (m, 2H, 7-CH_2), 2.01 (br.s, 1H, NH), 1.77–1.26 (m, 6H, 2,3,4-CH_2). Anal. calcd for ($C_{10}H_{15}N_3O \cdot C_2H_2O_4 0.2H_2O$): C, 49.92; H, 6.15; N, 14.55. Found: C, 50.02; H, 6.10; N, 14.26.

5.1.5. 8-Methyl-6β-(5-methyl-1,3,4-oxadiazol-5-yl)-8-azabicyclo[3.2.1]octane (7). In a similar way to the preparation of **4**, compound **7** was obtained from **6** as a colorless oil, yield 95%. **7**: CI-MS *m*/*z* 208 (MH^+); ^1H NMR ($CDCl_3$) δ 3.64 (br.s, 1H, 1-CH), 3.39 (dd, 1H, *J* = 8.8, 5.9 Hz, 6α-CH), 3.36 (br.s, 1H, 5-CH), 2.72–2.51 (m, 1H, 7β-CH), 2.37 (s, 3H, 5′CH_3), 2.32 (s, 3H, N–CH_3), 2.18 (dd, 1H, *J* = 13.7, 9.8 Hz, 7α-CH), 1.96–1.19 (m, 6H, 2,3,4-CH_2).

5.1.6. 8-Benzyl-6β-carbomethoxy-2,2-(1′,2′-ethylenedithiano)-8-azabicyclo[3.2.1]octane (9). *N*-Benzyl-2-oxo-6β-carbomethoxynortropane **8**[21] (1.1 g, 4 mmol) was dissolved in ethanedithiol (5 mL) and $BF_3 \cdot OEt_2$ (3 mL) was added dropwise with stirring. The mixture was stirred 24 h at room temperature under N_2, then poured into 1 N HCl (30 mL) and extracted with ether (2×30 mL). The aqueous layer was neutralized with saturated bicarbonate and extracted with ether (2×30 mL). The ether extract was washed with water, dried over sodium sulfate and evaporated to give a waxy solid consisting of three closely running spots. The mixture was purified by preparative thin layer chromatography (pentane/ether 4:1) to give **9** (629 mg, 45%) as colorless crystals, mp

98–100 °C. CI-MS *m/z* 350 (MH^+); EI-MS 349 (M+), 245, 230, 218 (100), 186, 170, 158, 91; ^{1}H NMR ($CDCl_3$) δ 7.42-7.26 (m, 5H, Ph), 3.73 (s, 3H, OCH_3), 3.39 (s, 2H, PhCH_2), 3.50 (d, 2H, 1,5 CH), 3.35-3.08 (m, 4H, –SCH_2CH_2S–), 2.84 (dd, J = 9.2, 6.0 Hz, 1H, 6α CH), 2.74 (p, 1H, 7β CH), 2.14–1.21 (m, 5H, 7α CH, 3, 4 CH_2). Anal. calcd for ($C_{18}H_{23}NO_2S_2$): C, 61.85; H, 6.63; N, 4.01. Found: C, 61.75; H, 6.62; N, 3.95.

5.1.7. 8-Benzyl-6β-carbomethoxy-8-azabicyclo[3.2.1]octane (10). Dithiolane **9** (238 mg, 0.68 mmol) was dissolved in THF (20 mL) and Raney nickel (3 g) was added. The mixture was refluxed under H_2 (1 atm) for 1 h. Filtration and removal of solvent afforded 128 mg (73%) of **10** as a colorless oil. CI-MS *m/z* 260 (MH^+); ^{1}H NMR ($CDCl_3$) δ 7.38–7.20 (m, 5H, Ph), 3.71 (s, 3H, OCH_3), 3.63 (s, 2H, PhCH_2), 3.56 (s, H, 1 CH), 3.32 (br-s, 2H, 5 CH), 2.87 (dd, J = 9.4, 5.8 Hz, 1H, 6α CH), 2.57 (p, 1H, 7β CH), 1.91–1.33 (m, 7H, 7α CH, 2, 3, 4 CH_2).

5.1.8. 8-(*tert*-Butoxycarbonyl)-6β-carbomethoxy-8-azabicyclo[3.2.1]octane (14). 6β-Carbomethoxynortropane **13**[21] (76 mg, 0.36 mmol) was dissolved in THF (1 mL) and H_2O (3 mL), and stirred with Boc_2O (119 mg, 0.55 mmol) and $NaHCO_3$ (100 mg, 1.2 mmol) at room temperature overnight, then extracted with ether (2×5 mL). The solvent was removed by rotary evaporation, and the residue was flash chromatographed (40:1 CH_2Cl_2/MeOH) to give **14** (67 mg, 69%) as a colorless oil. **14**: CI-MS *m/z* 270 (MH^+); ^{1}H NMR ($CDCl_3$) δ 5.30–4.30 (m, 2H, 1,5-CH), 3.69 (s, 3H, OCH_3), 2.85 (dd, 1H, J = 9.3, 4.9 Hz, 6α-CH), 2.42–2.32 (m, 1H, 7β-CH), 2.39–1.55 (m, 7H, 7α-CH, 3,4,5-CH_2), 1.46 (s, 9H, $C(CH_3)_3$).

5.1.9. 8-(*tert*-Butoxycarbonyl)-6β-(3-methylisoxazol-5-yl)-8-azabicyclo[3.2.1]octane (15). To a solution of acetone oxime (110 mg, 1.50 mmol) in THF (1.0 mL) under argon at 0 °C was added dropwise a solution of BuLi (2.0 mL, 1.6 M in hexane) over 10 min. After stirring for 2 h at 0 °C, a solution of compound **14** (201 mg, 0.75 mmol) in THF (1 mL) was added slowly during 10 min. After stirring at 0 °C for 20 h, the reaction mixture was slowly transferred into a vigorously stirred solution of 10% HCl (25 mL, cooled to 0 °C) over 40 min, then neutralized with $NaHCO_3$, and extracted with CH_2Cl_2 (3×20 mL). The solvent was removed by rotary evaporation, and the residue was flash chromatographed (40:1 CH_2Cl_2/MeOH) to give **15** (93 mg, 42%) as colorless crystals. **15**: mp 95–97 °C; CI-MS *m/z* 293 (MH^+); ^{1}H NMR ($CDCl_3$) (two rotamers, 1:1) δ 5.87, 5.81 (s, 1H, 4′-CH), 4.46–4.26 (m, 2H, 1,5-CH), 3.36 (dd, 1H, J = 9.2, 4.8 Hz, 6α-CH), 2.25, 2.24 (s, 3H, CH_3), 2.19–1.51 (m, 8H, 2,3,4,7-CH_2), 1.48, 1.42 (s, 9H, $C(CH_3)_3$).

5.1.10. 8-(*tert*-Butoxycarbonyl)-6β-(5-methyl-1,3,4-oxadiazol-5-yl)-8-azabicyclo[3.2.1]octane (16). Acetamide oxime[44] (44 mg, 0.60 mmol) suspended in THF (1.5 mL) under argon was heated at 60 °C for 1 h with NaH (28 mg, 0.70 mmol, 60% dispersion in oil) and 4 Å molecular sieves (4 g). Then compound **14** (67 mg, 0.25 mmol) in THF (1.5 mL) was added, and the mixture was refluxed with stirring for 1.5 h. After cooling, the reaction mixture was filtered through Celite 535, and the solvent was removed in vacuum, then added H_2O (2 mL), and extracted with CH_2Cl_2 (2×5 mL). The solvent was removed by rotary evaporation, and the residue was flash chromatographed (40:1 CH_2Cl_2/MeOH) to give **16** (59 mg, 80%) as a colorless oil. **16**: CI-MS *m/z* 294 (MH^+); ^{1}H NMR ($CDCl_3$) δ 4.50 (br.s, 1H, 1-CH), 4.46 (br.s, 1H, 5-CH), 3.47 (dd, 1H, J = 9.8, 4.9 Hz, 6α-CH), 2.36 (s, 3H, CH_3), 2.21–1.51 (m, 8H, 2,3,4,7-CH_2), 1.49, 1.46 (s, two rotamers, 9H, $C(CH_3)_3$).

5.2. In vitro studies

5.2.1. General. Enantiomeric *N*-methylepibatidines were prepared from the corresponding enantiomers of epibatidine using paraformaldehyde/formic acid as previously described[42] (±)-Epibatidine dihydrochloride, *S*-nicotine ditartrate, and (±)-epiboxidine hydrochloride were obtained from Sigma-RBI.

5.2.2. Cultured cells. The following cell lines were used for preparation of membranes for nicotinic binding assays and/or for functional ion flux assays: Rat pheochromocytoma PC12 cells were provided by the late Dr Gordon Guroff (NIH, Bethesda, MD, USA). Human rhabdomyosarcoma TE-671 cells, human neuroblastoma IMR-32 cells and a human neuroblastoma SH-SY5Y cells, were all from the American Type Culture Collection (Rockville, MD, USA). Human embryonic kidney (HEK-293) cells stably transfected with human α4 and β2 nicotinic subunits (K-177 cell line)[22] were provided by Abbott Laboratories (Abbott Park, IL, USA) and HEK 293 cells stably transfected with rat α3 and β4 nicotinic subunits (KXα3β4R2 cell line)[23] were provided by Dr. Kenneth J. Kellar (Georgetown University, Washington, DC, USA). Cell culture conditions have been described.[13,22,23,26]

5.2.3. Radioligand binding assays. Rat brains were obtained from Pel Freez Biological (Rogers, AK, USA) and cerebral cortical tissue was homogenized and membranes prepared as described.[13] Cultured cells expressing nicotinic receptors were harvested and homogenized and cell membranes were prepared essentially as described.[20] Membrane aliquots were stored at −70 °C until use. Inhibition of binding of 0.2 nM [^{3}H]nicotine to rat cerebral cortical membranes was essentially as described.[2] S-Nicotine ditartrate (1 μM) was used to determine nonspecific binding and a Brandel M24R cell harvester with Whatman GF/B filters presoaked for 30 min in 0.3% polyethylenimine was used to isolate membranes for scintillation counting. Inhibition of binding of 0.5 nM [^{3}H]epibatidine to membranes from cell lines was performed by incubation of cell membranes prepared as described for KXα3β4R2 cells.[25]

5.2.4. ^{22}Na influx assays. Stimulation of sodium-22 influx in PC-12, TE-671, and K-177 cells was assayed after 2 min at room temperature essentially as described[2] with agonist and ^{22}NaCl in the influx buffer. Stimulation of sodium-22 influx in KXα3β4R2 and TE-671

cells was assayed in a 96-well format. Briefly, cells were seeded into 96-well plates coated with poly-D-lysine and grown to near confluence, then removed from the incubator and allowed to reach room temperature over ten min. Each stage of the assay was conducted row wise using a 12-channel pipettor each subsequent row being assayed at 20-s intervals and each subsequent plate assayed at 9-min intervals. Media was removed by *gentle* aspiration, replaced with 30 μL Hank's Balanced Salt Solution, supplemented with 20 mM 4-hydroxyethylpiperazine-1-sulfonic acid (HBSS/HEPES, adjusted to pH 7.4) and preincubated 10 min. The buffer was then exchanged with 30 μL of otherwise identical buffer containing the compound to be evaluated, 8.3 μCi/mL (0.25 μCi/well) of ^{22}NaCl, and 5 mM ouabain (to inhibit Na/K-ATPase). After allowing influx for three min, the buffer was removed and cells were *gently* washed three times with ~300 μL HBSS/HEPES by means of a Cornwall syringe equipped with a 12-channel manifold. The upper wall of each well was then cleaned of traces of isotope by means of a moistened cotton-tipped applicator and the entire top surface of the plate was wiped with a moistened paper towel and dried. The plate was then exposed to a phosphorimaging screen (Fuji BAS IP-MS 2040, 20×40 cm) for 18–24 h. Screens were then read using a Fuji FL-3000IR phosphorimager. Influx was calculated from image density for each well normalized to a standard 96-well plate containing fixed dpm ^{22}Na from the same lot. While this adaptation of the assay is new, comparisons with the six-well assay using TE-671 were favorable (Table 2).

5.2.5. Calcium fluorescence measurements. The method used for analysis was an adaptation of the method developed by investigators at Sibia/Merck for fluo-3 calcium measurements.[33] Cell cultures in 96-well plates were removed from the incubator, allowed to reach room temperature over the course of 10 min, and washed twice with 100 μL of HBSS/HEPES. Subsequently, the medium was replaced with 30 μL of otherwise identical buffer, containing 15 μM Fluo-4 AM (Molecular Probes, Eugene, OR, USA, prepared as described in the package literature) and the cells were loaded for 1 h in the dark. Subsequently, the cells were again washed to remove extracellular dye. Buffer exchange was performed using a multichannel pipettor, rather than a vacuum manifold as use of the latter led to stimulation of anomalous calcium responses and unreliable readings. Stimulated calcium fluorescence was then measured using a Cytofluor 4000 fluorescence plate reader (Applied Biosystems) to which had been attached a holder arm, allowing a multichannel pipettor to be positioned above the wells, thus allowing addition of reagents while reading. Excitation and emission wavelengths were set to 485 and 525 nm, respectively. Measurements were made at ~6-s intervals. Basal fluorescence was measured for 15–30 s (3–5 scans), followed by addition of 30 μL of a 2× solution of test compound (to assess agonist activity), and measurement of fluorescence for 90–180 s (15–30 scans). Subsequently, 30 μL of a 3× solution of nicotine di-D-tartrate was added to afford a final concentration of 100 μM and fluorescence was measured for 30–60 s (5–10 scans). Finally, 30 μL of a 4× calibrant solution was added to afford a final concentration of 5 μM ionomycin, 100 μM carbamylcholine chloride, and 20 μM FCCP and the resultant fluorescence was measured for 30–60 s (5–10 scans) to maximize signal and correct for dye loading and cell count. Addition of compounds was facilitated by interruption of the scan sequence by computer control, followed by addition of reagents with simultaneous restarting of the scan sequence. Such interruptions did not cause spontaneous variation in fluorescence as measured by addition of blank buffer or restarts without addition of reagents. Following data collection, responses were calculated as follows:

$$\text{Compound response} = (F_{Max}(\text{Test compound}) - F_{\text{Basal}})/(F_{\text{Max}}(\text{Calibrant}) - F_{\text{Basal}})$$

$$\text{Nicotine response} = (F_{\text{Max}}(\text{Nicotine}) - F_{\text{Basal}})/(F_{\text{Max}}(\text{Calibrant}) - F_{\text{Basal}})$$

Absolute calcium concentrations could be determined by subsequent treatment with 10 mM $MnCl_2$ to quench fluorescence and calcium concentration calculated by the method of Grinkiewicz et al.[45] However, in most cases this was not determined, as calcium concentrations tend to be unreliable with intensity dyes and such measurements were unnecessary for determination of pharmacological parameters.

Calcium measurements were also made using a proprietary no-wash calcium dye on a Flexstation fluorescence plate reader (Molecular Devices, Sunnyvale, CA, USA). Results were nearly identical to those obtained using Fluo-4 and the Cytofluor plate reader (data not shown).

5.2.6. Membrane potential fluorescence measurements. Membrane potential studies were conducted essentially as previously described[34] and in very similar fashion to the calcium fluorescence assay. Cells were equilibrated and washed as before, followed by loading for 45 min with 30 μL of Membrane Potential Dye solution (Molecular Devices). Fluorescence measurements were read on the Flexstation fluorescence plate reader. Excitation and emission wavelengths were set to 535 and 560 nm, respectively, and with a cutoff of 550 nm. Readings were taken at ~1-s intervals. Basal fluorescence was measured for 15 s, followed by addition of the test compound and measurement for an additional 105 s. Nicotine was added as before and the fluorescence was measured for 40 s. Finally, the ionomycin/carbachol/FCCP calibrant solution was replaced by a 4× calibrant solution containing 160 mM KCl. Responses were calculated as before with KCl serving as the calibrant. The baseline for nicotine responses was taken as that immediately prior to the nicotine addition.

5.3. In vivo studies

5.3.1. Analgesia activity in mice. Sensitivity to painful stimuli was assessed with male NIH Swiss strain mice (25–30 g) using a hot plate assay essentially as previously described.[2,41] Briefly, agents were dissolved in 20:80 (v:v) mixture of Emulphor EL-60 (Rhone Poulenc, Cranbury, NJ, USA) and 0.9% saline solution and injected intraperitoneally in a volume corresponding to 5 mL/kg body weight. The mouse was then placed on a 55 °C hot plate (Columbus Instruments, Columbus, OH, USA) and latency time to the first hind-paw response (licking or shaking) recorded. Latency times were determined at 5, 10, 20, 30, 45, 60, and 90 min post injection.

5.3.2. Data analysis. Pharmacological data were fitted using Prism (V. 3.0, Graph Pad Software, San Diego, CA, USA). Dose-response data from ion flux and calcium fluorescence experiments were normalized to the maximum response of (±)-epibatidine. In cases where bell-shaped curves were observed, data points on the ascending side were used for determination of potency and efficacy.

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