Nicotinic Acetylcholine Receptor Efficacy and Pharmacological Properties of 3-(Substituted phenyl)-2β-substituted Tropanes

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Abstract

There is a need for different and better aids to tobacco product use cessation. Useful smoking cessation aids, bupropion (**2**) and varenicline (**3**), share some chemical features with 3-phenyltropanes (**4**), which have promise in cocaine dependence therapy. Here we report studies to generate and characterize pharmacodynamic features of 3-phenyltropane analogues. These studies extend our work on the multiple molecular target model for aids to smoking cessation. We identified several new 3-phenyltropane analogues that are superior to **2** in inhibition of dopamine, norepinephrine, and sometimes serotonin reuptake. All of these ligands also act as inhibitors of nicotinic acetylcholine receptor (nAChR) function with a selectivity profile that favors, like **2**, inhibition of α3β4*-nAChR. Many of these ligands also block acute effects of nicotine-induced antinociception, locomotor activity, and hypothermia. Importantly, all except one of the analogues tested have better potencies in inhibition of nicotine conditioned place preference than **2**. We have identified new compounds that have utility as research tools and possible promise for treatment of nicotine dependence.

Keywords

Introduction

Tobacco product use, principally through cigarette smoking, is the greatest preventable cause of premature mortality, contributing in the United States to over 435,000 deaths annually.[1] Tobacco use cessation can halt and reverse the biological damage caused by smoking.[2] It is now commonly accepted that smoking behavior is maintained by the reinforcing effects of nicotine (**1**) and aversive effects of nicotine withdrawal.[3–6] Both nonpharmacological and pharmacological interventions have demonstrated efficacy in smoking cessation.[7] At present, first-line pharmaceutical treatments include nicotine

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replacement therapy (NRT),[8] bupropion (**2**) and varenicline (**3**).[9,10] While these treatments are useful in helping 5–20% of smokers abstain long-term, new pharmacotherapies are needed that are either more effective or can impact those individuals not helped by existing treatments.

The effectiveness of varenicline in smoking cessation is thought to be due to its action as a partial agonist at α4β2-containing nicotinic acetylcholine receptors (nAChR).[9,10] The mechanism for bupropion's effectiveness as a smoking-cessation aid appears to be more multi-faceted.[11] Its behavioral and neurophysiological effects resemble those of psychomotor stimulants,[12] and similar to other psychomotor stimulants, **2** inhibits the reuptake of dopamine (DA).[13,14] It also inhibits the reuptake of norepinephrine (NE)[13,14] and is a noncompetitive inhibitor of α3β4*-(where the * indicates that nAChR subunits are known or possible assembly partners in addition to those indicated) and α4β2-nAChR.[14] In animal behavioral pharmacology studies, **2** induced locomotor activity,[15,16] generalized to cocaine and amphetamine in drug discrimination studies,[17,18] produced conditioned place preference (CPP),[19] and was self-administered by both rats[20] and nonhuman primates.[21]

Over the last several years, we have synthesized a large number of 3-phenyltropane analogues and evaluated them for binding at monoamine transporters.[22,23] Similar to **2**, some analogues were better at inhibiting the dopamine (DA) transporter (DAT) and the norepinephrine (NE) transporter (NET) than the serotonin (5HT) transporter (SERT). Others were selective for DAT relative to NET and SERT, whereas others showed similar inhibition at all three transporters.[22,23] In 1995, Lerner-Marmarosh et al. reported that a number of 3-phenyltropane analogues were effective in blocking nicotine-induced seizures in mice and that a good correlation was observed between pharmacological potencies and abilities to block [^{3}H]mecamylamine binding to brain membranes.[24] Based on these results, Lerner-Marmorosh et al. concluded that the 3-phenyltropane analogues are neuronal nicotinic antagonists acting on a similar site to that of mecamylamine, a noncompetitive nicotinic antagonist. In another study, we reported that several 3-phenyltropane analogues blocked nicotine-induced antinociception in the tail-flick test in mice with potencies greater than that of **2**.[25] These intriguing results suggested that an additional pharmacological study of the 3-phenyltropane class of monoamine uptake inhibitor might provide information about the mechanism of action of noncompetitive nicotinic antagonists like **2** and could provide lead compounds for development as aids to smoking cessation or as drugs for treatment of neurological or psychiatric disorders involving nicotinic mechanisms.

In this study, we report the synthesis and biological evaluation of 3-phenyltropane analogues **4a–4l**. All analogues show inhibitory potency at human DAT and NET and functional antagonism of human α3β4*-nAChR. Similar to **2**, the compounds antagonize the antinociceptive, hyperlocomotor, and/or hypothermic effects induced by an acute injection of nicotine in mice and blocked nicotine CPP after repeated injection.

Chemistry

The 3-phenyltropane analogues **4a–e**, **4g**, and **4l** were synthesized as previously reported.[26–29] Scheme 1 outlines the synthetic route used to prepare 3β-(4-chlorophenyl)-2β-(4′,5′-dimethylbenzimidazol-2′-yl)tropane (**4f**). 3β-(4-Chlorophenyl)tropane-2β-carboxylic acid (**5**)[28] is converted to the acid chloride and then treated with 4,5-dimethyl-1,2-phenylenediamine to give amide **6**. Treatment of **6** with phosphorus oxychloride gave the desired **4f**. The nortropane analogue **4h** was prepared from **4c** as shown in Scheme 2. Treatment of **4c** in 1,2-dichloroethane containing excess 1-chloroethyl chloroformate (ACE-Cl) followed by refluxing the intermediate urethane in methanol gave **4h**.

The synthesis of the 3β-(4-chloro-3-methylphenyl)-2β-(3′-substituted isoxazol-5′-yl)tropanes (**4i** and **4j**) is also outlined in Scheme 2. A solution of **4c** was added to the dilithium salt of the appropriate ketone oxime in tetrahydrofuran (THF) at 0 °C under nitrogen, and the reaction mixture was allowed to warm to 25 °C. After a few hours, the reaction mixture was added to a THF solution containing sulfuric acid and refluxed for 1 h to give the desired products **4i** and **4j**.

The synthesis of the 3α,2β-tropane **4k** is outlined in Scheme 3. Addition of a solution of (1*R*,5*S*)-2-(3′-methyl-1′,2′,4′-oxadiazol-5′-yl)-8-methyl-8-azabicyclo[3.2.1]oct-2-ene (**7**)[30] in anhydrous THF at −78 °C to a solution of the 3-chloro-4-methylphenyllithium (prepared from the appropriate aryl bromide and butyl lithium) followed by quenching with 1 N hydrochloric acid at −78 °C formed the 3α-(substituted phenyl)tropane-2α-(3′-methyl-1′,2′, 4′-oxadiazol-5′-yl)tropanes (**8**). In addition to the desired isomer, the 2α,3β-isomer was also formed, which was removed by flash chromatography or carried through and removed at the next reaction. Transformation of oxadiazole **8** to the desired methyl ester **4k** was accomplished by reduction with nickel boride (generated *in situ* by reaction of sodium borohydride and nickel tetraacetate) and hydrochloric acid in refluxing methanol. Under such conditions, a complete epimerization of C-2 to form the 3α,2β-stereoisomer was observed.

***In Vitro* Assays**—The 3-phenyltropane analogues **4a–4l** were evaluated for their ability to block reuptake of [^{3}H]dopamine ([^{3}H]DA), [^{3}H]serotonin ([^{3}H]SERT), and [^{3}H]norepinephrine ([^{3}H]NE) using human (h) DAT, hSERT, and hNET stably expressed in HEK293 cells using conditions similar to those previously reported.[31],[32] The results are given in Table 1.

The 3-phenyltropane analogues **4a–4l** were also evaluated for their ability to antagonize functional responses of α3β4*-, α4β2-, α4β4-, and α1*-nAChR and mechanisms involved for functional inhibition using previously reported methods.[33] Results are given in Table 1 and in Figures 1–2.[32]

In Vivo Assays

Acute nicotine testing—The 3-phenyltropane analogues **4a–4l** were also evaluated for their ability to antagonize behavioral responses to acute nicotine administration as previously described.[32] Results are given in Table 2.

Nicotine reward using the CPP test—Selected 3-phenyltropane analogues were also evaluated for their ability to antagonize the development of nicotine-induced CPP in mice using an unbiased paradigm.[34] Results are given in Table 2.

Results

Effects on Monoamine Uptake

Compound **2** is a relatively weak monoamine uptake inhibitor. In contrast, all of the 3-phenyltropane analogues studied here have higher inhibitory potencies for DA uptake inhibition. 3β-(4-Methylphenyl)tropane-2β-carboxylic acid isopropyl ester (**4a**) and 3β-(4-methylphenyl)-2β-(3′-ethylisoxoazol-5′-yl)tropane (**4c**), with IC$_{50}$ values of 0.83 and 0.75 nM, respectively, were the most potent analogues as inhibitors of DA uptake. However, 3β-(4-chloro-3-methylphenyl)-2β-(3′-methylisoxazol-5′-yl)tropane (**4i**), 3β-(4-chlorophenyl-3-methyl)tropane-2β-carboxylic acid methyl ester (**4g**), and its nortropane analogue (**4h**), with IC$_{50}$ values of 1.5, 2.0, and 2.3 nM, are almost as potent at inhibition of DA uptake as **4a** and **4c**. In addition, all of the 3-phenyltropane analogues were potent at NE uptake

inhibitors. The most potent NE uptake inhibitors were the nor-tropane analogues 3α-(4-fluoro-3-methylphenyl)nortropane-2β-carboxylic acid methyl ester (**4l**) and 3β-(4-chloro-3-methylphenyl)nortropane-2β-carboxylic acid methyl ester (**4h**), with an IC_{50} values of 0.5 and 0.9 nM, respectively. 3α-(4-Chloro-3-methylphenyl)tropane-2β-carboxylic acid methyl ester (**4g**), 3β-(4-chloro-3-methylphenyl)-2β-(3′-methylisoxazol-5′-yl)tropane (**4i**), and 3α-(4-chloro-4-methylphenyl)tropane 2β-carboxylic acid methyl ester (**4k**), with IC_{50} values of 1.1, 6.3, and 6.5, respectively, also are very potent NE uptake inhibitors.

Most of the 3-phenyltropane analogues are inactive or have IC_{50} values greater than 100 nM for 5HT uptake inhibition. However, the 4-chloro-3-methyl carboxylic acid methyl ester **4g** and its nortropane analogue **4h** have IC_{50} values of 1 and 6.2 nM for 5HT uptake inhibition. Since these two analogues also have high potency for DA and NE uptake inhibition, they have high potency uptake inhibition at all three monoamine transporters.

Analogue **4g** has a slight (~2-fold) preference for inhibition of 5HT and NE over DA uptake inhibition. Compound **4j** has comparable inhibitory potencies for DA and NE uptake. Ligand **4h** has slight (~2-fold) preference for NE over DA uptake inhibition. Compound **4l** has 14-fold preference for inhibition of NE over DA uptake. Otherwise, all the other compounds have preference for inhibition of DA uptake over other monoamine transporters, as low as 2-fold for **4k** and **4f** to >60-fold for **4a**.

Effects on nAChR Function

The effects of 3-phenyltropane analogues **4a**–**4l** on function of diverse human nAChR subtypes naturally or heterologously expressed by human cell lines were assessed using $^{86}Rb^+$ efflux assays that are specific only for nAChR function in the cells used. None of the analogues has activity as agonists at α1*-, α3β4*-, α4β2-, or α4β4-nAChR, because $^{86}Rb^+$ efflux in the presence of these ligands alone at concentrations from ~5 nM to 100 μM (data not shown here) was indistinguishable from responses in cells exposed only to efflux buffer.

$^{86}Rb^+$ efflux assays also were used to assess whether ligands had activity as antagonists at human nAChR. Representative concentration-response curves for selected ligands (Fig. 1) illustrate nAChR *in vitro* inhibitory profiles (see also Table 1).

Compound **2** has IC_{50} values of 1.8, 12, 15, and 7.9 μM for functional antagonism of α3β4*-, α4β2-, α4β4-, and α1β1*-nAChR, respectively. Analogues **4a**, **4b**, **4c**, **4e**, **4f**, **4i**, and **4j** all have IC_{50} values equal to or lower than that for **2** at α3β4*-nAChR. The most potent inhibitors of α3β4*-nAChR function are **4f** and **4i** (IC_{50} = 0.57 and 0.73 μM, respectively). Similar to **2**, all the 3-phenyltropane analogues show preference for functional inhibition of α3β4*-nAChR over the other nAChR subtypes tested, with greatest overall preference for **4e** (8-, >56-, and 11-fold over α4β2-, α4β4-, or α1*-nAChR), and **4i** (11-, 8-, and 18-fold over α4β2-, α4β4-, or α1*-nAChRs). Only **4f** had significantly better potency as a functional antagonist of α4β2-nAChR than **2**. Analogue **4j** had lowest overall preference for α3β4*-nAChR (~2-fold), in part because it (and **4f**) had the highest inhibitory potencies at α4β4- and α1*-nAChR. Compounds **4f** and **4g** had the lowest preference (~3-fold), and compounds **4i** and **4j** had the highest preference (10–13-fold) for α3β4*- over α4β2-nAChR.

As was the case for **2**, all of the 3-phenyltropane analogues tested inhibited nAChR function via an apparently noncompetitive mechanism, lowering apparent agonist efficacy without altering agonist EC_{50} values [representative data for compounds acting at α3β4*-nAChR are shown (Fig. 2)].

Comparing inhibitory potencies across classes of targets, **4g**, **4h**, and **4l** have 3500–5700-fold selectivity for inhibition of NE uptake over α3β4*-nAChR, and selectivity for inhibition of DA uptake over α3β4*-nAChR ranges from 78–100-fold for **4f** and **4j** to >2000-fold for **4a** and **4c**.

In Vivo Effects

Compound **2** blocks nicotine-induced antinociception in the tail-flick and hot-plate tests with AD_{50} values of 1.2 and 15 mg/kg, respectively. Ten of the 3-phenyltropane analogues were more potent in blocking nicotine's effects in the tail-flick assay than **2**, having AD_{50} values between 0.002 to 0.28 mg/kg. The most potent analogue in the tail-flick test was 3α-(4-fluoro-3-methylphenyl)-2β-carboxylic acid methyl ester (**4l**), AD_{50} = 0.002 mg/kg. Six analogues, **4b**, **4c**, **4g**, **4h**, **4i**, and **4l**, with AD_{50} values of 0.35 to 9.8 mg/kg were more potent than **2** in blocking nicotine's acute effects in the hot-plate test, with **4g** being most potent (AD_{50} = 0.35 mg/kg, ~42-fold more potent than **2**).

Compound **2** blocks nicotine-induced increases in locomotor activity with an AD_{50} value of 4.9 mg/kg. Compounds **4a**, **4b**, **4c**, **4g**, **4h**, **4i**, and **4l** with AD_{50} values of 0.11 to 1.65 mg/kg are more potent than **2**. The most potent analogue was **4g**, which has an AD_{50} value of 0.11 mg/kg.

Nicotine-induced hypothermia is blocked by **2** with an AD_{50} of 9.2 mg/kg. Compounds **4a**, **4b**, **4c**, **4g**, **4h**, **4i**, **4k**, and **4l** with AD_{50} values of 0.14 to 7.4 mg/kg are all more potent than **2**. Compound **4g** and its nortropane analogue **4h** with IC_{50} values of 0.14 and 0.23 mg/kg, respectively, were the two most potent analogues in the hypothermia test.

Pre-treatment with **2** at different doses decreased the development of nicotine-induced CPP in mice conditioned with 0.5 mg/kg nicotine, with an estimated AD_{50} value of 0.35 mg/kg. Nine of the 3-phenyltropane analogues were tested for their ability to block nicotine's preference. Six of the compounds, **4c, 4d, 4i, 4j, 4k and 4l**, with AD_{50} values of 0.013 to 0.15 mg/kg are more potent than **2**.

Discussion

The results from this study show that the 3-phenyltropane analogues have monoamine uptake inhibition and nAChR antagonism profiles similar to **2**. Some of the analogues also have slightly higher potency as antagonists of α3β4*-nAChR, and all analogues retain preference across nAChR subtypes for blockade of α3β4*-nAChR. Moreover, some of these compounds show better potency than **2** as inhibitors of acute effects of nicotine and nicotine-induced CPP, which measures the acute rewarding effect of the drug.

Regardless of the type of substituent at the 2β- and 3β-position, all the 3-phenyltropane analogues (see structures **4a–4l**) had high potency in DA uptake inhibition. In addition all the analogues except the 3β-cyclobutyl ester **4b** and the 3β-4-chlorophenylisoxazole **4d** also had high potency in NE uptake inhibition. From a structural perspective, extensive modifications of the 2β-group (see structures **4a–4l**) lowered analogue potency as 5HT uptake inhibitors more than they altered inhibitory potency as DA and NE uptake inhibitors. Only analogues with smaller 2β-substituents (**4g** and **4h**) retained high efficacy for 5HT uptake inhibition. Based on the limited comparisons, stereochemistry seems to influence activity for 5HT and NE uptake inhibition more than for DA uptake inhibition and α3β4*-nAChR antagonist potency (compare **4g** and **4k**). With the exception of the 3β-4-chlorophenylisoxazole analogue **4d**, all the analogues had similar relative potencies at α3β4*- and α4β2-nAChRs.

It is interesting to note that analogue **4c** has 150-times more potency than **2** as an inhibitor of nicotine's analgesic action in the tail-flick assay, 9-fold better activity in the hot-plate assay, 24-fold better activity in hypolocomotion studies, and a marginally more potent effect on body temperature, whereas it has ~8-fold better activity in the CPP assay. Analogue **4c** does not differ much from **2** in its antagonistic potency at any of the nAChR subtypes studied, and it has 480-fold better activity as a DA uptake inhibitor, ~170-fold better activity as NE uptake inhibitor and no less than 5-fold better activity as a 5HT uptake inhibitor than **2**. Since analogue **4g** ($AD_{50} = 0.25$ mg/kg) has activity comparable to **2** ($AD_{50} = 0.35$ mg/kg) in the CPP assay, is 6-fold more potent than **2** in the tail-flick assay, 40-, 44-, and 65-fold more potent than **2** in the hot-plate locomotor and hypothermia tests and has about the same nAChR inhibition profile as **2** while having ~300/~2000/>10,000-times more potency at DA/NE/5HT uptake inhibition, its CPP effects appears to be more related to antagonism of nAChR. A similar analysis of **4i** and **4j** shows that the 4- to 5-fold better CPP activity relative to **2** correlates better with their nAChR inhibition profile than with their monoamine transporter and acute nicotinic effects. Compound **4d** with an $AD_{50} = 0.013$ mg/kg was the most potent analogue in the CPP test and, thus, was 27 times more potent than **2**. Somewhat surprisingly, unlike **2**, compound **4d** was inactive in the acute mouse tail-flick, hot-plate, and hypothermia tests and less potent than **2** in the hypolocomotor test. However, it retained high antagonist potency against α3β4*-nAChR and in DA uptake inhibition. In previous studies, we found that even though **4d** was a potent and selective inhibitor of the DAT relative to the NET and SERT, it did not show locomotor activity in rats even though in vivo binding studies showed that appreciable brain levels were achieved.[8,26] We also found that **4d** was a positive allosteric modulator of the CB-1 cannabinoid receptor and suggested that its interaction with this receptor might in part be the reason for its unusual behavioral pharmacological properties.[8] However, the reason for this lack of locomotor activity is still not fully understood. One possibility is that **4d** is interacting with some as yet unidentified target, possibly a nAChR subtype other than those studied, that is responsible for its lack of locomotor activity and potent activity in the CPP test.

Although many analogues have much better potency in behavioral assays than **2**, it is challenging to determine how those effects relate to ligand actions at molecular targets, in part because improvements over **2** for a given ligand can vary quite widely depending on the behavioral assay. Correlation plots comparing the in vitro and in vivo data did not provide any useful insights. Some of these challenges may be due to metabolism of analogues to more or less active forms. In addition, **2** in mice is converted in humans to hydroxy metabolites, one of which expresses much of the drug's behavioral activity.[35]

In summary, ligands have been developed that can serve as useful research tools, having different inhibitory potency profiles across nAChR and monoamine transporter targets. For example, 3β-(4-methylphenyl)-2β-(3′-ethylisoxazol-5′-yl)tropane (**4c**) has IC_{50} values of 0.75, 11, and 2600 nM for inhibition of DA, NE, and 5HT uptake, and an IC_{50} of 1.7 μM for antagonism of α3β4*-nAChR. Like **2**, **4c** is active in all four acute tests of nicotine action in mice. Importantly, it is 150-, 9-, and 5-times more potent in the tail-flick, hot-plate, and locomotor tests, respectively, than **2**. It also is eight times more potent than **2** in blocking nicotine-CPP. Three other compounds (**4g**, **4h**, **4i**) have higher potencies than **4c** as inhibitors in all four tests of acute nicotine effects, and these compounds as well as **4d**, **4j**, **4k**, and **4l** also have potency as antagonists of nicotine-CPP significantly higher than that of **2**. Compounds **4d** and **4j** are inactive or are less active than **2** in the acute test of nicotinic action. However, they retain high antagonist potency against α3β4*-nAChR and DA uptake inhibition. The current studies support the idea that nAChR antagonism, particularly inhibition of α3β4*-nAChR function, and inhibitory actions at monoamine transporters are pharmacodynamic features of 3-phenyltropanes. Perhaps most importantly, and certainly

warranting further investigation, is the possible utility of these ligands as aids to smoking cessation, especially given their ability to inhibit acute effects of nicotine and a test of nicotine preference with better potencies than **2**, which is a useful pharmacotherapy for nicotine dependence.

Experimental Section

Nuclear magnetic resonance (^{1}H NMR and ^{13}C NMR) spectra were recorded on a 300 MHz (Bruker AVANCE 300) spectrometer. Chemical shift data for the proton resonances were reported in parts per million (δ) relative to internal $(CH_3)_4Si$ (δ 0.0). Elemental analyses were performed by Atlantic Microlab, Norcross, GA. Purity of compounds (>95%) was established by elemental analysis. Analytical thin-layer chromatography (TLC) was carried out on plates precoated with silica gel GHLF (250 μM thickness). TLC visualization was accomplished with a UV lamp or in an iodine chamber. All moisture-sensitive reactions were performed under a positive pressure of nitrogen maintained by a direct line from a nitrogen source. Anhydrous solvents were purchased from Aldrich Chemical Company or VWR. CMA80 is a mixture of 80% chloroform, 18% methanol, and 2% concentrated ammonium hydroxide.

3β-(4-Chlorophenyl)-2β-(4′,5′-dimethylbenzimidozol-2′-yl)tropane (4f) Dihydrochloride

Compound **6** (3.8 g, 0.0095 mol) and POCl$_3$ (50 mL) were mixed and stirred at reflux for 90 min. The reaction mixture was cooled and added to pet. ether (2 L). Most of the solvent was decanted, and concentrated NH$_4$OH-water (1:1) was added until basic to litmus paper then extracted with CH$_2$Cl$_2$. The organic layer was separated, dried (Na$_2$SO$_4$), and concentrated to yield 3.0 g of a tan amorphous solid. This solid was chromatographed on silica gel, eluting with EtOAc then EtOAc-CMA80 (1:1) to afford 1.7 g (47%) of **4f.** The free base was dissolved in ether and treated with 1 M ethereal HCl to give 1.65 g of the dihydrochloride salt of **4f** as a white solid: mp 224–227 °C; [α]$_D$ −166.2 °C (c 0.69, MeOH). ^{1}H NMR (CDCl$_3$, free base) δ 1.73 (bd, 1H), 1.88 (q, J = 9.0 Hz, 2H), 2.22–2.35 (m, 4H), 2.31(s, 3H), 2.33 (s, 3H), 2.35 (s, 3H), 3.20–3.48 (m, 4H), 6.67 (d, J = 9.0 Hz, 2H), 7.00 (d, J = 9.0 Hz, 2H), 7.26 (s, 2H). Anal. (C$_{23}$H$_{28}$Cl$_3$N$_3$•1.25 H$_2$O) C, H, N.

(−)-N-Nor-3β-(3-methyl-4-chlorophenyl)tropane-2β-carboxylic Acid Methyl Ester (4h) Tartrate

Compound **4c** (0.85 g, 2.76 mmol) was dissolved in anhydrous CH$_2$Cl$_2$ (30 mL), and 1-chloroethyl chloroformate (ACE-Cl, 4.2 mL, 39 mmol) was added. The mixture was refluxed for 8 h. The reaction mixture was concentrated, the residue dissolved in MeOH (30 mL), and the solution refluxed overnight. The MeOH solution was concentrated and the residue partitioned between CH$_2$Cl$_2$ and 25 mL of NH$_4$OH-H$_2$O (1:1). The layers were separated and the aqueous layer extracted twice with CH$_2$Cl$_2$. The combined organic extracts were dried (Na$_2$SO$_4$), filtered, and concentrated to give 0.85 g of yellow solid, which was chromatographed on 50 g of silica gel using 25% CMA80 in CH$_2$Cl$_2$ to obtain 0.54 g (67%) of **4h**. This material was converted to the tartrate salt by dissolving 520 mg (1.77 mmol) of **4h** in MeOH and adding a MeOH solution of D-tartaric acid 0.286 mg (1.77 mmol). The salt was crystallized from MeOH-ether to give 0.70 g of **4h**•tartrate: mp 156–158 °C; [α]^{25}$_D$ −100.0 °C (c 1.00, MeOH). C$_{20}$H$_{25}$ClNO$_8$: C, 54.12; H, 5.90; N, 3.16; Cl, 7.99. Found: C, 53.88; H, 6.01; N, 3.13; Cl, 7.88. ^{1}H NMR (CD$_3$OD) δ 7.30 (d, 1H), 7.18 (s, 1H), 7.03 (d, 1H), 4.40 (s, 2H), 4.22 (m, 2H), 3.50 (m, 1H), 3.38 (s, 3H), 3.05 (d, 1H), 2.61 (t, 1H), 2.35 (s, 3H), 2.12–2.28 (m, 4H), 1.91 (d, 1H). Anal. (C$_{20}$H$_{26}$ClNO$_8$) C, H, N.

(−)-3β-(3-Methyl-4-chlorophenyl)tropane-2β-(3′-methylisoxazol-5′-yl (4i) Hydrochloride

Acetone oxime (262 mg, 359 mmol) was dissolved under N_2 in 7.5 mL of THF, and the solution was cooled to 0 °C. Butyl lithium, 25 M in hexanes (2.9 mL, 7.18 mmol), was slowly added, and the solution was stirred in an ice bath for 2–3 h. Compound **4c** (85 mg, 2.76 mmol) was dissolved in 4 mL of THF and slowly added to the cold reaction mixture. The mixture was allowed to warm to room temperature and remain for 16 h. A solution of 1.65 g of 36 N H_2SO_4, 2.1 mL of H_2O, and 7.65 mL of THF was slowly added, and the mixture was refluxed for 6 h before being stirred at room temperature for 16 h. The reaction mixture was concentrated and basified with NH_4OH-H_2O (1:1) to pH 10–11 and extracted with CH_2Cl_2 (3×10 mL). The combined organic extracts were dried (Na_2SO_4), filtered, and concentrated to give 0.87 g of product, which was purified by flash chromatography on silica gel using 10% Et_2O-Et_3N (9:1) in 90% hexanes to give 0.26 g of **4c** and 0.27 g (44% adjusted) of the desired **4i**. The product was converted to the HCl salt by adding 1M HCl in Et_2O to an Et_2O solution of **4i**. Recrystallization from MeOH-Et_2O gave **4i**•HCl: mp 150 °C (dec.); $[\alpha]^{25}_D$ −112.0 °C (c 1.00, MeOH). ^{1}H NMR (CD_3OD) δ 7.20 (d, 1H), 7.08 (s, 1H), 6.94 (d, 1H), 5.70, (s, 1H), 4.22 (m, 1H), 4.12 (m, 1H), 3.87 (m, 1H), 3.68 (m, 1H), 2.89 (s, 3H), 2.16–2.66 (m, 16H). Anal. ($C_{19}H_{25}Cl_2N_2O$) C, H, N.

(−)-3β-(3-Methyl-4-chlorophenyl)tropane-2β-(3′-phenylisoxazol-5′-yl (4j) Hydrochloride

Acetophenone oxime (617 mg, 4.56 mmol) was dissolved under N_2 in 10 mL of THF and cooled to 0 °C. Butyl lithium, 2.5 M in hexanes (3.65 mL, 9.12 mmol), was slowly added, and the solution was stirred in an ice bath for 2 h. Compound **4c** (1.08 g, 0.0035 mol) was dissolved in THF (8 mL) and slowly added to the cold reaction mixture. The mixture was allowed to warm to room temperature for 16 h. A solution of 2.00 g of 36 N H_2SO_4, 2.5 mL of H_2O, and 10.5 mL of THF was slowly added, and the mixture was refluxed for 6 h before being stirred at room temperature 16 h. The reaction mixture was concentrated and basified with conc. NH_4OH-H_2O (1:1) to pH 10–11 and extracted with CH_2Cl_2 (3×25 mL). The combined organic extracts were dried (Na_2SO_4), filtered, and concentrated to give 1.23 g of product, which was purified by flash chromatography on silica gel using 10% (Et_2O-Et_3N) (9:1) in 90% hexanes to give 0.39 g of **4c** and 0.71 g (80% adjusted yield) of **4j**. The **4j** was crystallized from petroleum ether to give 0.59 g of fine white crystals: mp 139–141 °C. The product was converted to the HCl salt by adding 1M HCl in Et_2O to an Et_2O solution of **4j**. Recrystallization from MeOH-Et_2O gave 0.467 g of **4j**•HCl: mp 278 °C (dec.); $[\alpha]_D$ −110.2 °C (c 1.00, MeOH). ^{1}H NMR (CD_3OD) δ 7.66 (m 2H), 7.43 (m, 3H), 7.21 (d, 1H), 7.12 (s, 1H), 7.00 (d, 1H), 6.20 (s, 1H), 4.30 (m, 1H), 4.16 (m, 1H), 3.98 (m, 1H), 3.75 (m, 1H), 2.93 (s, 3H), 2.04–2.74 (m, 9H). Anal. ($C_{24}H_{27}Cl_2N_2O$) C, H, N.

3α-(4-Chloro-3-methylphenyl)tropane-2β-carboxylic acid Methyl Ester (4k) Tosylate

To $Ni(OAc)_2$ (9.21 g, 0.037 mol) in MeOH (50 mL) was added $NaBH_4$ (1.4 g, 0.037 mol) in MeOH (20 mL). Compound **8** (2.20 g, 0.0074 mol) in MeOH (20 mL) was added followed by concentrated HCl (3.1 mL, 0.037 mol) in MeOH (5 mL). The black heterogeneous reaction mixture was stirred at reflux for 17 h. The mixture was filtered through celite, and the filtrate was concentrated *in vacuo*. The residue was partitioned between Et_2O and conc. NH_4OH-H_2O (1:1). The ether was separated, dried (Na_2SO_4), and concentrated *in vacuo* to give an orange oil. This oil was chromatographed on silica gel, eluting with hexane-Et_2O-Et_3N (50:45:5), to afford 0.92 g (46%) of **4k** as a colorless oil. To a solution of **4k** in EtOAc (25 mL) was added one equivalent of p-toluene sulfonic acid in a minimal amount of EtOAc. The resulting solids were separated by filtration and dried to give 1.25 g of **4k**•$CH_3C_6H_4SO_3H$ as a white solid: mp 170–171 °C. ^{1}H NMR ($CDCl^3$, free base) δ 1.32 (t, J = 5.8 Hz, 1H), 1.59 (m, 3H), 2.10 (m, 1H), 2.22 (s, 3H), 2.32 (s, 3H), 2.46 (m, 2H), 3.32

(m, 3H), 3.59 (s, 3H), 6.95 (d, J = 3.0 Hz, 1H), 6.98 (s, 1H), 7.25 (d, J = 5.5 Hz). Anal. ($C_{24}H_{30}ClNO_5S•0.25 H_2O$) C, H, N.

3β-(4-Chlorophenyl)tropane-2β-*N*-(3′-amino-4,5-dimethylphenyl Carboxamide) (6)

To compound **5** (5.3 g, 0.0189 mol) in CH_2Cl_2 (100 mL) was added oxalyl chloride (19.0 mL, 0.0378 mol, 2 M in CH_2Cl_2). The reaction mixture was stirred at room temperature for 2 h, then concentrated *in vacuo*. The resulting acid chloride was dissolved in CH_2Cl_2 (60 mL) and added to 4,5-dimethyl-1,2-phenylenediamine (6.4 g, 0.0473 mol) in CH_2Cl_2 (50 mL). The reaction mixture was stirred, under nitrogen, for a period of 17 h. The solvent was decanted from a gummy residue, and 10% $NaHCO_3$-CH_2Cl_2 was added. The organic layer was separated, dried (Na_2SO_4), and concentrated *in vacuo* to give 5.4 g of a foam. This material was chromatographed on silica gel, eluting with EtOAc then EtOAc-CMA80 (1:1) to afford 3.8 g (51%) of **6** as a yellow amorphous solid. ^{1}H NMR ($CDCl_3$) δ 1.71–1.80 (m, 3H), 2.12 (s, 3H), 2.14 (s, 3H), 2.13–2.39 (m, 3H), 2.39 (s, 3H), 2.66 (d, J = 3.0 Hz, 1H), 3.19 (p, J = 3.0 Hz, 1H), 3.40–3.60 (bm, 5H), 6.51 (s, 1H), 6.81 (s, 1H), 7.21 (s, 4H).

3α-(4-Chloro-3-methylphenyl)-2β-(3′-methyl-1′,2′,4′-oxadiazol-5-yl)tropane (8)

n-Butyl lithium (12.0 mL, 0.030 mol, 2.5 M) in hexane was added to 5-bromo-2-chlorotoluene (6.0 g, 0.0292 mol) in THF (50 mL) at –78 °C. The resulting creamy white suspension was stirred for 15 min, and anhydroecgonine oxadiazole (**7**) (3.0 g, 0.0146 mol) in THF (50 mL) was added. The orange reaction mixture was stirred for an additional 3 h, allowing the reaction to come to room temperature. TFA (3.9 mL, 0.050 mol) was added, the mixture was stirred for 15 min and concentrated *in vacuo*. The resulting residue was treated with conc. NH_4OH-H_2O (1:1) (100 mL) and CH_2Cl_2 (100 mL). The organic layer was separated, dried (Na_2SO_4), and concentrated *in vacuo* to afford 5.7 g of an orange oil. This oil was chromatographed on silica gel, eluting with ether-Et_3N (9:1) to afford 2.0 g (46%) of a mixture that was used without further purification to prepare **4k**.

Transporter Assays

The abilities of **2** and its analogues to inhibit uptake of [3H]dopamine ([3H]DA), [3H]serotonin ([3H]5-HT), or [3H]norepinephrine ([3H]NE) by the respective, human transporters were evaluated using the appropriate HEK-293 cell line as previously reported. [31]

Cell lines and culture

Human embryonic kidney (HEK-293) cells stably expressing human DAT, NET or SERT were maintained as previously described.[31]

Use was made, as previously described,[33] of TE671/RD cells naturally expressing human muscle-type nAChR (α1β1γδ- or α1*-nAChR), SH-SY5Y neuroblastoma cells naturally expressing human autonomic α3β4*-nAChRs (containing α3, β4, probably α5, and sometimes β2 subunits), or transfected SH-EP1 epithelial cells heterologously expressing either human α4β2-nAChR, which are thought to be the most abundant, high affinity nicotine-binding nAChR in mammalian brain, or α4β4-nAChR.[36,37]

nAChR Functional Assays

Cells were harvested, seeded onto 24-well plates, and subjected to [86]Rb^{+} efflux assays as previously described.[33] Specific [86]Rb^{+} efflux was assessed as the response to a fully efficacious concentration of carbamylcholine alone less that in the presence of efflux buffer alone. Any intrinsic agonist activity of test compounds was normalized, after subtraction of non-specific efflux, to specific efflux. Antagonism of carbamylcholine-evoked [86]Rb^{+} efflux

was assessed in samples containing the full agonist at a concentration where it stimulates 80–90% of maximal function. For studies of mechanism of antagonism, concentration-response curves were obtained using samples containing the full agonist, carbamylcholine, at the indicated concentrations alone or in the presence of a concentration of the test ligand close to its IC_{50} value for inhibition of nAChR function. Ion flux assay results were fit using Prism (GraphPad) to the Hill equation, $F = F_{max}/(1 + (X/Z)^n)$, where F is the test sample specific ion flux as a percentage of control, F_{max} is specific ion flux in the absence of test drug (i.e., for control samples), X is the test ligand concentration, Z is the EC_{50} (n>0 for agonists) or IC_{50} (n<0 for antagonists), and n is the Hill coefficient. All concentration-ion flux response curves were simple and fit well allowing maximum and minimum ion flux values to be determined by curve fitting, but in cases where antagonists had weak functional potency, minimum ion flux was set at 0% of control. Note that because agonist concentrations used for test ligand antagonism assessments were EC_{80}–EC_{90} values, not all of the data, even at the lowest concentrations of test antagonist, approaches 100% of specific efflux as separately determined in sister samples exposed to fully efficacious concentrations of agonist. Note also that it has been repeatedly verified that functional parameters for nicotinic ligands and mechanisms of their action as determined using efflux assays are like those determined using whole-cell current recording techniques.[38]

Behavior

All animal experiments were conducted in accordance with the NIH Guide for the Care and Use of Laboratory Animals and Institutional Animal Care and Use Committee guidelines.

Animals

Male Institute of Cancer Research (ICR) mice (weighing 20–25 g) obtained from Harlan (Indianapolis, IN) were used throughout the study. Animals were housed in an Association for Assessment and Accreditation of Laboratory Animal Care-approved facility, were placed in groups of six, and had free access to food and water. Studies were approved by the Institutional Animal Care and Use Committee of Virginia Commonwealth University.

Tail-Flick Test

Antinociception for pain mediated at the spinal level was assessed by the tail-flick method of D'Amour and Smith.[39] In brief, mice were lightly restrained while a radiant heat source was shone onto the upper portion of the tail. To minimize tissue damage, a maximum latency of 10 s was imposed. Latency to remove the tail from the heat source was recorded for each animal. A control response (2–4 s) was determined for each mouse before treatment, and a test latency was determined after drug administration (nicotine as an analgesic 5 min after subcutaneous administration at 2.5 mg/kg; nicotine administration 15 min after exposure to saline of 3-phenytropane analogue to assess the latter drug's ability to block nicotine-mediated antinociception). Antinociceptive response was calculated as the percentage of maximum possible effect (%MPE), where %MPE = [(test control)/(10 control)] × 100.

Hot-Plate Test

Mice were placed into a 10-cm wide glass cylinder on a hot plate (Thermojust Apparatus) maintained at 55 °C for assessment of pain responses mediated at supraspinal levels. To minimize tissue damage, a maximum exposure to the hot plate 40 s was imposed. Measures of control latencies (time until the animal jumped or licked its paws; typically 8–12 s) were done twice for stimuli applied at least 10 min apart for each mouse. Antinociceptive responses after test drug administrations were determined and calculated as the %MPE, where %MPE = [(test latency in s − control latency in s)/(40 s − control latency in s) × 100].

Groups of 8 to 12 animals were used for each drug condition. Antagonism studies were carried in mice pretreated with either saline or 3-phenytropane analogues 15 min before nicotine. The animals were then tested 5 min after administration of a subcutaneous dose of 2.5 mg/kg nicotine.

Locomotor Activity

Mice were placed into individual Omnitech photocell activity cages (28×16.5 cm; Omnitech Electronics, Columbus, OH) 5 min after subcutaneous administration of either 0.9% saline or nicotine (1.5 mg/kg). Interruptions of the photocell beams (two banks of eight cells each) were then recorded for the next 10 min. Data were expressed as the number of photocell interruptions. Antagonism studies were carried out by pretreating the mice with either saline or 3-phenytropane analogues 15 min before nicotine.

Body Temperature

Rectal temperature was measured by a thermistor probe (inserted 24 mm) and digital thermometer (YSI Inc., Yellow Springs, OH). Readings were taken just before and 30 min after subcutaneous injection of either saline or 2.5 mg/kg nicotine. The difference in rectal temperature before and after treatment was calculated for each mouse. The ambient temperature of the laboratory varied from 21 to 24 °C from day to day. Antagonism studies were carried out by pretreating the mice with either saline or 3-phenytropane analogues 15 min before nicotine. The animals were then tested 30 min after administration of a subcutaneous dose of 2.5 mg/kg nicotine.

Nicotine CPP Assessment

An unbiased CPP paradigm was utilized in this study as described in Kota et al.[33] Briefly, place-conditioning chambers consisted of two distinct compartments separated by a smaller intermediate compartment with openings that allowed access to either side of the chamber. On day 1, adult male ICR mice were confined to the intermediate compartment for a 5 min habituation period, and then allowed to move freely between compartments for 15 min. Time spent in each compartment was recorded. These data were used to separate the animals into groups of approximately equal bias. Days 2–4 were the conditioning days during which the saline group received saline in both compartments and drug groups received s.c. vehicle or 3-phenyltropane analogues 15 min before nicotine (0.5 mg/kg, s.c.) in one compartment and saline in the opposite compartment for 20 min. Drug-paired compartments were randomized among all groups. Day 5 was the drug free test day and the procedure was the same as day 1. Activity counts and time spent on each side were recorded via photosensors using Med Associates interface and software. Separate groups of mice were conditioned with saline or 3-phenyltropane analogues alone to investigate if they induce CPP using the same procedure described above. Data were expressed as time spent on drug-paired side minus time spent on saline-paired side. A positive number indicated a preference for the drug-paired side, whereas a negative number indicated an aversion to the drug-paired side. A number at or near zero indicated no preference for either side.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

Abbreviations

NRT	nicotine replacement therapy
DA	dopamine
5HT	serotonin
NE	norepinephrine
HEK	human embryonic kidney
DAT	dopamine transporter
SERT	serotonin transporter
NET	norepinephrine transporter
nAChR	nicotine acetylcholine receptor
VTA	ventral tegmental area
MPE	maximum possible effect
CPP	conditioned place preference

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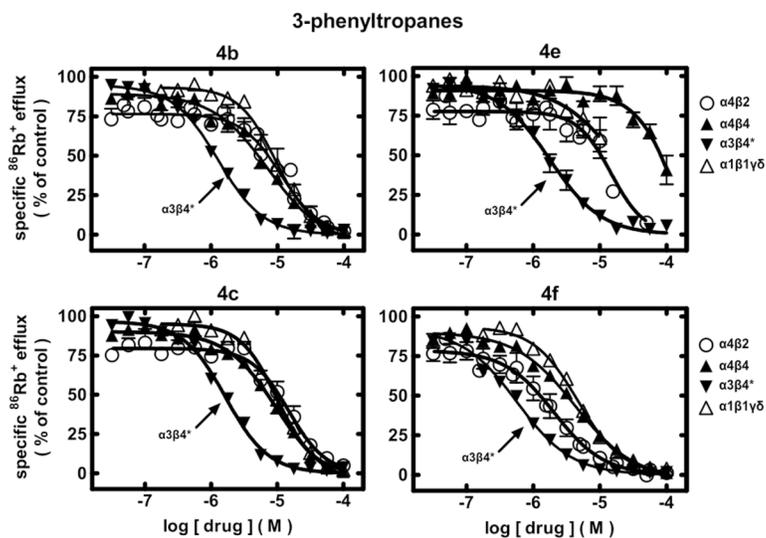39. D'Amour FE, Smith DL. A method for determining loss of pain sensation. J Pharmacol Exp Ther. 1941; 72:74–79.

Fig. 1.
Specific $^{86}Rb^+$ efflux (ordinate; percentage of control) was determined for functional, human muscle-type $\alpha1\beta1\gamma\delta$-nAChR ($\triangle$), ganglionic $\alpha3\beta4^*$-nAChR ($\blacktriangledown$), $\alpha4\beta2$-nAChR ($\bigcirc$), or $\alpha4\beta4$-nAChR ($\blacktriangle$) naturally or heterologously expressed in human cell lines in the presence of a receptor subtype-specific, EC_{80}–EC_{90} concentration of the full agonist, carbamylcholine, either alone or in the presence of the indicated concentrations (abscissa, log molar) of 3-phenyltropane analogues **4b, 4c, 4e,** or **4f** as indicated. Mean micromolar IC_{50} values and SEM as a multiplication/division factor of the mean micromolar IC_{50} value are provided in Table 1.
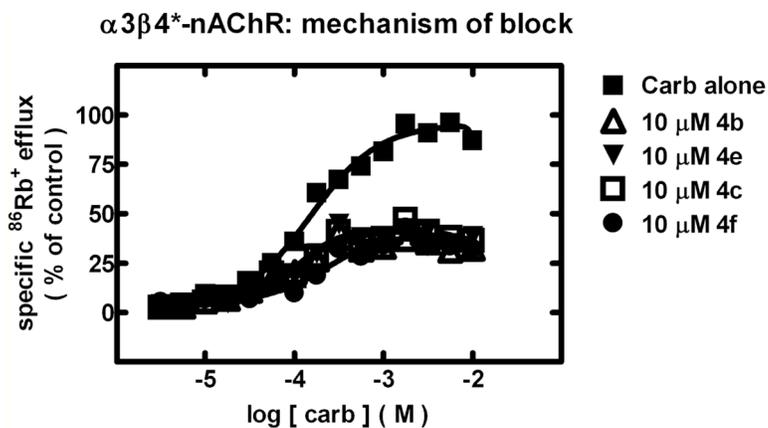
Fig. 2.
Specific $^{86}Rb^+$ efflux (ordinate; percentage of control) was determined for functional, human ganglionic α3β4*-nAChR naturally expressed by SH-SY5Y human neuroblastoma cells in the presence of the full agonist, carbamylcholine, at the indicated concentrations (abscissa, log molar) either alone (■) or in the presence of 10 µM 3-phenyltropane analogues **4b** (△), **4c** (□), **4e** (▼), or **4f** (●) as indicated. The reduction of apparent agonist efficacy without an effect on potency is consistent with a noncompetitive mechanism for nAChR block.
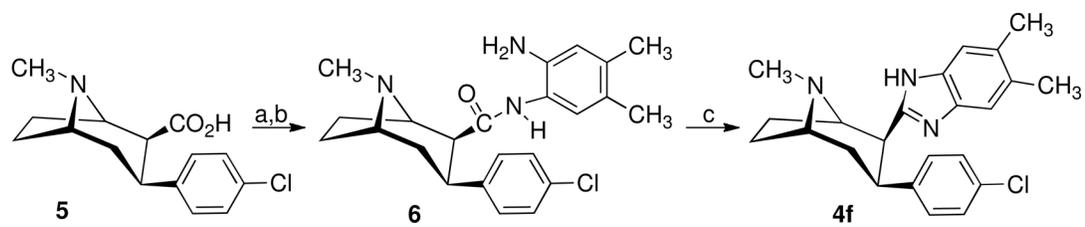
Scheme 1a.
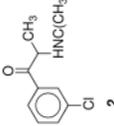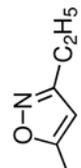[a]Reagents: (a) (COCl)$_2$, CH$_2$Cl$_2$; (b) 4,5-dimethyl-1,2-phenylenediamine, CH$_2$Cl$_2$; (c) POCl$_3$.
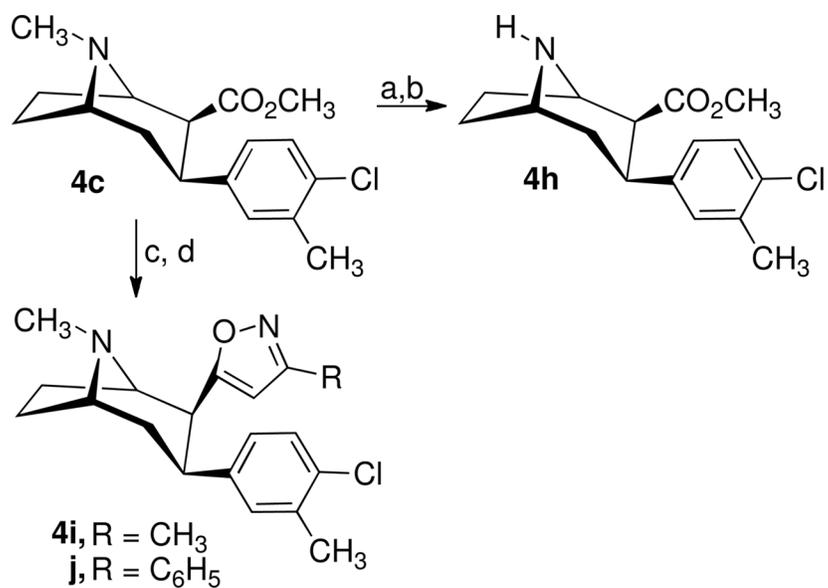
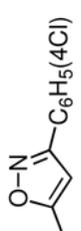**Scheme 2**[a]

[a]Reagents: (a) ACE-Cl, DCE, reflux; (b) CH_3OH, reflux; (c) $CH_3(C=NOH)R$, n-C_4H_9Li, THF, 0 °C; (d) H_2SO_4, THF.
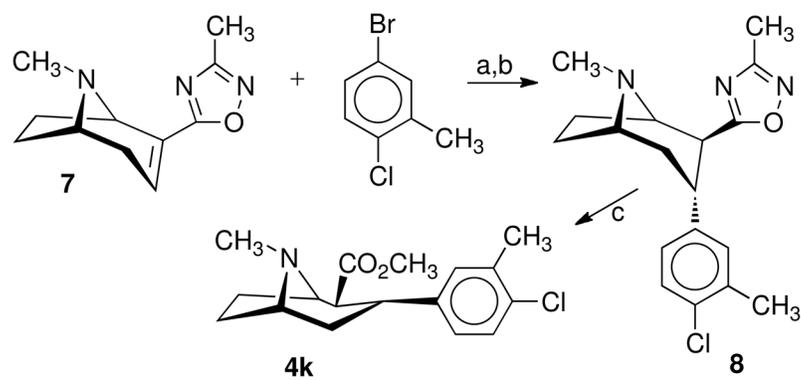
Scheme 3^a
^{a}Reagents: (a) n-C$_4$H$_9$Li, THF, −78 °C, 4 h; (b) TFA; (c) Ni$_2$B, CH$_3$OH, HCl.

Table 1

3-Phenyltropane Analogue Inhibition of Monoamine Uptake and Nicotinic Acetylcholine Receptor (nAChR) Function

Compd	Stereo-chemistry	R_1	R_2	X	Y	Monoamine Uptake Inhibition IC$_{50}$ (nM)[a]			nAChR Inhibition IC$_{50}$, (μM)[b]				
						[³H]DA	[³H]NE	[³H]5HT	α3β4*-	α4β2-	α4β4-	α1*-
2						658 ± 180	1850 ± 300	IA	1.8 (1.15)	12 (1.15)	15 (1.07)	7.9 (1.12)
4a	2β,3β	CH$_3$	CO$_2$CH(CH$_3$)$_2$	CH$_3$	H	0.83 ± 0.20	52 ± 10	2830 ± 500	1.8 (1.07)	12 (1.15)	8.8 (1.13)	12 (1.07)
4b	2β,3β	CH$_3$	CO$_2$ (cyclobutyl)	CH$_3$	H	4.8 ± 0.5	122 ± 20	2010 ± 800	1.3 (1.06)	11 (1.12)	7.3 (1.15)	9.5 (1.05)
4c	2β,3β	CH$_3$	isoxazole–C$_2$H$_5$	CH$_3$	H	0.75 ± 0.1	11 ± 2	2600 ± 1300	1.7 (1.05)	15 (1.10)	9.9 (1.11)	9.9 (1.05)
4d	2β,3β	CH$_3$	isoxazole–C$_6$H$_5$(4Cl)	CH$_3$	H	11 ± 3	310 ± 130	IA	20 (1.12)	942 (1.74)	57 (4.47)	95 (1.06)
4e	2β,3β	CH$_3$	isoxazole–C$_6$H$_4$(4CH$_3$O)	Cl	H	3.9 ± 1	26 ± 7	2000 ± 300	1.8 (1.03)	15 (1.15)	>100	19 (1.03)
4f	2β,3β	CH$_3$	benzimidazole–CH$_3$	Cl	H	7.3 ± 2	19 ± 4	IA	0.57 (1.03)	1.8 (1.15)	4.3 (1.03)	4.7 (1.06)
4g	2β,3β	CH$_3$	CO$_2$CH$_3$	Cl	CH$_3$	2 ± 1	1.1 ± 0.3	1.0 ± 0.3	3.6 (1.10)	9.8 (1.18)	24 (1.16)	26 (1.07)
4h	2β,3β	H	CO$_2$CH$_3$	Cl	CH$_3$	2.3 ± 0.4	0.9 ± 0.3	6.2 ± 2	3.1 (1.12)	16 (1.06)	12 (1.04)	11 (1.07)
4i	2β,3β	CH$_3$	isoxazole–CH$_3$	Cl	CH$_3$	1.5 ± 0.3	6.3 ± 0.9	115 ± 20	0.73 (1.12)	7.7 (1.26)	5.8 (1.08)	13 (1.18)
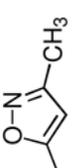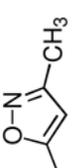
Compd	Stereo-chemistry	R₁	R₂	X	Y	Monoamine Uptake Inhibition IC₅₀ (nM)[a]				nAChR Inhibition IC₅₀, (μM)[b]			
						[³H]DA	[³H]NE	[³H]5HT	α3β4*-	α4β2-	α4β4-	α1*-
4j	2β,3β	CH₃	(O–N / CH₃ / C₆H₅ isoxazole)	Cl	CH₃	15 ± 5	14 ± 3	700 ± 200	1.4 (1.19)	14 (1.06)	4.6 (1.04)	3.0 (1.09)
4k	2β,3α	CH₃	CO₂CH₃	Cl	CH₃	3 ± 1	6.5 ± 2	105 ± 1	3.2 (1.08)	29 (1.04)	15 (1.04)	13 (1.05)
4l	2β,3α	H	CO₂CH₃	CH₃	F	6.4 ± 2	0.5 ± 0.1	110 ± 7	2.6 (1.07)	11 (1.12)	25 (1.36)	7.3 (1.04)

[a] Values for mean ± standard error of three independent experiments, each conducted with triplicate determination.

[b] Mean micromolar IC₅₀ values (to two significant digits) for bupropion and the indicated 3-phenyltropane analogues from three independent experiments for inhibition of functional responses to an EC₈₀–EC₉₀ concentration of carbamylcholine mediated by nAChR subtypes composed of the indicated subunits (where * indicates that additional subunits are or may be additional assembly partners with the subunits specified; see Methods and Materials). Numbers in parentheses indicate S.E.M. as a multiplication/division factor of the mean micromolar IC₅₀ values shown [i.e., the value 1.8 (1.15) reflects a mean IC₅₀ value of 1.8 μM with an S.E.M. range of 1.8 × 1.15 μM to 1.8/1.15 μM or 1.6–2.1 μM]. IA: IC₅₀ >100 μM.

Table 2

Comparison of behavioral potency for 3-phenyltropane analogues in blocking acute effects of nicotine (antinociception, hypomotility, and hypothermia) and development of nicotine CPP[a]

Compd	AD_{50} (mg/kg)				
	Tail-flick	Hot-plate	Locomotion	Hypothermia	CPP
2	1.2 (1–1.8)	15 (6–19)	4.9 (0.9–46)	9.2 (4–23)	0.35
4a	0.015 (0.001–0.16)	IA	1.5 (0.3–7)	1.6 (0.3–9)	NT
4b	0.28 (0.09–0.64)	9.8 (0.7–34)	1 (0.1–2.1)	7 (2.5–19)	NT
4c	0.008 (0.001–0.04)	1.6 (0.7–3.4)	0.2 (0.1–2.1)	6.9 (2.5–19)	0.045
4d	IA	IA	8.7 (6.5–11.2)	IA	0.013
4e	0.018 (0.002–0.16)	IA	IA	IA	NT
4f	0.11 (0.04–0.3)	19 (5.2–70)	IA	IA	0.45
4g	0.21 (0.06–2.7)	0.35 (0.1–1.2)	0.11 (0.03–0.3)	0.14 (0.1–0.18)	0.25
4h	0.003 (0.001–0.01)	0.78 (0.05–12)	0.41 (0.1–1.7)	0.23 (0.1–4)	0.25
4i	0.009 (0.002–0.03)	3.3 (1.4–7.8)	0.37 (0.06–1.5)	0.61 (0.24–1.5)	0.09
4j	2.5 (1.3–5.8)	IA	IA	IA	0.07
4k	0.026 (0.004–0.1)	IA	5.3 (0.5–48)	7 (3.3–18)	0.15
4l	0.002 (0.001–0.06)	2.6 (0.6–16)	1.65 (0.7–3.9)	7.4 (0.8–14.2)	0.03

[a]Results for nicotine acute effects were expressed as AD_{50} (mg/kg) ± confidence limits (CL). Dose-response curves were determined using a minimum of four different doses of test compound, and at least eight mice were used per dose group. AD_{50} (mg/kg) values were estimated in the CPP test using a minimum of three different doses of test compound, and at least eight mice were used per dose group. IA: AD_{50} > 20 mg/kg; n/a: not studied. NT is not tested.