Indolizidine (−)-235B’ and related structural analogs: Discovery of nicotinic receptor antagonists that inhibit nicotine-evoked [3H]dopamine release

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Although several therapeutic agents are available to aid in tobacco smoking cessation, relapse rates continue to be high, warranting the development of alternative pharmacotherapies. Nicotine-evoked dopamine release from its presynaptic terminals in the central nervous system leads to reward which maintains continued tobacco use. The ability of indolizidine (−)-235B’ and a sub-library of structurally related analogs to inhibit nicotine-evoked [3H]dopamine release in a concentration-dependent manner (IC50 = 42 nM, Imax = 55%). Compound (−)-237D, the double bond-reduced analog, afforded the greatest inhibitory potency (IC50 = 0.18 nM, Imax = 76%), and was 233-fold more potent than indolizidine (−)-235B’. The des-8-methyl aza-analog of indolizidine (−)-235B’, ZZ-272, also inhibited nicotine-evoked [3H]dopamine release (IC50 = 413 nM, Imax = 59%). Concomitant exposure to maximally effective concentrations of indolizidine (−)-235B’, ZZ-272 or (−)-237D with a maximally effective concentration of α-conotoxin MII, a selective antagonist for α6β2-containing nicotinic receptors, resulted in inhibition of nicotine-evoked [3H]dopamine release no greater than that produced by each compound alone. The latter results suggest that indolizidine (−)-235B’, (−)-237D, ZZ-272 and α-conotoxin MII inhibit the same α-conotoxin MII-sensitive nicotinic receptor subtypes. Thus, indolizidine (−)-235B’ and its analogs act as antagonists of α6β2-nicotinic receptors and constitute a novel structural scaffold for the discovery of pharmacotherapies for smoking cessation.

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1. Introduction

Several tobacco smoking cessation pharmacotherapies are available currently, but are limited by efficacy and high relapse rates (Hurt et al., 2003; George and O’Malley, 2004; Wileyto et al., 2004). Nicotine replacement reduces craving and withdrawal symptoms during tobacco cessation (Shiffman et al., 2002; Shiffman, 2008), but a meta-analysis of 132 clinical trials reported a lack of effectiveness after 8 weeks of treatment (Stead et al., 2008). Bupropion, a norepinephrine and dopamine transport inhibitor and nicotinic receptor antagonist (Ferris & Cooper, 1993; Ascher et al., 1995; Miller et al. 2002, Dwoskin et al., 2006), has limited utility as a tobacco cessation agent (Shiffman et al., 2000; Dwoskin et al., 2006; Sidhpura et al., 2007). Surprisingly, bupropion increases tobacco use in non-treatment seeking smokers (Cousins et al., 2001) and decreases seizure threshold, which is a limiting side-effect (Kuate et al., 2004). Varenicline, a partial agonist at α4β2 and full agonist at α7 nicotinic receptors (Coe et al., 2005; Mihalak et al., 2006), reduces nicotine reinforcement and craving (Gonzales et al., 2006; Oncken et al., 2006; West et al., 2008), but with side-effects including agitation, depression and suicidal ideation (Hays et al., 2008). Clearly, there is a need for more efficacious smoking cessation therapeutic.

Activation of nicotinic receptors by nicotine increases extracellular dopamine concentrations in mesolimbic and nigrostriatal systems, both of which mediate nicotine-induced reward leading to tobacco dependence (Corrigall et al., 1992; Rahman et al., 2007; Govind et al., 2009). Studies employing knockout mice identified six nicotinic receptor subtypes that mediate nicotine-evoked dopamine release from striatum (Salminen et al., 2004, 2007). These subtypes are classified as α-conotoxin MII-sensitive (α6β2, α6β2ε3, α6α4β2ε and α6α4εβ2ε) and α-conotoxin MII-insensitive (α4β2 and α4εβ2ε). The development of α6-containing nicotinic receptor antagonists is expected to decrease the rewarding and reinforcing properties of nicotine without producing peripheral side-effects (e.g.,...
constipation and hypotension) associated with nonselective antagonists (e.g., mecamylamine; Rose et al., 1999). Development of nicotinic receptor antagonists has been considered as a viable alternative approach to treating nicotine addiction (Dwoskin and Crooks, 2001; Drenan et al., 2008; Benowitz, 2009; Dwoskin and Bardo, 2009).

Indolizidines are defined as bicyclic heterocycles containing a 6-membered ring fused to a 5-membered ring with carbon and nitrogen bridging atoms (Michael, 2008). Previous research has demonstrated that the synthetic 5,8-disubstituted indolizidine (−)−235B acts as a potent (IC50 = 74 nM), noncompetitive antagonist at α4/2 nicotinic receptors expressed in Xenopus oocytes (Tsuneki et al., 2004; Toyooka et al., 2005). The purpose of the present study was to investigate the inhibitory effects of indolizidine (−)−235B and a small sub-library of related structures on nicotine-evoked [3H]dopamine release from superfused rat striatal slices, and to determine if c6-containing nicotinic receptors contribute to the observed inhibition.

2. Materials and Methods

2.1. Animals

Male Sprague–Dawley rats (200–225 g) were obtained from Harlan Industries, Inc. (Indianapolis, IN) and were housed two per cage with free access to food and water in the Division of Laboratory Animal Resources at the University of Kentucky (Lexington, KY). Experimental protocols involving the rats were in accordance with the NIH 1996 Guide for the Care and Use of Laboratory Animals and were approved by the Institutional Animal Care and Use Committee at the University of Kentucky.

2.2. Materials

[3H]Dopamine (dihydroxyphenylethylamine, 3,4-[7-3H], specific activity 28.0 Ci/mmol) was purchased from PerkinElmer Life Sciences, Inc. (Boston, MA). L-Ascorbic acid, α,α′-glucose, S(−)-nicotine dihydrate (nicotine), nomifensine maleate and pargyline hydrochloride were obtained from Sigma–Aldrich Corporation (St. Louis, MO). TS-2 Tissue solubilizer and scintillation cocktail were purchased from Research Products International Corporation (Mount Prospect, IL). All other chemicals used in the in vitro assay buffers were purchased from Fisher Scientific International Corporation (Pittsburgh, PA). Chemical structures of the compounds are shown in Fig. 1. Indolizidine (−)−235B−[5R,8R,8aS]−(−)−5−(hept-6-enyl)−8−methyloctahydroindolizine, (−)−B−6−[5R,8R,8aS]−(−)−5−(hex-5-enyl)−8−methyloctahydroindolizine, (−)−B−8−[5R,8R,8aS]−(−)−5−(oct-7-enyl)octahydroindolizine, (−)−207A−[5R,8R,8aS]−(−)−5−(pent-4-enyl) octahydroindolizine, (−)−205A−[5R,8R,8aS]−(−)−8−methyl-5−(pent-4-enyl)octahydroindolizine, (−)−237D−[5R,8R,8aS]−(−)−5−heptyl-8−methyloctahydroindolizine and ZZ-257 (methyl 8-oxo-5,6,7,8-tetrahydropyridino-diol-5-carboxylate) were synthesized as previously reported (Jefford et al. 1995; Toyooka et al., 2005). Indolizidine (−)−235B− fragments, ZZ-261 (1,8-bis-(2-methyl-6-piperidinyl)octane) and ZZ-262 (2-methyl-6-oxypiperidine), were prepared through catalytic hydrogenation of the corresponding ammonium precursors. Indolizidine (−)−235B− fragments, ZZ-294B (methyl N−(oct-7-en-1-yl)pypyridoline and HRS-1-97 N-oxypyrrolylizine), were prepared through direct N-alkylation of pyrroline. Catalytic reduction of ZZ-257 afforded ZZ-270A (methyl octahydroindolizine-5-carboxylate) and ZZ-270B (methyl 8-hydroxyoctahydroindolizine-5-carboxylate). ZZ-270A was further reduced using disobutylaluminium hydride to the corresponding alcohol. The resulting aldehyde was subjected to reductive amination to afford ZZ-272 (N-((octahydroindolizin-5-yl)methyl)heptan-1-amine), and the Wittig reaction to afford ZZ-275 ((E)-5−(hept-1-en-yl) octahydroindolizine), and ZZ-254 (5-heptyloctahydroindolizine) upon subsequent catalytic reduction. All analogs were characterized by [3H- and 13C-NMR spectroscopy, mass spectroscopy and elemental analysis.

2.3. [3H]Dopamine Overflow Assay

Rat striatal slices (~6 mg wet weight) were incubated for 30 min in Krebs’ buffer (118 mM NaCl, 4.7 mM KCl, 1.2 mM MgCl2,1.0 mM NaH2PO4, 1.4 mM CaCl2, 11.1 mM glucose, 25 mM NaHCO3, 0.11 mM l-aspartic acid and 0.004 mM disodiumlactenamidetetraacetate, pH 7.4, saturated with 95% O2/5% CO2) in a metabolic shaker at 34 °C for 30 min. Slices were incubated with for 30 min with 0.1 μM [3H] dopamine (final concentration), which is taken up into presynaptic terminals. Slices were transferred to superfusion chambers maintained at 34 °C (Brandel Suprafusion system 2500, Biomedical Research & Development Laboratories, Inc., Gaithersburg, MD) and superfused (flow rate = 0.6 ml/min) with oxygenated Krebs’ buffer for a 60-min washout period. Buffer contained both 10 μM nomifensine (a dopamine uptake inhibitor) and 10 μM parglyline (a monoamine oxidase inhibitor), to prevent reuptake and metabolism of [3H] dopamine respectively, and to assure that the [3H] collected in superfusate primarily represented parent neurotransmitter. Two samples (2.4 ml/sample) were collected at 4-min intervals to determine basal [3H] dopamine outflow, followed by inclusion of 100 nM analog in the buffer to determine analog-induced intrinsic activity. Control slices were superfused for 36 min in the absence of analog to determine nicotine-evoked [3H] dopamine overflow. Nicotine (10 μM) was added to the buffer, and slices were superfused for an additional 36 min in the absence (control) and presence of analog. The analog was present throughout the superfusion period including during exposure to nicotine. If the analog (100 nM) inhibited >30% of the nicotine-evoked [3H] dopamine overflow, then the full concentration response was determined. For concentration–response studies, two samples (2.4 ml/sample) were collected at 4-min intervals to determine basal [3H] dopamine outflow. Then, each slice from an individual rat was superfused for 36 min in either the absence (control) or presence of a single concentration of indolizidine (−)−235B− (1 nM–10 μM), (−)−237D− (0.01–100 nM), ZZ-272 (1 nM–10 μM), α-conotoxin MII (0.1–30 nM), or mecamylamine (1 nM–10 μM). Subsequently, nicotine (10 μM) was added to the buffer for 36 min. A control slice in each experiment was superfused for 36 min in the absence of compound, followed by addition of nicotine to the buffer to determine nicotine-evoked [3H] dopamine overflow. At the end of each experiment, slices were solubilized, and the [3H]-content of the tissue and superfusate samples was determined using liquid scintillation spectrometry.

To determine if indolizidine (−)−235B−, (−)−237D or ZZ-272 interact with α-conotoxin MII-sensitive nicotinic receptors, duplicate striatal slices were superfused for 36 min in the absence (nicotine control) and presence of maximally inhibitory concentrations of α-conotoxin MII (1 nM) and/or indolizidine (−)−235B− (300 nM), (−)−237D− (100 nM) or ZZ-272 (10 μM) followed by superfusion with 10 μM nicotine added to the buffer for an additional 36 min. In each experiment, duplicate slices were superfused with a maximally inhibitory concentration (10 μM) of the nonselective nicotinic receptor antagonist, mecamylamine, which is expected to produce complete inhibition of the effect of nicotine. At the end of the experiment, slices and superfusate samples were processed as previously described.

2.4. Data Analysis

Fractional release for each superfusate sample was calculated by dividing the amount of tritium in each sample by the total tissue-[3H] at the time of sample collection. Basal [3H] dopamine overflow was calculated as the average fractional release in the two samples just before addition of analog to the superfusion buffer. Total [3H] dopamine overflow was calculated by summing the fractional release resulting from exposure to nicotine, either in the absence or presence of analog,
and subtracting the basal $[^3]H$[dopamine outflow. For analysis of inhibitor concentration response, data were fit by nonlinear least-squares regression using a variable slope, sigmoidal function. $I_{50}$ and $I_max$ values were determined using the Prism 5.0 program (GraphPad Software Inc., San Diego, CA). Statistical analyses were conducted using SPSS (version 17.0; SPSS Inc., Chicago, IL). Two-way analysis of variance (ANOVA) was used to analyze the effect of the inhibitors on fractional $[^3]H$[DA release, with inhibitor concentration and time as within-subjects factors. One-way repeated-measures ANOVAs were used to analyze the concentration-dependent effect of each inhibitor on nicotine-evoked $[^3]H$[dopamine overfl ow. To determine if indolizidine $(-)-235B'$, $(-)-235B'$ analogs, indolizidine $(-)-235B'$ analogs without 8-methyl group and indolizidine $(-)-235B'$ fragments.

3. Results

3.1. Indolizidine $(-)-235B'$ Inhibition of Nicotine-Evoked $[^3]H$[Dopamine Release

Only the highest (10 µM) concentration of indolizidine $(-)-235B'$ evoked $[^3]H$[dopamine overflow (Table 1). The time course of the inhibitory effect of indolizidine $(-)-235B'$ (0.001–1 µM) on nicotine-evoked fractional release is illustrated in Fig. 2 (inset). Fractional release peaked at 12 min following the addition of nicotine to the superfusion buffer and subsequently decreased toward basal levels despite the presence of nicotine in the buffer throughout the remainder of the experiment. Indolizidine $(-)-235B'$ produced a
Intrinsic activity of indolizidine (-)-235B', (-)-237D and ZZ-272. Ability of analogs to evoke [3H]dopamine overflow from superfused rat striatal slices. Slices were preloaded with [3H]dopamine and superfused for 60 min with buffer containing nomifensine (10 μM) and pargyline (10 μM) to inhibit the reuptake of released [3H]dopamine and to prevent metabolism of [3H]dopamine, respectively. Subsequently, slices were superfused for 36 min either in the absence (control) or presence of analog, which was added to the superfusion buffer. Each slice was exposed to only one concentration of analog.

Data are mean±S.E.M. total [3H]dopamine overflow expressed as a percentage of tissue tritium, n=3–5 rats/analog.

* Indicates P<0.01 compared to control (absence of analog).

### 3.2. Inhibition of Nicotine-Evoked [3H]Dopamine Release by Indolizidine (-)-235B' Analogs and Fragments

The inhibitory effect of indolizidine (-)-235B' analogs and its structural fragments were determined at an initial probe concentration of 100 nM (Fig. 3). None of the analogs or fragments exhibited intrinsic activity at the probe concentration (Table 2). The C5-oct-7-en-1-yl analog of indolizidine 235B, compound (−)-B-8, did not inhibit nicotine-evoked [3H]dopamine overflow. However, the C5-hex-5-en-1-yl and C5-pent-4-en-1-yl analogs (−)-B-6 and (−)-207A inhibited nicotine-evoked [3H]dopamine overflow by 20% and 36%, respectively. Compound (−)-205A, the C5-pent-4-yn-1-yl analog of indolizidine (−)-235B', inhibited nicotine-evoked [3H]dopamine overflow by 39%. Compound 237D, the reduced C5-hept-1-yl analog of indolizidine (−)-235B', inhibited nicotine-evoked [3H]dopamine overflow by 58%. Although ZZ-254 and ZZ-275, which lack the chiral 8-methyl group in indolizidine (−)-235B', did not inhibit nicotine-evoked [3H]dopamine overflow, ZZ-272, which similarly lacks the 8-methyl group, inhibited nicotine-evoked [3H]dopamine overflow by 31% (Fig. 3). Importantly, none of the structural fragments (ZZ-270A, ZZ-270B, ZZ-257, ZZ-261, ZZ-262, ZZ-249B, and HRS-1-97), inhibited nicotine-evoked [3H]dopamine overflow when evaluated at the probe concentration.

Full concentration–response curves were generated for (−)-237D, an analog having the greatest inhibitory activity (58%) at 100 nM, and for ZZ-272, an analog lacking the 8-methyl group and having 31% inhibitory activity at 100 nM (Fig. 4 top and bottom, respectively). Intrinsic activity was not observed at any concentration of either analog (Table 1). Both (−)-237D (0.01–100 nM) and ZZ-272 (1–10 μM) produced concentration-dependent inhibition of nicotine-evoked fractional release. For (−)-237D, two-way ANOVA revealed a main effect of time (F_{8,240}=40.2, P<0.001) and a time x concentration interaction (F_{56,240}=1.64, P<0.01). Evaluation of the inhibition of nicotine-evoked total [3H]dopamine overflow by nonlinear regression revealed sigmoidal functions for (−)-237D and ZZ-272 with IC_{50} values of 0.18 nM (confidence interval = 0.022–1.48 nM) and 413 nM (confidence interval = 20–8620 nM), respectively, and with I_{max} values of 76±7% and 59±7%, respectively. One-way ANOVAs revealed concentration-dependent effects of (−)-237D (F_{8,55}=8.44, P<0.001) and ZZ-272 (F_{4,41}=3.947, P<0.01). Maximal inhibition of nicotine-evoked [3H]dopamine overflow was observed at 1 nM (−)-237D and at 10 μM ZZ-272.
Table 2
Intrinsic activity of indolizidine (−)-235B′ structural analogs and fragments. Ability of analogs to evoke \[^3H\]dopamine overflow from superfused rat striatal slices. Slices were preloaded with \[^3H\]dopamine and superfused for 60 min with buffer containing nomifensine (10 μM) and pargyline (10 μM) to inhibit the reuptake of released \[^3H\]dopamine and to prevent metabolism of \[^3H\]dopamine, respectively. Subsequently, slices were superfused for 36 min either in the absence (control) or presence of analog (100 nM), which was added to the superfusion buffer.

<table>
<thead>
<tr>
<th>Indolizidine (−)-235B′ analogs without 8′-methyl group</th>
<th>Intrinsic activity</th>
<th>Indolizidine (−)-235B′ analogs without 8′-methyl group</th>
<th>Intrinsic activity</th>
<th>Indolizidine (−)-235B′ fragments</th>
<th>Intrinsic activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>−B-8</td>
<td>0.00 ± 0.00</td>
<td>ZZ-254</td>
<td>0.01 ± 0.01</td>
<td>ZZ-270A</td>
<td>0.16 ± 0.11</td>
</tr>
<tr>
<td>−B-8</td>
<td>0.00 ± 0.00</td>
<td>ZZ-272</td>
<td>0.00 ± 0.00</td>
<td>ZZ-270B</td>
<td>0.03 ± 0.01</td>
</tr>
<tr>
<td>−205A</td>
<td>0.01 ± 0.01</td>
<td>ZZ-275</td>
<td>0.02 ± 0.02</td>
<td>ZZ-257</td>
<td>0.00 ± 0.00</td>
</tr>
<tr>
<td>−207A</td>
<td>0.05 ± 0.05</td>
<td>HRS-1-97</td>
<td>0.00 ± 0.00</td>
<td>ZZ-249B</td>
<td>0.05 ± 0.04</td>
</tr>
<tr>
<td>−237D</td>
<td>0.01 ± 0.01</td>
<td>ZZ-261</td>
<td>0.00 ± 0.00</td>
<td>ZZ-262</td>
<td>0.01 ± 0.01</td>
</tr>
</tbody>
</table>

Data are mean ± S.E.M. total \[^3H\]dopamine overflow expressed as a percentage of tissue tritium, n = 3–5 rats/analog.

3.3. α-Conotoxin MII and Mecamylamine Inhibition of Nicotine-Evoked \[^3H\]Dopamine Release

Concentration–response curves were generated to determine the concentrations of α-conotoxin MII and mecamylamine that maximally inhibit nicotine-evoked \[^3H\]dopamine overflow. α-Conotoxin MII inhibited nicotine-evoked \[^3H\]dopamine overflow from rat striatal slices in a concentration-dependent manner (\(F_{6,35} = 3.42, P < 0.001\); \(IC_{50} = 0.026 \text{nM}, \text{confidence interval} = 0.005–0.13 \text{nM}; \(I_{\text{max}} = 66 ± 5\%; \text{Fig. 5}\)). Maximal inhibition of nicotine-evoked \[^3H\]dopamine overflow was observed at 0.3 μM. Mecamylamine also inhibited nicotine-evoked \[^3H\]dopamine overflow in a concentration-dependent manner (\(F_{5,36} = 24.1, P < 0.001\) with an \(IC_{50} \text{ value of } 14.5 \text{nM, confidence interval = 3.69–57.1 nM and an } I_{\text{max}} \text{ of 95 ± 5% (data not shown).}

3.4. Interaction of Indolizidine (−)-235B′, ZZ-272 and (−)-237D with α-Conotoxin MII-Sensitive Nicotinic Receptors

In order to determine whether indolizidine (−)-235B′ interacts with α-conotoxin MII-sensitive nicotinic receptors, rat striatal slices were exposed to concentrations of α-conotoxin MII (1 nM), or indolizidine (−)-235B′ (300 nM), which produced maximal inhibition, or the combination of α-conotoxin MII plus indolizidine (−)-235B′ at the respective concentrations. Both α-conotoxin MII and indolizidine (−)-235B′ inhibited nicotine-evoked \[^3H\]dopamine overflow compared to the within-subject nicotine control (Fig. 6). One-way repeated measures ANOVA revealed a significant effect of inhibitor (\(F_{4,27} = 16.0, P < 0.001\). Post hoc analysis revealed that inhibition of nicotine-evoked \[^3H\]dopamine overflow resulting from concomitant exposure of α-conotoxin MII and indolizidine (−)-235B′ was not different from that produced by either inhibitor alone (Fig. 6, top). Further, the time course shows that inhibition produced by

![Fig. 4. (−)-237D (top) and ZZ-272 (bottom)](image)

![Fig. 5. α-Conotoxin MII concentration-dependent inhibition of nicotine-evoked \[^3H\]dopamine overflow from superfused rat striatal slices. Overflow data are expressed as mean ± S.E.M. α-Conotoxin MII concentration–response curve was generated by nonlinear regression. Control represents \[^3H\]dopamine overflow in response to 10 μM nicotine in the absence of antagonist. n = 6–7 rats.](image)
the concomitant exposure of inhibitors was not different from that following either inhibitor alone (Fig. 6, bottom). Two-way ANOVA revealed main effects of time (\(F_{2,205} = 17.5, P < 0.001\)) and concentration (\(F_{4,2} = 19.1, P < 0.001\)) and a time x concentration interaction (\(F_{4,205} = 2.21, P < 0.01\)). Thus, indolizidine (−)−235B interacts with α-conotoxin MII-sensitive nicotinic receptors. Mecamylamine (10 μM) was included as a positive control and inhibited completely (97%) nicotine-evoked [H]dopamine overflow.

In order to determine whether (−)−237D and ZZ-272 interact with α-conotoxin MII-sensitive nicotinic receptors, individual striatal slices were exposed to maximally inhibitory concentrations of (−)−237D (100 nM) or ZZ-272 (10 μM) either in the absence or presence of a maximally inhibitory concentration of α-conotoxin MII (1 nM). In the experiment with (−)−237D, one-way repeated measures ANOVA revealed an inhibitor-induced decrease in nicotine-evoked [H]dopamine overflow (\(F_{2,220} = 10.68, P < 0.001\)). Importantly, inhibition of nicotine-evoked [H]dopamine overflow by (−)−237D and α-conotoxin MII was no greater than that observed when striatal slices were exposed concurrently to these inhibitors (Fig. 7, top). In the experiment with ZZ-272, one-way repeated measures ANOVA also revealed an effect of inhibitor (\(F_{2,205} = 10.38, P < 0.001\)). Similarly, inhibition of nicotine-evoked [H]dopamine overflow produced by ZZ-272 or α-conotoxin MII alone was no greater than that observed with concurrent exposure to both inhibitors (Fig. 7, bottom). Analyses of the time courses from these experiments revealed that inhibition produced by concurrent exposure to analog plus α-conotoxin MII was not different from that following analog or α-conotoxin MII alone (data not shown). In the experiment with (−)−237D, two-way ANOVA revealed a main effect of time (\(F_{2,236} = 29.1, P < 0.0001\)) and a time x inhibitor interaction (\(F_{8,236} = 2.26, P < 0.001\)). In the experiment with ZZ-272, two-way ANOVA also revealed a main effect of time (\(F_{2,236} = 9.73, P < 0.001\)) and a time x inhibitor interaction (\(F_{8,236} = 2.00, P < 0.005\)). Thus, similar to indolizidine (−)−235B, (−)−237D and ZZ-272 interacts with α-conotoxin MII-sensitive nicotinic receptors.

4. Discussion

5,8-Disubstituted indolizines have been shown to noncompetitively inhibit carbamylcholine-evoked 22Na\(^+\)-influx in nicotinic receptor assays employing pheochromocytoma cells (Daly et al., 1991). Indolizidine (−)−235B, which contains an 8-methyl group and CS-hept-6-en-1-yl moiety, acts as a potent, voltage- and use-dependent antagonist at α4β2 nicotinic receptors expressed in Xenopus oocytes (Tsukui et al., 2004). In the latter study, indolizidine (−)−235B was found to be 6-, 51- and 54-fold more selective for α4β2 nicotinic receptors than for α7, α3β2 and α3β4 nicotinic receptors, respectively. The current results extend previous studies by demonstrating that indolizidine (−)−235B also inhibits \(K_{50} = 42 \text{nM}, I_{\text{max}} = 55\%\) nicotine-evoked [H]dopamine release from rat striatal slices, and moreover, acts as an antagonist at α-conotoxin MII-sensitive α6β2 nicotinic receptors mediating nicotine-evoked dopamine release. Similar to indolizidine (−)−235B, (−)−237D, the CS-hept-1-yl analog, and ZZ-272, a defunctionalized des-methylaza-analog, inhibited nicotine-evoked [H]dopamine release via α-conotoxin MII-sensitive α6β2 nicotinic receptors. These results provide support for the further structural elaboration of this novel structural scaffold for the discovery of selective antagonists at α6β2 nicotinic receptors as pharmacotherapies for smoking cessation.
To provide information on which elements of the indolizidine (−)-235B′ pharmacophore might be necessary for inhibition of nicotine-evoked [3H]dopamine release, a small series of C5 analogs and structural fragments of indolizidine (−)-235B′ were synthesized and evaluated for inhibition of nicotine-evoked [3H]dopamine release at 100 nM probe concentration. In one subset of analogs the C5-alkenyl moiety was varied in length to afford C5, C6 and C8 analogs, which retained the terminal olefin moiety in their structures (compounds (−)-207A, (−)-B-6, and (−)-B-8, respectively). Analog (−)-B-8, which contains an oct-7-en-1-yl substituent at C5 did not inhibit nicotine-evoked [3H]dopamine release. In contrast, analogs (−)-B-6 and (−)-207A, the C5-substituted hex-5-en-1-yl and pent-4-en-1-yl analogs, respectively, inhibited nicotine-evoked [3H]dopamine release by 15% and 36%, respectively. Thus, the carbon length of the C5-substituent may be a limiting structure-activity factor, and is optimal at C7. At the probe concentration, the reduced C5-hept-1-yl analog of indolizidine (−)-235B′, (−)-237D, produced 58% inhibition of nicotine-evoked [3H]dopamine overflow, which was not different from inhibition (62%) produced by the probe concentration of the parent compound. Full concentration response for (−)-237D revealed a 233-fold greater potency (IC50 = 0.18 nM, Imax = 76%) for inhibition of nicotine-evoked [3H]dopamine compared to indolizidine (−)-235B′ (IC50 = 42 nM, Imax = 55%), whereas maximal inhibition was not different. These results suggest that the terminal olefinic bond is not a structural requirement for activity, and that saturation of the terminal olefinic bond of indolizidine (−)-235B′ robustly increases potency at nicotinic receptors that mediate nicotine-evoked [3H]dopamine release.

Another subset of analogs evaluated the effect of removing the chiral 8-methyl group in indolizidine (−)-235B′. Both ZZ-254, the des-methyl analog of (−)-237D, and the related analog ZZ-275 were devoid of inhibitory activity at the probe concentration. However, the des-methyl analog ZZ-272, which contains a C5-2-azahexan-1-yl moiety, modestly decreased (31%) nicotine-evoked [3H]dopamine release. ZZ-272, the only analog in this subset to show inhibitory activity at the probe concentration, was selected for full concentration–response analysis and showed 10-fold lower potency (IC50 = 413 nM, Imax = 59%) and maximal inhibitory activity not different from indolizidine (−)-235B′. These findings suggest that removal of the chiral 8-methyl group from indolizidine (−)-235B′ is detrimental to inhibitory potency.

The final subset of compounds constituted structural fragments of indolizidine (−)-235B′. ZZ-270A, ZZ-270B and ZZ-257, and are simple des-methyl indolizidines bearing a carboxymethyl ester moiety at C5, and either a hydrogen, hydroxy group or keto group at C8. None of these fragment structures exhibited inhibitory activity at the probe concentration. Fragments HRS-1-97, ZZ-249B, ZZ-262 and ZZ-261 also showed no inhibitory activity at the probe concentration, demonstrating that the indolizidine ring system is a structural requirement for inhibitory activity.

Comprehensive studies employing transgenic mice that lack a single nicotinic receptor subunit suggest that 6 different receptor subtypes mediate nicotine-evoked dopamine release in mouse striatum (Salminen et al., 2004; Gotti et al., 2005; Salminen et al., 2007; Grady et al., 2002). These subtypes are subdivided into α-conotoxin MII-sensitive subtypes (i.e., α6β2*, α6β23*, α4α6β2* and α4α6β23*) and α-conotoxin MII-insensitive subtypes (i.e., α4β2* and α4α6β23*). Thus, α-conotoxin MII acts as an antagonist at α6β2*-containing nicotinic receptors, which is in contrast to mecamylamine, a nonselective nicotinic receptor antagonist inhibiting all known nicotinic receptor subtypes. In the current study, α-conotoxin MII potently (IC50 = 0.026 nM) inhibited nicotine-evoked [3H]dopamine release from rat striatal slices, consistent with our previous studies (IC50 = 0.03 nM; Smith et al., 2010). Also, in the current study, maximal inhibition produced by α-conotoxin MII was 66% of control, whereas mecamylamine produced ~95% inhibition, supporting the interpretation that α-conotoxin MII acts more selectively as an antagonist at a subpopulation of nicotinic receptors mediating nicotine-evoked [3H]dopamine release (Azam and McIntosh, 2005).

The amount of inhibition produced by indolizidine (−)-235B′, (−)-237D and ZZ-272 (Imax = 51%, 66% and 49%, respectively) is less than inhibition produced by mecamylamine, suggesting that these novel antagonists also act at a subpopulation of nicotinic receptors mediating nicotine-evoked [3H]dopamine release. Moreover, concomitant exposure to maximally inhibitory concentrations of indolizidine (−)-235B′ and α-conotoxin MII did not result in greater inhibition of nicotine-evoked [3H]dopamine release than that observed with either antagonist alone, suggesting that both act at α-conotoxin MII-sensitive nicotinic receptors (i.e., α6β2*-containing). Concomitant exposure of (−)-237D or ZZ-272 with α-conotoxin MII similarly did not result in greater inhibition than that observed with either antagonist alone. Based on the results of these interaction studies, indolizidine (−)-235B′, (−)-237D and ZZ-272 act at α6β2-containing nicotinic receptor subtypes that mediate nicotine-evoked dopamine release. The lack of an additive effect of concurrent exposure to α-conotoxin MII and indolizidine (−)-235B′ appears incongruous with previous findings showing that indolizidine (−)-235B′ also acts as an antagonist at α4β2 nicotinic receptors (Tsuneki et al., 2004). One interpretation of these results is that indolizidine (−)-235B′ does not inhibit α4β2 nicotinic receptors expressed in rat striatum. Support for this interpretation comes from comparison of previous results with dihydro-β-erythroidine (an antagonist at α4-containing nicotinic receptors). When dihydro-β-erythroidine is concomitantly exposed with a maximally inhibitory concentration of α-conotoxin MII, significantly greater inhibition of nicotine-evoked [3H]dopamine release is observed than with either antagonist alone (Smith et al., 2010). Thus, additivity of inhibitory activity has been found with α-conotoxin MII and an α4 antagonist, i.e., dihydro-β-erythroidine. Future studies employing genetically modified mice or in vitro expression systems will be important for providing additional evidence to support the suggestion that these analogs act as α6β2 subtype-selective antagonists.

In conclusion, the present study suggests that the potency of indolizidine (−)-235B′ to inhibit nicotine-evoked dopamine release is dependent upon specific structural elements within the molecule, including an intact indolizine ring and a 7-carbon n-alkyl or n-alkene chain at C5 of the indolizidine (−)-235B′ structure. Further, indolizidine (−)-235B′ and the structurally related analogs, (−)-237D and ZZ-272, interact with α-conotoxin MII-sensitive nicotinic receptor subtypes that mediate nicotine-evoked striatal dopamine release. Thus, the current findings suggest that 5,8-disubstituted indolizine ring represents a new structural scaffold for discovery of a novel class of potent nicotinic receptor antagonists that inhibit nicotine-evoked dopamine release.

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