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Subtype-selective nicotinic receptor antagonists: potential as tobacco use cessation agents

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Abstract—*N*-*n*-Alkylpicolinium and *N,N*′-alkyl-bis-picolinium analogues were assessed in nicotinic receptor (nAChR) assays. The most potent and subtype-selective analogue, *N,N*′-dodecyl-bis-picolinium bromide (bPiDDB), inhibited nAChRs mediating nicotine-evoked [^{3}H]dopamine release ($IC_{50} = 5$ nM; I_{max} of 60%), and did not interact with α4β2* or α7* nAChRs. bPiDDB represents the current lead compound for development as a tobacco use cessation agent.
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1. Introduction

Several lines of evidence suggest that subtype-selective nicotinic acetylcholine receptor (nAChR) antagonists may be efficacious tobacco use cessation agents. Bupropion, an antidepressant and nAChR antagonist that inhibits nAChR subtypes mediating nicotine-evoked dopamine (DA) release[1] has some efficacy as a tobacco use cessation agent.[2–4] Also, mecamylamine, a non-selective nAChR antagonist, shows some efficacy as a tobacco use cessation agent, although its use is limited by peripherally mediated autonomic side effects, mainly constipation.[5,6] Furthermore, the efficacy of nicotine replacement therapy may be derived from its ability to desensitize nAChRs, rather than due to its effects as a nAChR agonist. Based on these observations and hypotheses, we predict that subtype-selective nAChR antagonists will be efficacious tobacco use cessation agents with therapeutic advantages over currently available therapies.

Evidence suggests that the acute reinforcing actions of drugs of abuse, including nicotine, may be mediated by dopaminergic systems in the striatopallidal and extended amygdala systems.[7–9] Nicotine's rewarding properties are believed to result from activation of nAChRs located presynaptically on cell bodies and terminals of DA neuronal pathways, resulting in the release of DA.[10–16]

Subtype assignment of native nAChRs mediating nicotine-evoked DA release has not been conclusively established, but is based largely on inhibition of agonist-induced response by subtype-selective antagonists. The subtype selectivity of these antagonists has been defined based on their ability to inhibit nAChR subtypes of known subunit composition when expressed in cell systems. Thus, for example, neuronal-bungarotoxin (*n*-BTX) and α-conotoxin-MII (α-CTX MII) selectively inhibit acetylcholine-induced electrophysiological responses in *Xenopus* oocytes expressing the α3β2 subtype.[17,18] Moreover, n-BTX and α-CTX MII inhibit nicotine-evoked [^{3}H]DA overflow from striatal preparations,[18–23] implicating α3β2*-containing nAChR subtypes as mediating nicotine-stimulated DA release.

Importantly, α-CTX MII inhibited only 50% of nicotine-evoked [^{3}H]DA overflow, implicating the involvement of at least two nAChR subtypes in this response.[22,24] These additional subtypes may contain α4- and/or β4-subunits.[20,22,25,26] Studies using β2 or α4 knockout mice also implicate β2- and α4-containing nAChR subtypes in the mediation of DA release.[21,27–29] Rat substantia nigra neurons express mRNA for α3, α4, α5, α6, α7, β2, β3 and β4 subunits,[30–34] such that multiple nAChR subtypes may be involved in nicotine-evoked striatal DA release. Since DA-containing neurons in the substantia nigra express high levels of α6 and β3 mRNA,[31,33,35,36] their combination with α3 and β2 in the mediation of nicotine-evoked DA release is likely. More recently, nAChR subtypes containing α6 subunits

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have also been suggested to mediate nicotine-evoked DA release,[29,37–39] although multi-subunit combinations may be involved.

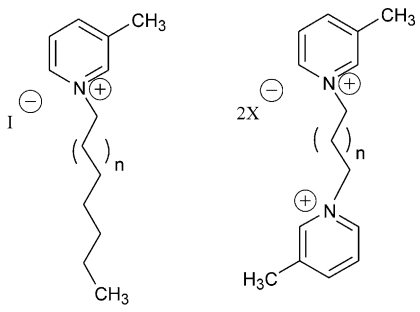
High μM concentrations of nicotine have also been shown to activate α7* nAChRs to stimulate [^{3}H]DA release, via an indirect mechanism, that is, stimulation of glutamate release, which in turn indirectly stimulates DA release.[12,40] Glutamate receptor antagonists inhibited ~20–50% of nicotine-evoked [^{3}H]DA release from striatal slices, but not from synaptosomes,[41] indicating that local circuitry within the slice is sufficient to reveal the indirect actions of nicotine on DA release.

The availability of novel compounds which selectively antagonize specific nAChR subtypes, would be invaluable for unraveling the complexity of nicotine's response. Furthermore, such subtype-selective nAChR antagonists may have potential as treatments for nicotine addiction.[42–45] Pyridine *N*-*n*-alkylation of nicotine affords *N*-*n*-alkylnicotinium analogues with alkyl chains of C_{1-12}. This structural modification converts nicotine from an agonist into high affinity, subtype-selective nAChR antagonists. *N*-*n*-Octylnicotinium iodide (NONI, C_8 analogue) competitively inhibits nicotine-evoked [^{3}H]DA overflow ($IC_{50} = 0.62$ μM), but does not inhibit [^{3}H]nicotine binding to rat brain membranes; while *N*-*n*-decylnicotinium iodide (NDNI, C_{10} analogue) is a competitive inhibitor of [^{3}H]nicotine binding and nicotine-evoked $^{86}Rb^+$ efflux, but does not inhibit nicotine-evoked [^{3}H]DA overflow from superfused striatal slices.[45,46] Thus, NONI and NDNI are selective and relatively potent nAChR antagonists. *N*-*n*-Dodecylnicotinium iodide (NDDNI, C_{12} analogue) has even higher affinity at nAChR subtypes mediating nicotine-evoked [^{3}H]DA overflow, however selectivity of this analogue for the nAChR subtype(s) mediating nicotine-evoked DA release is diminished, relative to that for NONI and NDNI.

The present report describes further optimization within the *N*-*n*-alkylnicotinium series. The current structure–activity approach is based on classical observations that hexamethonium chloride and decamethonium bromide, nAChR antagonists with bis-quaternary ammonium structures, distinguish between neuromuscular and ganglionic nAChR subtypes.[47–52] Thus, in the current study, nAChR subtype selectivity of the *N*-*n*-alkylpicolinium and *N,N*′-alkyl-bis-picolinium series was determined. The latter series has afforded our most promising lead candidate to date, that is, *N,N*′-dodecyl-bis-picolinium bromide (bPiDDB, C_{12} analogue), which inhibits nAChRs mediating nicotine-evoked [^{3}H]DA release with an $IC_{50} = 5$ nM and an I_{max} of 60%, but does not inhibit α4β2* nAChRs or α7* nAChRs, as determined in [^{3}H]nicotine and [^{3}H]methyllycaconitine (MLA) binding assays, respectively.

2. Chemistry

N-*n*-Alkylpicolinium analogues shown in Figure 1 (left) were prepared by reacting 3-picoline with the appropriate *n*-alkyl iodide utilizing previously described conditions.[42] The *N,N*′-alkyl-bis-picolinium analogues shown in Figure 1 (right) were prepared by reacting an excess of 3-picoline with a variety of diiodo- or dibromoalkanes for 24 h in the absence of solvent. The resulting solid was collected by filtration, dissolved in water and the aqueous solution was washed with diethyl ether (3×50 mL). The aqueous solution was then lyophilized to afford either a solid or viscous hygroscopic oil. All compounds were characterized by ^{1}H and ^{13}C NMR spectroscopy, mass spectroscopy and elemental analysis.[45]



NOPiI: n = 2
NDPiI: n = 4
NDDPiI: n = 6

bPiHxI: n = 4, X = I
bPiOI: n = 6, X = I
bPiNB: n = 7, X = Br
bPiDI: n = 8, X = I
bPiUB: n = 9, X = Br
bPiDDB: n = 10, X = Br

Figure 1. Structures of *N*-*n*-alkylpicolinium (left) and *N,N*′-alkyl-bis-picolinium (right) analogues.

3. Biological assays

Male Sprague–Dawley rats (225–250 g) were obtained from Harlan Industries (Indianapolis, IN) and housed two per cage with free access to food and water in the Division of Lab Animal Resources in the College of Pharmacy at the University of Kentucky. All experiments were carried out in accordance with the 1996 NIH 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.

[^{3}H]DA release assays were performed according to previously published methods,[1,45,53] with minor modifications. Striatal slices (500 μm, 4–6 mg) were incubated for 30 min in Krebs' buffer (in mM: 118 NaCl, 4.7 KCl, 1.2 $MgCl_2$, 1 NaH_2PO_4, 1.3 $CaCl_2$, 11.1 glucose, 25 $NaHCO_3$, 0.11 L-ascorbic acid and 0.004 disodium EDTA, pH 7.4, saturated with 95% O_2/5% CO_2) in a metabolic shaker at 34 °C. Slices were incubated with 0.1 μM (final concentration) [^{3}H]DA during the latter 30 min of the 60-min incubation period. Each slice was transferred to a glass superfusion chamber maintained at 34 °C and superfused (1 mL/min) with Krebs' buffer containing nomifensine (10 μM) and pargyline (10 μM) to inhibit [^{3}H]DA reuptake and metabolism, respectively, after release into the extracellular space, ensuring that [^{3}H]overflow primarily represents [^{3}H]DA.[54] Sample collection (5-min; 5 mL) began after 60 min

of superfusion. The ability of *N*-*n*-alkylpicolinium and *N,N′*-alkyl-bis-picolinium analogues to evoke [^{3}H]DA release (that is, exhibit intrinsic activity) and to inhibit nicotine-evoked [^{3}H]DA release (that is, act as nAChR antagonists) was determined. To establish that these analogues act as nAChR antagonists, by definition, inhibition of the response to nicotine must be observed at analogue concentrations that do not evoke a response. Thus, the ability of these analogues to elicit intrinsic activity (evoke [^{3}H]DA overflow) was determined. At the end of the experiment, each slice was solubilized and [^{3}H]content of the tissue determined. Release during each min was normalized for total [^{3}H]content of the slice. Analogue-induced intrinsic activity and inhibitory activity were determined using slices from the same rat (repeated-measures design).

Fractional release was calculated by dividing the total tritium collected in each sample by the total tritium in the tissue at the time of sample collection. The sum of all the increases in [^{3}H]DA fractional release resulting from either exposure to analogue or nicotine equaled 'total [^{3}H]DA overflow'. 'Overflow', rather than 'release', is the more correct terminology because the neurotransmitter measured is the net result of release and reuptake. Typically, data were analyzed by weighted, least squares regression analysis of sigmoidal concentration–effect curves to obtain EC_{50} and IC_{50} values.

Interaction of the analogues with nAChR subtypes probed by [^{3}H]nicotine binding (α4β2*) and [^{3}H]MLA binding (α7*) to rat brain membranes was determined to assess nAChR subtype selectivity of the analogues.[46] [^{3}H]Nicotine and [^{3}H]MLA binding assays were performed using whole brain, excluding cortex and cerebellum. Whole brain was homogenized in 20 vol of ice-cold buffer (in mM: 2 HEPES, 11.8 NaCl, 0.48 KCl, 0.25 $CaCl_2$ and 0.12 $MgSO_4$, pH 7.5). Homogenates were centrifuged (25,000*g*, 15 min, 4 °C). Pellets were resuspended in 20 vol of buffer and incubated at 37 °C, for 10 min, cooled to 4 °C and centrifuged (25,000*g*, 15 min, 4 °C). Pellets were resuspended and centrifuged again using the same conditions. Final pellets were stored at −70 °C in assay buffer (in mM: 20 HEPES, 118 NaCl, 4.8 KCl, 2.5 $CaCl_2$, and 1.2 $MgSO_4$, pH 7.5). Upon use, final pellets were resuspended in ∼20 vol assay buffer. Samples (250 μL) contained 100–140 μg of membrane protein, 3 nM [^{3}H]nicotine or 3 nM [^{3}H]MLA, and a range of concentrations (0.1 μM–1 mM) of analogue in assay buffer containing 50 mM Tris. Control was in the absence of analogue. In [^{3}H]nicotine and [^{3}H]MLA binding assays, nonspecific binding was determined in the presence of 10 μM nicotine and 10 μM MLA, respectively. Incubation proceeded for 60 min at room temperature using 96-well plates and was terminated by harvesting on Unifilter-96 GF/B filter plates, presoaked in 0.5% polyethylenimine, using a Packard FiterMate harvester. After washing 5 times with 350 μL ice-cold assay buffer, filter plates were dried (60 min, 4 °C), bottom-sealed, and filled with Packard's MicroScint 20 cocktail (40 μL/well). After 60 min, filter plates were top-sealed, and radioactivity determined. Protein concentration was determined using BSA as the standard.[55]

4. Results and discussion

Table 1 shows the inhibitory activity of the *N*-*n*-alkylpicolinium analogues with carbon chain lengths of C_8–C_{12} in the nicotine-evoked [^{3}H]DA overflow assay and in the [^{3}H]nicotine and [^{3}H]MLA binding assays. This series of analogues was relatively selective for the nAChR subtype(s) mediating nicotine-evoked [^{3}H]DA overflow, in that, these analogues did not inhibit binding of either [^{3}H]nicotine or [^{3}H]MLA to rat brain membranes, indicating low affinity for or no interaction with α4β2* and α7* nAChRs.

In the nicotine-evoked [^{3}H]DA overflow assay, NDDPiI at concentrations of ≥1.0 μM evoked [^{3}H]DA overflow, and thus, exhibited intrinsic activity; whereas the C_8 and C_{10} analogues demonstrated no intrinsic activity up to 1.0 μM (data not shown). Importantly, as the carbon chain length was increased the inhibitory potency at nAChRs mediating nicotine-evoked [^{3}H]DA overflow increased with a rank order of NDDPiI > NDPiI > NOPiI (Table 1). The most potent analogue in the series was NDDPiI (IC_{50} = 30 nM). Furthermore, NDDPiI inhibited nicotine-evoked [^{3}H]DA overflow by 63%, indicating that this analogue may interact with a single subtype of nAChR that mediates this effect of nicotine.

Table 2 illustrates the ability of a series of *N,N′*-bis-alkylpicolinium analogues with carbon chain lengths of C_6–C_{12} to inhibit nicotine-evoked [^{3}H]DA overflow and the binding of [^{3}H]nicotine and [^{3}H]MLA to rat brain

Table 1. Inhibitory activity of *N*-*n*-alkylpicolinium iodide analogues at native nAChRs

Compd	Nicotine-evoked [^{3}H]DA overflow IC_{50} μM[a]	[^{3}H]Nicotine binding K_i μM[b]	[^{3}H]MLA binding K_i μM[b]
NOPiI	1.0 (±0.09)	na	na
NNPiI	nd	62 (±17)	na
NDPiI	0.3 (±0.05)	26 (±3.6)	na
NUPiI	nd	na	na
NDDPiI	0.03 (±0.02)	na	na

[a] Values are means of three to five independent experiments, standard error is given in parentheses (nd = not determined).
[b] Values are means of four independent experiments, standard error is given in parentheses (na = not active).

Table 2. Inhibitory activity of *N,N′-bis*-picolinium salts at native nAChRs

Compd	Nicotine-evoked [^{3}H]DA overflow (IC_{50} μM)[a]	[^{3}H]Nicotine binding (K_i μM)[b]	[^{3}H]MLA binding (K_i μM)[b]
bPiHxI	1.66 (±0.85)	na	na
bPiOI	0.01 (±0.009)	na	na
bPiNB	1.52 (±0.34)	80 (±17)	na
bPiDI	0.03 (±0.01)	na	na
bPiUB	1.61 (±1.08)	69 (±29)	na
bPiDDB	0.005 (±0.003)	49 (±17)	na

[a] Values are means of four to six independent experiments, standard error is given in parentheses.
[b] Values are means of four independent experiments, standard error is given in parentheses (na = not active).

membranes. None of the analogues in this series inhibited either [^{3}H]nicotine or [^{3}H]MLA binding with high affinity, indicating no affinity at α7* nAChRs and only low affinity at α4β2* nAChRs.

Furthermore, none of the analogues in this series evoked [^{3}H]DA overflow (data not shown), and thus, did not exhibit intrinsic activity at subtypes mediating nicotine-evoked DA release. Interestingly, the most active analogues in this series are those with the longer *n*-alkyl chain length and with an even number of carbon atoms (Table 2). Moreover, bPiDDB (C_{12} analogue) was the most potent ($IC_{50} = 5$ nM) inhibitor of the nAChR subtype mediating nicotine-evoked [^{3}H]DA overflow. bPiDDB is also selective for the nAChR subtype mediating nicotine-evoked [^{3}H]DA overflow, since it has low or no affinity for the α4β2* and α7* nAChR subtypes (Table 2). Thus, bPiDDB is a selective inhibitor of the nAChR subtype that mediates nicotine-evoked [^{3}H]DA overflow. Furthermore, bPiDDB is ~320-fold more potent than DHβE in inhibiting nicotine-evoked [^{3}H]DA overflow.[45] Importantly, bPiDDB and the other analogues in this series inhibited [^{3}H]DA overflow by a maximum of 60%, similar to the maximum inhibition observed for α-conotoxin-MII,[22,24] suggesting that this small synthetic molecule (bPiDDB) and the neurtoxic *Conus* peptide of higher molecular weight may be acting at the same nAChR subtype to inhibit nicotine-evoked DA release.

bPiDDB appears to be an excellent candidate for further study, since it has 4–5 orders of magnitude higher affinity for the nAChR subtype mediating nicotine-evoked [^{3}H]DA overflow compared to its affinity at both α4β2* and α7* nAChRs. Thus, based on this preclinical data, bPiDDB or related analogues have the potential to diminish the rewarding effects produced by nicotine self-administration, and as such may serve as tobacco use cessation agents.

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