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# Development of subtype-selective ligands as antagonists at nicotinic receptors mediating nicotine-evoked dopamine release

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**Abstract**—*N*-*n*-Alkylation of nicotine converts it from an agonist into an antagonist at neuronal nicotinic acetylcholine receptor subtypes mediating nicotine-evoked dopamine release. Conformationally restricted analogues exhibit both high affinity and selectivity at this site, and are able to access the brain due to their ability to act as substrates for the blood–brain barrier choline transporter.


## 1. Introduction

Considerable effort has been focused on the development of neuronal nicotinic receptor (nAChR) agonists as therapeutic agents.[1–6] On the other hand, relatively few studies have focused on the therapeutic development of nAChR antagonists. Thus, only a few subtype-selective antagonists are currently available for use as pharmacological tools to investigate the physiological roles of specific nAChR subtypes.[7,8]

The rationale for the current research is based on the finding that nicotine stimulates all known nAChR subtypes, and that *N*-quaternization of nicotine converts it from an agonist into an antagonist with enhanced nAChR subtype selectivity. Several classical nAChR antagonists are bis-quaternary ammonium structures. Hexamethonium chloride and decamethonium bromide, both bis-quaternary ammonium salts, are considered simplified analogues of D-tubocurarine.[9,10] The latter drugs have been used to distinguish between peripheral nAChR subtypes, specifically neuromuscular and ganglionic nAChRs.[10–14] More recently, quaternary ammonium *N*-*n*-alkyl analogues of nicotine have been reported to be nAChR subtype-selective antagonists.[15–20] These latter findings prompted the evaluation of a series of quaternary ammonium compounds containing cationic azaaromatic head groups for their interaction with α4β2* and α7* nAChRs (asterisks indicate the putative nature of the designation of the native nAChR subtype[8]), as well as their interaction with the as yet to be elucidated nAChR subtype mediating nicotine-evoked dopamine release.

While it is generally believed that quaternary ammonium compounds do not easily access the brain due to their charge and polarity, precedence from the literature shows that such compounds have the ability to access the brain via several mechanisms, including the blood–brain barrier choline transporter.[21]

The hypothesis for the current study is that nAChR subtype-selective and brain-bioavailable antagonists will result from modification of the nicotine molecule by (1) quaternization of the pyridine-*N* atom with a lipophillic substituent to afford *N*-substituted analogues, and (2) modifying the structure of the nicotinium cationic head group.

## 2. Chemistry

Compounds **1–3** (Fig. 1) were prepared by reacting *S*-(−)-nicotine with the appropriate *n*-alkyl iodide in

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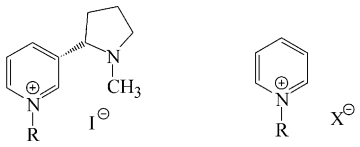
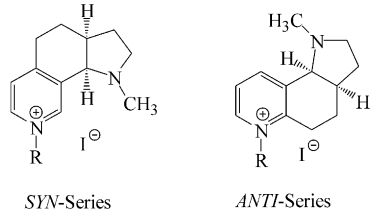


**1.** NONI: R = $nC_8H_{17}$
**2.** NDNI: R = $nC_{10}H_{21}$
**3.** NDDNI: R = $nC_{12}H_{25}$
**4.** NDPI: R = $nC_{10}H_{21}$, X = I
**5.** NDDPI: R = $nC_{12}H_{25}$, X = I
**6.** NPDPB: R = $nC_{15}H_{31}$, X = Br
**7.** NEcPB: R = $nC_{20}H_{41}$, X = Br

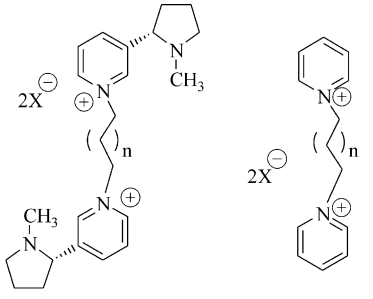


*SYN*-Series *ANTI*-Series

**8.** ACO: R = $nC_8H_{17}$
**9.** ACN: R = $nC_9H_{19}$
**10.** ACD: R = $nC_{10}H_{21}$
**11.** ACU: R = $nC_{11}H_{23}$
**12.** ACDD: R = $nC_{12}H_{25}$
**13.** BCO: R = $nC_8H_{17}$
**14.** BCN: R = $nC_9H_{19}$
**15.** BCD: R = $nC_{10}H_{21}$
**16.** BCU: R = $nC_{11}H_{23}$
**17.** BCDD: R = $nC_{12}H_{25}$

**Figure 1.** Structures of *N*-*n*-alkylnicotinium (**1**–**3**), *N*-*n*-alkylpyridinium (**4**–**7**), and conformationally restricted *N*-*n*-alkylnicotinium (**8**–**17**) iodides and bromides.

glacial acetic acid, utilizing the procedure described by Crooks et al.[15] The *N*-*n*-alkylpyridinium salts **4**–**7** (Fig. 1) were obtained via *n*-alkylation of pyridine with the appropriate *n*-alkyl halide.[22] The conformationally restricted racemic *syn*- and *anti*-nicotine analogues **8**–**12** and **13**–**17**, respectively (Fig. 1), were prepared via regiospecific alkylation of the pyridine-*N* atom of the corresponding free base.[20,23,24] The *syn*-free base was synthesized from 7-aza-1-tetralone,[23] and the *anti*-free base was prepared from 5-aza-1-tetralone.[25] *N,N′*-Dodecanediyl-bis-nicotinium dibromide, **18** (Fig. 2), was prepared by dissolving *S*-(−)-nicotine in glacial acetic acid, stirring the mixture for 5 min and then adding dibromododecane. The solution was stirred under reflux for three days, the solvent was evaporated under reduced pressure, and the resulting residue was treated with an aqueous saturated solution of $NaHCO_3$. The resulting mixture was then extracted with diethyl ether (3×50 mL), and then with chloroform (3×50 mL). The aqueous layer was collected and lyophilized for 24 h, and the resulting solid was triturated with chloroform. After filtration, the filtrate was dried over anhydrous $MgSO_4$ and removal of solvent afforded the bis-nicotinium salt, **18**. *N,N′*-Dodecanediyl-*bis*-pyridinium dibromide, **19** (Fig. 2) was prepared by reacting an excess of pyridine with 1,12-dibromododecane for 24 h at 65 °C in the absence of solvent. The resulting solid was collected by filtration, dissolved in water, and the aqueous solution washed with diethyl ether (3×50 mL). The aqueous solution was then lyophilized to afford **19** as a crystalline solid.



**18.** bNDDB: n=12, X=Br **19.** bPDDB n=12, X=Br

**Figure 2.** Structures of *N,N′*-dodecanediyl-bis-nicotinium dibromide (**18**, bNDDB) and *N,N′*-dodecanediyl-*bis*-pyridinium dibromide (**19**, bPDDB).

All compounds were characterized by $^1H$ and $^{13}C$ NMR spectroscopy, mass spectroscopy and elemental analysis.

## 3. Biological assays

### 3.1. Subjects

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.

### 3.2. [$^3$H]-Dopamine overflow from superfused striatal slices

[$^3$H]-Dopamine release assays were performed according to previously published methods,[26–28] with minor modifications. Striatal coronal slices (500 μm, 4–6 mg) were obtained and 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) of [$^3$H]-dopamine 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]-dopamine reuptake after release into the extracellular space, ensuring that [$^3$H]-overflow primarily represented [$^3$H]-dopamine.[29] Sample collection (5-min; 5 mL) began after 60 min of superfusion. The ability of *N*-*n*-alkylated nicotinium, *N*-*n*-alkylated pyridinium, conformationally restricted *N*-*n*-alkylated nicotinium and bis-azaaromatic quaternary ammonium analogues to evoke [$^3$H]-dopamine release (intrinsic activity) and to inhibit nicotine-evoked [$^3$H]-dopamine release (act as antagonists) were determined. At the end of the experiment, each slice was solubilized, and the [$^3$H]-content of the tissue determined. Release during each min was normalized for total tissue [$^3$H]-content. Analogue-induced intrinsic activity and inhibitory activity were determined using slices from the same rat (repeated-measures design).

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

### 3.3. [$^3$H]-Nicotine ($\alpha4\beta2$* subtype) and [$^3$H]-methyllycaconitine ($\alpha7$* subtype) binding assays

Whole brain, excluding cortex and cerebellum, was homogenized in 20 vol of ice-cold buffer, containing (in mM): 2 HEPES, 11.8 NaCl, 0.48 KCl, 0.25 $CaCl_2$ and 0.12 $MgSO_4$, pH 7.5. Homogenate was 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 using the same conditions. Final pellets were stored in assay buffer, containing (in mM): 20 HEPES, 118 NaCl, 4.8 KCl, 2.5 $CaCl_2$, and 1.2 $MgSO_4$) pH 7.5 at −70 °C. 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]-methyllycaconitine, and analogue (0.1 μM–1 mM) in assay buffer containing 50 mM Tris. Control was in the absence of analogue. In [$^3$H]-nicotine and [$^3$H]-methyllycaconitine binding assays, nonspecific binding was determined in the presence of 10 μM nicotine, and 10 μM methyllycaconitine, respectively. Incubations proceeded for 60 min at room temperature using 96-well plates and were 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 using a Packard TopCount. Protein concentrations were determined using bovine serum albumin as the standard.[30]

### 3.4. Blood–brain barrier choline transporter affinity assays

Quaternary ammonium analogues were evaluated for their ability to inhibit [$^3$H]-choline uptake into brain providing an indication of the ability of these analogues to interact with the choline transporter. These assays were conducted using the in situ rat brain perfusion method of Takasato et al.,[31] as modified by Allen and Smith.[32,33] Inhibition coefficients ($K_i$, concentration of analogue inhibiting 50% of [$^3$H]-choline uptake into brain) were determined using a single inhibitor concentration as previously described.[34] $K_i$ values were compared by ANOVA followed by Bonferoni's multiple comparisons test to determine if an analogue inhibits [$^3$H]-choline uptake.

## 4. Results

During the development of nAChR receptor antagonists, we discovered that structural modification of the nicotine molecule converted nicotine into a series of *N*-*n*-alkylnicotinium analogues exhibiting antagonist activity at specific nAChR subtypes, and several of these antagonists showed both high affinity and subtype-selectivity (Table 1).[15–19] Furthermore, when the pyridine-*N* atom of nicotine is *n*-alkylated with chain lengths $\geq C_6$, no intrinsic activity at native nAChRs in the dopamine release and $^{86}Rb^+$ efflux assays was observed. Moreover, nicotine-evoked dopamine release from striatal slices and nicotine-evoked $^{86}Rb^+$ efflux from thalamic synaptosomes (a functional assay for the $\alpha4\beta2$* receptor) was inhibited by these analogues.[17,19]

### 4.1. Analogue inhibition of nicotine-evoked dopamine overflow

Analogue-induced inhibition of nicotine-evoked dopamine release was striking, in that inhibitory potency was linearly related to *n*-alkyl chain length. Thus, the greater the number of carbons in the *n*-alkyl chain, the greater the potency (that is, the lower the $IC_{50}$ value) for inhibition of nicotine-evoked [$^3$H]-dopamine overflow. Compounds bearing *n*-alkyl groups from $C_1$ to $C_4$ were low potency antagonists ($IC_{50} > 10$ μM); the most potent compound was the $C_{12}$ analogue, NDDNI ($IC_{50} = 9$ nM) (Table 1), indicating that a relatively long *n*-alkyl chain is necessary for potent inhibition. Analogues with chain lengths $> C_{12}$ were not examined, due to poor water solubility. In the regression analysis of linearity between *n*-alkyl chain length and inhibitory activity, an orderly progression in potency from $C_1$ (*N*-methylnicotinium iodide; NMNI) to $C_{12}$ (NDDNI) was observed, with the exception of the *n*-decyl analogue, NDNI, which unexpectedly did not exhibit inhibitory activity at the nAChR subtype mediating nicotine-evoked dopamine release (Table 1). NDNI is believed to exist in solution in a unique conformation that is different from the conformations of the other *N*-*n*-alkylnicotinium analogues.[18] The most potent antagonists in this series of analogues produced 80–100% maximal inhibition of the response to nicotine. Thus, these *N*-*n*-alkylnicotinium analogues represent a new class of nAChR antagonist, and the most potent compound, NDDNI, is at least two orders of magnitude more potent than the classical antagonist, dihydro-β-erthyroidine (DHβE) as

**Table 1.** Evaluation of *N*-*n*-alkylnicotinium 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] |
|---|---|---|---|
| NONI | 0.62 (0.20–1.9) | 20 (15–25) | 12 (9.2–16) |
| NDNI | na | 0.09 (0.08–0.11) | na |
| NDDNI | 0.009 (0.003–0.03) | 0.14 (0.11–0.17) | na |

[a] Values are means of 3–10 independent experiments, 95% confidence interval is given in parentheses (na = not active).
[b] Values are means of 4–10 independent experiments, 95% confidence interval is given in parentheses (na = not active).

an inhibitor of nAChRs mediating nicotine-evoked dopamine release. In addition, kinetic studies (Schild analysis) indicate that the $C_8$ compound, NONI interacts in a competitive manner with these receptors.[18]

Unlike agonist molecules, antagonists which are generally larger molecules, have been proposed to dock onto the agonist-binding site, but extend beyond the region of agonist binding.[35] The additional structural bulk associated with antagonist molecules is proposed to prevent the receptor protein from achieving the open channel form.[36] In this respect, the active *N*-*n*-alkylnicotinium analogues are of significantly larger molecular weight than nicotine, and the sterically bulky *N*-*n*-alkyl chain may interact within a hydrophobic cavity extending outside the normal volume for agonist binding to the receptor. *N*-*n*-Alkylnicotinium analogues have been proposed to interact with the nAChR mediating nicotine-evoked dopamine release in the unprotonated form, leading to a reversal in the role of the pharmacophoric *N*-containing moieties.[15] Thus, the quaternary pyridinium *N*-atom of the antagonist molecule is believed to interact with the binding site that normally accommodates the protonated pyrrolidine *N*-atom in the agonist binding mode, and the unprotonated pyrrolidine *N*-atom of the antagonist molecule substitutes for the pyridine *N*-atom of the agonist molecule at the hydrogen-bonding site of the nAChR. Alternatively, these *N*-*n*-alkylnicotinium molecules may interact with the nicotine binding site in a manner allowing free positioning of the *n*-alkyl chain into the receptor ion channel, thereby sterically inhibiting ion flux through the channel.

### 4.2. Analogue inhibition of [$^3$H]-nicotine and [$^3$H]-MLA binding

The above *N*-*n*-alkylnicotinium analogues have also been evaluated for their affinity for α4β2* and α7* nAChRs (Table 1). These compounds generally exhibited low affinity for α7* nAChRs. In addition, these *N*-*n*-alkylnicotinium analogues exhibited affinity for [$^3$H]-nicotine binding sites across a ~200-fold concentration range, from ~90 nM (NDNI) to ~20 μM (NONI).[20] A simple linear relationship was observed between the length of the *n*-alkyl chain and affinity for the [$^3$H]-nicotine binding site, with the exception of the $C_8$ analogue, NONI. Thus, analogues with longer chain lengths ($C_9$, $C_{10}$ and $C_{12}$) were more potent inhibitors than analogues with shorter chain lengths ($C_1$–$C_7$). Higher affinity of longer *n*-alkyl chain analogues for [$^3$H]-nicotine binding sites may reflect a stronger association with the nAChR due to an increased lipophilic interaction of the carbon chain with a region of the protein near the [$^3$H]-nicotine binding pocket, likely rich in hydrophobic amino acid residues. Thus, the lipophilic interaction may stabilize the analogue–receptor complex, increasing the inhibitory potency of longer chain analogues.

Scatchard analyses of [$^3$H]-nicotine saturation binding in the absence and presence of NONI and NDNI indicated that affinity for [$^3$H]-nicotine binding sites decreased in the presence of increasing concentrations of either NDNI or NONI, with no change in $B_{max}$ value, indicating that these compounds interact with the high affinity [$^3$H]-nicotine binding site in a competitive manner, suggesting interaction with specific amino acid residues involved in [$^3$H]-nicotine binding or with nearby residues allowing for steric hindrance of [$^3$H]-nicotine binding.

The high affinity of NONI to inhibit nicotine-evoked [$^3$H]-dopamine release from superfused striatal slices and its low affinity for the [$^3$H]-nicotine and [$^3$H]-methyllycaconitine binding sites, indicate that NONI has selectivity for nAChR subtypes mediating nicotine-evoked dopamine release in striatum. The high affinity of NDNI combined with its lack of affinity for the [$^3$H]-methyllycaconitine binding sites and its lack of inhibition of nicotine-evoked [$^3$H]-dopamine release from superfused rat striatal slices, indicates that NDNI has selectivity for α4β2* nAChRs. Thus, *N*-*n*-alkylnicotinium analogues may be useful ligands for defining structural topographies of recognition sites of nAChR subtypes through computer-assisted modeling.

In subsequent studies, simple *N*-*n*-alkylpyridiniums, with alkyl chain lengths ranging from $C_1$ to $C_{20}$, exhibited moderate to low affinity for nAChR subtypes mediating nicotine-evoked DA release and for α4β2* nAChRs ($IC_{50}$ = 90–530 nM and $K_i$ = 9–44 μM, respectively; Table 2).[22]

These compounds also lacked affinity for α7* nAChRs. These data demonstrate the importance of the 3-(2′-pyrrolidino) moiety in the *N*-*n*-alkylnicotinium series of compounds for potent inhibition of nAChR subtypes mediating nicotine-evoked dopamine release.

We have recently reported the synthesis and nAChR receptor properties of some conformationally restricted analogues of the above *N*-*n*-alkylnicotinium compounds (Table 3).[20] These analogues were designed to assess the rotameric preference about the C3–C2′ bond of NONI and NDNI for interaction with nAChR subtypes. Two classes of bridged nicotinium analogues, representing extreme rotameric conformations (i.e., *syn*- and *anti*-rotamers, Fig. 1) were examined. Interestingly, these conformationally restrained analogues lacked affinity for α4β2* and α7* nAChRs, as determined from [$^3$H]-nicotine and [$^3$H]-methyllycaconitine binding assays. More importantly, with the exception of BCDD, these

**Table 2.** Inhibitory activity of simple *N*-*n*-alkylpyridinium 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] |
|---|---|---|---|
| NDPI | 0.13 (0.02–0.87) | 17 (14–20) | na |
| NDDPI | 0.26 (0.02–4.23) | na | na |
| NPDPB | 0.32 (0.11–0.87) | 38 (28–51) | na |
| NEcPB | 0.12 (0.01–2.38) | na | na |

[a] Values are means of 5–6 independent experiments, 95% confidence interval is given in parentheses.
[b] Values are means of four independent experiments, 95% confidence interval is given in parentheses (na = not active).

**Table 3.** Inhibitory activity of simple *N*-*n*-alkylpyridinium analogues at native nAChRs

| Compd | Nicotine-evoked [$^3$H]DA overflow $IC_{50}$ μM[a] | [$^3$H]Nicotine binding[b] | [$^3$H]MLA binding[b] |
|---|---|---|---|
| ACO | 0.08 (±0.04) | na | na |
| ACN | 0.66 (±0.03) | na | na |
| ACD | 0.58 (±0.55) | na | na |
| ACU | 0.04 (±0.02) | na | na |
| ACDD | 0.22 (±0.15) | na | na |
| BCO | 0.04 (±0.02) | na | na |
| BCN | 0.31 (±0.15) | na | na |
| BCD | 0.03 (±0.01) | na | na |
| BCU | 0.04 (±0.03) | na | na |
| BCDD | na | na | na |

[a] Values are means of 6–8 independent experiments for the DA release assay, standard error is given in parentheses (na = not active).
[b] Determined at a single 10 μM concentration in four independent experiments.

analogues potently and selectively inhibited nicotine-evoked [$^3$H]-dopamine release ($IC_{50}$ values 30–660 nM), although no clearly defined structure–activity trends could be determined. These analogues may be interacting with multiple nAChR subtypes mediating nicotine-evoked dopamine release. Alternatively the lack of clear structure–activity relationships within this group may be due to the fact that these molecules are racemic in nature. It is conceivable that the optical isomers of each compound may exhibit different affinities at the nAChR subtypes examined, thus confounding the structural analysis.

The bis-azaaromatic quaternary ammonium analogues: *N,N′*-dodecanediyl-bis-nicotinium dibromide (**18**, Fig. 2), and *N,N′*-dodecanediyl-*bis*-pyridinium dibromide (**19**, Fig. 2), have been synthesized and evaluated for their inhibitory properties at nAChR subtypes (Table 4).[37] $C_{12}$ analogues were studied, based on our previous findings that the $C_{12}$ analogue (NDDNI) in the *N*-*n*-alkylnicotinium series was the most potent as an inhibitor of the nAChR subtype mediating nicotine-evoked [$^3$H]-dopamine overflow. These compounds were not potent or selective for the subtype mediating nicotine-evoked dopamine release.

### 4.3. Analogue inhibition of [$^3$H]-choline uptake by the blood–brain barrier choline transporter

Importantly, several *N*-*n*-alkylnicotinium analogues have been shown to have good affinity for the blood–brain barrier choline transporter.[21] Specifically, NONI, inhibited [$^3$H]-choline uptake (Table 5) with an apparent $K_i$ value of 49 μM (±24 μM). The $C_1$ analogue, NMNI, showed low affinity for the transporter, with an apparent $K_i$ value of >1000 μM. The $C_{10}$ analogue, NDNI, inhibited [$^3$H]-choline uptake into brain with an apparent $K_i$ value of 27 μM (±2 μM), similar to NONI and choline (data not shown). The $C_4$ analogue (*N*-*n*-butylnicotinium iodide; NBNI) had an apparent $K_i$ value of 777 ± 590 μM. These results suggest that increasing the length of the *N*-*n*-alkyl chain in these *N*-*n*-alkylnicotinium analogues may facilitate transporter binding, and thus, enhance brain uptake via the blood–brain barrier choline transporter.

**Table 4.** Inhibitory activity of *N,N′-bis*-azaaromatic quaternary ammonium $C_{12}$ analogues, bNDDB and bPDDB, 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] |
|---|---|---|---|
| bNDDB | 0.17 (±0.13) | 1.95 (±0.19) | na |
| bPDDB | 1.00 (±0.38) | 9.15 (±0.17) | 33 (±4.6) |

[a] Values are means of 4–6 experiments, standard error is given in parentheses.
[b] Values are means of 3–4 experiments, standard error is given in parentheses (na = not active).

**Table 5.** Affinity of *N*-*n*-alkylcholine analogues and *N*-*n*-alkylnicotinium analogues for the blood brain barrier choline transporter

| Compd | Concentration μM[a] | $K_i$ μM[b] |
|---|---|---|
| *N*-*n*-Octylcholine | 10 | 1.7 (±0.3) |
| *N*-*n*-Hexylcholine | 10 | 2.2 (±0.1) |
| *N*-*n*-Octylpyridinium iodide | 250 | 32 (±22) |
| NBNI | 250 | 777 (±590) |
| NONI | 250 | 49 (±24) |
| NDNI | 250 | 27 (±0.1) |

[a] Values are means of 3–5 independent experiments, standard error is given in parentheses.
[b] In vitro therapeutic index ($IC_{50}$ cytotoxicity/$IC_{50}$ complement inhibition).

### 4.4. [$^3$H]-NONI brain uptake

Considering the above $K_i$ value for NONI, experiments were performed to verify that this compound gains access to brain via the blood–brain barrier choline transporter. Thus, brain distribution parameters of [$^3$H]-NONI were determined.[21] Uptake of [$^3$H]-NONI (1 μCi/mL) into rat brain was evaluated from 0–60 s in the absence of unlabeled NONI. The transfer coefficient value ($K_{in}$) for [$^3$H]-NONI uptake was $1.59 \pm 0.14 \times 10^{-3}$ mL/s/g, calculated from the slope of the compound accumulating in brain versus time.[36] An uptake time of 45 s was chosen as within the linear portion of the brain uptake curve in order to evaluate [$^3$H]-NONI brain uptake in the presence of unlabeled NONI. Unlabeled NONI (250 μM) in the perfusion fluid resulted in 46% inhibition of [$^3$H]-NONI brain uptake, suggesting saturable kinetic parameters associated with NONI transport into brain.

The ability of choline to inhibit [$^3$H]-NONI uptake into brain and [$^3$H]-NONI distribution parameters were also determined. The permeability-suface area product (PA; mL/s/g) for [$^3$H]-NONI with no inhibitors present was $1.64 \pm 0.37 \times 10^{-3}$ mL/s/g, determined as a single time point PA value. If NONI is transported in total, or in part, by the blood–brain barrier choline transporter, then addition of choline to the perfusion fluid should reduce brain uptake of [$^3$H]-NONI. When 500 μM choline was added to the perfusion fluid the PA for [$^3$H]-NONI decreased ~25% to $1.24 \pm 0.5 \times 10^{-3}$ mL/s/g. A higher concentration (5 mM) of choline further reduced the uptake of [$^3$H]-NONI to $7.55 \pm 3.30 \times 10^{-4}$ mL/s/g,

which was $<50\%$ of the uptake in the absence of choline. These results suggest that a significant component of NONI uptake occurs via the blood–brain barrier choline transporter.

## 5. Summary

In summary, rotameric restriction of *N*-*n*-alkylnicotinium iodides eliminates inhibitory activity at both $\alpha4\beta2^*$ and $\alpha7^*$ nAChRs, and affords high affinity and selectivity for nAChRs mediating nicotine-evoked dopamine release from striatum. Thus, conformational restriction of *N*-*n*-alkyl analogues of nicotine appears to be a useful approach in the development of subtype-selective inhibitors for nAChRs mediating nicotine-evoked dopamine release.

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