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ORIGINAL PAPER

r-bPiDI, an α6β2* Nicotinic Receptor Antagonist, Decreases Nicotine-Evoked Dopamine Release and Nicotine Reinforcement

Joshua S. Beckmann[1] · Andrew C. Meyer[1] · M. Pivavarchyk[2] · David B. Horton[2] · Guangrong Zheng[3] · Andrew M. Smith[2] · Thomas E. Wooters[1] · J. Michael McIntosh[4] · Peter A. Crooks[3] · Michael T. Bardo[1] · Linda P. Dwoskin[2]

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Abstract α6β2* nicotinic acetylcholine receptors (nAChRs) expressed by dopaminergic neurons mediate nicotine-evoked dopamine (DA) release and nicotine reinforcement. α6β2* antagonists inhibit these effects of nicotine, such that α6β2* receptors serve as therapeutic targets for nicotine addiction. The present research assessed the neuropharmacology of 1,10-*bis*(3-methyl-5,6-dihydropyridin-1(2*H*)-yl)decane (r-bPiDI), a novel small-molecule, tertiary amino analog of its parent compound, *N*,*N*-decane-1,10-diyl-*bis*-3-picolinium diiodide (bPiDI). bPiDI was previously shown to inhibit both nicotine-evoked DA release and the reinforcing effects of nicotine. In the current study, r-bPiDI inhibition of [^{3}H]nicotine and [^{3}H]methyllycaconitine binding sites was evaluated to assess interaction with the recognition binding sites on α4β2* and α7* nAChRs, respectively. Further, r-bPiDI inhibition of nicotine-evoked DA release in vitro in the absence and presence of α-conotoxin MII and following chronic in vivo nicotine administration were determined. The ability of r-bPiDI to decrease nicotine self-administration and food-maintained responding was also assessed. Results show that r-bPiDI did not inhibit [^{3}H]nicotine or [^{3}H]methyllycaconitine binding, but potently (IC_{50} = 37.5 nM) inhibited nicotine-evoked DA release from superfused striatal slices obtained from either drug naïve rats or from those repeatedly treated with nicotine. r-bPiDI inhibition of nicotine-evoked DA release was not different in the absence or presence of α-conotoxin MII, indicating that r-bPiDI acts as a potent, selective α6β2* nAChR antagonist. Acute systemic administration of r-bPiDI specifically decreased nicotine self-administration by 75 %, and did not alter food-maintained responding, demonstrating greater specificity relative to bPiDI and bPiDDB, as well as the tertiary amino analog r-bPiDDB. The current work describes the discovery of r-bPiDI, a tertiary amino, α-conotoxin MII-like small molecule that acts as a potent and selective antagonist at α6β2* nAChRs to specifically decrease nicotine self-administration in rats, thus, establishing r-bPiDI as a lead compound for development as a treatment for nicotine addiction.

Keywords Nicotine · Abuse · Dopamine · Reinforcement

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✉ Linda P. Dwoskin
ldwoskin@email.uky.edu

[1] Department of Psychology, University of Kentucky, Lexington, KY, USA

[2] Department of Pharmaceutical Sciences, College of Pharmacy, University of Kentucky, 789 S. Limestone St., Lexington, KY 40536-0596, USA

[3] Department of Pharmaceutical Sciences, University of Arkansas for Medical Sciences, Little Rock, AR, USA

[4] George E. Wahlen Veterans Affairs Medical Center and Departments of Psychiatry and Biology, University of Utah, Salt Lake City, UT, USA

Introduction

Despite the knowledge that tobacco smoking has serious health and economic consequences and is the most preventable cause of death in the US, cessation remains elusive and those addicted for the most part continue to use tobacco [1–4]. The major psychoactive alkaloid in tobacco predominantly associated with its abuse liability is nicotine [5]. Nicotine acts directly as an agonist on nicotinic acetylcholine receptors (nAChRs) located on dopaminergic

neurons. Activation of nAChRs evokes dopamine (DA) release within the neuronal circuitry producing reward [6]. DA release is well known to underlie the reinforcing properties of nicotine and other drugs of abuse [7]. Therefore, tobacco smoking is reinforced and maintained at least in part by nicotine activation of nAChRs within the DA reward circuitry.

Results from comprehensive molecular genetics studies, in which an individual nAChR subunit gene (α4, α5, α6, α7, β2, β3, and β4) was deleted, suggest that nicotine-evoked DA release is mediated by six different nAChR subtypes, including α-conotoxin MII (α-CtxMII)-sensitive (α6β2β3*, α4α6β2β3*, α6β2*, and α4α6β2*) and α-CtxMII-insensitive (α4β2* and α4α5β2*) subtypes (* denotes possible assembly with other subunits), while deletion of α7 and β4 subunits had no effect [8, 9]. Thus, these studies suggest that multiple nAChR subtypes mediate nicotine-evoked DA release.

Expression of the α6 subunit is largely limited to dopaminergic neurons, and the DA-selective neurotoxin, 6-hydroxydopamine, produces >90 % decrease in α6 mRNA expression in the nigrostriatal tract [10, 11]. Moreover, α6 knockout mice do not self-administer nicotine [12, 13] and do not show nicotine conditioned-place preference [14]. Consistent with these findings, mice with gain-of-function α6 nAChRs show enhanced DA release in nucleus accumbens and are hypersensitive to the locomotor activating effects of a novel environment, an effect that is exaggerated further when nicotine is administered [15, 16]. Also, animals chronically treated with nicotine show down-regulation of α6β2* nAChRs in both dorsal and ventral striatum [17–19].

α-conotoxin MII (α-CtxMII), a peptide that selectively inhibits α6β2* nAChRs, but does not cross the blood–brain barrier, inhibits nicotine-evoked DA release from mouse striatal synaptosomes [6, 20] and from rat nucleus accumbens slices [21, 22]. Also, α-CtxMII when locally infused into the ventral tegmental area inhibits phasic DA release in the nucleus accumbens in anesthetized rats [23], and when infused intraventricularly or into nucleus accumbens decreases nicotine conditioned place preference [14, 24]. Further, following microinjection into nucleus accumbens shell, α-CtxMII decreases motivation for nicotine reinforcement using a progressive ratio schedule of nicotine self-administration [25]. Also, microinjection of α-CtxMII into the ventral tegmental area decreases nicotine self-administration, without causing alterations in food-maintained behavior [22]. Together, these results suggest that selective inhibition of α6β2* nAChRs decreases nicotine reinforcement and reward, indicating that this nAChR subtype is a valid target for development of smoking cessation therapies [26–28]. Thus, α6β2* nAChRs play a major role in mediating nicotine-evoked DA release and the corresponding reinforcement and reward produced by nicotine.

Small molecule antagonists that selectively inhibit α6β2* nAChR subtypes that act as brain-bioavailable surrogates for α-CtxMII would be expected to inhibit nicotine-evoked DA release and to decrease nicotine reinforcement. Importantly, such α-CtxMII-like small molecules presumably would have greater drug-likeness compared to α-CtxMII, and could be developed as novel tobacco smoking cessation therapeutics. Toward this goal, our laboratories have discovered a group of *N*,*N*'-alkanediyl-*bis*-3-picolinium compounds with varying $C_{6\text{-}12}$ methylene linker lengths that act as selective antagonists of α6β2* nAChRs. Importantly, the C_{12} analog, bPiDDB (Fig. 1), inhibits nicotine-evoked DA release from superfused rat striatal slices [29]. Concurrent superfusion with maximal inhibitory concentrations of bPiDDB and α-CtxMII resulted in inhibition of nicotine-evoked DA release no greater than inhibition with either compound alone, indicating that bPiDDB inhibits nicotine-evoked DA release by interacting with α-CtxMII-sensitive, α6β2* nAChRs [29]. Additionally, bPiDDB completely inhibits nicotine-evoked DA release in rat nucleus accumbens, measured using in vivo microdialysis [30]. These neurochemical findings translated to whole animal behavioral analyses, in that bPiDDB specifically decreased nicotine self-administration [31]. The C_{10} analog bPiDI (Fig. 1) exhibited a pharmacological profile similar to that for bPiDDB [29, 32]. These results provide proof-of-concept

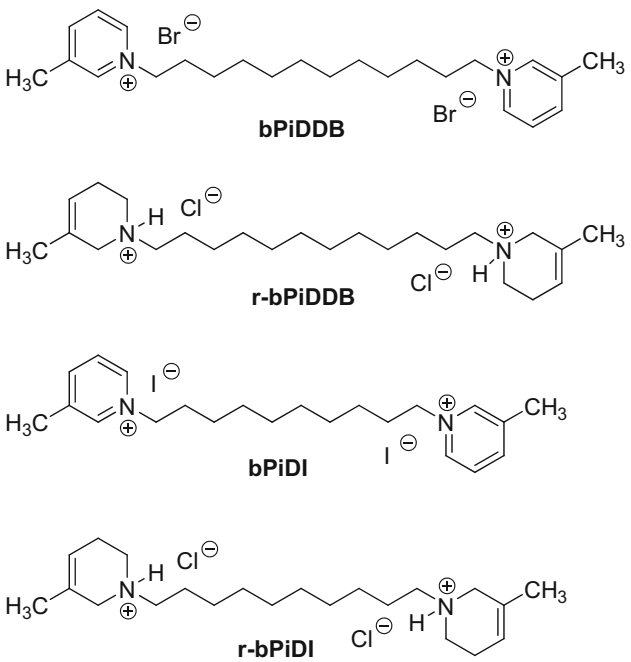

Fig. 1 Chemical structures of bPiDDB, r-bPiDDB, bPiDI, and r-bPiDI

supporting the discovery of a new class of small molecules with potential as novel pharmacotherapies for nicotine addiction.

Based on these previous efforts using *bis*-quaternary ammonium analogs (bPiDDB and bPiDI), we recently investigated a chemically-reduced, tertiary amino derivative, 1,12-*bis*(3-methyl-5,6-dihydropyridin-1(2*H*)-yl)dodecane dihydrochloride (r-bPiDDB, bis-THP3, Fig. 1) [26, 33], designed as a more drug-like analog of the parent compound, bPiDDB. When striatal slices were obtained from rats 24 h after either repeated nicotine or saline administration (once daily for 10 days), concurrent superfusion with maximal inhibitory concentrations of r-bPiDDB and α-CtxMII resulted in inhibition of nicotine-evoked DA release no greater than inhibition with either compound alone [33]. These results indicate that r-bPiDDB inhibits nicotine-evoked DA release by interacting with α-CtxMII-sensitive, α6β2* nAChRs in both naive and repeatedly treated rats. Further, r-bPiDDB decreased nicotine self-administration by 60 % [26]. However, the effect at the higher doses of r-bPiDDB was not specific, i.e., also decreasing responding maintained by food. The current research investigates the pharmacological effects of the novel, tertiary amino, small molecule, 1,10-*bis*(3-methyl-5,6-dihydropyridin-1(2*H*)-yl)decane dihydrochloride (r-bPiDI) as an α6β2*-selective antagonist with greater drug-likeness when compared with its parent compound, bPiDI.

Materials and Methods

Materials

[^{3}H]DA (dihydroxyphenylethylamine, 3,4-[7-^{3}H]); specific activity 28.0 Ci/mmol) and S(−)[^{3}H]nicotine [S(−)[*N*-methyl-^{3}H]; specific activity 81.0 Ci/mmol) were purchased from PerkinElmer Life and Analytical Sciences (Boston, MA). [^{3}H]Methyllycaconitine (MLA; [1α,4(S),6β,14α,16β]-20-ethyl-1,6,14,16-tetramethoxy-4-([{2-([3-^{3}H]-methyl-2,5-dioxo-1-pyrrolidinyl)benzoyl}-oxy]methyl)aconitane-7,8-diol; specific activity 25.8 Ci/mmol) was purchased from Tocris Cookson, Inc. (Bristol, UK). S(−)Nicotine ditartrate (nicotine), nomifensine maleate, pargyline hydrochloride, mecamylamine hydrochloride, MLA, cytisine hydrochloride, EDTA, EGTA, HEPES, sucrose, magnesium sulfate, and polyethyleneimine (PEI) were obtained from Sigma-Aldrich (St. Louis, MO, USA). TS-2 tissue solubilizer and scintillation cocktail were purchased from Research Products International (Mt. Prospect, IL, USA). L-Ascorbic acid and sodium bicarbonate were obtained from Aldrich Chemical Co. (Milwaukee, WI). Other assay buffer constituents were purchased from Thermo Fisher Scientific (Waltham, MA, USA).

α-CtxMII, and r-bPiDI (Fig. 1) were synthesized as previously described [34, 35]. r-bPiDI was characterized by ^{1}H and ^{13}C NMR spectroscopy, mass spectrometry, and elemental analysis. Chemical purity of all products was >98 %. r-bPiDI, mecamylamine and nicotine were dissolved in saline and administered (s.c., 1 ml/kg) 15 min prior to behavioral sessions. Nicotine solutions were adjusted to pH 7.4. Nicotine dose represents freebase; r-bPiDI doses represent the dihydrochloride salt.

Animals

All animal care and experimental protocols were in accordance with the Institute of Laboratory Animal Resources Commission on Life Sciences National Research Council (1996), and approved by the University of Kentucky Institutional Animal Care and Use Committee. Male Sprague–Dawley rats (Harlan Industries, Indianapolis, IN, USA) were housed in a temperature- and humidity-controlled colony with a 12/12 h light/dark cycle. Experiments were conducted during the light phase. Unless stated otherwise, rats had ad libitum access to food and water in the home cage.

[^{3}H]DA Release

Nicotine-evoked [^{3}H]DA overflow was determined using superfused rat striatal slices preloaded with [^{3}H]DA according to previous methods [32, 36]. Coronal slices of dorsal striatum (not including nucleus accumbens core or shell; 500 μm, ~5 mg) were incubated for 30 min in Krebs' buffer with 0.1 μM [^{3}H]DA (final concentration). Slices were transferred to a 2500 Suprafusion system (Brandel, Inc.; Gaithersburg, MD, USA) and superfused (0.6 ml/min) for 60 min with Krebs' buffer. Krebs' buffer contained 118 mM NaCl, 4.7 mM KCl, 1.2 mM $MgCl_2$, 1.0 mM NaH_2PO_4, 1.3 mM $CaCl_2$, 11.1 mM α-D-glucose, 25.0 mM $NaHCO_3$, 0.11 mM L-ascorbic acid 0.004 mM disodium EDTA, pH 7.4, saturated with 95 % O_2/5 % CO_2 at 34 °C. Nomifensine (10 μM) and pargyline (10 μM) were included in the buffer to assure that the [^{3}H] collected primarily represented [^{3}H]DA released into superfusate rather than [^{3}H]DA metabolites [37].

To determine the concentration-dependent effect of r-bPiDI to inhibit nicotine (10 μM)-evoked [^{3}H]DA overflow from rat striatal slices, each slice from an individual rat was superfused for 36 min with and without one of six r-bPiDI concentrations (1 nM–1 μM). This initial superfusion period determined if the analog itself had an effect on DA release, as an antagonist by pharmacological definition should have no effect. r-bPiDI remained in the

superfusion buffer until the end of the experiment. Following 36 min of superfusion with r-bPiDI, nicotine (10 μM) was added to the buffer and superfusion continued for 36 min. The nicotine concentration (10 μM) was chosen because it consistently and reproducibly provides sufficient dopamine release to allow investigation of antagonist concentration–response curves, and because it was employed in previous studies determining inhibitory effects of the structurally-related analogs [26, 29, 32, 33]. At the end of each experiment, slices were solubilized and the [^{3}H]-content of the tissue and superfusate samples was determined using liquid scintillation spectrometry.

r-bPiDI inhibition of nicotine-evoked [^{3}H]DA overflow was assessed in rats following repeated administration of nicotine (0.4 mg/kg, s.c.) or saline daily for 10 days. Striatal slices were obtained 24 h after the last injection. Concentration–response for r-bPiDI (0.1 nM–1 μM) to inhibit [^{3}H]DA overflow evoked by 10 μM nicotine was determined as described above. Concentration of nicotine (10 μM) used to determine r-bPiDI inhibition was selected based on previous findings demonstrating that prior repeated administration of nicotine (0.4 mg/kg, s.c.) does not alter the concentration–response for nicotine-evoked [^{3}H]DA overflow [33]. The latter findings are in agreement with earlier studies demonstrating that nicotine-evoked striatal DA release, either in vitro or in vivo, is not altered following repeated nicotine administration [38, 39].

To determine if r-bPiDI interacts with α-CtxMII-sensitive nAChRs, a series of experiments was conducted in which maximally inhibitory concentrations of α-CtxMII (1 nM), r-bPiDI (1 μM), or α-CtxMII plus r-bPiDI were superfused for 36 min in duplicate slices from each animal. Slices were superfused for 36 min in the absence of antagonist(s), followed by superfusion with 10 μM nicotine (nicotine control). To determine maximal inhibition produced by blockade of nAChRs, slices were superfused with mecamylamine (10 μM) [40], an antagonist at all known nAChRs. A repeated-measures design was used for this series of experiments.

[^{3}H]Nicotine and [^{3}H]MLA Binding

Inhibition of [^{3}H]nicotine and [^{3}H]MLA binding was determined using previously published methods [41]. Whole brain, excluding cortex and cerebellum, was homogenized in 20 vol of ice-cold modified Krebs'-HEPES buffer (2 mM HEPES, 14.4 mM NaCl, 0.15 mM KCl, 0.2 mM $CaCl_2 \cdot 2H_2O$, and 0.1 mM $MgSO_4 \cdot 7H_2O$, pH 7.5). Homogenates were centrifuged at 31,000*g* for 17 min at 4 °C (Avanti J-301 centrifuge; Beckman Coulter, Fullerton, CA). Pellets were resuspended by sonication (Vibra Cell; Sonics and Materials Inc., Danbury, CT) in 20 vol Krebs'-HEPES buffer and incubated at 37 °C for 10 min (Reciprocal Shaking Bath model 50; Precision Scientific, Chicago, IL). Suspensions were centrifuged using the above conditions. Resulting pellets were resuspended by sonication in 20 vol buffer and centrifuged at 31,000 g for 17 min at 4 °C. Final pellets were stored in incubation buffer containing 40 mM HEPES, 288 mM NaCl, 3.0 mM KCl, 4.0 mM $CaCl_2 \cdot 2H_2O$, and 2.0 mM $MgSO_4 \cdot 7H_2O$, pH 7.5. Membrane suspensions (100–140 μg of protein/100 μl) were added to tubes containing r-bPiDI (7-9 concentrations, 0.001 nM–1 mM) and 3 nM [^{3}H]nicotine or 3 nM [^{3}H]MLA in a final assay volume of 250 μl. Samples were incubated for 60 min at room temperature. Non-specific binding was determined in the presence of 10 μM cytisine or 10 μM nicotine for the [^{3}H]nicotine and [^{3}H]MLA assays, respectively. Reactions were terminated by harvesting samples on Unifilter-96 GF/B filter plates presoaked in 0.5 % PEI. Samples were washed 3 times with 350 μl of ice-cold buffer. Filter plates were washed 5 times with 350 μl of ice-cold buffer, dried and bottom sealed, and each well was filled with 40 μl of scintillation cocktail (MicroScint 20; PerkinElmer Life and Analytical Sciences, Waltham, MA).

Nicotine Self-Administration

Throughout nicotine self-administration training, rats were fed 15–20 g of food post-session in the home cage to maintain ~85 % free feeding body weight. Rats were initially trained to press a lever for food pellets (45 mg) in operant conditioning chambers (ENV-008, MED Associates, St. Albans, VT, USA). Responding on one lever (active) resulted in delivery of a food pellet, while presses on another lever (inactive) had no scheduled consequence. Following food training, rats were returned to a free feeding schedule for 3 days and then jugular vein catheters were implanted. After 5 days of recovery, rats were again returned to a food-restriction schedule (15–20 g post-session) and nicotine self-administration was initiated during daily 1 h sessions.

During nicotine self-administration, responding on the active lever, according to a fixed ratio 5 (FR 5) schedule, resulted in simultaneous delivery of nicotine (0.03 mg/kg/infusion over 5.9 s) and activation of the cue lights, which initiated a 20 s time-out period, where responding on either lever was not reinforced. Inactive lever responses were recorded, but had no consequence. Behavior was defined as stable once rats earned at least 10 infusions per session, produced less than 20 % variability in the number of infusions earned, and had a minimum of 2:1 active to inactive response ratio over 3 consecutive sessions. Effects of acute r-bPiDI (19.4–109 μmol/kg, s.c.) and saline pretreatment (15 min before each treatment session) on nicotine self-administration were determined using a within-

subjects Latin Square design. At least two maintenance sessions separated each treatment session.

Food-Maintained Responding

Food-maintained responding was carried out using the same procedures as nicotine self-administration, except responding resulted in the delivery of a 45 mg food pellet and rats were not subjected to catheter-implant surgery. Effects of acute r-bPiDI (2.5–74 μmol/kg, s.c.) and saline pretreatment (15 min before each treatment session) on food-maintained responding were determined using a within-subjects Latin Square design, with at least two maintenance sessions between each treatment session. Response stability criteria were the same as those for nicotine self-administration. Of note, the dose range for r-bPiDI varied slightly between food and nicotine self-administration experiments. Smaller starting doses were used in the food self-administration experiments, because these experiments were performed first to ensure that there were no adverse consequences. Since no adverse effects were observed across a large r-bPiDI dose range in the food self-administration study, the nicotine self-administration study began using a higher dose of r-bPiDI.

Data Analysis

Data are presented as mean (±SEM). In nicotine-evoked [^{3}H]DA overflow assays, fractional release was calculated by dividing [^{3}H] in each sample by total tissue-[^{3}H] at time of sample collection. Basal [^{3}H]outflow was the average fractional release in the two samples before r-bPiDI addition to the buffer. Total [^{3}H]overflow was the sum of increases in fractional release above basal [^{3}H]outflow resulting from r-bPiDI or nicotine exposure, with [^{3}H]outflow for equivalent periods of drug exposure subtracted. r-bPiDI concentration–response curves were generated by nonlinear fit to the sigmoidal dose–response. IC_{50} for r-bPiDI inhibition of nicotine-evoked [^{3}H]DA overflow was determined using Prism 5.0 (GraphPad Software Inc., San Diego, CA, USA). For the binding assays, specific [^{3}H]nicotine and [^{3}H]MLA binding were determined by subtracting the nonspecific binding from total binding. Inhibition constants (K_i values) were determined using the Cheng-Prusoff equation [42]. To determine if r-bPiDI interacts with α-CtxMII-sensitive nAChRs, the effect of concomitant exposure to maximally inhibitory concentrations of r-bPiDI and α-CtxMII was compared with inhibition produced by r-bPiDI or α-CtxMII alone using a one-way ANOVA. Pairwise comparisons ($\alpha = 0.05$) were used to compare [^{3}H]overflow after mecamylamine treatment to [^{3}H]overflow following r-bPiDI alone, α-CtxMII alone, and r-bPiDI plus α-CtxMII. The effects of r-bPiDDB and r-bPiDI on the number of nicotine infusions and food pellets earned during nicotine self-administration and food-maintained behavior, respectively, were analyzed by one-way, repeated-measures ANOVA, followed by Dunnett's test ($\alpha = 0.05$).

Results

r-bPiDI Inhibits Nicotine-Evoked [^{3}H]DA Overflow From Rat Striatal Slices

Total nicotine-evoked [^{3}H]DA overflow as a function of concentration of r-bPiDI is illustrated in Fig. 2 (top). In a concentration-dependent manner, r-bPiDI potently, but

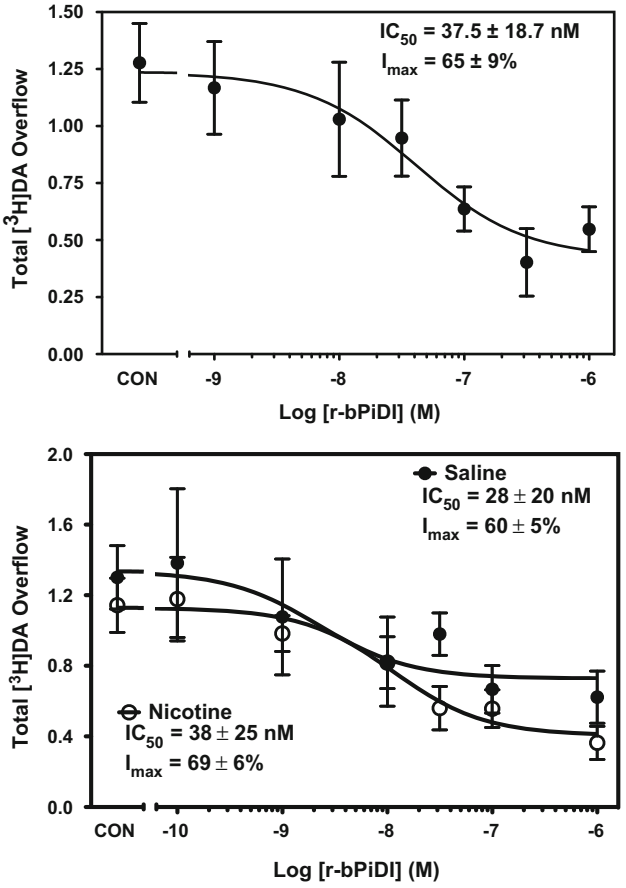

Fig. 2 In a concentration-dependent manner, r-bPiDI inhibits nicotine-evoked [^{3}H]DA overflow from rat striatal slices obtained from drug naive (*top*) and repeated nicotine- or saline-treated rats (*bottom*). Control (CON) represents [^{3}H]DA overflow in response to nicotine (10 μM) in the absence of r-bPiDI. Data are expressed as mean ± SEM total [^{3}H]DA overflow as a percentage of tissue-[^{3}H] content. For repeated-treatment groups, nicotine (0.4 mg/kg, s.c.) or saline were administered once daily for 10 days and striatum obtained 24 h after the last injection. (n = 4–6/group)

incompletely (IC_{50} = 37.5 ± 18.7 nM; I_{max} = 65 ± 9 %) inhibited nicotine-evoked [^{3}H]DA overflow. Furthermore, repeated nicotine treatment (0.4 mg/kg, s.c., once daily for 10 days) did not alter either r-bPiDI potency or maximal inhibition of nicotine-evoked [^{3}H]DA overflow compared to repeated saline injection (IC_{50} = 38 ± 25 and 28 ± 20 nM; I_{max} = 69 ± 6 and 60 ± 5 %, respectively; Fig. 2, bottom).

r-bPiDI Does Not Inhibit Binding of [^{3}H]nicotine or [^{3}H]MLA Binding

[^{3}H]Nicotine binding to rat brain membranes as a function of nicotine and r-bPiDI concentration is illustrated in Fig. 3 (top). r-bPiDI did not inhibit [^{3}H]nicotine binding. [^{3}H]MLA binding to rat brain membranes as a function of MLA and r-bPiDI concentration is illustrated in Fig. 3 (bottom). r-bPiDI also did not inhibit [^{3}H]MLA binding to rat brain membranes.

r-bPiDI Interacts with α-CtxMII-Sensitive α6β2*-Containing nAChRs

Inhibition of nicotine-evoked (10 μM) total [^{3}H]DA overflow by mecamylamine (MEC; 10 μM), α-CtxMII (1 nM), r-bPiDI (1 μM), or α-CtxMII + r-bPiDI is illustrated in Fig. 4 (top). Mecamylamine inhibition of [^{3}H]DA overflow was nearly complete (>90 %). In contrast, inhibition of [^{3}H]DA overflow was ~60–70 % for both α-CtxMII and r-bPiDI given alone. Concomitant administration of α-CtxMII and r-bPiDI did not result in greater inhibition of nicotine-evoked [^{3}H]DA overflow than either antagonist alone. Repeated nicotine treatment (0.4 mg/kg, s.c., once daily for 10 days) did not alter inhibition produced by

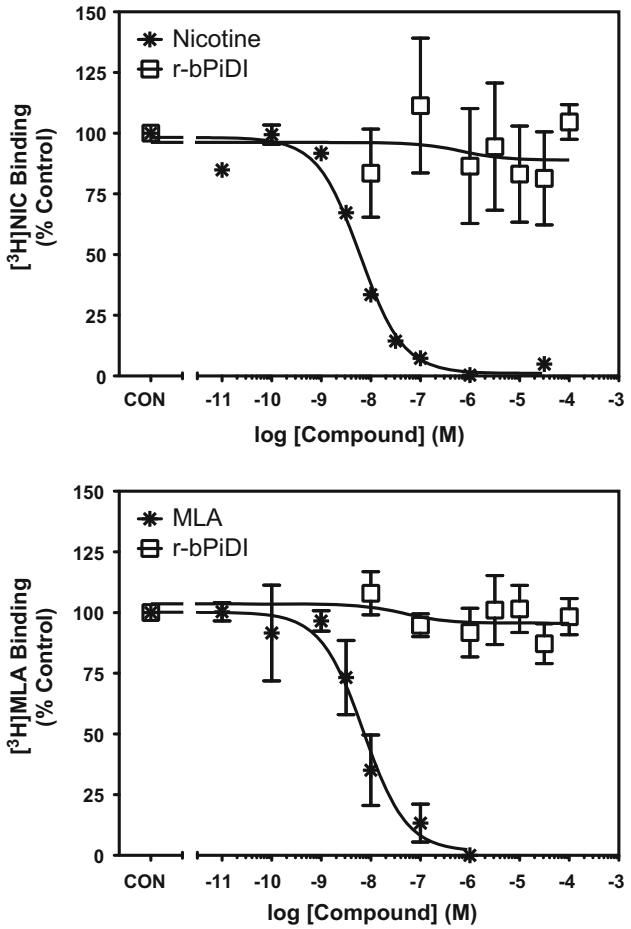

Fig. 3 r-bPiDI does not inhibit [^{3}H]nicotine ([^{3}H]NIC) or [^{3}H]MLA binding. Nonspecific binding in the [^{3}H]nicotine and [^{3}H]MLA binding assays was determined in the presence of 10 μM cytisine and 10 μM nicotine, respectively. Control (CON) represents [^{3}H]nicotine and [^{3}H]MLA binding in the absence of r-bPiDI (47.9 ± 4.50 and 47.4 ± 4.80 fmol/mg protein, respectively). Data are mean ± S.E.M. (n = 4–5 rats/compound)

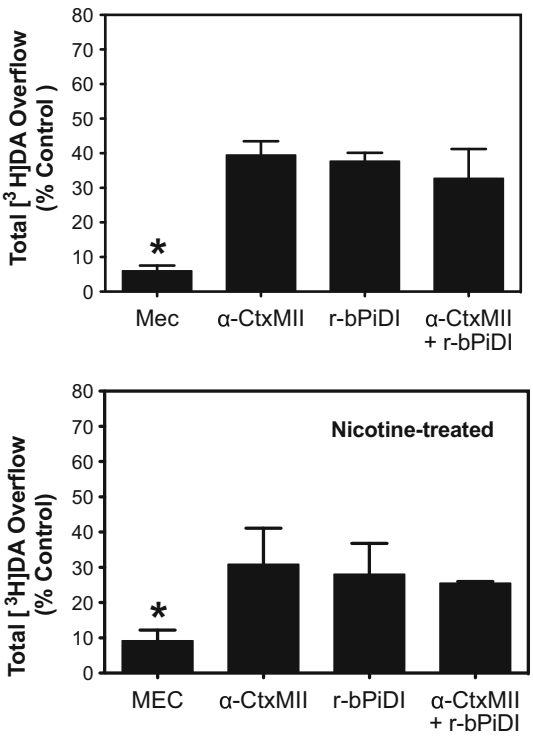

Fig. 4 Concomitant exposure to maximal inhibitory concentrations of r-bPiDI (1 μM) + α-CtxMII (1 nM) results in inhibition of nicotine-evoked [^{3}H]DA overflow not different from that produced by either antagonist alone. Striatal slices were obtained from either drug-naïve rats (*top*) or from rats treated once daily for 10 days with nicotine (0.4 mg/kg, s.c.; *bottom*). Control represents [^{3}H]DA overflow (drug-naïve = 1.74 ± 0.42; nicotine-treated = 1.06 ± 0.29 total [^{3}H]DA overflow as a percentage of tissue-[^{3}H] content) in response to nicotine (10 μM) in the absence of r-bPiDI, α-CtxMII and mecamylamine (MEC, 10 μM). Data are expressed as mean ± SEM total [^{3}H]DA overflow as a percent of control. (n = 4/group). *Asterisks* indicate significant differences between the mecamylamine condition and all other antagonist conditions ($p < 0.05$)

MEC (10 μM), α-CtxMII (1 nM), r-bPiDI (1 μM), or α-CtxMII + r-bPiDI (Fig. 4, bottom).

r-bPiDI Specifically Decreases Nicotine Reinforcement

The dose-related effect of r-bPiDI on the number of nicotine infusions (0.03 mg/kg/infusion) earned during self-administration is illustrated in Fig. 5 (top). One-way ANOVA revealed an effect of r-bPiDI dose [$F(3,21) = 14.91$, $p < 0.05$]. Dunnett's test revealed that, relative to saline control, r-bPiDI doses of 58.3 and 109 μmol/kg decreased the number of nicotine infusions earned. The dose-related effect of r-bPiDI on the number of food pellets earned during food-maintained responding is illustrated in Fig. 5 (bottom). One-way ANOVA indicated no significant effect of r-bPiDI dose.

Discussion

Relative to the maximal inhibition of nicotine-evoked DA release induced by the nonselective nAChR antagonist mecamylamine (>90 %), r-bPiDI produced incomplete maximal inhibition (60–70 %), an effect similar to that produced by parent and related compounds (bPiDI, bPiDDB, and r-bPiDDB) [26, 29, 32, 33]. The incomplete inhibition of nicotine-evoked DA release induced by r-bPiDI suggests that this compound acts at a subset of nAChRs mediating nicotine-evoked DA release. The incomplete maximal inhibition of nicotine-evoked DA release produced by r-bPiDI is consistent with incomplete inhibition produced by α-CtxMII [20, 43]. Further, r-bPiDI had little affinity for, and does not interact with, the agonist recognition site on either α4β2* or α7* nAChRs. Concomitant exposure to maximally effective concentrations of r-bPiDI (1 μM) and α-CtxMII (1 nM) produced inhibition of nicotine-evoked DA release that was comparable to inhibition produced by either antagonist alone, suggesting that these compounds act at the same α6β2* nAChR sites. Finally, relative to the quaternary compounds, bPiDDB and bPiDI, and relative to the tertiary amino analog r-bPiDDB, acute systemic administration of r-bPiDI resulted in greater specificity for decreasing nicotine reinforcement. Collectively, these results suggest that r-bPiDI functions as a small molecule antagonist at α-CtxMII-sensitive, α6β2*-containing nAChRs to inhibit nicotine-evoked DA release and specifically decreases nicotine reinforcement.

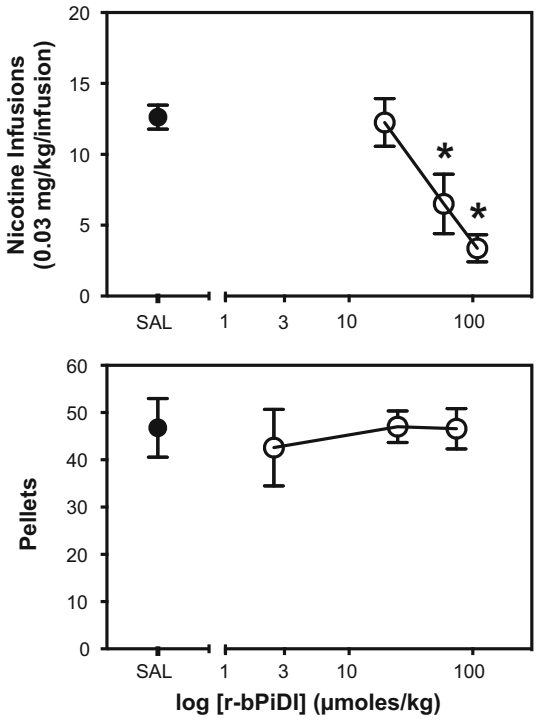

Fig. 5 Acute r-bPiDI specifically decreases nicotine self-administration. r-bPiDI (19.4–109 μmol/kg, s.c., 15-min pretreatment) decreased the number of nicotine infusions (0.03 mg/kg/infusion) earned (*top*). ($n = 8$). r-bPiDI (2.47–74.0 μmol/kg; s.c., 15-min pretreatment) did not alter the number of food pellets earned (*bottom*) (n = 5). Data are expressed as mean ± SEM food pellets earned. *Asterisks* indicate a significant difference relative to saline ($\alpha = 0.05$)

While the potency of the parent compound bPiDI to inhibit nicotine-evoked DA release was increased by ~3-orders of magnitude following repeated nicotine treatment (0.4 mg/kg, s.c., once daily for 10 days) [32], the concentration response for the tertiary amino analog r-bPiDI to inhibit nicotine-evoked DA release was not altered by the same repeated nicotine treatment (current findings). These results parallel those for bPiDDB and its tertiary amino analog r-bPiDDB, where repeated nicotine treatment increased the potency of bPiDDB to inhibit nicotine-evoked DA release by 3-orders of magnitude, but no change in potency was found for r-bPiDDB following repeated nicotine treatment [33]. Similar to r-bPiDI and r-bPiDDB, no change in inhibitory potency was found for α-CtxMII following the same repeated nicotine treatment [33]. Thus, although bPiDI, bPiDDB, r-bPIDDB and r-bPiDI all act as antagonists at α-CtxMII-sensitive α6β2*-containing nAChRs based on the lack of additivity upon concomitant exposure in the nicotine-evoked DA release assay, differences between the parent compounds (bPiDI and bPiDDB) and the tertiary amino analogs (r-bPiDI and r-bPiDDB) are revealed following repeated nicotine administration.

As we have described previously [33], these findings can be explained in part based on the different subtypes of α-CtxMII-sensitive α6β2* nAChRs, i.e., the α6(non-α4)-containing subtypes (α6β2β3* and α6β2*) and the α4-containing subtypes (α6α4β2β3* and α6α4β2*). Repeated nicotine treatment in vivo results in differential changes in

nAChR composition, conformation, and stoichiometry, as well as alterations in receptor maturation, by increasing subunit oligomerization and folding of these α-CtxMII-sensitive nAChRs subtypes [44–46]. For example, repeated nicotine down-regulates α4α6β2-containing subtypes by 50 %, while up-regulating α6β2-containing subtypes by 25 % [47]. The changes in α4α6β2 and α6(non-α4)β2-containing subtypes following repeated nicotine imply a corresponding functional change in these receptor subtypes, perhaps reflected by altered inhibitory effects of bPiDI and bPiDDB in the DA release assay. Furthermore, the β3 subunit promotes resistance to receptor regulation (α6β2β3* and α6α4β2β3*) by chronic nicotine [48, 49]. Thus, r-bPiDI and r-bPIDDB may be interacting with α6β2β3* and α6α4β2β3* subtypes following repeated nicotine administration. The α4α6β2β3* nAChR subtype is of particular importance, as it constitutes up to 50 % of the nAChRs on striatal DA terminals [8].

The tertiary amino analog r-bPiDI investigated herein specifically decreased nicotine reinforcement. r-bPiDI had no effect on food-maintained responding at doses demonstrated to reduce nicotine self-administration by >50 %. In contrast to these findings with r-bPiDI, pretreatment with bPiDI, bPiDDB and r-bPiDDB showed nonspecific suppressive effects on food maintained responding within the same dose range that decreased nicotine self-administration [26, 32]. In the latter studies, an FR 5 schedule of reinforcement with a 120-s timeout for food-maintained responding was employed in an attempt to match rates of responding for nicotine and food reinforcement [32]. In the current study, an FR 5 schedule of reinforcement with a 20-s signaled timeout was used to investigate the effects of r-bPiDI on both nicotine self-administration and food-maintained behavior. Thus, the differential effects on food-maintained responding may have been due to the difference in schedules used. However, given that the FR 5 schedule with the accompanying 20-s timeout used for r-bPiDI resulted in higher rates of responding, these higher response rates should have had a greater susceptibility to disruption [50]. Thus, given the lack of r-bPiDI-induced disruption in food-maintained behavior, the change in schedule is unlikely to have contributed to the specificity of the r-bPiDI-induced decrease in nicotine reinforcement reported herein. Thus, r-bPiDI exhibits the greatest efficacy and specificity in the nicotine self-administration assay, compared to the other three structurally related analogs.

In conclusion, the current evidence indicates that r-bPiDI acts at α-CtxMII-sensitive, α6β2* nAChRs to inhibit nicotine-evoked striatal DA release, and relative to bPiDDB, bPiDI and r-bPiDDB, it has the significant advantage of being highly specific for decreasing nicotine reinforcement. One of the goals in drug discovery research is to generate a pipeline of compounds within the same structural class that serve as backups and alternatives in the event there are problems with the initial lead. r-bPiDI is one such compound. Our observations that r-bPiDI has a greater efficacy and specificity in the behavioral assay relative to r-bPiDDB, despite its similar profile in the neurochemical assays, exemplifies the need to test a number of structurally-related compounds to arrive at the best lead compound to take forward through the drug discovery pathway into clinical trials. As such, the findings in the current manuscript describing effects of r-bPiDI represents a significant step in identifying the lead compound in the development of the current class of nAChR antagonists as potential treatments for nicotine addiction. Furthermore, the results of the present study indicate that α6β2* nAChRs play an important role in the reinforcing effects of nicotine. Thus, targeting α6β2* nAChRs with small molecule α-CtxMII-sensitive antagonists may offer a novel and effective route that constitutes an alternative approach to the development of therapeutics for nicotine addiction.

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Compliance with Ethical Standards

Disclosure Statement The University of Kentucky holds patents on bPiDDB, r-bPiDDB, bPiDI, r-bPiDI, and novel analogs of these compounds. A potential royalty stream to Dwoskin and Crooks may occur consistent with University of Kentucky policy.

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