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ORIGINAL INVESTIGATION

Substituted heteroaromatic compounds: effect on nicotine self-administration in rats

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Abstract
Rationale Certain compounds that nonselectively inhibit a prominent human nicotine-metabolizing enzyme (i.e., human cytochrome P-450 2A6, hCYP 2A6) showed inhibition of smoking in humans. However, a comprehensive examination of hCYP 2A6 inhibitors to decrease nicotine self-administration in rats has not been reported.
Objectives We tested substituted heteroaromatic compounds designed to selectively inhibit hCYP 2A6 in a model system to (a) examine selective hCYP 2A6 inhibitors to decrease cotinine formation in vivo in rats administered with nicotine and (b) examine their efficacy to decrease nicotine self-administration in rats.
Methods Rats were trained to IV self-administer nicotine in 1-h sessions. Nicotine self-administration was carried out at a unit dose of 0.03 mg/kg/infusion in 0.1 ml/s. Pretreatment with substituted heteroaromatic test compounds (0.5–25 mg/kg, i.p., 30 min prior to nicotine self-administration sessions) resulted in dose-dependent decreases of nicotine self-administration. Using operant conditioning techniques, nicotine- vs. food-reinforced responding was evaluated for compounds **10** and **11**.
Results Compounds **10** and **11** selectively decreased nicotine self-administration with estimated ED_{50} values 4 and 2.8 mg/kg, respectively. Of the test compounds examined, none showed significant affinity for mammalian $\alpha4\beta2$- or $\alpha7$-neuronal nicotinic acetylcholine (nAChR) receptors and none were inhibitors of the human dopamine transporter (hDAT); thus, neither the endogenous nAChRs nor DAT apparently plays a role in decreasing nicotine self-administration for this series of compounds.
Conclusion The results indicate that chemical analogs of nicotine can play a role in nicotine self-administration harm reduction but a non-nAChR and a non-hDAT mechanism are likely involved.

Keywords Nicotine self-administration · Nicotine metabolism · Cytochrome P-450 2A6 · Neuronal nicotinic acetylcholine receptors · Dopamine transporter

Introduction

In the USA, tobacco smoking is the leading cause of preventable death (Taylor and Bettcher 2000). Currently, almost 25% of Americans smoke some form of tobacco product (Rock et al. 2007) despite convincing evidence that smoking cigarettes is the primary cause of lung cancer (Doll and Hill 1952) and that sidestream smoke is also unquestionably linked to lung cancer (Hirayama 1981) and heart disease. Worldwide, death from tobacco smoking is predicted to increase to over six million individuals per year by 2015 and account for 10% of all deaths (Mathews and Loncar 2006).

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The pharmacological effects of smoking are mediated by the alkaloid (*S*)-nicotine (Balfour 2002). Treatments for tobacco smoking addiction have included nicotine replacement therapy and use of the antidepressant bupropion (Hughes 2007). However, these therapies only double the placebo quit rate (West 2007) and the success rate for people using these therapies is only about double that achieved with no pharmacological treatment (Stead et al. 2008). Recently, a smoking reduction agent, varenicline (Chantix, Pfizer), based on partial agonism of the α4β2-nicotinic acetylcholine receptor (nAChR) was developed (Coe et al. 2005) and has shown utility in humans (Cahill et al. 2009). Although varenicline has promise as a therapeutic and has shown clinical utility and a role for a nicotine receptor partial agonist in smoking reduction, in view of the side effects, additional drugs and approaches are needed.

In humans, the main way administered nicotine is cleared is by metabolism via hepatic cytochrome P-450 2A6 (CYP 2A6)-mediated oxidation to cotinine via aldehyde oxidase (Cashman et al. 1992). Nicotine is primarily eliminated by metabolism that is quite variable among individuals (Benowitz and Jacob 1984; Benowitz 1988; Feyerabend et al. 1985). In rats, rCYP 2B1-mediated oxidation to cotinine via aldehyde oxidase is the major way that nicotine is metabolized and cleared (Miksys et al. 2000).

Various lines of evidence suggest that decreased CYP 2A6-dependent metabolism of nicotine is associated with decreased risk for smoking and thus harm reduction. If humans modulate their smoking to control nicotine consumption (Benowitz and Jacob 1984), it is possible that individuals with decreased nicotine metabolism will be predisposed to smokeless tobacco and have decreased addiction liability. In fact, the CYP 2A6 inhibitor, methoxsalen (administered at 30 mg, orally) attenuated nicotine clearance, increased nicotine bioavailability, and decreased cigarette smoking by 24% in humans compared with placebo (Sellers et al. 2000). Additional reports have suggested that individuals with defective CYP 2A6 smoke fewer cigarettes (Pianezza et al. 1998). While new, potent and selective CYP 2A6 inhibitors hold promise as chemopreventative agents against *N*-nitrosamine-induced lung tumorigenesis and possibly as nicotine harm reduction agents (Shin et al. 2008; Takeuchi et al. 2003), considerably more studies need to be done.

Kinetic studies of CYP 2A6 inhibition (Denton et al. 2005) and X-ray co-crystallographic studies with CYP 2A6 (Yano et al. 2006) of numerous substituted 3-heteroaromatic pyridine analogs of nicotine provided considerable insight into structure–activity relations (SAR) for interaction with CYP 2A6. Certain pyridyl imidazoles were potent inhibitors of CYP 2A6 (Denton et al. 2005). Based on this information and previous studies of the effect of inhibitors on human CYP 2A6 (Denton et al. 2004, 2005; Berkman et al. 1995) and inhibition studies of cigarette smoking (Sellers et al. 2000, 2003), we extended the investigation to 1-alkyl- or 1-benzyl imidazoles as inhibitors of human CYP 2A6 and studied the effects of these agents on nicotine self-administration in rats. The goals of the work described herein included the following: (1) examine a role of selective CYP 2A6 inhibitors in nicotine self-administration and clearance in rats and (2) examine a role of nAChRs and the dopamine transporter (DAT) in mediating this outcome. We determined that an association between potency of inhibition of nicotine metabolism by rat CYP 2B1 or nicotine clearance in rats and efficacy of nicotine self-administration in vivo was not observed. However, a new class of potent nicotine self-administration blockers have been discovered and characterized.

Methods

Chemicals

(−)-(*S*)-Nicotine hydrogen tartrate salt was purchased from Aldrich Chemical Company (Milwaukee, WI) and dissolved in isotonic saline solution, and the pH was adjusted to pH 7 before use in vivo. Mecamylamine hydrochloride salt was obtained from Aldrich and dissolved in isotonic saline solution. Heparinized saline and Timentin® were purchased from SavMart Pharmaceuticals, (San Diego, CA). Drug concentrations refer to the salt form. The synthesis of compounds **1**, **2**, and **4–6** and **8** was previously described (Denton et al. 2005; Yano et al. 2006) and used as HCl salts.

Overview

Eleven compounds were tested and each compound was referred to by a number between **1** and **11**.

General procedure for the synthesis of 1-alky and 1-benzyl imidazoles

The alkyl or benzyl halide (1.0 equiv) and imidazole (3.0 equiv) were placed together, heated to 90°C for 3 h, cooled to room temperature and the resulting residue was partitioned between 5 M NaOH and CH_2Cl_2 (Lu et al. 1997). The organic portion was concentrated in vacuo and the product was purified by silica gel chromatography and fully characterized as described in the Appendix for each compound.

Biological material

Wistar-derived male rats (250–300 g, Harlan (Livermore, CA)) were housed in groups of two and maintained in a temperature-controlled environment on a 12-h:12-h light/dark cycle (0600 hours on–1800 hours off) upon arrival to

the laboratory, given free access to food and water during a 1-week habituation period to the laboratory, and handled, housed, and killed in accordance with current NIH guidelines. Animals were handled daily for several days to desensitize them to handling stress before experimental testing. The cell sizes (n=5–8) provided reliable estimates of drug effects. The rats in this experiment were previously injected with a test compound and, therefore, were not drug naive. However, an appropriate washout period (14 days) was given before compounds were tested, and no carry-over effect was apparent.

Test compound treatment

In the study, separate groups of rats received compounds **1**, **3**, **4**, **7**, or **9** (vehicle, 15 mg/kg, i.p.), compounds **2**, **5**, and **6** (vehicle, 15 and 25 mg/kg, i.p.), compound **8** (vehicle, 15, 20 and 25 mg/kg, i.p.), compound **10** (vehicle, 0.5, 1.58, and 5 mg/kg, i.p.), compound **11** (vehicle, 1.6, 2.8, and 5 mg/kg, i.p.), or a positive control compound, mecamylamine (3.0 mg/kg, subcutaneously, s.c.). Each test compound and mecamylamine as hydrochloride salts was dissolved in isotonic saline, and administered to separate groups in a volume of 1 ml/kg. Test compounds were administered 30 min prior to the start of self-administration (SA) sessions. The experimental design was a within-subjects Latin square design, and each rat in a full dose–response study received all doses of test compound. Mecamylamine was tested after the dose–response studies with the above test compounds using a cross-over dose design.

Apparatus

Food training and nicotine self-administration took place in eight standard Coulbourn operant conditioning chambers (standard "rat" chambers) housed in a sound-attenuated box. Operant conditioning chambers were equipped with two levers mounted 2 cm above the floor and a cue light mounted 2 cm above the right lever (active lever) on the back wall of the chamber. For food training, a food hopper was located 2 cm to the left/right of either lever in the middle of the front wall. Intravenous infusions were delivered in a volume of 0.1 ml over a 1-s interval via an infusion pump (Razel, CT) housed outside of the sound-attenuated operant conditioning chamber.

Food training—IVSA study

Lever pressing was established using a previously described method (Hyytia et al. 1996). Initially, rats were restricted to 15 g of food daily (approximately 85% of their free-feeding body weight). After the second day of food restriction, rats were trained to respond for food under a fixed ratio 1 (FR1) schedule of reinforcement (one food pellet for each lever press) with a 1-s time-out (TO-1 s) after each reinforcement. The schedule was gradually increased to achieve an FR1-TO-20s schedule of reinforcement. Training sessions were given two times per day, and each session lasted for 30 min. Once the rats obtained steady baseline responding (defined as less than 20% variability across three consecutive sessions) at a FR1-TO-20s schedule of reinforcement, they were returned to ad libitum food to allow for preparation of studies using IV jugular catheter implant surgery.

Food training—food study

For food studies, two separate cohorts of rats (n=8) were used to test compounds **10** and **11**. Lever pressing was established as described above (Hyytia et al. 1996). Initially, rats were restricted to 15 g of food daily (approximately 85% of their free-feeding body weight). After the second day of food restriction, rats were trained to respond for food under a FR1 schedule of reinforcement (one food pellet for each lever press) with a 1-s time-out (TO-1 s) after each reinforcement. The schedule was gradually increased to achieve an FR1-TO-20s schedule of reinforcement. Training sessions were given two times per day, and each session lasted for 30 min. Once the rats obtained steady baseline responding (defined as less than 20% variability across three consecutive sessions) at an FR1-TO-20s schedule of reinforcement, dose response testing commenced. Rats were baselined for 1–3 days (until stable) between each dose probe.

Surgery

Rats were anesthetized with an isoflurane–oxygen mixture (1–3% isoflurane), and chronic silastic jugular catheters were inserted into the external jugular and passed subcutaneously to a polyethylene assembly mounted on the animal's back. The catheter was passed subcutaneously from the back to the jugular vein of the rat where it was inserted and secured with a nonabsorbable silk suture. Upon successful completion of surgery, rats were given 5 days to recover before baseline self-administration sessions started, remained on ad libitum food access, and had their catheter lines flushed daily with heparinized saline containing 66 mg/ml of Timentin® to prevent blood coagulation and infection.

Nicotine self-administration

Following successful recovery from catheter implant surgery, rats were again food deprived to 85% of their free-feeding body weight. Once self-administration sessions started, rats were trained to IV self-administer nicotine (0.03 mg/kg/infusion, 0.1 ml in 1 s) in 1-h baseline sessions.

Rats were studied 5–7 days per week, under an FR1-TO-20s schedule of reinforcement, until stable responding was achieved. A 20-s time-out (TO) period, concurrent with the beginning of the infusion, was signaled by illumination of the cue light above the active lever. Responding during the TO period was not reinforced. After stable responding for nicotine was achieved, various doses of test compound were tested using a within-subjects Latin square design. Rats were treated with one of several doses of test compound for each test session and subsequently retested to establish that baseline session values were obtained for 1–3 days before rats were administered the next dose of test compound.

Effect of compounds **4–6** and **8** on pharmacokinetics of nicotine clearance

The effect of pretreatment of compounds **4–6** and **8** (i.e., 25 mg/kg, 30 mins prior, i.p.) on the clearance of nicotine (3.0 mg/kg, IV) was determined by investigating the area under the concentration–time profile (AUC) in male Wistar rats (350–375 g, Harlan (Livermore, CA)). Briefly, blood was obtained from an IV jugular catheter of rats at various time points and centrifuged at 10,000×*g* at 4°C and the plasma was extracted as previously described (Denton et al. 2005); nicotine and cotinine were quantified by liquid chromatography–mass spectrometry (LC–MS). The mean and standard deviation of the time that the greatest or peak plasma concentration (C_{max}) of nicotine or cotinine occurred defined as t_{max}, and AUC were calculated using the WinNonlin-Pro program (Version 1.5, Pharsight, Inc.).

Data compilation, processing and analysis

Animal data were collected online simultaneously from multiple operant conditioning chambers. Results of the (IV self-administration) IVSA and food operant conditioning procedures, using the Latin square design, were reported as mean cumulative number of reinforced responses for nicotine and food. In general, tests for homogeneity of variance were first conducted on the data. If the scores did not violate the assumption of homogeneity of variance, appropriate analyses of variance (ANOVA) were conducted. "Order effects" were examined by conducting repeated measures ANOVA on the mean reinforcement values within each dose day across the dose response testing. Order effects were reported only if a significant finding was observed. Test data were analyzed using the StatView statistical package on a PC-compatible computer. For the analysis of the dose response testing and individual dose testing, a repeated measures ANOVA was conducted, with number of reinforced responses as the dependant variable and drug dose as the independent variable. Post hoc analysis, using the Student–Newman–Keuls test, was conducted on dose–response curve data, where appropriate.

In vitro pharmacology

Triplicate assays were done with each incubation containing radioligand (1.5 nM [^{3}H]cytisine for α4β2 and 1 nM [^{125}I] alpha-bungarotoxin for α7 nicotinic receptors), cell membrane (10 μg rat cerebral cortex protein) and assay buffer (pH 7.5, 50 mM HEPES, 100 mM NaCl, 5 mM $MgCl_2$), and test compounds **9–11** as before (Sharples et al. 2000). Non-specific binding was determined in the presence of nicotine bitartrate salt (10 μM) for α4β2 and α-bungarotoxin (1 μM) for α7 nicotinic receptors. Single determinations (1 μM) of test agent binding were done at each nicotinic receptor and were compared to those of the standard nicotinic reference compounds examined at the same time. Membranes were incubated with the test compounds for 75 min at 4°C (for the α4β2 receptor) and 150 min at 37°C (for the α7 nicotinic receptor) and centrifuged (1,500 rpm), and binding was assessed using scintillation counting.

Enzyme inhibition

To measure human CYP 2A6 functional activity, coumarin 7-hydroxylation was determined as previously described (Denton et al. 2005) using commercially available enzyme (BD Biosciences, Woburn, MA). The IC_{50} value was converted to an inhibition constant (K_i) value as described below. To measure the effect of compounds **1–11** on rat CYP 2B1 functional activity, cotinine formation was determined by a method previously described (MacDougall et al. 2003) using commercially available rat CYP 2B1 (BD Biosciences). The inhibitory effect of each test compound was determined at two concentrations (i.e., 25 μM and 100 μM).

Dopamine transporter binding

Dopamine transporter binding was determined as described previously (Feng et al. 2003). Briefly, membranes from the human dopamine transporter transfected into HEK cells (HEK-hDAT) were incubated in the presence of [^{125}I]-RTI-55 and test compounds in HEPES with pH 7.4, and specific binding was determined as the difference in radioactivity observed in the presence and absence of mazindol using a filtration assay. IC_{50} values for binding to the hDAT were determined using GraphPad Prism (GraphPad Software, San Diego, CA) and converted to K_i values as described before (Cheng and Prusoff 1973). K_i values determined in the presence of the hDAT were listed in Table 7.

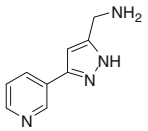
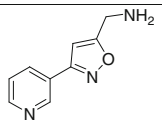
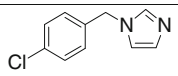
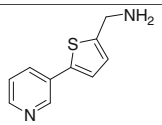
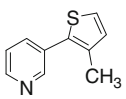
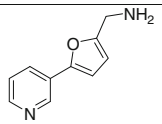
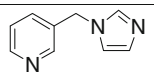
The specific ligand binding to the receptors was determined as the difference between the total binding and the nonspecific binding determined in the presence of an excess

of unlabelled ligand. The results were expressed as a percent of control specific binding ((measured specific binding / control specific binding)×100) and as a percent inhibition of control specific binding (100−((measured specific binding / control specific binding)×100)) obtained in the presence of each test compound. The IC_{50} values (concentration causing a half-maximal inhibition of control specific binding) were determined by non-linear regression analysis of the competition curves generated with mean replicate values using Hill equation curve fitting ($Y=D+[(A-D)/(1+(C/C_{50})^{nH})]$, where Y=specific binding, D=minimum specific binding, A=maximum specific binding, C=compound concentration, $C_{50}=IC_{50}$, and nH=slope factor). For both receptor-binding studies and enzyme inhibition, the analysis was done using SigmaPlot® 4.0 for Windows®. The K_i values were calculated using the Cheng–Prusoff equation: ($K_i = = IC_{50}/(1+(L/K_D))$, where L=concentration of radioligand in the assay, and K_D= affinity of the radioligand for the receptor). A Scatchard plot was used to determine the K_D (Cheng and Prusoff 1973).

Results

The synthesis of substituted 3-heteroaromatic pyridine or 1-alkyl- or 1-benzyl imidazole analogs of nicotine (compounds **1–11**, Tables 1 and 3) followed literature procedures for related compounds (Lu et al. 1997), or alkylation of imidazole was done using an alkyl or benzyl halide in the

Table 1 Effect of substituted heteroaromatic compounds on nicotine self-administration

Compd	Structure	Vehicle[a]	Compound[a] (15 mg/kg)	% Decrease	Vehicle	Compound[a] (25 mg/kg)	% Change	N[b]
1		13.6 ±2.6	11.6 + 2.9	14.7	NA[c]	NA		5
2		10.4 + 2.7	8.4 + 1.7	19.2	14.0 + 3.3	5.6 + 1.6[d]	60.	5
3		10.8 + 3.2	13.0 + 2.7	23.6	NA	NA		5
4		15.4 + 1.5	11.8 + 3.2	23.4	NA	NA		5
5		13.2 + 2.5	8.8 + 1.6	33.3	10.4 + 3.1	1.6 + 0.7$_d$	84.6	5
6		23.2 + 1.8	16.8 + 2.3[d]	27.6	23.2 + 1.8	8.3 + 2.1[d]	64.2	8
7		10.5 + 1.3	7.5 + 1.9[d]	28.6	NA	NA		8

NA not available

[a] Number refers to the effects of the vehicle or compound (15.0 mg/kg or 25 mg/kg), i.p. 30 min before IV self-administration of 0.03 mg/kg/infusion of nicotine under an FR1 schedule of reinforcement as described in the "Methods" section

[b] The number of animals used in the self-administration study test group

[c] Statistically different than vehicle-treated animals (p=0.05)

presence of excess imidazole (Baggaley et al. 1975; Kruse et al. 1990). If only the benzyl alcohol was commercially available, it would have been converted to the benzyl chloride using thionyl chloride, followed by alkylation of imidazole as described above. This methodology resulted in efficient preparation of a variety of analogs in good yield from readily available precursors. In addition to CYP inhibition, selected 1-alkyl- or 1-benzyl imidazoles were also studied for binding to α4β2- and α7-nicotinic acetylcholine receptors (nAChRs) and inhibition of binding to the hDAT. The effects of certain test compounds (i.e., **4**–**6** and **8**) on clearance of nicotine administered to rats was also examined in an attempt to relate in vitro CYP inhibition potency to in vivo nicotine clearance. Finally, compounds **1**–**11** were examined in an animal model of nicotine self-administration.

Effect of access to nicotine self-administration

Rats trained in the IVSA paradigm treated with test compounds **1**–**7** at 15 mg/kg, i.p., showed decreases in nicotine self-administration ranging from 14% to 33% (Table 1). Certain compounds (i.e., **2**, **5**, and **6**) tested in rats at 25 mg/kg showed decreases in nicotine self-administration ranging from 60% to 85% (Table 1). However, compounds **8**–**11** were more potent at decreasing nicotine self-administration and, accordingly, were examined in more detail along with mecamylamine (as a positive control) (Jones et al. 2010).

Effect of mecamylamine on nicotine self-administration

Mecamylamine (3.0 mg/kg, s.c., 30 min before the session) decreased nicotine self-administration (IVSA of 0.03 mg/kg/infusion of nicotine under an FR1 schedule of reinforcement). ANOVA of these data revealed a significant decrease in the number of nicotine infusions during 1-h self-administration sessions (F=11.9, df 1,7, p<0.02). At 3.0 mg/kg, responding for nicotine was decreased by 62% in rats when compared to control (i.e., vehicle-treated) conditions.

Effect of compounds **1**–**7** on nicotine self-administration

Initially, a select group of human CYP 2A6 inhibitors were examined at one dose to examine a SAR on IVSA of nicotine. Thus, pretreatment of **1**–**7** (15 mg/kg, i.p., 30 min prior to 1-h IVSA sessions) showed that the test compounds were effective in decreasing the number of nicotine infusions compared to vehicle-treated rats. Compounds **2**, **5**, and **6** were tested at 25 mg/kg and showed a dose-dependent decrease in the number of nicotine infusions compared to vehicle-treated rats (Table 1).

Effect of compounds **8**–**11** on nicotine self-administration

The dose-effect function of compound **8** on nicotine IVSA is depicted in Fig. 1. At stable criteria, rats responded with an average of 18.5±1.7 infusions (data not shown). That value was not significantly different from the zero dose within the Latin square design (i.e., the average vehicle dose value was 23.5±3.1). An ANOVA of the treatment data (i.e., compound **8** dose response) failed to reveal a significant effect of compound **8** on nicotine intake during the 1-h self-administration sessions (F=2.81, df 3,21, p=0.0644), although the analysis did reveal a "near-significant effect". As with all the compounds examined in this study, there was no carry-over effect. That is, there was no carry-over effect 24 h after administration (i.e., during baseline sessions between drug test days) of compounds **1**–**11**, indicating that whatever effect that caused blockade of nicotine self-administration was reversible. However, because the efficacy of compound **8** was low, it was not investigated further. Pretreatment of a single dose (n=8) with compound **9** (15 mg/kg, i.p., 30 min prior) significantly decreased nicotine self-administration (0.03 mg/kg/infusions of nicotine under an FR1 schedule of reinforcement, F=16.8, df 1,7, p=0.0046).

Analysis of the data revealed that 15 mg/kg of compound **9** produced a statistically significant decrease in IV nicotine intake during a 1-h self-administration session (F=16.8, df 1,7, p=0.0046) when compared to control (i.e., vehicle-treated) conditions. However, because the efficacy of

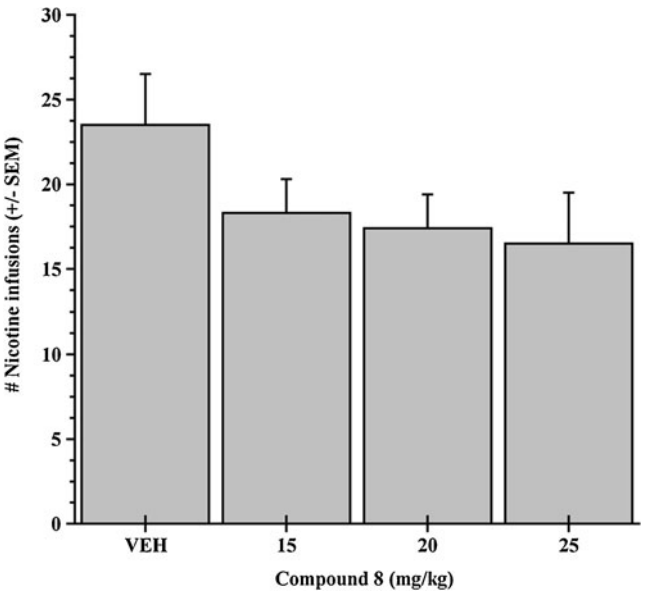


Fig. 1 Effects of compound **8** on IV self-administration of 0.03 mg/kg/infusions of nicotine under an FR1 schedule of reinforcement. The data are expressed as mean (±S.E.M.) number of infusions per session (n=8). Analysis of dose response revealed a near-significant effect of compound **8** (p=0.064)

compound **9** was low, it was not investigated further. Pretreatment with compound **10** (Fig. 2) revealed a significant dose effect on nicotine intake (F=15.5, df 3,21, p<0.0001). Post hoc Student–Newman–Keuls test of individual doses of compound **10** revealed that a dose of 5 mg/kg produced a statistically significant difference from control (vehicle-treated) conditions, with a corresponding p value of <0.0001. A dose-effect function on nicotine self-administration for treatment with compound **11** is shown in Fig. 3. As apparent from Fig. 3, pretreatment with compound **11** produced a significant effect of dose on nicotine self-administration (F=7.3, df 3,21, p=0.0021). Post hoc Student–Newman–Keuls test of individual doses of compound **11** revealed that doses of 2.8 and 5 mg/kg were significantly different from control (vehicle-treated) conditions, with corresponding p values of 0.0017 and 0.009, respectively. There was a near-significant effect of 1.6 mg/kg of compound **11** on nicotine intake (p=0.06).

Effect of compounds **10** and **11** on operant conditioning food responding

The effect of the two most potent compounds was examined for effects on food intake using operant conditioning techniques. Table 4 shows the effects of compounds **10** and **11** on food intake during 30-min operant conditioning food sessions. Results revealed that there was no effect of drug dose order within the repeated measures of the dose design.

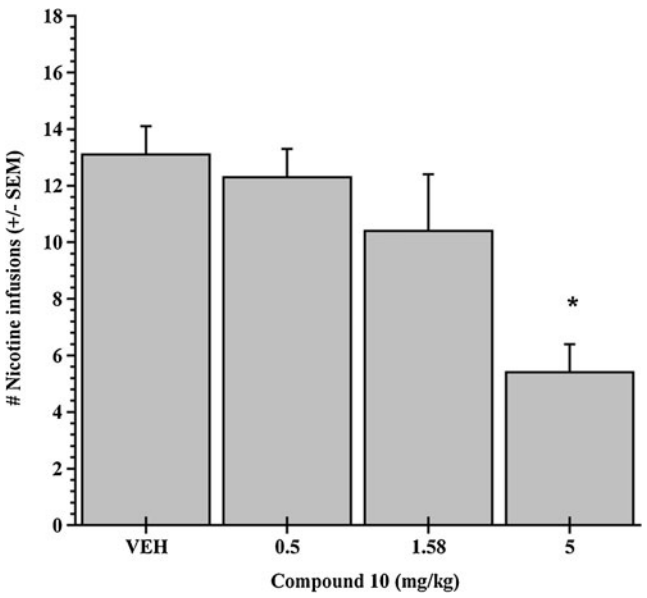


Fig. 2 Effect of compound **10** on IV self-administration of 0.03 mg/kg/infusions of nicotine under an FR1 schedule of reinforcement. The data are expressed as mean (±S.E.M.) number of infusions per session (n=8). As indicated by a *star*, statistical significance from vehicle-treated rats was p<0.05

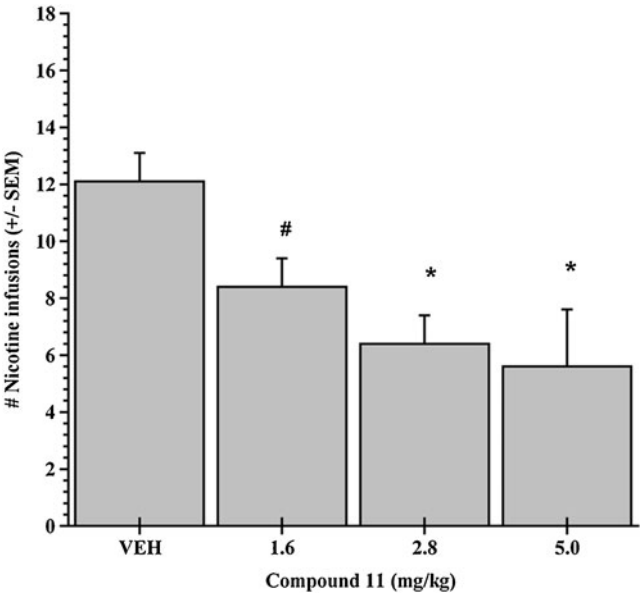


Fig. 3 Effect of compound **11** on IV self-administration of 0.03 mg/kg/infusions of nicotine under an FR1 schedule of reinforcement. The data are expressed as mean (±S.E.M.) number of infusions per session (n=7). As indicated by a *star,* statistical significance from vehicle-treated rats was p<0.05. There was a near-significant effect of 1.6 mg/kg of compound **11** (#, p=0.06)

However, studies revealed that only the highest dose of compound **11** (i.e., 5 mg/kg) produced a significant suppression on food intake (p<0.05) in rats.

Pharmacodynamic profiles of selected test compounds with CYPs, nicotinic receptors and the dopamine transporter

In the presence of compounds **3**–**6** and **10**, significant inhibition of human CYP 2A6 was observed (Tables 2 and 3). Compared to tranylcypromine, compounds **3**–**6** and **10** were more potent human CYP 2A6 inhibitors. Compounds **1**, **2**, **7**, and **11** were less potent inhibitors of human CYP 2A6 compared with tranylcypromine (Tables 2 and 3). The selectivity of human CYP 2A6 inhibition was examined by studying the effect of compounds **3**, **10**, and **11** on the selective functional activity of human CYP 3A4, 2E1, 2 C9, 2 C19, 2D6, and 2B6 at a concentration of 10 μM (Table 4). As shown in Table 5, compounds **3**, **10**, and **11** showed some inhibition of human CYP 2E1. Compounds **3**, **10**, and **11** also inhibited human CYP 2B6 to a certain extent. However, a 16- to 125-fold greater concentration of compounds **3**, **10**, and **11** was required to achieve the same degree of inhibition of human CYP 2A6. The results indicated that compounds **3**, **10**, and **11** were relatively selective inhibitors of human CYP 2A6. However, even though compounds **3**, **10**, and **11** were selective inhibitors of human CYP 2A6, the degree of blockade of nicotine self-administration in rats for these compounds did not exactly follow suit.

Table 2 Inhibition of human CYP2A6 and rat CYP2B1 in the presence of substituted heteroaromatic compounds and physicochemical properties of compounds **1–11**

Compound	hCYP2A6 IC_{50} (μM)	hCYP2B6 IC_{50} (μM)	rCYP2B1 % inhibition[a]	rCYP2B1 % inhibition[b]	tPSA	CLogP
1	0.6	66.4	ND	53.8	62.8	0
2	0.8	67.4	ND	55.3	59.9	−0.2
3	0.16	12	ND	35.5	15.6	2.5
4	0.16	52.2	ND	42.7	38.4	1.4
5	0.2	3.9	ND	47.9	12.4	2.6
6	0.2	191	ND	46.6	47.6	0.9
7	>200	ND	ND	43.3	27.9	0.3
8	800	103	NA	NA	36.7	0.7
9	NA	NA	ND	53.5	27.9	0.6
10	170	11	ND	56.9	15.6	2.6
11	618	>20	ND	67.7	15.6	2.1

tPSA topological polar service area, *CLogP* log of the partition ratio, *ND* no detectable inhibition, *NA* not available

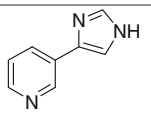
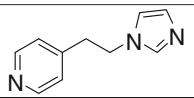
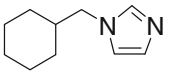
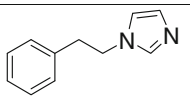
[a] Inhibitor present at 25 μM

[b] Inhibitor present at 100 μM

We examined the inhibition of rat CYP 2B1 by compounds **1–7** and **9–11** because CYP 2B1 is the prominent nicotine metabolizing enzyme in rat liver and largely responsible for nicotine clearance in rat. As shown in Table 3, compounds **1–7** and **9–11** were ineffective at inhibiting rat CYP 2B1. With the possible exception of compound **11**, all the compounds examined possessed an IC_{50} value estimated to be greater than 100 μM. Accordingly, at the doses used in the in vivo experiments, compounds **1–7** and **9–11** were unlikely to significantly inhibit rat CYP 2B1 and decrease nicotine metabolism in the rat by this mechanism. To examine this point in greater detail, the influence of selected test compounds on nicotine clearance was studied in rats.

After pretreatment with compounds **4–6** and **8** (i.p. administration 30 min before IV nicotine administration), the pharmacokinetics of cotinine formation was examined as a surrogate for nicotine clearance because the clearance of nicotine was very rapid and quantitation of cotinine was more reliable. Compounds **4–6** and **8** were studied because they represented highly potent and non-potent inhibitors of human CYP 2A6, respectively. LC–MS analysis of cotinine

Table 3 Interaction of *N*-alkyl-substituted imidazoles with CYP2A6 and nicotinic acetylcholine receptors

Compd	**Structure**	**CYP2A6 Inhibition IC_{50}, nM**	**[a]Nicotinic receptors μM**
	Tranylcypromine	233	**-**
8		800	**NA**
9		NA	**ND**
10		170	**ND**
11		618	**ND**

NA not available, *ND* no detectable binding at 1 μM

[a] Mammalian α-7 and α4ß2 nicotinic receptors

Table 4 Order and food effects of compounds **10** and **11** on operant responding for food in rats

Compound **10**				
Order effects	Day 1	Day 2	Day 3	Day 4
	75±3.3[a]	78±1.7	79±3.2	80±1.9
Food effects	Dose[b]	Dose	Dose	Dose
	Vehicle	0.5	1.58	5.0
	77±3.0	80±1.8	79±2.5	77±3.2
Compound **11**				
Order effects	Day 1	Day 2	Day 3	Day 4
	76±4.1	78±2.3	76±3.7	71±4.8
Food effects	Dose[a]	Dose	Dose	Dose[c]
	Vehicle	1.6	2.8	5.0
	79±1.0	81±1.2	79±1.3	63±5.6

[a]Average number of nicotine self-administrations in 1 h ± SEM

[b]Dose, mg/kg

[c]Statistically different than vehicle-treated animals ($p<0.05$)

formation from organic extracts of plasma samples taken after nicotine administration was examined in rats (Table 6). Compared to vehicle-pretreated animals, rats treated with test compounds **4–6** and **8** (25 mg/kg, i.p. route of administration) 30 min before nicotine administration (3 mg/kg, IV route of administration) did not decrease cotinine AUC or significantly change the t_{max} for cotinine formation (Table 6). The data indicated that the AUC for cotinine formation was increased possibly due to increased blood flow to the liver or some other pharmacological mechanism.

To explain the pharmacological mechanism of action, compounds **3**, **10**, and **11** were studied for their interaction with the α4β2- and α7-nicotinic receptors and compounds **1–5**, **7**, and **9–11** were examined as inhibitors of the hDAT. Compounds **3**, **10**, and **11** showed no apparent affinity for α4β2- and α7-nicotinic receptors (Table 3). In contrast, nicotine and α-bungarotoxin had K_D values of 1.8 nM and 0.3 nM, respectively, for α4β4- and α7-nicotinic receptors. The results indicated that compounds **3**, **10**, and **11** were not agonists or antagonists of the nAChRs examined. As shown in Table 7, compared with cocaine and mazindol, compounds **1–5**, **7**, and **9–11** had no significant effect on inhibition of [^{125}I]-RTI-55 binding to the hDAT and therefore no detectable affinity for the hDAT. We conclude that inhibition of dopamine binding to the hDAT was not responsible for the pharmacological activity of compounds **1–5**, **7**, and **9–11**.

Discussion

1-Benzyl imidazole and 1-methylcyclohexylimidazole inhibit bacterial CYP (Verras et al. 2004) and pyridyl imidazoles inhibit human CYP 2A6 (Denton et al. 2005). We extended the investigation to 1-alkyl- and 1-benzyl imidazoles as CYP 2A6 inhibitors and as antagonists of nicotine self-administration. A survey of potent and inactive human CYP 2A6 inhibitors and in vivo efficacy (Table 1) did not reveal any distinct SAR features that predicted in vivo efficacy for the inhibition of nicotine self-administration. However, the results showed that highly polarizable nucleophilic imidazoles are associated with potent inhibition of human CYP 2A6 and, in some cases, efficacious decrease in nicotine self-administration in animals. Based on the results (Tables 1 and 2), we conclude that structural features required for human CYP 2A6 inhibition could be related to inhibition of nicotine self-administration, but rat CYP 2B1 is not the main pharmacological target responsible for inhibition of nicotine self-administration in rats.

A comparison of Tables 1 and 2 showed no clear relationship between potency of human CYP 2A6 inhibition determined in vitro on the basis of kinetic studies of selective functional substrates and percent decrease in nicotine self-administration in vivo. This study shows that the use of human CYP 2A6 inhibition SAR data is a useful guide, but

Table 5 Percent inhibition of CYP3A4, 2E1, 2 C9, 2 C19, or 2D6 by alkyl- or aryl-imidazoles[a]

Compound	CYP3A4	CYP2E1	CYP2C9	CYP2C19	CYP2D6
	Percent inhibition				
3	23	87	32	43	48
10	27	89	44	24	34
11	63	88	13	21	62

[a] 10 μM concentration of alkyl or aryl imidazole used. Values are the mean of triplicate determinations

Table 6 Effect of heteroaromatic aryl compounds on nicotine distribution in vivo[a]

Treatment[a]	AUC[b] (ng×h/ml)	t_{max} (min)	Number of rats
Vehicle	119.1±23.4	90	4
4	411.6±117.6[c]	230	3
5	287.6±84.2[c]	93	4
6	357.5±77.9[c]	89	4
8	180.0±45.8	112	4

[a] Preadministration of 25 mg/kg of compounds **4–6** and **8** was done by the i.p. route of administration 30 min before nicotine administration. Nicotine was administered at 3.0 mg/kg by the IV route of administration

[b] Area under the concentration–time profile (AUC) was the mean ± SD

[c] Statistically different than vehicle-treated animals ($p<0.05$)

not an absolute indicator of the utility of a small molecule as a blocker of nicotine self-administration.

While not as extensively studied herein, compared to human CYP 2A6, rat CYP 2B1 possesses a distinct inhibitor binding profile based on the lack of nicotine metabolism inhibitory potency of compounds **1–7** and **9–11** (Table 2). Moreover, at the dose of compounds **10** and **11** that are effective at decreasing nicotine self-administration (i.e., IC_{50} values of 4 and 2.8 mg/kg, respectively), it is unlikely that any significant degree of CYP 2B1 inhibition occurs in rats administered compounds **10** or **11**. Accordingly, inhibition of rat CYP 2B1-mediated nicotine metabolism cannot explain the pharmacological or behavioral response of compounds **10** and **11** observed in rats. This point is further reinforced because pharmacokinetic studies of nicotine metabolism after the pretreatment of rats with compounds **4–6** or **8** did not show a decrease on the AUC for cotinine formation compared to vehicle administration (Table 6). Thus, relatively potent CYP 2A6 inhibitors (i.e., compounds **4–6**) afforded similar effects on the PK of cotinine formation as a relatively nonpotent CYP 2A6 inhibitor (i.e., compound **8**).

CYP 2A6 inhibitors could have two additional effects in humans in addition to its main mechanism of action: (a) CYP 2A6 inhibition could decrease nicotine metabolism, elevate nicotine levels and possibly provide an aversive effect on nicotine self-administration, and (b) CYP 2A6 inhibition could decrease metabolism of the inhibitor and increase the efficacy as an inhibitor of nicotine self-administration. Notwithstanding the CYP inhibitory properties of compounds **1–11**, these compounds possess the appropriate polar surface area and lipophilicity values to enable good blood–brain barrier penetration and efficacy (Table 2). Furthermore, the mechanism of substances **1–11** does not involve irreversible or covalent modification of a biomacromolecule because there is no apparent carry-over effect in rats. For compounds **10** and **11**, no apparent order effect was apparent. Compounds **10** and **11** did not have a food effect, except possibly at the largest dose of compound **11** (i.e., 5 mg/kg) (Table 4).

Because compounds **1–11** structurally resemble nicotine, we investigated possible molecular targets of action. Accordingly, we examined binding potency of certain compounds to nicotinic receptors as a possible target to explain the efficacy of inhibition of nicotine self-administration in vivo. As shown in Table 3, some of the most potent inhibitors of nicotine self-administration examined did not have any detectable direct binding affinity for mammalian α-7 and α4ß2-nAChRs. However, this does not rule out indirect or allosteric mechanisms. Agents that agonize nAChRs have shown utility in decreasing smoking. For example, the α4ß2 nAChR partial agonist varenicline blocks nicotine self-administration with slightly greater efficacy (i.e., ED_{50} of 2–3 mg/kg; Rollema et al. 2007) than compounds **10** and **11**. Thus, despite the structural relatedness of the test compounds described herein to nicotine, direct binding to the nAChR apparently does not explain the complex interaction responsible for the pharmacological efficacy of the test compounds observed.

The data in Table 7 shows that compounds **1–5**, **7**, and **9–11** had no detectable affinity for the hDAT. Generally, small molecules that show no affinity for the hDAT also do not inhibit dopamine reuptake to the hDAT (Eshleman et al. 1999) and we conclude that the DAT is not the biological target of compounds **1–5**, **7**, and **9–11**.

It is likely that additional neurotransmitter and/or receptor systems in addition to behavioral and environmental secondary effectors contribute to the efficacy of the test compounds. The mechanism by which 3-heteroaromatic

Table 7 Inhibition of radioligand binding in HEK-hDAT cells by compounds **1–5**, **7**, and **9–11**

Compound	Number[a]	Reuptake inhibition K_i (nM)±SEM
Cocaine	5	450±128[b]
Mazindol	5	38±15[b]
1	3	>10,000
2	3	>10,000
3	3	>9,525
4	3	>10,000
5	3	>10,000
7	3	>10,000
9	3	>10,000
10	3	>10,000
11	3	>9,874

[a] Number of independent determinations

[b] Hill slope and standard error of the mean (SEM) for cocaine and mazindol was −1.02±0.08 and −0.89±0.13, respectively

pyridine or 1-alkyl- or 1-benzyl imidazole analogs of nicotine decrease nicotine self-administration remains unknown. Because blockers of nicotine self-administration described herein do not apparently have an irreversible effect (i.e., 24 h after administration of **1–11**, no apparent residual effect was observed), we favor a direct effect on some specific molecular target. However, substantial further research will be required to determine this point.

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