

# AT-1001: A High Affinity and Selective $\alpha 3\beta 4$ Nicotinic Acetylcholine Receptor Antagonist Blocks Nicotine Self-Administration in Rats

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Genomic and pharmacologic data have suggested the involvement of the  $\alpha 3\beta 4$  subtype of nicotinic acetylcholine receptors (nAChRs) in drug seeking to nicotine and other drugs of abuse. In order to better examine this receptor subtype, we have identified and characterized the first high affinity and selective  $\alpha 3\beta 4$  nAChR antagonist, AT-1001, both *in vitro* and *in vivo*. This is the first reported compound with a  $K_i$  below 10 nM at  $\alpha 3\beta 4$  nAChR and >90-fold selectivity over the other major subtypes, the  $\alpha 4\beta 2$  and  $\alpha 7$  nAChR. AT-1001 competes with epibatidine, allowing for [<sup>3</sup>H]epibatidine binding to be used for structure-activity studies, however, both receptor binding and ligand-induced  $Ca^{2+}$  flux are not strictly competitive because increasing ligand concentration produces an apparent decrease in receptor number and maximal  $Ca^{2+}$  fluorescence. AT-1001 also potently and reversibly blocks epibatidine-induced inward currents in HEK cells transfected with  $\alpha 3\beta 4$  nAChR. Importantly, AT-1001 potently and dose-dependently blocks nicotine self-administration in rats, without affecting food responding. When tested in a nucleus accumbens (NAc) synaptosomal preparation, AT-1001 inhibits nicotine-induced [<sup>3</sup>H]dopamine release poorly and at significantly higher concentrations compared with mecamylamine and conotoxin MII. These results suggest that its inhibition of nicotine self-administration in rats is not directly due to a decrease in dopamine release from the NAc, and most likely involves an indirect pathway requiring  $\alpha 3\beta 4$  nAChR. In conclusion, our studies provide further evidence for the involvement of  $\alpha 3\beta 4$  nAChR in nicotine self-administration. These findings suggest the utility of this receptor as a target for smoking cessation medications, and highlight the potential of AT-1001 and congeners as clinically useful compounds.

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## INTRODUCTION

Neuronal nicotinic acetylcholine receptors (nAChRs) are ligand-gated ion channels in the same family as the GABA, glycine, and 5-HT<sub>3</sub> receptors and mediate cation flux (Gotti *et al*, 1997; McGehee *et al*, 1995a). In mammalian neural tissue, eight  $\alpha$ -subunits and three  $\beta$ -subunits have been identified. The receptor is usually made up of a combination of subunit types (McGehee and Role, 1995b), although there are also fully functional homomeric

receptor proteins, particularly the prominent  $\alpha 7$  nAChR. The  $\alpha 4\beta 2$  and the  $\alpha 7$  receptors are by far the most prevalent in the CNS (Perry *et al*, 2002; Xiao and Kellar, 2004).

Nicotine addiction, like addiction to other psychomotor stimulants, is thought to be due to the activation of the dopaminergic mesocorticolimbic pathway (Picciotto, 1998; Stolerman and Shoaib, 1991). The nicotinic receptor subtype most prevalent in this region is the  $\alpha 4\beta 2$  nAChR, although there is a significant level of the  $\alpha 6$  subunit (Perry *et al*, 2002, 2007). Furthermore, mRNA from multiple subunits is known to exist in small brain regions and even in single cells (Klink *et al*, 2001). Significant evidence suggests that the  $\alpha 4\beta 2$  receptor is involved in nicotine dependence. In particular,  $\beta 2$ -knockout mice do not self-administer nicotine (Epping-Jordan *et al*, 1999; Picciotto *et al*, 1998), the somewhat selective  $\alpha 4\beta 2$  antagonist DH $\beta$ E blocks nicotine self-administration (Watkins *et al*, 1999), and the  $\alpha 4\beta 2$  partial agonist varenicline is clinically used as a smoking cessation medication. More recently, the

Preliminary reports of these data were presented at the annual meetings of the Society for Neuroscience.

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importance of the  $\alpha 6$  subunit to nicotine self-administration has also been demonstrated (Pons *et al*, 2008).

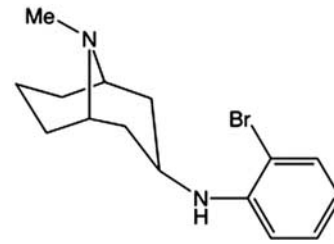
After  $\alpha 4\beta 2$  and  $\alpha 7$  nAChR, the next most prominent receptor subtype is the  $\alpha 3\beta 4$  nAChR. The  $\alpha 3\beta 4$  receptor, which predominates in sensory and autonomic ganglia and in the adrenal gland, is sometimes referred to as the 'ganglionic nAChR' (Mandelzys *et al*, 1994; Poth *et al*, 1997). In the CNS, the mesolimbic dopamine pathway contains some  $\alpha 3\beta 4$  receptors; however, the majority of this receptor subtype resides in the medial habenula (MHb), interpeduncular nucleus (IPN), and pineal gland (Perry *et al*, 2002; Quick *et al*, 1999). Interestingly, the MHb and IPN have recently been implicated in nicotine self-administration and reward along with the  $\alpha 3$ ,  $\alpha 5$ , and/or  $\beta 4$  nAChR subunits. In  $\alpha 5$  null mice, there is an increase in nicotine conditioned place preference (CPP) and nicotine self-administration at higher nicotine doses, indicating that nAChRs containing this subunit may be involved in limiting the reward mediated by higher nicotine doses (Fowler *et al*, 2011; Jackson *et al*, 2010). Genome-wide association studies (GWAS) have implicated variants in the  $\alpha 3$ - $\alpha 5$ - $\beta 4$  gene cluster on chromosome 15 to be associated with an increased risk of whether a smoker becomes nicotine dependent, and to smoking a greater number of cigarettes per day (Berrettini *et al*, 2008; Saccone *et al*, 2008). These GWAS studies, therefore, also suggest a possible involvement of the  $\alpha 3\beta 4$  nAChR subtype in tobacco dependence.

Another line of evidence also implicates the  $\alpha 3\beta 4$  nAChR subtype and the MHb in self-administration of not only nicotine, but also a variety of abused drugs. Glick *et al* have identified a compound, 18-methoxycoronaridine (18-MC), which inhibits the reward induced by a large number of abused drugs (Maisonneuve and Glick, 2003). 18-MC is a derivative of the alkaloid ibogaine, a compound that has considerable anecdotal evidence for utility as a drug abuse medication, but no demonstration of clinical efficacy. 18-MC reduces methamphetamine, nicotine, and morphine self-administration in rats (Glick *et al*, 2000a, 2006). It also attenuates alcohol consumption and blocks acquisition of cocaine place preference (McCallum and Glick, 2009; Rezvani *et al*, 1997). 18-MC apparently mediates these actions through inhibition of  $\alpha 3\beta 4$  nAChR (Glick *et al*, 2011; Maisonneuve and Glick, 2003). However, although 18-MC may be more selective than ibogaine, which binds to a large number of receptors, 18-MC is neither a high affinity nor a selective  $\alpha 3\beta 4$  nAChR antagonist. To better demonstrate the involvement of the  $\alpha 3\beta 4$  nAChR in drug abuse, we have synthesized a series of high affinity and selective  $\alpha 3\beta 4$  nAChR antagonists. Here, we demonstrate that AT-1001 from this series, has nanomolar affinity for  $\alpha 3\beta 4$ , low affinity at  $\alpha 4\beta 2$  and  $\alpha 7$  nAChR, and acts as a non-competitive antagonist at  $\alpha 3\beta 4$  nAChR *in vitro*. Furthermore, AT-1001 potently blocks nicotine self-administration in rats.

## MATERIALS AND METHODS

### Drugs

AT-1001 (*N*-(2-bromophenyl)-9-methyl-9-azabicyclo[3.3.1]nonan-3-amine) (Figure 1), was synthesized by methods to be published elsewhere. Nicotine tartrate was purchased



**Figure 1** Chemical structure of AT-1001.

from Sigma (St Louis, MO), dissolved in saline and adjusted to neutral pH (7.2–7.4), with doses reported as free base. Mecamylamine HCl (Sigma) was dissolved in saline with doses reported as salt.

### *In vitro* Characterization

**Cell culture.** KX $\alpha 3\beta 4$ R2 and KX $\alpha 4\beta 2$ R2 cells (HEK cells transfected with rat  $\alpha 3\beta 4$  and  $\alpha 4\beta 2$  respectively, obtained from Dr Kenneth Kellar, Georgetown University) were cultured in Dulbecco's modified Eagle's medium, supplemented with 10% fetal bovine serum, 0.5% penicillin/streptomycin, 0.4 mg/ml of geneticin, and were maintained in an atmosphere of 7.5% CO<sub>2</sub> at 37 °C. For binding assays, cells were passaged on 150-mm dishes and harvested when confluent. For functional assays, the cells were seeded into 96-well collagen-coated plates (Becton Dickinson Biocoat) at a density of approximately 50 000 cells per well. Cells seeded at this density grow into a confluent monolayer in 24 to 30 h.

**Binding assays.** Binding to  $\alpha 3\beta 4$  and  $\alpha 4\beta 2$  nAChR was conducted, as described previously (Zaveri *et al*, 2010). Briefly, cells were harvested by scraping the plates with a rubber policeman, suspended in Tris buffer, homogenized in a Polytron homogenizer, and washed twice by centrifugation at 20 000  $\times g$  for 20 min. For binding, the cell membranes were incubated with test compounds at concentrations ranging from 10<sup>-5</sup> to 10<sup>-10</sup> M in the presence of 0.3 nM of [<sup>3</sup>H]epibatidine. After a 2-h incubation at room temperature, samples were filtered through glass fiber filters by using a Tomtec cell harvester, and then counted. Nonspecific binding was determined with 0.1  $\mu$ M of the unlabeled epibatidine. IC<sub>50</sub> values and Hill coefficients were determined using the program Graphpad/PRISM. K<sub>i</sub> values were calculated using the Cheng Prusoff transformation (Cheng and Prusoff, 1973). Binding to  $\alpha 7$  nAChR was conducted with 1.0 nM [<sup>125</sup>I]- $\alpha$ -Bungarotoxin binding to rat brain membranes (Francis *et al*, 2001). Filtration and data analysis were conducted as described for [<sup>3</sup>H]epibatidine binding.

**Ca<sup>2+</sup> fluorescence assays.** Ca<sup>2+</sup> uptake was determined using FLIPR, as described previously (Zaveri *et al*, 2010). For dye loading, the no-wash dye (Molecular Devices Calcium Assay Kit, R8033) was reconstituted in assay buffer (Hank's Balanced Salt Solution with 20 mM of HEPES, pH 7.4) containing 2.5  $\mu$ M of probenecid (Sigma). For antagonist assays, test compounds were diluted in assay buffer containing 100 nM of epibatidine. For calcium measurements,

the FLIPR was equipped with a 5-W argon-ion laser source with an excitation wavelength of 488 nM, and a 510–570 nM bandpass emission filter was used for detection. For the assay, the FLIPR was set up to make fluorescence readings every 1 s for the first minute, and every 6 s for the next 2 min. The compounds were added at 10 s into the fluorescence readings. The change in fluorescence represents the maximum response, minus the minimum response for each well. Graphpad/PRISM was used to determine the  $EC_{50}$ s and  $IC_{50}$ s for the compounds by plotting the changes in fluorescence vs the logs of the compound concentrations.

**Patch clamp recordings.** Stably transfected HEK293 cells expressing  $\alpha 3\beta 4$  nAChR were plated on poly-D-lysine-coated coverslips and grown in 35 mm dishes for 1–2 days before patch clamp recordings. Voltage-clamp recordings were made in the whole-cell configuration using an Axopatch 700A patch-clamp amplifier (Axon Instruments, Unit City, CA). Thin-walled borosilicate glass microelectrodes (TW150F, World Precision Instruments, Sarasota, FL) with resistances of 3–5 M $\Omega$  when filled with an internal solution containing (in mM): 135 CsCl, 10 CsF, 10 HEPES (*N*-[2-hydroxyethyl] piperazine-NV-[2-ethanesulfonic acid]), 5 EGTA (ethylene glycol-bis[*h*-aminoethyl ether]-*N,N,NV,NV*-tetraacetic acid), 1 MgCl<sub>2</sub>, 0.5 CaCl<sub>2</sub>, pH 7.2. Whole-cell capacitance and series resistance were recorded and automatically compensated using the circuitry of the amplifier and PClamp 9.0 software. Cells were continuously superfused with extracellular solution containing (in mM): 150 NaCl, 3 KCl, 5 HEPES, 1 MgCl<sub>2</sub>, 1.8 CaCl<sub>2</sub>, 10 glucose, pH 7.3–7.4. Control, agonist, and drug solutions were applied to individual cells by a local, rapid perfusion driven by gravity for 500 ms or 2 s, each time with a minimum 30-s interval between applications.

**Dopamine release experiments.** The effect of nicotine and other compounds on release of [<sup>3</sup>H]dopamine was determined on synaptosomes prepared from rat nucleus accumbens (NAc). Synaptosomes were produced by a modification of the method of Quik *et al* (2003). Briefly, the NAc was dissected from freshly harvested Sprague-Dawley rat brain, and placed into ice cold Hibernate A medium. Dissected NAc was homogenized in 8.0 ml of 0.32 M sucrose, buffered to pH 7.35 with 5 mM HEPES. A crude synaptosome preparation was obtained by centrifugation at 12 000  $\times$  *g* for 20 min. To initialize uptake, a single pellet was drained and resuspended into 800  $\mu$ l of uptake buffer (128 mM NaCl, 2.4 mM KCl, 1.2 mM KH<sub>2</sub>PO<sub>4</sub>, 25 mM HEPES, pH 7.5, 10 mM glucose, 3.2 mM CaCl<sub>2</sub>, 1.2 mM MgSO<sub>4</sub>, 1 mM ascorbic acid, 10  $\mu$ M pargyline, pH 7.5). The solution was then immediately placed in a 37 °C water bath for 10 min. Test compounds were also added at this point. Next, [<sup>3</sup>H]dopamine was added to a final concentration of 125 nM and synaptosomes were incubated another 5 min at 37 °C. Triplicate samples of 80  $\mu$ l each were then applied to the perfusion chambers. From this point on, washes and release were carried out in perfusion buffer (uptake buffer containing 0.1% BSA and 10  $\mu$ M nomifensin). Unbound test compounds and any uninternalized [<sup>3</sup>H]-DA were removed with this wash. Perfusion was carried out with a flow rate of 1 ml/min, and the first 20 ml were typically discarded. Finally, 12  $\times$  15 drop (~20 s) fractions were collected. At

the fourth fraction, 10  $\mu$ M nicotine, or nicotine plus readministered test compound were passed through the chamber. Subsequent fractions were collected by rinsing in perfusion buffer and subsequently counted in a liquid scintillation counter.

### Self-Administration Experiments

**Food training.** Male Sprague-Dawley rats (age P81, Charles River Labs, Hollister, CA; group housed two per cage) were trained to lever-press to earn a food pellet (45 mg rodent purified diet; Bio-Serv, Frenchtown, NJ) in an operant testing chamber (Med Associates, St Albans, VT). The chamber contained two levers; reinforced (R), responses at which led to reward delivery, non-reinforced (NR), responses at which were recorded but had no consequence. On earning a reward, a cue light above the R lever was illuminated and the house light extinguished for the entire duration of the timeout period. Food training began with a fixed-ratio 1, 1-s timeout (FR1TO1) protocol, followed by FR1TO10, FR2TO20, and FR5TO20. Rats progressed to the next ratio after earning at least 50 pellets during the 30-min session. Throughout the food training protocol, animals were food restricted to maintain approximately 85% of their free-feeding body weight.

**Surgery.** After completing food training, rats were anesthetized with equithesin (0.0035 ml/g body weight), and implanted with an indwelling catheter into the right external jugular vein using previously published procedures (Belluzzi *et al*, 2005). Catheters were flushed daily with heparinized saline solution (0.6 ml of 1000 units/ml heparin in 30 ml saline) to maintain patency. Patency was verified by infusing propofol (0.1 ml, Abbott Laboratories, Chicago, IL) to test for rapid anesthesia.

**Nicotine self-administration.** Following 2 days of recovery, animals were maintained at 90–95% of free-feeding weight and allowed to self-administer nicotine (30  $\mu$ g/kg/infusion, 100  $\mu$ l i.v. over 5.6 s) in the same chambers as used for food training, during daily 1-h sessions. Rats self-administered nicotine under the FR5TO20 schedule for a minimum of 10 days until reaching stable responding ( $R \pm 20\%$  of the mean over 3 days;  $R \geq 6$ ;  $R \geq 2 \times NR$ ). Following stable responding, AT-1001 (0, 0.75, 1.5, or 3 mg/kg, s.c., 10 min before testing  $n = 6$  per group) was administered to all animals in a within-subjects latin-square design, where all animals received all test doses in randomized order. Animals returned to baseline responding for a minimum of 3 days, or until reaching stable responding, before testing the next dose. Following the last test dose, we examined the effects of mecamylamine (3 mg/kg, s.c., 15 min before testing) on nicotine self-administration. Animals were allowed to self-administer nicotine for an additional day to confirm that responding returned to baseline.

**Food self-administration.** To assess any nonspecific effects of AT-1001 treatment a separate group of animals was trained to respond for food pellets. The same testing procedures were used as described above except these animals were given a 3-day rest period rather than catheter implantation surgery and allowed to respond for food

pellets during the 1-h test sessions rather than nicotine. They underwent the same drug treatments as above.

All procedures were conducted in accordance with the National Institutes of Health guidelines for the Care and Use of Animals (2002) and were approved by the Institutional Animal Care and Use Committees.

**Data analysis.** The effect of AT-1001 on the number of responses was analyzed by repeated-measures ANOVA, with drug dose as the within-subjects factor. Significant main effects were further analyzed by paired *t*-tests, Bonferroni corrected for multiple comparisons. AT-1001 effects on nicotine self-administration and food-maintained responding were analyzed separately.

## RESULTS

### Receptor Binding and $Ca^{2+}$ Fluorescence Assays

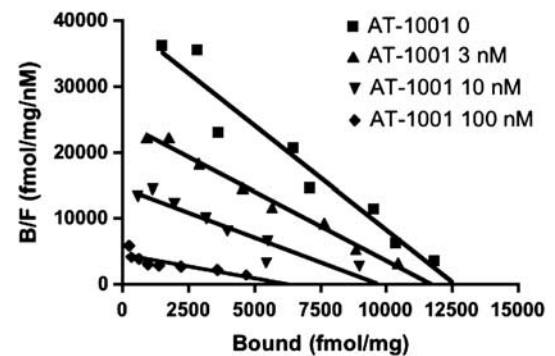
AT-1001 binds to  $\alpha 3\beta 4$  nAChRs in membranes of HEK cells transfected with this receptor type. It has high affinity and selectivity for the  $\alpha 3\beta 4$  nAChR, with  $K_i$  values of 2.4, 476, and 221 nM at  $\alpha 3\beta 4$ ,  $\alpha 4\beta 2$ , and  $\alpha 7$  nAChR, respectively (Table 1). The affinity of AT-1001 is less than that of epibatidine, but is far higher than the other agonists nicotine and cytosine. The nAChR antagonists mecamylamine and 18-MC did not compete with [ $^3$ H]epibatidine binding. As seen in Figure 2, increasing concentrations of AT-1001 did not induce a parallel shift in the Scatchard Plot derived from a [ $^3$ H]epibatidine saturation experiment. Rather, AT-1001 induced a decrease in  $B_{max}$  as well as an increase in  $K_d$ . Similar data are shown with the direct binding saturation curves in Supplementary Figure S1. This demonstrates that AT-1001 is not strictly a competitive antagonist of epibatidine binding to  $\alpha 3\beta 4$  nAChR. Nevertheless, it competes with [ $^3$ H]epibatidine, unlike mecamylamine and 18-MC.

Efficacy at  $\alpha 3\beta 4$  nAChR was determined by measuring  $Ca^{2+}$  flux in HEK cells transfected with  $\alpha 3\beta 4$  nAChR (Table 2). AT-1001 has no agonist activity of its own but potently inhibits an epibatidine-induced increase in intracellular  $Ca^{2+}$ -induced fluorescence. Epibatidine treatment produced a 1000-fold increase in intracellular  $Ca^{2+}$ -induced fluorescence, and this was inhibited by

several nicotinic antagonists (see Figure 3 and Table 2). AT-1001 ( $IC_{50}$   $35.2 \pm 8.1$  nM) was more than twice as potent as mecamylamine and nearly 30 times more potent than 18-MC. Once again, inhibition was non-competitive, as AT-1001 induced a decrease in maximal  $Ca^{2+}$  flux rather than a parallel shift to the right of the epibatidine dose response curve (Figure 3). Unlike with [ $^3$ H]epibatidine binding, both mecamylamine and 18-MC were able to inhibit epibatidine-induced  $Ca^{2+}$  flux in these cells, indicating that both of these compounds are also  $\alpha 3\beta 4$  nAChR antagonists. However, neither of these compounds was nearly as potent as AT-1001.

### Assessment of Off-Target Activity of AT-1001

AT-1001 has been evaluated against 48 different receptor and ion channel targets in the NIMH Psychoactive Drug Screening Program (Supplementary Table T1). The only



**Figure 2** Inhibition of [ $^3$ H]epibatidine binding to  $\alpha 3\beta 4$ -containing HEK cell membranes by AT-1001. Scatchard analysis was plotted from saturation binding to HEK cell membranes, using [ $^3$ H]epibatidine concentrations ranging from 0.04 to 3.2 nM. Saturation experiments were conducted in the presence of 0 (■) 3 nM (▲), 10 nM (▼), and 100 nM (◆) AT-1001. Data are from a single experiment repeated twice with similar results.  $K_d$  and  $B_{max}$  values were: 0.32 nM, 12 620 fmol/mg protein; 0.48 nM, 11 700 fmol/mg protein; 0.66 nM, 9723 fmol/mg protein; and 1.40 nM, 6320 fmol/mg protein for Scatchard analysis in the presence of 0, 3, 10, and 100 nM AT-1001, respectively. Therefore, increasing concentrations of AT-1001 show a decrease in  $B_{max}$  and increase in  $K_d$ , suggesting non-competitive, or mixed inhibition of binding. As inhibition was not strictly competitive, a  $pA_2$  value could not be determined.

**Table 1** Binding Affinity  $K_i$  (nM) of AT-1001, Compared with Other Prototypical Agonists at  $\alpha 3\beta 4$ ,  $\alpha 4\beta 2$ , and  $\alpha 7$  Receptors

Compound	Ki (nM)		
	$\alpha 3\beta 4$	$\alpha 4\beta 2$	$\alpha 7$
Epibatidine	0.15 $\pm$ 0.05	0.06 $\pm$ 0.0	4.16 $\pm$ 0.47
Nicotine	480.69 $\pm$ 59.38	11.13 $\pm$ 1.11	
Cytosine	202.89 $\pm$ 18.85	1.53 $\pm$ 0.20	
AT-1001	2.64 $\pm$ 0.24	476.52 $\pm$ 98.75	221.72 $\pm$ 9.88
SRI6584	507.86 $\pm$ 162.39	> 10K	> 10K
18-MC	> 10K	> 10K	

Data show mean  $\pm$  SD for at least two experiments conducted in triplicate.

**Table 2** Effect of nAChR Agonists and Antagonists on  $Ca^{2+}$ -Induced Fluorescence in  $\alpha 3\beta 4$ -Containing HEK Cells

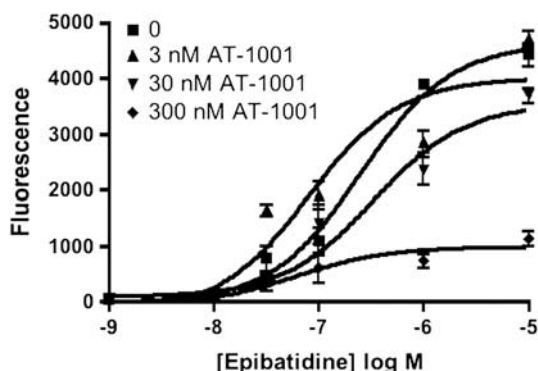
Compound	$\alpha 3\beta 4$
	$EC_{50}$ (nM) $\pm$ SEM
Epibatidine	43.5 $\pm$ 12
	$IC_{50}$ (nM) $\pm$ SEM
AT-1001	35.2 $\pm$ 8.1
18-MC	1058 $\pm$ 530
Mecamylamine	83.7 $\pm$ 13.3
DH $\beta$ E	> 10000

Data show mean  $\pm$  SD for at least two experiments conducted in quadruplicate.

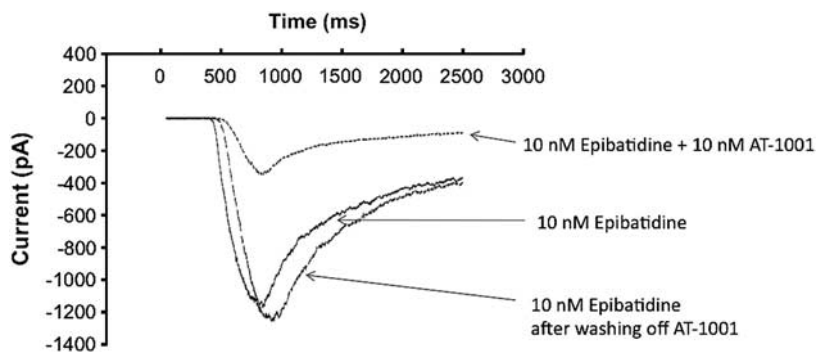
receptors for which AT-1001 had affinity below a  $K_i$  of 1  $\mu\text{M}$ , are the 5HT<sub>3</sub> ( $K_i$  600 nM), histamine H<sub>1</sub> ( $K_i$  201 nM), muscarinic M<sub>4</sub> ( $K_i$  661 nM) and M<sub>5</sub> ( $K_i$  325 nM), SERT (271 nM) sigma<sub>2</sub> ( $K_i$  418 nM), and sigma<sub>1</sub> ( $K_i$  84 nM). Given the high binding affinity of AT-1001 for its intended target, AT-1001 demonstrates a selectivity of >100-fold for everything except other  $\beta 4$ -containing nAChRs and sigma<sub>1</sub>, and still maintains nearly 50-fold selectivity over these binding sites.

### Whole-Cell Voltage Clamp Experiments

Inward currents were induced in HEK cells transfected with  $\alpha 3\beta 4$  nAChR by either nicotine or epibatidine in a concentration-dependent manner (Supplementary Figure S2). Epibatidine had an  $EC_{50}$  of approximately 150 nM. AT-1001 potently inhibits the epibatidine-induced current in HEK cells. As shown in Figure 4, inward current, induced by 10 nM epibatidine, was inhibited  $66 \pm 15\%$  by the inclusion of 10 nM AT-1001. The inhibition induced by a 2-s



**Figure 3** Inhibition of epibatidine-induced  $\text{Ca}^{2+}$  fluorescence by AT-1001 in HEK cells containing  $\alpha 3\beta 4$  nAChR.  $\text{Ca}^{2+}$  fluorescence induced by increasing concentrations of epibatidine was measured as described in Materials and methods section. Epibatidine dose-response experiments conducted in the presence of 0 (■), 3 nM (▲), 30 nM (▼), and 300 nM (◆) of AT1001 induced a rightward shift in  $EC_{50}$  and progressive decrease in maximal effect suggesting a non-competitive mode of inhibition. Data shown are mean  $\pm$  SD from a single experiment conducted in quadruplicate that was repeated with similar results.



**Figure 4** Patch clamp recording in  $\alpha 3\beta 4$ -containing HEK cells. Sample trace of the blocking effect of 10 nM AT-1001 against 10 nM Epibatidine activation (66% block,  $n = 3$ ). Whole-cell voltage-clamp recordings were made using Axon 700A and PClamp 8.2. Experiments were conducted within 24–72 h after replating the stable cells. Cells were clamped at  $-60$  mV and drugs were delivered via local rapid perfusion for 500 ms, as described in Materials and methods section.

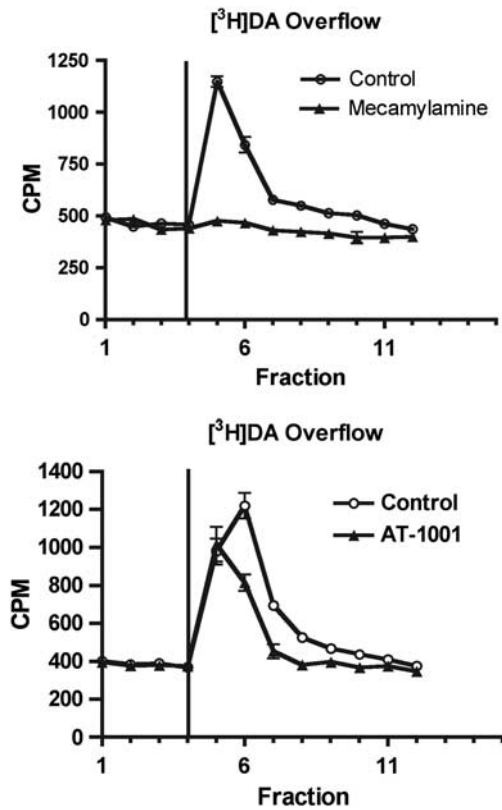
application of AT-1001 was fully reversed by a minimum of a 30-s perfusion of control solution, indicating that the inhibition by AT-1001 at the  $\alpha 3\beta 4$  nAChR-gated channel is fully reversible.

### Effect on [<sup>3</sup>H]dopamine Release *In Vitro*

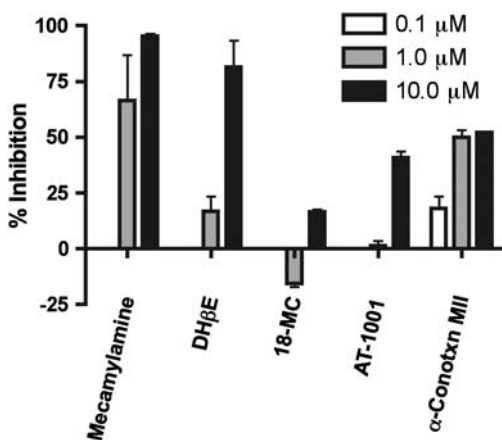
If  $\alpha 3\beta 4$  nAChRs have an effect on reward, they may be expected to inhibit release of dopamine from a striatal slice or synaptosome preparation. To test this hypothesis, we examined whether AT-1001 could inhibit nicotine-induced release of [<sup>3</sup>H]dopamine from NAc synaptosomes. In this preparation, synaptosomes were perfused with 10  $\mu\text{M}$  nicotine, inducing a 3–4-fold increase in release of previously accumulated [<sup>3</sup>H]dopamine (Figure 5). This release was inhibited at 1 and 10  $\mu\text{M}$  by the non-selective nAChR antagonist mecamylamine and the  $\alpha 4\beta 2$ -selective antagonist DH $\beta$ E, which has 100-fold higher affinity for  $\alpha 4\beta 2$  than  $\alpha 3\beta 4$  nAChRs (Chavez-Noriega *et al*, 1997). The selective  $\alpha 3\beta 4$  antagonist AT-1001 had a partial effect (40% inhibition) on [<sup>3</sup>H]dopamine release only at the high dose of 10  $\mu\text{M}$ , while 18-MC had a smaller effect, inhibiting [<sup>3</sup>H]dopamine release only approximately 15% at 10  $\mu\text{M}$ . Consistent with other studies, the  $\alpha 6$ -selective antagonist conotoxin MII inhibited approximately 50% at both 1 and 10  $\mu\text{M}$ , indicating roughly half of the receptors contain the  $\alpha 6$  subunit (Figure 6) (Grady *et al*, 2002).

### Effect of AT-1001 on Nicotine Self-Administration in Rats

AT-1001 dose-dependently decreased responding for nicotine, but not food pellets (Figure 7a). In the nicotine group, there was a significant main effect of drug dose ( $F_{3,15} = 46.685$ ;  $p < 0.001$ ). Further testing revealed that all AT-1001 doses significantly reduced responding compared with vehicle treatment. In contrast, vehicle administration did not produce a significant change from baseline responding. Although there was a significant main effect of AT-1001 treatment in the food-responding group ( $F_{3,15} = 7.582$ ;  $p = 0.003$ ), there were no significant differences in *post-hoc* tests ( $p = 0.054$  corrected, AT-1001, 3 mg/kg vs vehicle).

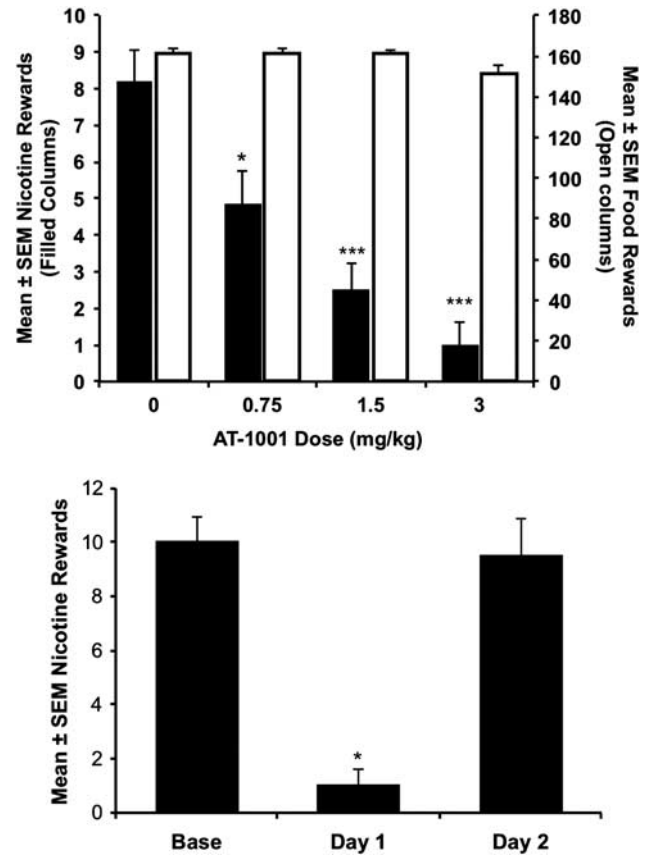


**Figure 5** Inhibition of nicotine-induced [ $^3\text{H}$ ]DA release from nucleus accumbens synaptosomes. Synaptosomes were perfused with buffer and then  $10\ \mu\text{M}$  nicotine was added as indicated by the vertical line at fraction 4. Nicotine-induced [ $^3\text{H}$ ]DA release was inhibited completely by  $10\ \mu\text{M}$  mecamylamine (a) and to a much lesser extent by  $10\ \mu\text{M}$  AT-1001 (b). Data shown are mean  $\pm$  SD from a single experiment conducted in triplicate that was repeated with similar results.



**Figure 6** Inhibition of nicotine-induced [ $^3\text{H}$ ]DA release by nAChR antagonists. Data shown represents mean inhibition  $\pm$  SD from three experiments conducted in triplicate. Mecamylamine and conotoxin MII significantly inhibited [ $^3\text{H}$ ]DA at both  $1$  and  $10\ \mu\text{M}$  while DH $\beta$ E and AT-1001 inhibited release only at  $10\ \mu\text{M}$ .

As 18-MC treatment has long-lasting effects (Glick *et al*, 2000a), we compared responding the day before testing with the  $3\ \text{mg/kg}$  dose of AT-1001, the day of testing, and the day after testing (Figure 7b). There was a significant main effect



**Figure 7** AT-1001 reduces nicotine self-administration in rats. (a) Acute effects of AT-1001 treatment. Each data point is the mean  $\pm$  SEM number of rewards earned in the 1-h session following AT-1001 treatment. Filled bars represent animals responding for nicotine ( $30\ \mu\text{g/kg/inj}$ , left axis), solid bars are animals responding for food pellets ( $45\ \text{mg}$  pellets, right axis). AT-1001 doses ( $\text{mg/kg}$ , s.c.) are indicated on the x axis. \* $p < 0.05$  vs vehicle, \*\*\* $p < 0.001$  vs vehicle. (b) AT-1001 ( $3\ \text{mg/kg}$ ) does not have long-lasting effects. Base is the number of reinforced responses on the day before AT-1001 treatment. Day 1 is the number of reinforced responses on the test day of AT-1001 ( $3\ \text{mg/kg}$ ) treatment, while day 2 is the day following the test day. \* $p < 0.05$  vs base and day 2.  $n = 6$  per group.

of day ( $F_{2,10} = 40.043$ ;  $p < 0.001$ ), with responding on the test day significantly lower than either other day ( $p < 0.001$ ). Animals responded the day after treatment at baseline levels, indicating that AT-1001 did not have long-lasting effects. Finally, we tested whether the nonspecific nAChR antagonist mecamylamine, which reduces nicotine self-administration in this paradigm (Corrigall and Coen, 1989), was able to reduce responding to a similar extent as AT-1001. Consistent with past reports, mecamylamine ( $3\ \text{mg/kg}$ ; s.c.) significantly reduced responding for nicotine ( $p = 0.011$ ). However, AT-1001 ( $3\ \text{mg/kg}$ ) reduced responding significantly more than did mecamylamine ( $90.5\% \pm 7.1$  vs  $48\% \pm 8.9$ ;  $p < 0.05$ ) (Supplementary Figure S3).

## DISCUSSION

Considerable research has been conducted to determine the nAChR subunits involved in drug abuse and nicotine abuse in particular. The  $\alpha 4\beta 2$  nAChR is the most prevalent in

brain tissue and it was demonstrated relatively early on that both the  $\alpha 4$  and  $\beta 2$  subunits have a role in nicotine-seeking activity.  $\beta 2$  nAChR knockout mice do not self-administer nicotine, an activity that can be restored by viral vector-induced replacement of this subunit into the VTA (Maskos *et al*, 2005; Picciotto *et al*, 1998; Pons *et al*, 2008). Complementary experiments demonstrated that selective activation of a mutated  $\alpha 4$  subunit that responds to lower nicotine concentrations, by low doses of nicotine that do not activate wild-type nAChR, is sufficient for nicotine-induced reward, as determined by CPP (Tapper *et al*, 2004). In addition, the clinically used medication for smoking cessation, varenicline, is a potent  $\alpha 4\beta 2$  nAChR partial agonist that decreases nicotine-induced increase in NAC dopamine, and attenuates nicotine self-administration (Rollema *et al*, 2007).

The  $\alpha 6$  subunit has been demonstrated to be equally important for nicotine self-administration. Deletion of the  $\alpha 6$  subunit blocks nicotine self-administration in mice, which can be restored by replacement of the  $\alpha 6$  subunit into the VTA (Pons *et al*, 2008). In addition, inhibition of  $\alpha 6$ -containing nAChR with  $\alpha$ -conotoxin MII (H9A;L15A) blocks both nicotine CPP and nicotine withdrawal-associated CPA and anxiety (Jackson *et al*, 2009; McIntosh *et al*, 2004). In fact, both  $\alpha 4$  and  $\alpha 6$  receptors are required in the VTA to induce the rewarding effects of nicotine, suggesting that the  $\alpha 4\alpha 6\beta 2$  receptor might be the most important player in mediating nicotine abuse (Pons *et al*, 2008).

More recently, the importance of the  $\alpha 5$  subunit to nicotine addiction has been demonstrated. However, deletion of the  $\alpha 5$  subunit modulates nicotine self-administration and reward in the opposite direction to the deletion of  $\beta 2$  or  $\alpha 6$ . Nicotine demonstrates an inverted U shaped dose response curve for self-administration and CPP, with higher doses being less rewarding. Deleting the  $\alpha 5$  subunit appears to block the aversive properties of higher nicotine doses, thereby inhibiting the falling arm of the dose response curve and increasing both self-administration and CPP at the higher nicotine doses (Fowler *et al*, 2011; Jackson *et al*, 2010). These results are particularly interesting because several studies have identified the  $\alpha 5$ - $\alpha 3$ - $\beta 4$  nicotinic receptor gene cluster found on chromosome 15 as associated with increased risk for tobacco dependence. Variants in this gene cluster lead to an increased likelihood of a smoker becoming dependent and to smoking a greater number of cigarettes per day (Berrettini *et al*, 2008; Caporaso *et al*, 2009; Saccone *et al*, 2008; Thorgeirsson *et al*, 2008). A variant (rs16969968) leading to a D398N change in amino acid sequence of the  $\alpha 5$  subunit may be particularly important in this regard as the N variant of the receptor appears to less efficiently induce  $\text{Ca}^{2+}$  flux than the more prevalent D-containing receptor in HEK cells containing  $\alpha 4\alpha 5\beta 2$  receptors transfected with the different receptor variants (Bierut *et al*, 2008).

Although the  $\alpha 5$  subunit of the nAChR can be found in the midbrain reward center, one location proven to be crucial for its effect on nicotine self-administration is the MHb to IPN pathway. The MHb sends projections through the fasciculus retroflexus to the IPN. Fowler *et al*. demonstrated that reintroduction of the  $\alpha 5$  subunit via a viral vector into the IPN restores self-administration to w.t. levels (Fowler *et al*, 2011). Inactivation of the MHb or IPN by

lidocaine microinjection produces self-administration levels similar to the  $\alpha 5$  KO animals. The MHb and IPN have a very high level of nAChRs. Furthermore, a large portion of these receptors represent the  $\alpha 3\beta 4$  nAChR subtype and these regions, along with the pineal gland, are the only brain regions with a high density of this subtype (Perry *et al*, 2002). However, most  $\alpha 3\beta 4$  subunits in the MHb-IPN are not complexed with  $\alpha 5$ , and the  $\alpha 3\beta 4$  nAChR controls ACh release in the fasciculus retroflexus (Grady *et al*, 2009).

In addition to the  $\alpha 4$ ,  $\alpha 5$ ,  $\alpha 6$ , and  $\beta 2$  containing receptors, the  $\alpha 3\beta 4$  nAChR has also been demonstrated to be involved in drug-seeking behavior, not only of nicotine but a variety of other drugs of abuse as well. As one example, deletion of the  $\beta 4$  subunit eliminated nicotine withdrawal symptoms (Salas *et al*, 2004). Glick *et al* (2000a, 2006) have demonstrated repeatedly that the ibogaine analog 18-MC is able to attenuate self-administration of a variety of abused drugs, including nicotine, morphine, cocaine, methamphetamine, and alcohol (Rezvani *et al*, 1997). It also blocked the acquisition of cocaine CPP, but surprisingly potentiated cocaine prime-induced reinstatement (McCallum and Glick, 2009). Unlike ibogaine, 18-MC appears to be a somewhat selective, though not particularly potent,  $\alpha 3\beta 4$  nAChR antagonist (Glick *et al*, 2000b).

Our studies were undertaken to identify new selective  $\alpha 3\beta 4$  nAChR antagonists and determine if this receptor is a viable target for treating tobacco dependence. AT-1001 is the first reported  $\alpha 3\beta 4$  antagonist with nearly 100-fold selectivity over  $\alpha 4\beta 2$  and  $\alpha 7$  nAChRs. It has very high binding affinity ( $K_i < 10$  nM) for the  $\alpha 3\beta 4$  nAChR, as determined by inhibition of [ $^3\text{H}$ ]epibatidine binding, and has about a 200-fold higher affinity for  $\alpha 3\beta 4$  nAChR than our original lead compound SR16584 (Zaveri *et al*, 2010). AT-1001 is not a congener of 18-MC and clearly binds differently to the  $\alpha 3\beta 4$  nAChR because 18-MC does not block [ $^3\text{H}$ ]epibatidine binding but in fact binds to the mecamlamine site in the central lumen of the receptor (Arias *et al*, 2010). Although AT-1001 displaces [ $^3\text{H}$ ]epibatidine, Scatchard analysis indicates at least partial non-competitive inhibition of [ $^3\text{H}$ ]epibatidine binding. Both AT-1001 and 18-MC inhibit  $\alpha 3\beta 4$  nAChR activity, with the ability to block epibatidine-induced  $\text{Ca}^{2+}$  flux in transfected HEK cells. However, AT-1001 is approximately 30-fold more potent than 18-MC as an inhibitor of  $\text{Ca}^{2+}$  flux. AT-1001 is also more potent than mecamlamine in inhibiting epibatidine-induced  $\text{Ca}^{2+}$  flux through  $\alpha 3\beta 4$  nAChR.

Consistent with past reports implicating the  $\alpha 3\beta 4$  nAChR subtype in drug-seeking behavior, AT-1001 potently blocked self-administration of nicotine in rats. In this model of addiction, AT-1001 (0, 0.75, 1.5, and 3.0 mg/kg) produced a dose-dependent inhibition of nicotine self-administration, almost completely blocking nicotine responding at 3 mg/kg. This ability to block nicotine self-administration was rapidly reversible, with nicotine responding returning to pre-drug levels by 24 h. In contrast, AT-1001 did not affect food-seeking behavior, suggesting that the drug acts specifically on nicotine-induced rather than natural reward processes. In addition, AT-1001 did not show any reward of its own up to 10 mg/kg in mice using the CPP paradigm (Supplementary Figure S4). We have not yet examined this compound for ganglionic blockade-induced hypotension or constipation. Based on previous studies by Glick *et al* (2006) and Fowler

*et al* (2011), and the discrete localization of  $\alpha 3\beta 4$  nAChR (Perry *et al*, 2002), we believe that AT-1001 is acting at the level of the MHb and IPN, supporting the hypothesis that both these brain regions and the  $\alpha 3\beta 4$  nAChR subtype are involved in nicotine self-administration. In addition, the fact that some animals at the highest dose did not self-administer any nicotine suggests that AT-1001 is affecting drive for the drug reward rather than antagonizing nicotine's rewarding effects. This is also consistent with studies by Glick *et al* (2000a, 2006) demonstrating a reward-attenuating effect of 18-MC for more than just nicotine (McCallum and Glick, 2009; Rezvani *et al*, 1997). On the other hand, varenicline, DH $\beta$ E, and mecamylamine, which probably inhibit nicotine reward directly at the level of the NAc/VTA, reduce rather than eliminate, responding for nicotine (Corrigall and Coen, 1989; Glick *et al*, 2011; Rollema *et al*, 2007; Watkins *et al*, 1999).

Since AT-1001 potentially blocked nicotine self-administration, we investigated whether AT-1001 had any effect on DA release in the NAc. AT-1001 was tested for its ability to block nicotine-induced [ $^3$ H]DA release from synaptosomes produced from tissue taken from rat NAc. In this system, mecamylamine was the most potent compound, and completely blocked [ $^3$ H]DA release when used at 10  $\mu$ M. AT-1001, which is selective for the  $\alpha 3\beta 4$  and far more potent than mecamylamine for inhibition of Ca $^{2+}$  flux in HEK cells transfected with the  $\alpha 3\beta 4$  nAChR, produces only partial inhibition of [ $^3$ H]DA release at 10  $\mu$ M, a far higher concentration than at which it inhibits Ca $^{2+}$  flux. nAChR in the striatum are mostly  $\alpha 4\beta 2$ ,  $\alpha 4\alpha 5\beta 2$ , and  $\alpha 4\alpha 6\beta 2$  nAChR (Gotti *et al*, 2010). Nicotine-induced [ $^3$ H]DA release from this brain region is attenuated by the antagonist DH $\beta$ E, which has 100-fold higher affinity for  $\alpha 4\beta 2$  than  $\alpha 3\beta 4$ , and partially inhibited by the  $\alpha 6$ -selective antagonist conotoxin MII. This is consistent with literature values and suggests that a subset of nAChR receptors in this brain region contain the  $\alpha 6$  subunit (Grady *et al*, 2002). Bivalent anionic compounds, such as *N,N*-alkane-diylbis-3-picolinium, and *N,N*-decane-1,10-diyl-bis-3-picolinium diiodide (bPiDI), which inhibit  $\alpha 4\beta 2$  and  $\alpha 6$ -containing nAChR have also been demonstrated to block DA release from a striatal brain slice preparation (Dwoskin *et al*, 2008; Wooters *et al*, 2011). The poor potency of inhibition of DA release by AT-1001 probably reflects inhibition of the  $\alpha 4\beta 2$  subtype at this high concentration. These results indicate that the inhibition of nicotine self-administration by AT-1001 is not a consequence of a decrease in striatal (NAc) DA levels by acting directly at this brain region, as is the case with the  $\alpha 6\beta 2$ -selective antagonist, bPiDI (Wooters *et al*, 2011), and suggest that the role of the  $\alpha 3\beta 4$  nAChR receptor subtype in decreasing nicotine self-administration still needs further investigation. Although the  $\alpha 3\beta 4$  nAChR has a relatively low affinity for nicotine, we believe that serum nicotine levels are sufficiently high to activate this receptor in the self-administration assay. We have previously demonstrated that two intravenous infusions of nicotine at the dose used in this study yield blood levels of 1.85  $\mu$ M that remain high over a 30-min period (Cao *et al*, 2007). Thus, the potent effect of AT-1001 on nicotine self-administration is a good starting point for the demonstration of the role of this important nAChR subtype, as a target for smoking cessation medications.

The mechanism by which  $\alpha 3\beta 4$  nAChR antagonism reduces nicotine self-administration is unclear. The fact that  $\alpha 3\beta 4$  nAChR antagonism reduces nicotine self-administration strongly suggests that, in some way,  $\alpha 3\beta 4$  receptor activation, probably originating in the MHb and/or IPN, promotes self-administration. Nevertheless, studies by Fowler *et al* (2011) clearly demonstrate that nAChRs containing  $\alpha 5$  subunits, in these same brain regions, seem to limit self-administration. Potentially, the  $\alpha 5$  subunits are on  $\alpha 4\beta 2$  nAChRs and therefore  $\alpha 3\beta 4$  and  $\alpha 4\alpha 5\beta 2$  may work against each other with respect to nicotine's rewarding properties. At this time, the relative levels of  $\alpha 3\beta 4$  and  $\alpha 4\beta 2$  nAChR in the MHb and IPN are somewhat difficult to determine and still in question (Gotti *et al*, 2010; Perry *et al*, 2002). Furthermore, the pathway by which the IPN projects to the VTA or NAc is not well understood. Resolving these issues may require new tools for identifying and labeling receptors. The family of compounds represented by AT-1001 may provide some of these tools. In addition to its potential as a drug abuse medication, AT-1001 and other compounds in this family will certainly have significant research benefits. We have recently synthesized an [ $^{125}$ I]-analog of AT-1001. Preliminary studies suggest that this radiolabeled ligand will be useful for radioligand binding and *in vitro* autoradiography to further characterize the localization of this receptor subtype in brain.

In conclusion, we have characterized the first high affinity and selective  $\alpha 3\beta 4$  nAChR antagonist, AT-1001. This compound has nanomolar affinity at the  $\alpha 3\beta 4$  nAChR, and nearly 100-fold selectivity vs  $\alpha 4\beta 2$  and  $\alpha 7$  nAChR. AT-1001 binds in a non-competitive manner to the  $\alpha 3\beta 4$  nAChR, but unlike channel blockers such as mecamylamine, it competes for the [ $^3$ H]epibatidine binding site, facilitating structure-activity studies. In addition to its selectivity, AT-1001 is much more potent than either 18-MC or the classical nicotinic antagonist mecamylamine in inhibiting receptor activation *in vitro*. Finally, when administered systemically, AT-1001 dose-dependently blocks nicotine self-administration in rats without affecting food responding. This compound shows great potential as a novel pharmacotherapy for smoking cessation and validates the  $\alpha 3\beta 4$  nAChR as a target for tobacco dependence medications.

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## DISCLOSURE

The authors declare no conflict of interest.



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Supplementary Information accompanies the paper on the Neuropsychopharmacology website (<http://www.nature.com/npp>)