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RESEARCH PAPER

AT-1001: a high-affinity α3β4 nAChR ligand with novel nicotine-suppressive pharmacology

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BACKGROUND AND PURPOSE

The α3β4 subtype of nicotinic acetylcholine receptors (nAChRs) has been implicated in mediating nicotine reinforcement processes. AT-1001 has been recently described as a high-affinity and selective α3β4 nAChR antagonist that blocks nicotine self-administration in rats. The aim of this study was to investigate the mechanism of action underlying the nicotine-suppressive effects of AT-1001.

EXPERIMENTAL APPROACH

Effects of AT-1001 were determined using *in vitro* assays and rat models of nicotine addiction, and compared with varenicline.

KEY RESULTS

AT-1001 and its analogue AT-1012 were functionally selective as antagonists for α3β4 over α4β2 nAChRs, but not to the same extent as the binding selectivity, and had partial agonist activity at α3β4 nAChRs. In contrast, varenicline was a partial agonist at α4β2, a weak agonist at α3β4 and inhibited α4β2 at a much lower concentration than it inhibited α3β4 nAChRs. AT-1001 and varenicline also had very different *in vivo* properties. Firstly, AT-1001 did not exhibit reinforcing properties *per se* while varenicline was self-administered. Secondly, systemic treatment with AT-1001 did not induce reinstatement of nicotine seeking but rather attenuated reinstatement induced by varenicline, as well as nicotine. Finally, unlike varenicline, AT-1001 selectively blocked nicotine self-administration without altering alcohol lever pressing as assessed in an operant co-administration paradigm.

CONCLUSIONS AND IMPLICATIONS

These findings describe a more complex AT-1001 *in vitro* profile than previously appreciated and provide further support for the potential of AT-1001 and congeners as clinically useful compounds for smoking cessation, with a mechanism of action distinct from currently available medications.

Abbreviations

EXT, extinction; FR, fixed ratio; HBSS, Hank's balanced salt solution; MP, membrane potential; NAc, nucleus accumbens; nAChRs, nicotinic acetylcholine receptors; PR, progressive ratio; TO, time out

Tables of Links

TARGETS	
α3 nAChR	α6 nAChR
α4 nAChR	α7 nAChR
α5 nAChR	β2 nAChR
	β4 nAChR

LIGANDS	
[3H]-epibatidine	Mecamylamine
Alcohol (ethanol)	Morphine
Cocaine	Nicotine
Dopamine	Varenicline

These Tables list key protein targets and ligands in this article which are hyperlinked to corresponding entries in http://www.guidetopharmacology.org, the common portal for data from the IUPHAR/BPS Guide to PHARMACOLOGY (Pawson *et al.*, 2014) and are permanently archived in the Concise Guide to PHARMACOLOGY 2013/14 (Alexander *et al.*, 2013).

Introduction

Nicotine is the main active ingredient contained in tobacco leaves that is responsible for the addictive properties associated with smoking. Nicotine binds to nicotinic acetylcholine receptors (nAChRs) found in the brain and the periphery. There is considerable reason to believe that several nAChR subtypes are involved in nicotine addiction. The greatest amount of evidence relates to the α4β2 nAChR, by far the most abundant in the brain and found in high concentrations in reward centres such as the nucleus accumbens (NAc) and ventral tegmental area (Perry *et al.*, 2002; 2007). Knockout of the β2 subunit eliminates nicotine self-administration in mice (Picciotto *et al.*, 1998; Epping-Jordan *et al.*, 1999), while knocking in a mutant α4 subunit induces higher sensitivity of the α4β2 nAChR to nicotine and renders mice more susceptible to self-administration (Tapper *et al.*, 2004). Consistent with a role for α4β2 nAChR in mediating nicotine reward, varenicline, a partial agonist at this receptor, reduces nicotine self-administration in rats (Rollema *et al.*, 2007; George *et al.*, 2011).

In addition to the α4β2, recent genetic and knockout studies have shown the importance of other nAChR subunits in nicotine dependence including the α6 and the α5 nAChR subunit (Pons *et al.*, 2008; Fowler *et al.*, 2011). There is now considerable evidence that the α3β4 nAChR, highly expressed in the medial habenula and interpeduncular nucleus, but very sparsely in most other regions (Marks *et al.*, 2002; Perry *et al.*, 2002), is also involved in drug reward. Studies using the α3β4 nAChR antagonist 18-methoxycoronaridine have demonstrated that α3β4 inhibition blocks self-administration of a large number of abused drugs including nicotine, morphine, cocaine and alcohol (Glick and Maisonneuve, 2000; Maisonneuve and Glick, 2003). In agreement, we have recently reported that AT-1001, a novel α3β4 nAChR ligand with high affinity and selectivity for this receptor, blocked nicotine self-administration in rats without affecting operant responding for food (Toll *et al.*, 2012). In contrast, transgenic mice with targeted overexpression of β4 nAChRs showed aversion for nicotine (Frahm *et al.*, 2011).

Currently, the most efficacious smoking cessation medication used clinically is varenicline (Chantix; Jorenby *et al.*, 2006; Galanti, 2008). This compound has been hypothesized to reduce smoking by selectively partially activating α4β2 nAChR (Rollema *et al.*, 2007). However, although binding studies support the selectivity of varenicline for the α4β2 nAChR, *in vitro* functional studies have indicated that it fully activates α3β4 and α7 nAChR at virtually the same concentration at which it partially activates α4β2 nAChR (Mihalak *et al.*, 2006; Rollema *et al.*, 2007; Chatterjee *et al.*, 2011). These and other studies have led to the suggestion that the ability of this compound to block nicotine- and alcohol-mediated behaviours may be due to stimulation of α3β4 nAChR rather than, or in addition to, partial activation of α4β2 (Steensland *et al.*, 2007; Baldwin *et al.*, 2011; Chatterjee *et al.*, 2011).

To better understand the mechanism of action underlying the nicotine-suppressive properties of AT-1001, we further analysed the *in vitro* activities of this compound and its analogue AT-1012, and compared these with varenicline. AT-1001 and varenicline were also compared with regard to abuse potential, vulnerability to relapse and selectivity of nicotine-suppressive effects.

Methods

Cell culture

KXα3β4R2 and KXα4β2R2 cells containing rat α3β4 and α4β2 nAChRs, respectively (obtained from Drs Kenneth Kellar and Yingxian Xiao, Georgetown University, Washington, DC, USA), were cultured as described by Wu *et al.* (2014).

Binding assays

Binding assays were conducted using 0.3 nM [^{3}H]-epibatidine as the radioligand (Toll *et al.*, 2012). IC_{50} values and Hill coefficients were determined by using GraphPad Prism (GraphPad Software, Inc, La Jolla, CA, USA). K_i values were calculated using the Cheng–Prusoff transformation (Cheng and Prusoff, 1973).

Calcium (Ca^{2+}) fluorescence and membrane potential (MP) assays

nAChR functional activity was determined by measuring nAChR-induced Ca^{2+} mobilization (for α3β4 nAChR) or MP change (for α4β2 nAChR) using the Molecular Devices Ca^{2+} dye or Membrane Potential Assay Kit (Blue Dye; Molecular Devices, Sunnyvale, CA, USA) using the FlexStation 3® microplate reader (Molecular Devices). Briefly, HEK cells transfected

with α3β4 or α4β2 nAChRs were seeded in a 96-well plate (4000 cells per well) 1 day before the experiments. For agonist assays, after brief washing, cells were loaded with 225 μL of HBSS assay buffer (Hank's balanced salt solution with 20 mM of HEPES, pH 7.4) containing the appropriate dye, and incubated at 37°C. After 30 min, 25 μL solutions of test compounds were dispensed into the wells by the FlexStation and resulting MP change or Ca^{2+} fluorescence was recorded every 3 s for 120 s by reading at 565 nM fluorescence excited at 530 nM wavelength for MP, and reading at 525 and excited at 485 for Ca^{2+} flux. For the antagonist assay, cells were loaded with 200 μL HBSS buffer containing dye and incubated at 37°C. After 15 min, 25 μL of test compounds was added, and after another 15 min, 25 μL of epibatidine was added by the FlexStation to a final concentration of 100 nM, and fluorescence measured as described above. The change in fluorescence represents the maximum response, minus the minimum response for each well. GraphPad Prism was used to determine the EC_{50} and IC_{50} values by plotting the changes in fluorescence versus the logs of the compound concentrations.

Animals

Fifty-seven male Sprague-Dawley rats (200–225 g) were obtained from Charles River (Portage, MI, USA). Rats were housed in a room with a reverse 12 h light/12 h dark cycle (lights off at 07:30 h). All experiments were conducted during the dark phase of the cycle. Animals were acclimatized for 7 days with water and chow (Teklad Diets, Madison, WI, USA) and handled for 3 days before the experiments were conducted. Throughout all operant procedures, rats were food restricted and received 16–20 g of chow daily with water freely accessible. All studies involving animals are reported in accordance with the ARRIVE guidelines for reporting experiments involving animals (Kilkenny *et al.*, 2010; McGrath *et al.*, 2010).

Animal welfare and ethical statement

All animal experiments were carried out in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals. Consistent with these guidelines, ongoing statistical testing of data collected was used to minimize the number of animals used within the constraints of necessary statistical power. All methods used were pre-approved by the Institutional Animal Care and Use Committee at the Torrey Pines Institute for Molecular Studies (Port Saint Lucie, FL, USA).

Apparatus

Self-administration and reinstatement experiments were conducted in operant conditioning chambers (see Supporting Information).

Food training

One week after arrival, all rats were trained for 3 days to lever-press for 45 mg food pellets (Test Diet 5-TUM, Richmond, IN, USA) under a fixed ratio-1 (FR-1) schedule of reinforcement in 30 min sessions.

I.v. catheterization

I.v. catheterization was performed after operant food training as previously described (de Guglielmo *et al.*, 2013), except that rats were anaesthetized via inhalation of isoflurane (Vetamac, Inc., Rossville, IN, USA. To maintain patency for the duration of the experiment, catheters were flushed daily with 0.2 mL of heparin (1000 UPS $U{\cdot}mL^{-1}$)-containing saline solution, which also contained enrofloxacin (0.7 $mg{\cdot}mL^{-1}$).

Nicotine self-administration

Following recovery from surgery, animals were trained to self-administer nicotine (30 $\mu g{\cdot}kg^{-1}$ per infusion) in the same chambers as the food training sessions using an FR-3 [20 s time out (TO)] schedule during daily 2 h sessions (Gilpin *et al.*, 2014) conducted over 7 days. TO was concurrent with illumination of a cue light located above the active lever to signal delivery of the positive reinforcement. An intermittent tone (7 kHz, 70 dB) was sounded throughout the session. Responses to the inactive lever were recorded and served as a measure of non-specific motor behaviour. After 7 days on this reinforcement schedule, the task was switched to progressive ratio (PR) schedule that better measures motivational properties rather than rate of drug intake. For the PR procedure, the response requirement for successive injections was 3, 6, 9, 12, 17, 24, 32, 42, 56, 73, 95, 124, 161, 208, 268, etc., based on the formula 'Response ratio (rounded to nearest integer) = $[5e^{(0.25\times\text{inj. number})}] - 5$' (Richardson and Roberts, 1996; Gamaleddin *et al.*, 2012). The PR schedule used a 20 s TO following each drug infusion. The break point was defined as the highest ratio completed before a 60 min period during which no injections were earned and lasted a maximum of 3 h.

Assessment of the reinforcing properties of AT-1001 and varenicline

Following initial nicotine self-administration using FR3TO20 and PR reinforcement schedules, rats (n = 17) were given access to saline self-administration under the FR3TO20 schedule for 2 h over 4 consecutive days. This protocol is sufficient to significantly decrease nicotine-reinforced lever pressing (Paterson *et al.*, 2010). Saline self-administration under PR was tested on day 5. Following saline substitution, one group of animals (n = 9) was allowed to lever-press for increasing doses of AT-1001 where each dose (0.3, 3.0 and 30 $\mu g{\cdot}kg^{-1}$) was made available weekly according to the 4 day FR3TO20 and 1 day PR design, whereas another group of animals (n = 8) was allowed to self-administer varenicline (20 $\mu g{\cdot}kg^{-1}$). In this second group of animals, nicotine (30 $\mu g{\cdot}kg^{-1}$) self-administration was also re-established.

Effect of AT-1001 on reinstatement of nicotine seeking

A new cohort of rats was initially trained to self-administer nicotine under the FR3TO20 schedule in 2 h sessions for 16 days. After the training period, an extinction (EXT) phase was conducted for 15 consecutive days. During 1 h EXT sessions, the lever presses were no longer associated with nicotine delivery while all cues (i.e. cue light, tone) were presented to allow for their concomitant EXT. On the day after the last EXT session, drug prime reinstatement of extinguished nicotine self-administration was examined. Reinstatement sessions were conducted under conditions identical to that of EXT. To evaluate the effect of AT-1001 on nicotine or varenicline priming-induced reinstatement, rats (n = 6 and 7)

were administered AT-1001 (0.75, 1.5 and 3.0 mg·kg^{-1}) or its vehicle in a counterbalanced order (Latin square design) 10 min before a nicotine or varenicline injection (0.15 mg·kg^{-1}, s.c., for both). Ten minutes following the final injection (nicotine or varenicline), animals were placed in the self-administration chambers and tested for 60 min. To determine whether an AT-1001 prime alone would induce reinstatement, 10 min before the beginning of the session, AT-1001 (0.75, 1.5 and 3.0 mg·kg^{-1}) was administered and animals (n = 6) tested as described above. A 3 day interval occurred between drug tests, during which the animals were subjected to EXT sessions. AT-1001, varenicline and nicotine doses, time of injection and experimental design were as described in previous studies (Forget *et al.*, 2010; Le *et al.*, 2010; Le Foll *et al.*, 2012; Toll *et al.*, 2012).

Assessment of motor behaviour using SmartCage™

Motor behaviour in freely moving rats (n = 8) was assessed using SmartCage technology (AfaSci, Inc., Redwood City, CA, USA; Khroyan *et al.*, 2012; Vazquez-DeRose *et al.*, 2013). SmartCage uses a USB-cable linked, non-invasive rodent behaviour monitoring system in conjunction with the animal's home cage. Data were analysed automatically using the Windows-based program CageScore™ (AfaSci, Inc., Burlingame, CA, USA). Home cage activity variables including activity counts (counts of breaks in x-, y- and z-axes photo beams), activity time (min) and distance travelled (cm) were calculated for 60 min following treatment with AT-1001 (1.5 and 3.0 mg·kg^{-1}) or its vehicle. Doses of AT-1001 were s.c. administered in a counterbalanced order (Latin square design) every 3 days.

Effect of AT-1001 and varenicline on co-administration of i.v. nicotine and p.o. alcohol

One week after arrival, a new group of rats was exposed to an intermittent (every other day) 10% (v/v) alcohol exposure procedure. Alcohol exposure was conducted in the rat home cages as previously described (Cippitelli *et al.*, 2012). The procedure lasted until animals attained seven alcohol exposures. Then animals were moved to self-administration chambers for food training followed by i.v. catheter implantation. After recovery from surgery, rats were moved to a food-restricted regimen for the duration of the experiment. Animals were then subjected to co-administration of i.v. nicotine and p.o. alcohol. Operant conditions used in this experiment were chosen from previous work (Le *et al.*, 2010). Following each nicotine infusion, a 20 s TO period occurred, during which responses at the lever that delivered nicotine (right lever) did not lead to programmed consequences. Nicotine reinforcements were accompanied by concurrent illumination of a cue light to signal delivery of nicotine. Alcohol reinforcements were accompanied by a flashing house light (0.5 s on, 0.5 s off) with a TO period of 20 s during which responses at the lever that delivered alcohol (left lever) did not lead to programmed consequences. An intermittent tone (7 kHz, 70 dB) was sounded throughout the 60 min session. These self-administration sessions were conducted for 10 days under an FR-1 schedule and 6 days under an FR-3 schedule for both reinforcers. Following stable responding, AT-1001 (0.0, 0.75, 1.5 and 3.0 mg·kg^{-1}) or varenicline (0.0 and 1.5 mg·kg^{-1}) was s.c. administered to rats 10 min before sessions using a within-subject Latin square design (n = 6 and 7 respectively). Test sessions were 4 days apart and conducted after two consecutive self-administration sessions.

Data analysis

To establish that nicotine replacement by saline led to decreased responding, the last day of nicotine self-administration was compared with the last day of saline-reinforced lever pressing by one-way within-subject ANOVA. Reinforcing properties of AT-1001 were analysed by comparing the last day of nicotine self-administration to the last self-administration day of each drug dose by one-way within-subject ANOVA. The same approach was employed to analyse reinforcing properties of varenicline and nicotine, also under the FR-3 schedule. The non-parametric Friedman's ANOVA & Kendall's concordance analysis followed by Wilcoxon matched pairs test was carried out for PR data. To establish that reinstatement was successfully induced, responding during the last EXT session and the respective reinstatement session were separately compared with the vehicle-treated group by one-way within-subject ANOVA. The effect of systemic AT-1001 on reinstatement experiments and locomotor activity was analysed using one-way repeated measures ANOVA with treatment (drug dose) as a within-subject factor. The same approach was used to examine the effect of AT-1001 and varenicline on nicotine and alcohol co-administration while patterns of nicotine and alcohol intake during their co-administration were analysed by means of two-way within-subject ANOVA with 'day' and 'reinforcer' used as within factors. The level of significance was set at $P < 0.05$. ANOVAs were followed, where appropriate, by Student–Newman-Keuls *post hoc* tests.

Drugs

AT-1001 [*N*-(2-bromophenyl)-9-methyl-9-azabicyclo [3.3.1] nonan-3-amine] and AT-1012 were synthesized at Astraea Therapeutics, Mountain View, CA, USA. (–)-Nicotine hydrogen tartrate salt and alcohol were purchased from Sigma (St. Louis, MO, USA). Varenicline tartrate was purchased from Tocris Bioscience (Bristol, UK). Alcohol was diluted in water and made available orally. Solutions of nicotine, varenicline and AT-1001 for i.v. injections were obtained by dissolving drugs in 0.9% saline and the pH adjusted to 7.0–7.4 with 3 M sodium hydroxide. Nicotine and varenicline self-administration doses are reported as free base concentrations. Systemic solutions of nicotine and varenicline were obtained by dissolving drugs in 0.9% saline and AT-1001 was suspended in a vehicle containing 2% DMSO, 1% HCl and 97% hydroxypropyl cellulose. All drugs for systemic treatment were administered in a 1 mL·kg^{-1} volume injection and given by s.c. route of administration.

Results

In vitro *functional activity*

Previous binding studies have demonstrated that AT-1001 and AT-1012 have high affinity and are selective for α3β4 nAChRs, whereas nicotine and varenicline show greater

Table 1

Functional activity of nicotinic compounds in HEK cells

	Inhibition		Stimulation			
	α3β4	α4β2	α3β4		α4β2	
Compound	IC_{50} (nM)	IC_{50} (nM)	EC_{50} (nM)	Stimulation (%)	EC_{50} (nM)	Stimulation (%)
Epibatidine	30.5 ± 4.8	0.10 ± 0.02	42.5 ± 6.40	85.60 ± 11	10.0 ± 0.6	106.1 ± 15.4
Nicotine	ND	4.00 ± 0.47	32 995 ± 1735	100 ± 0	1 285 ± 65	100 ± 0
Acetylcholine	ND	37.8 ± 1.05	548 ± 190	125 ± 9.8	1 645 ± 505	111.9 ± 3.6
AT-1001	91 ± 41	276 ± 185	1722 ± 330	38.1 ± 0.90	>10 000	≈25
AT-1012	62 ± 46	167 ± 51	976 ± 5.21	33.5 ± 1.61	>10 000	≈25
Varenicline	ND	0.61 ± 0.18	>10 000	≈25	569 ± 123	51.2 ± 1.2

The experimental data are presented as mean ± SEM. The experiments were performed in triplicate and repeated three to four times. ND, not determined.

binding selectivity for α4β2 nAChRs (Supporting Information Table S1; Toll *et al.*, 2012; Wu *et al.*, 2014). To examine the *in vitro* functional profile of these compounds, experiments were conducted on intact α3β4 nAChRs transfected into HEK cells using a Ca^{2+} dye to measure increases in intracellular Ca^{2+}. In these experiments, both AT compounds exhibited partial agonist activity when tested alone. AT-1001 and AT-1012 had EC_{50} values of 1.7 and 0.98 μM, respectively, with approximately 35% stimulation compared with nicotine, whereas varenicline induced approximately 25% stimulation at the α3β4 nAChR at 10 μM, the highest concentration that could be tested due to solubility (Table 1 and Figure 1A). This is different than published data showing full agonist activity at approximately 1.0 μM in cells transfected with human α3β4 nAChRs (Chatterjee *et al.*, 2011). AT-1001, AT-1012 and varenicline were also tested for functional activity on HEK cells transfected with α4β2 nAChRs. Because Ca^{2+} mobilization cannot be measured when using the α4β2 nAChR-transfected HEK cells (Fitch *et al.*, 2003), functional experiments with α4β2 nAChRs were conducted using the MP assay. AT-1001 and AT-1012 displayed very little agonist activity at α4β2 nAChRs, compared with epibatidine and varenicline, stimulating approximately 30% at 10 μM, the highest concentration that could be tested (Figure 1B). Consistent with previous evidence (Rollema *et al.*, 2007), varenicline was a partial agonist with approximately 50% efficacy at α4β2 nAChR and an EC_{50} of 570 nM.

HEK cells containing nAChRs were then pretreated with compounds to determine their ability to block epibatidine-induced cellular activation. AT compounds and varenicline blocked epibatidine-induced Ca^{2+} mobilization in α3β4 nAChR-transfected HEK cells (Figure 1C), as reported previously (Toll *et al.*, 2012). Inhibitory activity of AT-1001 and AT-1012 against the α4β2 nAChR occurred at IC_{50} concentrations a few fold higher than on α3β4 nAChR HEK cells. Varenicline, however, was very potent when inhibiting α4β2 nAChR (Figure 1D), with an IC_{50} of 0.6 nM, similar to its binding affinity at this site (Rollema *et al.*, 2007), indicating that it inhibits α4β2 nAChRs (probably by desensitization) at concentrations far lower than it stimulates α4β2, or inhibits α3β4 nAChRs (Chatterjee *et al.*, 2011).

In summary, AT-1001 and AT-1012 appear to have partial agonist activity at rat α3β4 nAChRs. These compounds can also stimulate α4β2 nAChRs but at considerably higher concentrations. They can also act as functional antagonists at both receptors with a threefold higher inhibitory potency at the α3β4 nAChR. At the α3β4 nAChR, it is possible that the 'antagonism' is a function of receptor desensitization as well as direct antagonism of the agonist response. In contrast, varenicline was a partial agonist at α4β2 nAChR and a weak agonist at α3β4 nAChRs, but it inhibits α4β2 at a much lower concentration than it inhibits α3β4 nAChRs, indicating significant selectivity as a functional antagonist.

Assessment of the reinforcing properties of AT-1001 and comparison with varenicline and nicotine

Both AT-1001 and varenicline potently block self-administration of nicotine in rats but varenicline has also been demonstrated to be self-administered as readily as nicotine (Paterson *et al.*, 2010). Accordingly, the reinforcing effects of AT-1001 were examined to determine whether the α3β4 or α4β2 nAChR partial agonist activity was sufficient to sustain nicotine self-administration. Following stable responding for nicotine using an FR-3 schedule of reinforcement (26.9 ± 2.2 response rate on the last day), saline was substituted for nicotine. As expected, saline decreased lever responding compared with nicotine ($F_{(1,8)} = 88.7$, $P < 0.001$). Following saline substitution, various doses of AT-1001 induced low levels of responding that were significantly lower than nicotine and comparable to saline controls ($F_{(4,32)} = 58.3$, $P < 0.001$, Figure 2A).

The findings obtained using the PR reinforcement schedule, with AT-1001, were similar to those obtained using the FR-3 schedule of reinforcement (Figure 2B). There was significantly less motivation for self-administration of AT-1001 or saline when compared with nicotine ($P < 0.05$). The break point obtained with all doses of AT-1001 was similar to that of saline.

In a separate group of animals, following stable responding for nicotine self-administration (25.8 ± 2.7, response rate on the last day) and decreased responding following

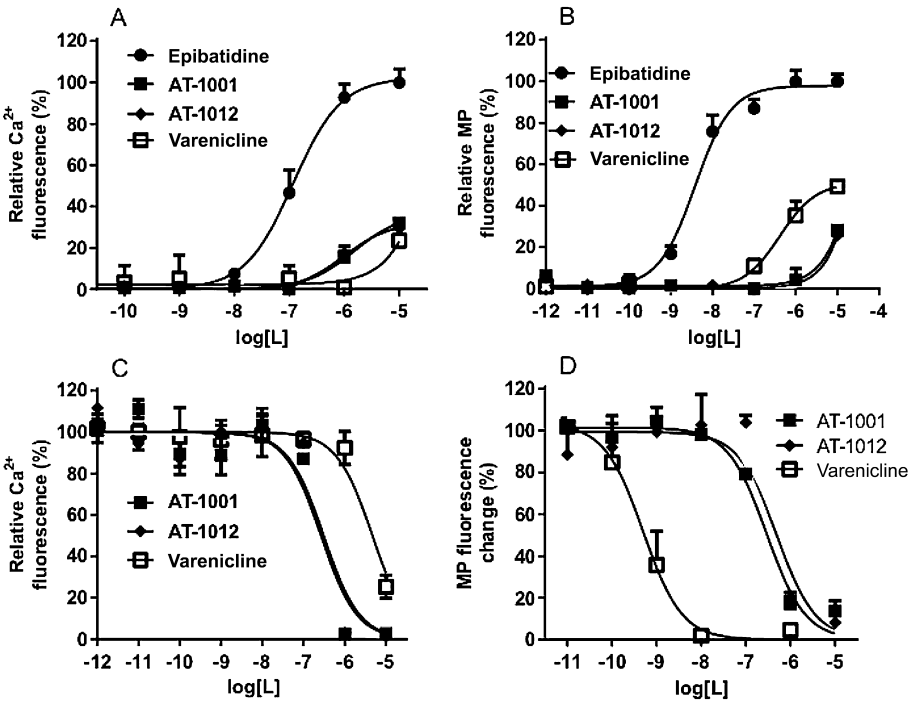


Figure 1

Stimulating and inhibitory activity of AT compounds and varenicline on α3β4 and α4β2 nAChRs stably expressed in HEK cells. (A) The stimulating effect on α3β4 nAChRs as measured by a calcium (Ca^{2+}) fluorescence assay. (B) Stimulating effect on α4β2 nAChRs measured by MP fluorescence assay. (C) Inhibition of 100 nM epibatidine-induced Ca^{2+} fluorescence by AT compounds and varenicline in HEK cells containing α3β4 nAChRs. (D) Inhibition of 100 nM epibatidine-induced MP fluorescence by AT compounds and varenicline in HEK cells containing α4β2 nAChRs. Data shown are mean ± SEM from a single experiment conducted in triplicate that was repeated at least three times with similar results.

saline substitution ($F_{(1,7)}$ = 77.7, P < 0.001), the reinforcing effects of varenicline under an FR-3 schedule of reinforcement were assessed (Figure 2C). Varenicline was self-administered ($F_{(3,21)}$ = 13.7, P < 0.001), where *post hoc* tests indicated a significant increase in responding compared with saline self-administration (P < 0.01). Similar to varenicline, using this 4 day schedule, re-exposure to nicotine also increased response rates (P < 0.01).

Using the PR schedule of reinforcement (Figure 2D), varenicline only showed a trend to increase the break point (P = 0.06) compared with saline, whereas break point levels following the second exposure to nicotine returned to those seen during the first nicotine self-administration session (P < 0.05).

Effect of AT-1001 on reinstatement of nicotine seeking

Lever pressing activity during nicotine self-administration training and EXT is shown in Supporting Information Fig. S1.

Treatment with AT-1001 in the absence of stimuli that triggered reinstatement caused decreased lever pressing on the nicotine-associated lever as compared with vehicle treatment ($F_{(3,15)}$ = 8.6, P < 0.01). A significant reduction at the dose of 3.0 mg·kg^{-1} (P < 0.01) was observed on *post hoc* analysis (Figure 3A).

As shown in Figure 3B, ANOVA analysis performed on the nicotine-associated lever indicated a main effect of 0.15 mg·kg^{-1} nicotine priming in inducing nicotine-seeking behaviour, as compared with EXT conditions ($F_{(1,5)}$ = 31.8, P < 0.01). In examining the effect of pretreatment of AT-1001 before the priming injection of nicotine, the overall ANOVA indicated a significant effect such that AT-1001 dose-dependently decreased responding to a nicotine prime ($F_{(3,15)}$ = 14.1, P < 0.001). *Post hoc* comparisons revealed that doses of 1.5 and 3.0 mg·kg^{-1} significantly decreased responding (P < 0.01 and P < 0.001 respectively).

Because of its α4β2 agonist activity, we examined the ability of a priming dose of varenicline to induce reinstatement of extinguished nicotine self-administration and whether AT-1001 could attenuate this effect. A priming dose of varenicline (0.15 mg·kg^{-1}) induced reinstatement of nicotine seeking conducted in a preliminary test after 12 EXT days (see Supporting Information Fig. S2). Therefore, in a subsequent experiment, we examined whether pretreatment of AT-1001 reduced reinstatement induced by varenicline. Reliably, ANOVA revealed that reinstatement of nicotine seeking was successfully induced by varenicline (0.15 mg·kg^{-1}) compared with EXT conditions as indicated by changes in lever pressing ($F_{(1,6)}$ = 16.3, P < 0.01). ANOVA analysis indicated that pretreatment with AT-1001 attenuated responding induced by a priming injection of varenicline ($F_{(3,18)}$ = 13.1, P < 0.001). *Post hoc* comparison tests revealed that all doses of AT-1001 resulted in a diminished number of responses as compared with those of varenicline alone (P < 0.01 for 0.75 and 1.5 mg·kg^{-1}, P < 0.001 for 3.0 mg·kg^{-1} dose, Figure 3C).

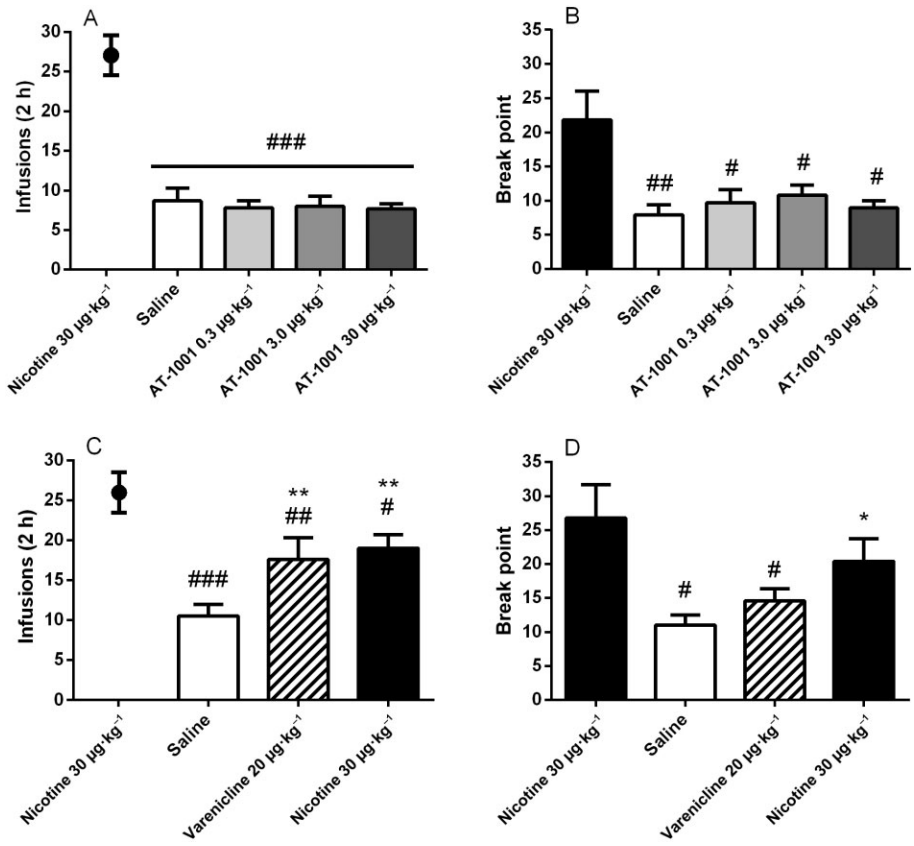


Figure 2

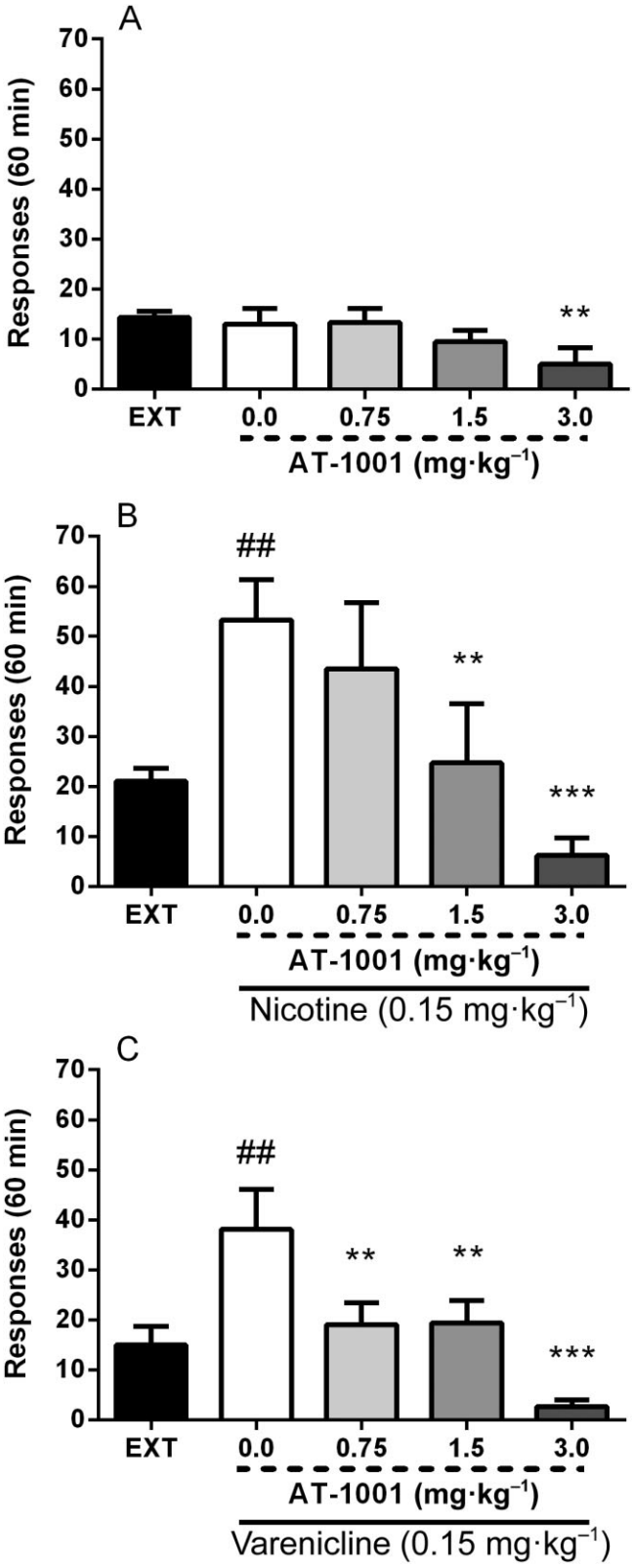
AT-1001 (0.3, 3.0 and 30 µg·kg^{-1}) made available i.v. is not self-administered more than saline under fixed ratio-3 (FR-3) and PR reinforcement schedules. Conversely, i.v. exposure to varenicline (20 µg·kg^{-1}) and nicotine (30 µg·kg^{-1}) increases self-administration in these tasks. (A) and (C): black circles represent baseline infusion values (±SEM) in 2 h of the last nicotine self-administration session at the training dose of 30 µg·kg^{-1}. Bars are the mean values (±SEM) of the last FR-3 self-administration session for the corresponding reinforcers. There was a significant increase in varenicline- and nicotine- but not in AT-1001-reinforced lever pressing as compared with responses of the same rats (n = 8 and 9, respectively) that self-administered saline; #P < 0.05, ##P < 0.01, ###P < 0.001 difference from mean of the last training day; **P < 0.01 difference from saline responses. (B) and (D): bars represent break point values (final ratio completed ±SEM) for the corresponding reinforcers during the PR schedule. There was no increase in motivation to obtain various doses of AT-1001 while varenicline and re-exposure to nicotine produced a trend to increase and significant increase, respectively, in break point as compared with responses of the same rats that self-administered saline under the PR schedule. #P < 0.05, ##P < 0.01 difference from nicotine responses; *P < 0.05 difference from saline responses. For detailed statistics, see 'Results'.

Locomotor activity following AT-1001 treatment

AT-1001 did not alter home cage activity at the doses examined. No significant changes were observed for active counts ($F_{(2,14)}$ = 2.3, NS), distance travelled ($F_{(2,14)}$ = 1.0, NS) and active time ($F_{(2,14)}$ = 2.3, NS) measured for 60 min following treatment (Supporting Information Fig. S3).

Effect of AT-1001 on operant co-administration of i.v. nicotine and p.o. alcohol

Because varenicline is effective at inhibiting alcohol consumption in addition to nicotine self-administration, AT-1001 was tested to determine whether it was equally effective in blocking nicotine and ethanol intake in a model of co-administration of i.v. nicotine and p.o. 10% ethanol. As shown in Figure 4A, 10 training sessions under an FR-1 schedule were sufficient for rats to concurrently self-administer nicotine and alcohol. Overall, ANOVA revealed a main effect of days ($F_{(9,45)}$ = 7.4, P < 0.001) accompanied by 'reinforcer' × 'day' interaction ($F_{(9,45)}$ = 6.3, P < 0.001) to suggest different patterns of intake for nicotine and alcohol. *Post hoc* analysis showed escalation of alcohol consumption, as a significantly different intake was found between the first and the last 4 days of exposure. However, nicotine-reinforced lever pressing did not change significantly over time. On FR-3 schedule, alcohol and nicotine were self-administered in a similar manner ['reinforcer' × 'day' interaction ($F_{(5,25)}$ = 0.4, NS)], which allowed us to conduct drug testing on comparable levels of lever pressing for nicotine and alcohol. Pretreatment with AT-1001 caused decreased responding on the nicotine-associated lever as compared with nicotine alone (0 mg·kg^{-1} treatment, $F_{(2,10)}$ = 11.9, P < 0.01) with *post hoc* comparisons



showing significance at 1.5 mg·kg^{-1} of AT-1001 ($P < 0.01$, Figure 4B). Conversely, alcohol lever pressing was not altered following AT-1001 treatment ($F_{(2,10)} = 0.7$, NS). Varenicline potently reduced nicotine ($F_{(1,6)} = 39.1$, $P < 0.001$) as well as alcohol ($F_{(1,6)} = 14.3$, $P < 0.01$) taking behaviour (Figure 4C).

Figure 3

(A) Systemic injection of AT-1001 (0.0, 0.75, 1.5 and 3.0 mg·kg^{-1}, s.c.) does not elicit reinstatement *per se*. Values represent the mean (±SEM) number of total responses of ($n = 6$) rats on the nicotine-associated lever in 60 min. AT-1001 at 3.0 mg·kg^{-1} reduced response levels shown during EXT. Systemic injection of AT-1001 (0.0, 0.75, 1.5 and 3.0 mg·kg^{-1}, s.c.) suppresses (B) nicotine priming- and (C) varenicline priming-induced reinstatement of nicotine seeking. Values represent the mean (±SEM) number of total responses on the nicotine-associated lever in 60 min. There was a significant reinstatement induced by nicotine (0.15 mg·kg^{-1}, s.c.) and varenicline (0.15 mg·kg^{-1}, s.c.), as indicated by responses in rats ($n = 6$ and 7, respectively) exposed to these stimuli (in the absence of reward delivery) compared with EXT responding; ##$P < 0.01$ versus last day of EXT. There was a significant main effect of AT-1001 treatment and a significant suppression of responding at dosages of 1.5 and 3.0 mg·kg^{-1} on *post hoc* analysis. AT-1001 0.75 mg·kg^{-1} dose significantly attenuated varenicline-induced reinstatement. **$P < 0.01$, ***$P < 0.001$ difference from vehicle (0.0 mg·kg^{-1}) treated controls. For detailed statistics, see 'Results'.

Discussion and conclusions

The novel compound AT-1001 previously demonstrated to block nicotine self-administration in rats (Toll *et al.*, 2012) was tested in comparison with the clinically used compound varenicline to better understand the mechanism of action of its nicotine-suppressive effects. We show that the two compounds have very different *in vitro* and *in vivo* properties. Unlike AT-1001, the α4β2 nAChR partial agonist varenicline is self-administered by rats and is able to reinstate extinguished nicotine self-administration. AT-1001 appears to be a more selective inhibitor of nicotine taking and seeking, as it reduces nicotine self-administration at doses that do not affect alcohol lever pressing and attenuates reinstatement induced by both nicotine and varenicline prime. The *in vitro* profiles are also significantly different, although both show discrepancies between binding and functional selectivity.

The *in vitro* profile of AT-1001, like that of varenicline and other nAChR ligands, is complicated. Although the original manuscript on AT-1001 (Toll *et al.*, 2012) discussed this compound as being a selective antagonist at α3β4 nAChRs, the present data suggest that it has partial agonist activity in cells that are made up of rat α and β subunits. Recent reports also show that AT-1001 has partial agonist activity at the human α3β4 nAChR (Bowman Dalley *et al.*, 2013; Zaveri *et al.*, 2014). In addition, although this compound and its analogue AT-1012 clearly have excellent selectivity in binding experiments, they appear far less selective when it comes to *in vitro* functional activity, acting as antagonists at the α4β2 nAChR at concentrations only threefold higher than antagonist potency at α3β4 nAChR. This is reminiscent of varenicline, which has nearly 1000-fold higher binding affinity at α4β2 than α3β4 nAChR, but can act as a partial agonist at α4β2 at virtually the same concentration at which it is an agonist at α3β4 nAChR (Chatterjee *et al.*, 2011). Measuring functional activity in transfected cells has another complicating factor. It is well known that the activity of ligands for both *in vitro* and *in vivo* actions can depend a great deal on the particular functional assay, and that potency and relative maximal

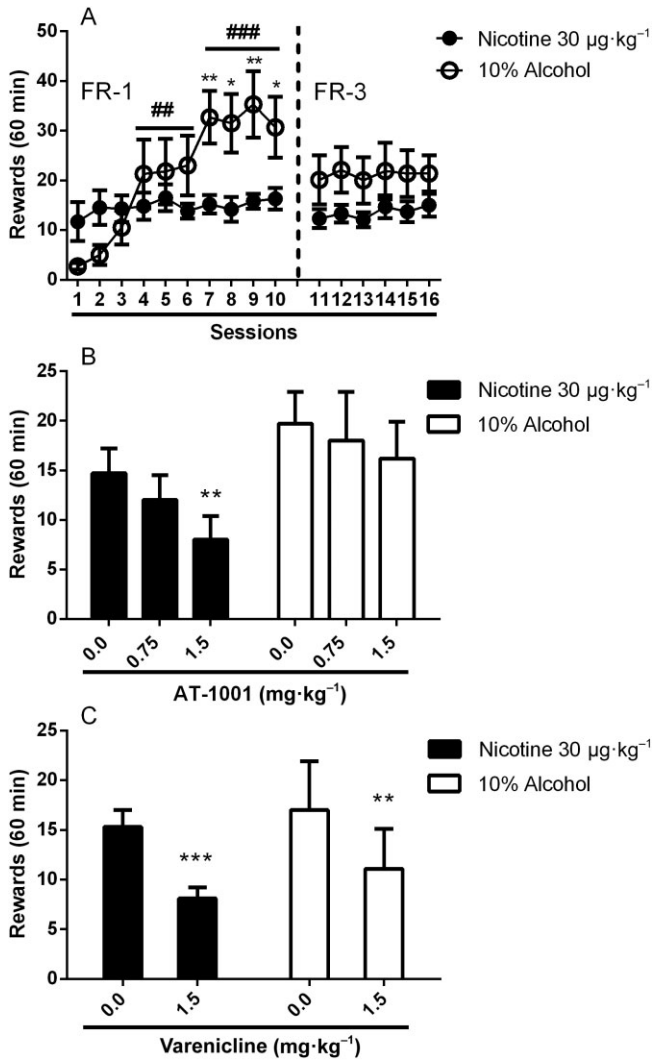


Figure 4

Rats ($n = 6$) self-administer both nicotine (30 µg·kg^{-1}) and 10% (v/v) alcohol in the operant co-administration paradigm. AT-1001 potently decreases nicotine-reinforced lever pressing leaving alcohol self-administration unaltered. (A) Training for co-administration of nicotine and alcohol under both FR-1 and FR-3 reinforcement schedules in 60 min sessions. Symbols represent the mean (±SEM) number of nicotine and alcohol rewards. *$P < 0.05$, **$P < 0.01$ difference from nicotine reinforcement; ##$P < 0.01$, ###$P < 0.001$ versus mean of the first co-administration session. (B) and (C) are mean (±SEM) of nicotine and alcohol rewards in 60 min obtained following systemic treatment with AT-1001 (0.0, 0.75 and 1.5 mg·kg^{-1}, $n = 6$) and varenicline (0.0 and 1.5 mg·kg^{-1}, $n = 7$) respectively. $P < 0.05$, **$P <$ 0.01, $P < 0.001$ difference from vehicle (0.0 mg·kg^{-1}) treatment. For detailed statistics, see 'Results'.

activity are a function of receptor number (Kenakin, 1985). This makes the determination of receptor potency and activity more variable than receptor binding affinity, and differences in receptor number in the transfected cells, or perhaps species differences, could explain why Chatterjee *et al*. (2011) found full agonist activity for varenicline in cells transfected with human α3β4 nAChRs, and we observed significantly weaker activity in cells transfected with rat α3β4 nAChRs.

The study of agonists and antagonists is also very difficult in nAChRs due to rapid desensitization. In fact, using assays such as FlexStation or Rb^+ flux measurements, it can be very difficult to differentiate between antagonist activity and desensitization. One method would be to follow the re-sensitization of the receptor. We conducted such experiments in which cells were pretreated with AT-1001 or the agonist nicotine, and then washed at various times before epibatidine-induced cellular activation. In these experiments, epibatidine was fully effective as soon as we could activate receptors after washing away the agonists (L. Toll, unpubl. obs.). Unfortunately, these experiments did not distinguish between receptor inhibition versus desensitization with the receptor rapidly re-sensitizing after AT-1001 treatment. Ultimately, it is unclear whether there is a functional difference between desensitization and antagonist activity. In either case, the receptor is in an inactive state and agonists do not function. This is probably why both agonists and antagonists can have similar actions in a variety of behavioural responses including blockade of nicotine self-administration, as observed for the partial agonists varenicline and sazetidine-A and the antagonist mechamylamine (Watkins *et al*., 1999; Rollema *et al*., 2007; Levin *et al*., 2010). Accordingly, regardless of partial agonist activity, AT-1001 is a functional antagonist of α3β4 nAChRs.

The studies described here do not conclusively prove the mechanism of action of either AT-1001 or varenicline. However, they do demonstrate that the two compounds work differently when compared directly, despite the fact that they both block nicotine-taking behaviour at similar doses. This direct comparison is necessary because there seems to be some variability in the actions of varenicline. Le Foll *et al*. (2012) demonstrated that varenicline blocks cue-induced reinstatement of nicotine seeking, while O'Connor *et al*. (2010) found that varenicline attenuated reinstatement induced by nicotine prime, and prime plus cue, but not by cue alone. We found that AT-1001 also very potently blocked relapse-like behaviour when induced by nicotine priming. However, rather than blocking nicotine prime-induced reinstatement, varenicline actually induced reinstatement. This result is different from published data, which demonstrated no induction of nicotine seeking by varenicline (O'Connor *et al*., 2010). However, in that study, doses higher than 0.15 mg·kg^{-1} varenicline were examined, which may account for this discrepancy. As expected, AT-1001 also blocked varenicline-induced reinstatement of nicotine seeking without inducing reinstatement on its own. Rather, extinction responding was decreased by 3.0 mg·kg^{-1} AT-1001, a dose that decreased responding compared with the extinction level in each reinstatement experiment, perhaps suggesting the occurrence of non-specific effects at this dose. Testing the home cage activity using SmartCage (Khroyan *et al*., 2012; Vazquez-DeRose *et al*., 2013), we found no significant changes in the locomotor activity at any dose (Supporting Information Fig. S3).

The rewarding aspect of varenicline is also demonstrated in its ability to be self-administered *per se*, an effect consistent with evidence showing that partial α4β2 nAChR activation is sufficient to mimic discriminative stimulus and reinforcing properties of nicotine (Paterson *et al*., 2010). However, under a PR reinforcement schedule, rats worked for varenicline to a

lesser extent than for nicotine, indicating reduced motivation to obtain the reward, probably due to its lower efficacy at α4β2 nAChRs. AT-1001 is very different and is not self-administered by rats at all, consistent with very low *in vitro* efficacy at α4β2 nAChRs.

To further explore whether AT-1001 and varenicline act through distinct mechanisms, we sought to separate their effects by looking at selectivity in altering reinforcing properties of other drugs. Because varenicline can reduce nicotine as well as alcohol self-administration and reinstatement at similar doses (Steensland *et al.*, 2007; Chatterjee *et al.*, 2011), AT-1001 and varenicline were tested in an operant co-administration paradigm in which i.v. nicotine and p.o. alcohol were simultaneously available. When rats were performing comparable levels of nicotine and alcohol lever pressing, AT-1001 reduced responding for nicotine while pressing for alcohol was unaltered. Varenicline gave a different result, inhibiting both alcohol and nicotine responding at 1.5 $mg \cdot kg^{-1}$. These results suggest that α3β4 nAChR antagonism/desensitization attenuates nicotine but not alcohol intake and that the effects of varenicline on alcohol-related behaviours were probably not due to a α3β4-mediated mechanism, as has been hypothesized previously (Steensland *et al.*, 2007; Chatterjee *et al.*, 2011).

Based upon the *in vitro* profile, it is possible that AT-1001 blocks nicotine self-administration through inhibition of α4β2 rather than α3β4 nAChRs. However, we find this unlikely because the potency of AT-1001 at α4β2 is less than that of mecamylamine, yet it is more effective in blocking nicotine self-administration. AT-1001 is also far less potent than mecamylamine at blocking nicotine-induced dopamine release from synaptosomes derived from the NAc (Toll *et al.*, 2012), suggesting that AT-1001 is not acting through α4β2 nAChRs, the receptor subtype found most prevalent in the NAc (Gotti *et al.*, 2010). Furthermore, as discussed, unlike varenicline, AT-1001 did not block alcohol self-administration at doses at which it blocked nicotine.

In conclusion, because of agonist activity of AT-1001 at α3β4, and α4β2 inhibition at slightly higher concentrations *in vitro*, it may be difficult to definitively assign its ability to block nicotine self-administration and priming-induced reinstatement only to α3β4 nAChR antagonism. It is possible that inhibition of nicotine self-administration is due to partial agonism or potentially desensitization of α3β4 nAChRs, although functionally there may be no difference between antagonism and desensitization. Regardless of the exact mechanism of action, these studies indicate that it is clearly very different from varenicline, which is self-administered in nicotine-experienced rats, has sufficient agonist activity (probably through α4β2) to reinstate previously extinguished nicotine self-administration, and decreases alcohol as well as nicotine-taking behaviour. These studies also suggest very strongly that varenicline is not acting through α3β4 nAChRs in its ability to block nicotine self-administration, as has also been suggested previously (Baldwin *et al.*, 2011). In fact, varenicline very possibly blocks nicotine self-administration not because it is a partial agonist at α4β2 nAChRs, but because it very potently desensitizes α4β2 nAChRs (i.e. acts as a functional antagonist). Furthermore, varenicline probably does not block alcohol self-administration via α3β4 nAChRs because AT-1001 is ineffective in this procedure. Although selective for binding, the mechanisms of action of varenicline and AT-1001 may be very complex, and it is still unclear whether it would be more efficacious and safer for a pharmacotherapy to act at a single receptor or both α3β4 and α4β2 nAChRs. Regardless of the mechanism of action of varenicline, its long-term safety has been questioned due to suicidal ideation in patients (Ahmed *et al.*, 2013), although in another recent study varenicline was demonstrated to be well tolerated (Evins *et al.*, 2014). The findings described here provide further support for the potential of AT-1001 and congeners as potentially safer and clinically useful compounds for smoking cessation, with a mechanism of action distinct from currently available medications.

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Author contributions

A. C., J. W., K. A. G., D. M., J. S., M. G., A. R. and C.P. performed the research. A. C., J. W. and L. T. designed the research study. R. C., T. V. K., D. Y., N. T. Z. and X. S. X. contributed essential reagents or tools. A. C., J. W. and L. T. analysed the data. A. C. and L. T. wrote the paper.

Conflict of interest

The authors declare no conflict of interests.

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Supporting information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

http://dx.doi.org/10.1111/bph.13034

Figure S1 Lever pressing activity during nicotine self-administration and extinction (EXT) of responding. Left panel represents baseline levels of nicotine infusions during the last 120 min training session (±SEM) under an FR-3 schedule. Right panel represents the number of responses (±SEM) during 15 consecutive 60 min EXT sessions in rats used to examine the effects of AT- 1001 on responding in the absence of stimuli that triggered reinstatement (grey lines/bar), on nicotine (black lines/bar) and varenicline (white lines/bar) induced reinstatement of nicotine-seeking behaviour.

Figure S2 A s.c. priming injection of varenicline (0.15 $mg \cdot kg^{-1}$) induced reinstatement of nicotine seeking in a preliminary test carried out after 12 consecutive EXT sessions. $^{*}P < 0.05$ difference from the last EXT session.

Figure S3 Locomotor activity as measured by SmartCage™ over a 60 min period following treatment with AT-1001 (0.0, 1.5 and 3.0 $mg \cdot kg^{-1}$, s.c.). No significant changes were observed for home cage activity variables, including (A) active counts, (B) distance travelled and (C) active time.

Table S1 Binding affinity of nicotinic compounds on α3β4 and α4β2 nAChRs