

AT-1001 is a Partial Agonist with High Affinity and Selectivity at Human and Rat $\alpha 3\beta 4$ Nicotinic Cholinergic Receptors

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AT-1001, N-(2-bromophenyl)-9methyl-9azabicyclo[3.3.1] nonan-3-amine; [^3H]-epibatidine, [^3H]-EB); nAChR, nicotinic acetylcholine receptor

Abstract

AT-1001 is a high affinity and highly selective ligand at $\alpha 3\beta 4$ nicotinic cholinergic receptors (nAChRs) that was reported to decrease nicotine self-administration in rats. It was initially reported to be an antagonist at rat $\alpha 3\beta 4$ nAChRs heterologously expressed in HEK293 cells. Here we compared AT-1001 actions at rat and human $\alpha 3\beta 4$ and $\alpha 4\beta 2$ nAChRs similarly expressed in HEK 293 cells. We found that, as originally reported, AT-1001 is highly selective for $\alpha 3\beta 4$ receptors over $\alpha 4\beta 2$ receptors, but its binding selectivity is much greater at human than at rat receptors, due to a higher affinity at human than at rat $\alpha 3\beta 4$ nAChRs. Binding studies in human and rat brain and pineal gland confirmed the selectivity of AT-1001 for $\alpha 3\beta 4$ nAChRs and its higher affinity for human compared to rat receptors. In patch clamp electrophysiology studies, AT-1001 was a potent partial agonist with 65-70% efficacy at both human and rat $\alpha 3\beta 4$ nAChRs. It was also a less potent and weaker (18%) partial agonist at $\alpha 4\beta 2$ nAChRs. Both $\alpha 3\beta 4$ and $\alpha 4\beta 2$ nAChRs are up-regulated by exposure of cells to AT-1001 for 3 days. Similarly, AT-1001 desensitized both receptor subtypes in a concentration-dependent manner, but it was 10-times and 30-times more potent to desensitize human $\alpha 3\beta 4$ receptors than rat $\alpha 3\beta 4$ and human $\alpha 4\beta 2$ receptors, respectively. After exposure to AT-1001, the time to recovery from desensitization was longest for the human $\alpha 3\beta 4$ nAChR and shortest for the human $\alpha 4\beta 2$ receptor, suggesting that recovery from desensitization is primarily related to the dissociation of the ligand from the receptor.

Introduction

Neuronal nicotinic acetylcholine receptors (nAChRs) mediate important functions across the central and peripheral nervous systems. These receptors are pentameric cation channels composed from 12 subunits, α 2-10 and β 2-4, the combinations of which define the nAChR subtypes. Although there are a large number of theoretically possible nAChR subtypes, with possibly differing properties and characteristics, the predominant subtypes found in the central and peripheral nervous systems are α 4 β 2*, α 7* and α 3 β 4* (where * indicates that an additional subunit might be incorporated in the receptor). These nAChR subtypes are differentially expressed, with α 4 β 2* and α 7* receptors predominating in most areas of the forebrain and α 3 β 4* receptors predominating in certain areas of the midbrain and hindbrain, as well as in the pineal gland, adrenal gland and most autonomic ganglia (Perry et al., 2002; Hernandez et al., 2004; Gotti et al., 2006; Mao et al., 2006).

Most known ligands and drugs that target nAChRs have much higher affinity for the α 4 β 2* subtypes, and these receptors appear to be involved in several important functions, including essential aspects of nicotine addiction. However, increasing evidence suggests that the α 3 β 4* subtypes also may be important in nicotine addiction and withdrawal (Salas et al., 2004; Glick et al., 2011; Jackson et al., 2013; McCallum et al., 2012). Moreover, the genes coding for both of these constituent subunits are part of a gene cluster in which allelic variations have been associated with heavy smoking (Bierut et al., 2007; Saccone et al., 2009; Bierut et al., 2008; Berrettini et al., 2008). The possibility that the α 3 β 4* nAChR subtype contributes to nicotine addiction, and even to other addictions (Muldoon et al. 2014), as well as its importance in crucial physiological functions such as ganglionic neurotransmission underscores the need for selective ligands for this receptor that can be used as research tools and/or eventually as therapeutic drugs.

Recently, a new compound, AT-1001 (N-(2-bromophenyl)-9methyl-9azabicyclo[3.3.1] nonan-3-amine), was found to be a selective, high affinity ligand at rat $\alpha 3\beta 4$ nAChRs (Toll et al., 2012; Zaveri et al., 2015). Furthermore, AT-1001 was reported to be a potent antagonist or partial agonist at $\alpha 3\beta 4$ nAChRs (Toll et al. 2012; Zaveri et al. 2015), and it effectively reduced nicotine self-administration in rats (Toll et al., 2012). Rat and human nAChR subunits are, in general, very similar, but an important difference between the two species is found within the extracellular D loop of the $\beta 4$ subunits (Young et al., 2007). This structural variation confers differences in the pharmacological responses to certain drugs (Young et al. 2007; Zwart et al., 2008; Stokes and Papke, 2012). To further explore the pharmacological properties of AT-1001, we carried out comparative studies on the pharmacology of binding and function for rat and human $\alpha 3\beta 4$ and $\alpha 4\beta 2$ nAChRs expressed in HEK-293 cells, as well as binding in rat and human brain, which express primarily $\beta 2$ -containing nAChRs, and pineal gland, which express exclusively (rat) or primarily (human) $\alpha 3\beta 4$ nAChRs. Our results indicate that there are substantial species differences in the binding and functional characteristics of AT-1001 at rat and human $\alpha 3\beta 4$ nAChRs and, most important, that AT-1001 is a potent partial agonist with an efficacy of 65-70 % at these receptors. In addition, we found that AT-1001 desensitizes both $\alpha 3\beta 4$ and $\alpha 4\beta 2$ nAChRs, but is much more potent at the $\alpha 3\beta 4$ subtype.

Materials and Methods

Materials. AT-1001 (N-(2-bromophenyl)-9-methyl-9-azabicyclo[3.3.1] nonan-3-amine) was synthesized as described by Zaveri et al., 2011. [³H]-epibatidine ([³H]-EB) was purchased from Perkin-Elmer Life Sciences (Boston, MA). Unless otherwise indicated, all other chemicals were purchased from Sigma-Aldridge (St. Louis, MO).

Cell lines. Tissue culture medium and antibiotics were obtained from Invitrogen Corporation (Carlsbad, CA), unless otherwise stated. Fetal bovine serum and horse serum were provided by Gemini Bio-Products (Woodland, CA). The cell lines KX α 3 β 42R2 and KX α 4 β 2R2, expressing rat α 3 β 4 and α 4 β 2 nAChRs, respectively, were established and characterized previously (Xiao et al., 1998; Meyer et al., 2001). These cell lines were maintained in medium consisting of Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 100 units/ml penicillin G, 100 μ g/ml streptomycin, and 0.7 mg/ml Geneticin (G418). The cell lines YX α 3 β 4H1 and YX α 4 β 2H1, expressing human α 3 β 4 and α 4 β 2 nAChRs, respectively, were established by introducing human α 3 and β 4 subunit genes or human α 4 and β 2 subunit genes into HEK293 cells. These cell lines were maintained in a medium consisting of minimum essential medium supplemented with 10% fetal bovine serum, 100 units/ml penicillin G, 100 μ g/ml streptomycin, 0.7 mg/ml G418 (Geneticin), and 0.1 mg/ml hygromycin B.

Brain and pineal gland. Frozen rat brains and pineal glands from adult Sprague-Dawley rats (approximately 50% males and 50% females) were purchased from Zivic Miller Laboratories (Portersville, PA, USA). Human frontal cerebral cortex and pineal glands were obtained at autopsy from psychiatrically-normal individuals (males, average age 57). All procedures were carried out in compliance with an approved protocol from the University of Mississippi Medical

Center Institutional Review Board. Written informed consent was obtained from legally-defined next-of-kin for tissue collection and informant-based retrospective diagnostic interviews. Psychiatric assessments were performed as previously described (Cobb et al., 2013). Toxicology screens revealed nothing remarkable.

Radioligand binding. Cells were harvested at 90-100% confluency by first aspirating culture media and replacing it with 25 mL of 50 mM Tris-HCl buffer (pH 7.4), gently scraping cells from the culture flask surface and centrifuging this cell suspension at 1,000 x g for 10 min at 4°C. Cell membrane homogenates were then prepared by first re-suspending the whole cells in 50 mM Tris-HCl buffer, homogenizing them with a Polytron homogenizer, followed by centrifugation at 33,000 x g for 10 min at 4°C. The supernatant was discarded, and the pellet was re-suspended in fresh buffer and treated as above two more times. The final pellet was resuspended in 50 mM Tris-HCl buffer and used in subsequent experiments. Brain and pineal glands were homogenized in 50 mM Tris-HCl buffer (pH 7.4) and membranes were then prepared as described above for cells.

Radioligand competition and saturation binding assays were performed with [³H]-EB as described previously (Xiao et al, 1998) in 0.25 ml 50 mM Tris-HCl buffer, pH 7.4 at 23° C. The affinities of AT-1001 at the rat and human versions of the $\alpha 3\beta 4$ and $\alpha 4\beta 2$ nAChR subtypes expressed in HEK293 cells were determined in competition binding assays. Cell membrane homogenates were incubated for 4 hours with ~0.4 nM [³H]-EB in the absence or presence of increasing concentrations of AT-1001. The homogenates were then filtered through Whatman GF/C filters treated with 0.5% polyethylenimine and counted in a Beckman-Coulter LS6500 scintillation counter. Nonspecific binding was measured in the presence of 300 μ M (-) nicotine, and specific binding was defined as the difference between total and nonspecific binding. The AT-1001

binding competition curves were fit to one-site and two-site binding competition models by nonlinear least square regression analysis using GraphPad Prism 5 (San Diego, CA). The K_i of AT-1001 at each receptor was then calculated with the Cheng-Prusoff equation (Cheng and Prusoff, 1973) using the [^3H]-EB K_d for each receptor.

To determine how well AT-1001 binding affinities for rat and human $\alpha 3\beta 4$ and $\alpha 4\beta 2$ nAChR subtypes heterologously expressed in HEK293 cells correspond to its binding affinities in native tissues, we measured its competition for nAChRs labeled by [^3H]-EB in membrane homogenates from rat and human cerebral cortex and pineal gland. Immunoprecipitation studies with subunit-specific antibodies have established that the heteromeric nAChRs in rat cerebral cortex are predominantly (~90%) the $\alpha 4\beta 2$ subtype (Whiting et al., 1987; Flores et al., 1992; Mao et al., 2008), with ~16% of these receptors also containing an $\alpha 5$ subunit (Mao et al., 2008). The predominant nAChRs in human cerebral cortex are also the $\alpha 4\beta 2$ subtype (Gotti et al., 2006). The heteromeric nAChRs in rat pineal gland are virtually all the $\alpha 3\beta 4$ subtype (Hernandez et al., 2004). The human pineal gland appears to contain the $\alpha 3\beta 4$ nAChR subtype predominantly, but also one that includes the $\beta 2$ subunit (TT Olson and KJ Kellar, unpublished). Therefore, to measure binding competition at the $\alpha 3\beta 4$ subtype alone in human pineal gland, 10 nM sazetidine-A was included in the assay to block binding to essentially all $\beta 2$ -containing nAChRs. Under these conditions, AT-1001 competition curves for [^3H]-EB binding sites in the human pineal fit best to a model for one class of binding site consistent with $\alpha 3\beta 4$ nAChRs.

To investigate the type of binding inhibition exerted by AT-1001, [^3H]-EB binding saturation assays in membranes from HEK cells expressing $\alpha 3\beta 4$ and $\alpha 4\beta 2$ nAChRs were compared in the absence or presence of several concentrations of AT-1001. Cell membrane homogenates were

incubated for 4 h with increasing concentrations of [³H]-EB (from 0.5 pM to 3 nM for rat receptors or up to 20 nM for human receptors). The homogenates were then filtered through Whatman GF/C filters and counted as above. Nonspecific binding of [³H]-EB was determined in the presence of 300 μM (-) nicotine, and specific binding was defined as the difference between total and nonspecific binding. Free ligand concentrations were adjusted for amount bound, and the receptor densities (B_{max}) and dissociation constants (K_d) were then determined using nonlinear least square regression analysis in GraphPad Prism 5.

Up-regulation of nicotinic receptors. The effect of chronic exposure to AT-1001 on nAChR density was examined by culturing cells for three days in media containing either 0, 0.3 μM or 10 μM AT-1001, or, for comparison, 10 μM (-)nicotine. After 3 days, when the cells were 90-100% confluent, they were collected, homogenized and washed, as described above, except that the cell membranes were incubated at 37°C for 30 min after each homogenization and then centrifuged at 33,000 x g and re-suspended in buffer. This procedure was repeated 5 times to reduce the possibility of any AT-1001 remaining in the membranes. The cell homogenates were then resuspended and receptor binding site density was measured with a single, saturating concentration (~3 nM) of [³H]-EB.

Electrophysiology. Nicotinic receptor function was evaluated in HEK cell lines in the whole-cell voltage-clamp configuration using an Axopatch 200B amplifier (Axon Instruments, Burlingame, CA). Cells plated on a glass coverslip were placed in the recording chamber of a microfluidic chip (Dynaflow Resolve, Celectricon, Sweden) containing extracellular solution composed of (in mM): 130 NaCl, 5 KCl, 2 CaCl₂, 2 MgCl₂, 10 dextrose, and 10 HEPES. Voltage-clamp recordings ($V_{hold} = -70mV$) were made with patch electrodes (5-8 MΩ) with internal solution (pH

7.2) composed of the following (in mM): 145 K-gluconate, 5 EGTA, 5 MgCl₂, 10 HEPES, 5 ATP.Na, and 0.2 GTP.Na. Series resistance was typically <10 MΩ and was not compensated but was continuously monitored with a hyperpolarizing 5 mV pulse. Generation of voltage-clamp protocols and acquisition of data were carried out using pCLAMP 10 software (Axon Instruments, Inc., Burlingame, CA). Sampling frequency was 20 kHz and current signals were filtered at 5 kHz before digitization and storage. All experiments were performed at room temperature (23–25°C).

Drug application. In conjunction with whole-cell voltage-clamp recording, drugs were applied to cells via a microfluidic laminar stream solution exchange system (MLSSE; Dynaflo Resolve, Collectricon, Sweden) as described previously (Federov et al, 2012). Briefly, after establishing a steady-state laminar flow, a drug application protocol was initiated, which consisted of exposing a single voltage-clamped cell to various drugs at the indicated concentrations (200 ms drug exposure; 1 min inter-stimulus interval). Peak elicited currents in each individual cell were normalized to the peak current elicited by 1 mM ACh in the same cell. This concentration of ACh reliably stimulates maximum currents in these cells.

Rubidium efflux assays. The effect of AT-1001 on nAChR function was also examined by assessing ⁸⁶Rb⁺ efflux through the receptor channel, as described previously (Xiao et al., 1998; Meyer et al., 2001). Cells were first loaded with ⁸⁶RbCl by incubating them for 2-4 hrs with 0.5 mL media containing ~100,000 dpm ⁸⁶Rb⁺. To test agonist activity of AT-1001, the cells were rinsed gently 4 times with 1 mL buffer over 10 min and then either buffer alone or buffer containing 100 μM nicotine or different concentrations of AT-1001 was added for 2 mins. The background efflux was determined in the cells that received buffer alone, while maximal response was defined as the efflux elicited by 100 μM nicotine. The ⁸⁶Rb⁺ efflux was assessed using

Cerenkov counting on a Beckman-Coulter LS6500 Scintillation Counter. After subtracting background efflux, AT-1001 stimulation was expressed as the percent of efflux elicited by 100 μM nicotine, which stimulates maximum $^{86}\text{Rb}^+$ efflux in these cells.

To examine concentration-dependent desensitization, cells were loaded with $^{86}\text{Rb}^+$, rinsed gently 3 times over ~2 min with 1 mL buffer each time, and then treated for 10 min with different concentrations of AT-1001 in 0.5 mL buffer. After this 10 min treatment, the drug-containing buffer was aspirated and the cells were immediately stimulated with 100 μM nicotine in 0.5 mL buffer for 2 minutes. For analysis, nicotine-stimulated $^{86}\text{Rb}^+$ efflux in cells exposed to AT-1001 was compared to stimulated $^{86}\text{Rb}^+$ efflux in cells not exposed to AT-1001, which was set as 100%.

Results

Binding of AT-1001 to human and rat $\alpha 3\beta 4$ and $\alpha 4\beta 2$ nAChRs expressed in HEK293 cells.

The binding affinities of AT-1001 were examined in membrane homogenates from four separate HEK293 cell lines expressing either human or rat $\alpha 3\beta 4$ nAChRs or human or rat $\alpha 4\beta 2$ nAChRs. AT-1001 competed effectively with [^3H]-EB for binding at all of the nAChR subtypes tested. Figure 1 shows the competition curves from a representative experiment, and the results of five such binding studies are summarized in Table 1. As shown previously (Toll et al., 2012), AT-1001 competes with much higher affinity for $\alpha 3\beta 4$ than for $\alpha 4\beta 2$ nAChRs. Importantly, however, its affinity for the human $\alpha 3\beta 4$ receptor expressed in these cells is approximately 20-fold higher than its affinity for the rat $\alpha 3\beta 4$ receptor ($p < 0.001$), while its affinities for the human and rat $\alpha 4\beta 2$ nAChRs are similar. Consequently, the relative affinity (selectivity ratio) of AT-1001, calculated as the ratio of its K_i for $\alpha 4\beta 2$ nAChRs over its K_i for $\alpha 3\beta 4$ nAChRs, is ~ 40 for the rat receptors but more than 980 for the human receptors.

Binding of AT-1001 to human and rat $\alpha 3\beta 4$ and $\alpha 4\beta 2$ nAChRs in native tissues. To determine how closely AT-1001 binding in these heterologously expressed rat and human $\alpha 3\beta 4$ and $\alpha 4\beta 2$ nAChRs corresponds to its binding in native tissues, we examined AT-1001 competition for receptors in rat and human pineal gland, which in the rat are exclusively and in the human predominantly $\alpha 3\beta 4$ receptors, and in rat and human cerebral cortex, which are predominantly $\alpha 4\beta 2$ nAChRs in both species. As shown in figure 2 and summarized in Table 2, consistent with its higher affinity for $\alpha 3\beta 4$ nAChRs, AT-1001 competes with much higher affinity for nAChRs in rat and human pineal gland than for the receptors in rat and human cortex. Interestingly, the affinities of AT-1001 in these native tissues were 8- to 4-fold lower than the affinities found in the HEK cells heterologously expressing the corresponding nAChRs (compare

Tables 1 and 2). This might be due to differences such as lipids or accessory proteins in the cell types, or to differences in the stoichiometry of the receptors expressed in HEK cells vs the native cells. Nevertheless, as shown in Table 2, the relative selectivity ratio between human pineal ($\alpha3\beta4$) and human cortex ($\alpha4\beta2$), and rat pineal and rat cortex were similar to the selectivity ratios measured in the heterologously expressed receptors.

To determine the type of mechanism by which AT-1001 inhibits binding at nAChRs, [^3H]-EB binding saturation curves were compared in the absence and presence of AT-1001. To account for its higher affinity at the human $\alpha3\beta4$ receptor, we extended the concentration of [^3H]-EB used for the human receptors. As shown in figure 3A and 3B, at both human and rat $\alpha3\beta4$ nAChRs, low concentrations of AT-1001 shifted the [^3H]-EB saturation curves to the right, consistent with simple competitive inhibition; but at higher concentrations, AT-1001 appeared to also decrease the B_{max} of the saturation curves, which is usually indicative of a non-competitive mechanism. For example, at the highest AT-1001 concentration used here, the B_{max} of the human $\alpha3\beta4$ nAChR was decreased to $28 \pm 3\%$ of the control B_{max} , and it was decreased to about 20% at the rat $\alpha3\beta4$ nAChR. Even when the incubation time for the [^3H]-EB binding assays in the presence of 30 nM AT-1001 was extended to 24 h to ensure that equilibrium would be reached, the saturation curves were again shifted to the right, but the full B_{max} was still decreased (data not shown). A possible explanation for the apparent decrease in B_{max} of the $\alpha3\beta4$ nAChRs is related to the high affinity of AT-1001, which is similar to that of [^3H]-EB at these receptors; thus, even at the highest concentration, AT-1001 is probably acting competitively but at these high concentrations it may simply out-compete [^3H]-EB for re-binding to any unoccupied receptors, resulting in the appearance of a non-competitive mechanism.

In contrast to its apparently complex binding profile at $\alpha 3\beta 4$ nAChRs, at the $\alpha 4\beta 2$ nAChRs, where it has much lower affinity (and [^3H]-EB has higher affinity), AT-1001 shifted the [^3H]-EB saturation curves to the right without altering the B_{max} (Fig. 3C and 3D). This right shift, consistent with simple competitive inhibition, was found even in the presence of 30 μM AT-1001, a concentration used to achieve the receptor occupancy at $\alpha 4\beta 2$ nAChRs that would approximate the occupancy by the 30 nM concentration at human $\alpha 3\beta 4$ nAChRs, which have nearly 1000-fold higher affinity.

Up-regulation of nAChRs by AT-1001. Chronic administration of most nicotinic agonists and partial agonists *in vivo* up-regulate $\alpha 4\beta 2$ nAChRs in brain (Schwartz and Kellar; 1983; Marks et al., 1983; Flores et al., 1992; Mao et al., 2008; Marks et al., 2011). In contrast, the $\alpha 3\beta 4^*$ receptors found in autonomic ganglia, adrenal gland, retina and pineal gland are not up-regulated by chronic nicotine (Flores et al., 1997; Dávila-García et al., 2003). However, in cell lines that heterologously express various nAChR subunit combinations, $\alpha 3\beta 4$ nAChRs as well as all other putative nAChR subtypes tested, are increased by exposure for 1 to 5 days to several nicotinic ligands that bind to the agonist binding site (Wang et al., 1998; Meyers et al. 2001; Xiao and Kellar 2004). To determine if AT-1001 also up-regulates heterologously expressed nAChRs, we measured [^3H]-EB binding to human $\alpha 3\beta 4$ and $\alpha 4\beta 2$ nAChRs from cells treated for 3 days with 0.3 μM or 10 μM AT-1001, concentrations that would be expected to occupy virtually all $\alpha 3\beta 4$ nAChRs and 77% or 99% of the $\alpha 4\beta 2$ nAChRs. These treatments up-regulated both the $\alpha 3\beta 4$ and $\alpha 4\beta 2$ nAChRs (Fig. 4). The $\alpha 3\beta 4$ receptors were increased \sim two-fold by AT-1001 at both the 0.3 μM and 10 μM concentrations, similar to the increase induced by 10 μM nicotine. The $\alpha 4\beta 2$ nAChRs, which are more responsive than $\alpha 3\beta 4$ receptors to up-regulation by agonists, were increased two-fold by the 0.3 μM concentration of AT-1001 but six-fold by the 10 μM

concentration. The increase in $\alpha 4\beta 2$ nAChRs induced by 10 μM AT-1001 was similar to that induced by 10 μM nicotine (Fig. 4).

Agonist activity of AT-1001. Agonist activity of AT-1001 at nAChRs was determined in patch-clamp electrophysiology studies and $^{86}\text{Rb}^+$ efflux studies. Examples of the response traces elicited by AT-1001 in patch-clamp experiments with human and rat $\alpha 3\beta 4$ and human $\alpha 4\beta 2$ receptors are shown in figure 5A. AT-1001 concentration-response curves for these three receptors are shown in figure 5B and summarized in Table 3. The responses elicited by AT-1001 were normalized to the response of each cell to 1 mM ACh, which reliably elicited maximum responses in these cells. AT-1001 demonstrated 65-70% partial agonist activity at the two $\alpha 3\beta 4$ nAChRs and 18% agonist activity at the human $\alpha 4\beta 2$ receptor (see legend to figure 5 for the maximal current responses for each of these receptors). It also showed partial agonist activity at rat $\alpha 4\beta 2$ receptors, but because these receptors have a low maximal response to 1 mM ACh ($E_{\text{max}} = 48 \pm 7$ pA, $n=3$) and an even lower response to AT-1001 ($E_{\text{max}} 7.2 \pm 5$ pA), we could not determine a reliable EC_{50} value for AT-1001 at this rat receptor.

As shown in Table 3, of the three receptors at which we were able to calculate EC_{50} values in these patch-clamp studies, AT-1001 has the highest potency at human $\alpha 3\beta 4$ receptors ($\text{EC}_{50} = 0.4$ μM); its next highest potency is at rat $\alpha 3\beta 4$ ($\text{EC}_{50} = 1.4$ μM), and its lowest potency is at human $\alpha 4\beta 2$ receptors ($\text{EC}_{50} = 4.3$ μM). Thus, the agonist potency of AT-1001 is 3-times higher at human $\alpha 3\beta 4$ nAChRs than at the rat version, and it is ~ 10 -times higher than at human $\alpha 4\beta 2$ nAChRs.

Responses elicited by AT-1001 in $^{86}\text{Rb}^+$ efflux studies were normalized to the responses elicited by 100 μM nicotine; these nicotine-stimulated responses are shown in figure 6A. Consistent with the electrophysiological measurements, AT-1001 demonstrated partial agonist activity in these $^{86}\text{Rb}^+$ efflux measurements at both human and rat $\alpha 3\beta 4$ receptors (Fig. 6B). Although nicotine reliably induces $^{86}\text{Rb}^+$ efflux via the human $\alpha 4\beta 2$ nAChR channels (see Fig 6A), AT-1001 did not induce a measurable response in these receptors with this assay. Neither nicotine nor AT-1001 elicited responses at the rat $\alpha 4\beta 2$ receptor in this cell line. The EC_{50} and E_{max} values for the human and rat $\alpha 3\beta 4$ receptors calculated from these $^{86}\text{Rb}^+$ efflux studies are summarized in Table 3. Consistent with the electrophysiological studies, AT-1001 demonstrated significantly higher potency at the human $\alpha 3\beta 4$ nAChR than at the rat receptor ($p < 0.01$). In these $^{86}\text{Rb}^+$ efflux assays, the E_{max} value for the human receptor was significantly greater than for the rat receptor ($p < 0.01$).

Desensitization. Nicotinic agonists and partial agonists induce nAChR desensitization, which appears to depend primarily on a ligand's occupancy of the agonist binding site rather than its efficacy at the receptor. The potencies of AT-1001 to desensitize human and rat $\alpha 3\beta 4$ nAChRs and human $\alpha 4\beta 2$ nAChRs were determined using the $^{86}\text{Rb}^+$ efflux assay, which we find is a more suitable method than patch clamp for assessment of desensitization because it measures the responses in a large population of cells. Thus, nicotine-stimulated $^{86}\text{Rb}^+$ efflux was measured immediately following a 10-minute exposure to different concentrations of AT-1001. The 10-minute exposure to the highest concentrations of AT-1001 fully desensitized all three nAChRs tested, but the desensitization potency of AT-1001 varied across the receptors (Fig. 7 and Table 4). The concentration that decreased the nicotine-stimulated $^{86}\text{Rb}^+$ -efflux response by 50% is referred to here as the DC_{50} .

The order of potency for desensitization of these three receptors followed the same order of potency for activating the receptors and as the affinity for binding; but in the studies that allowed accurate assessments of the EC₅₀ values (all three receptors in patch clamp studies and two of the three receptors in ⁸⁶Rb⁺ efflux assays), the potencies to desensitize the receptors were closer to the potencies to activate than to the affinities for the agonist binding sites. Thus, under these conditions, the DC₅₀ concentrations of AT-1001 were similar to its EC₅₀ for activation at each of these receptors; while in contrast, these DC₅₀ concentrations of AT-1001 were 1200- to 600-times higher than its binding K_i values at the human and rat α3β4 receptors, respectively, and 36-times higher than the K_i at the human α4β2 receptor. It should be noted, however, that the similarities between the potencies to desensitize and to activate may, at least in part, reflect the similar incubation times under which the measurements were made, i.e, seconds and minutes for activation and desensitization, compared to hours for equilibrium binding.

As shown in Table 4, the Hill slopes for these desensitization measurements at the human α3β4 and α4β2 receptors were statistically greater than 1 (p<0.01), while measurement at the rat α3β4 receptors trended in that direction (p<0.1). This suggests that desensitization involves more than simple receptor occupancy by a ligand at a single site; it could, for example, indicate that two or more binding sites have to be occupied to induce full desensitization and that there is a cooperative step in the process.

AT-1001 at high concentrations completely desensitizes all of these nAChRs (Fig. 7). To determine the rates of recovery from AT-1001-induced desensitization for each receptor subtype, we measured responses to ACh using the patch clamp method because it allows a high degree of temporal resolution. Responses to 1 mM ACh in cells expressing human or rat α3β4 or human

$\alpha 4\beta 2$ receptors were recorded and the cells were then exposed for 10 min to AT-1001 at a concentration equal to 100-times its DC_{50} . Following a rapid wash in buffer, the cells were exposed to a rapid pulse of 1 mM ACh each minute followed by a wash in buffer to observe the recovery of function for up to ~35 min. As shown in figure 8, the human $\alpha 4\beta 2$ receptors display the fastest rate of recovery, with a half-time for recovery of 0.68 min; the rat $\alpha 3\beta 4$ receptors recovered next fastest, with a half-time of 3.3 min, and the human $\alpha 3\beta 4$ receptors displayed the slowest rate of recovery, with a half-time of 9.7 min. These results suggest that the rate of recovery from desensitization after exposure to AT-1001 is a function of the binding affinity of the ligand; that is, directly related to its dissociation rate.

Discussion

AT-1001 is likely to be an important tool for studying nAChRs because it is the first ligand with high selectivity for $\alpha 3\beta 4$ nAChR binding sites. Nevertheless, in assigning nAChR subtype(s) that mediate the effects of AT-1001, especially its effects *in vivo*, it is important to have as complete a pharmacological profile as possible.

Our results confirm earlier studies that AT-1001 binds with high affinity and selectivity to $\alpha 3\beta 4$ nAChRs (Toll et al., 2012) and that it displays partial agonist activity (Zaveri et al., 2015). In HEK cells heterologously expressing the rat versions of the $\alpha 3\beta 4$ and $\alpha 4\beta 2$ nAChR subtypes, we found that AT-1001 displays 41-fold selectivity for the $\alpha 3\beta 4$ subtype, which also is in general agreement with the initial report (Toll et al., 2012). Interestingly, however, in cells expressing the human versions of these nAChR subtypes, AT-1001 exhibits nearly 1000-fold selectivity for the $\alpha 3\beta 4$ subtype. This was due to approximately 20-fold higher affinity of AT-1001 for the human than for the rat $\alpha 3\beta 4$ receptor, while exhibiting similar affinities for the heterologously expressed rat and human $\alpha 4\beta 2$ receptors.

To further explore this difference between rat and human nAChRs, we compared the affinities of AT-1001 in rat and human pineal gland, which under the conditions used here represent nearly exclusively the $\alpha 3\beta 4$ nAChR subtype, and rat and human cerebral cortex, which represent predominantly ($\geq 90\%$) the $\alpha 4\beta 2^*$ nAChR subtype. Although the binding affinities of AT-1001 for the heterologously expressed receptors and their corresponding subtypes in native tissues differed, possibly because of different subunit stoichiometries or differences in accessory proteins or lipids between the cells in native tissues and HEK cells, the selectivity of AT-1001 for $\alpha 3\beta 4$

nAChRs was still very clear; in fact, the selectivity of AT-1001 for both human and rat $\alpha 3\beta 4$ over $\alpha 4\beta 2$ nAChRs was similar in the native tissues and in the cell lines.

An iodinated analog of AT-1001, [^{125}I]-AT-1012, was recently synthesized and shown to be an excellent radioligand for autoradiography studies of $\alpha 3\beta 4$ nAChRs in rat brain (Wu et al., 2014). If the same difference in affinity between human and rat $\alpha 3\beta 4$ nAChRs holds for [^{125}I]-AT-1012, it should be an even better probe for $\alpha 3\beta 4$ nAChRs in human CNS and peripheral tissues, where the density of nAChRs is much lower than in these cell lines.

At low concentrations of AT-1001 (up to about 3 nM) there is a clear rightward shift of [^3H]-EB saturation curves, consistent with a competitive mode of inhibition; but at higher concentrations of AT-1001 (30 nM and 300 nM), which are approximately 300-times and 150-times its K_i at human and rat $\alpha 3\beta 4$ nAChRs, respectively, there is also an apparent decrease in the B_{max} of binding, suggesting a non-competitive mode of inhibition at the receptor. However, this more likely results from a very slow dissociation rate of AT-1001 at $\alpha 3\beta 4$ nAChRs, which would be consistent with its very high affinity, and the high concentrations of AT-1001 out-competing [^3H]-EB for rebinding to the open receptors. In contrast to its inhibition of [^3H]-EB binding to $\alpha 3\beta 4$ nAChRs, AT-1001 inhibition of binding to $\alpha 4\beta 2$ nAChRs, where it has a much lower affinity, was consistent with a standard and simple competitive mechanism only.

The initial paper describing AT-1001 pharmacology determined that it was a selective antagonist at rat $\alpha 3\beta 4$ nAChRs (Toll et al, 2012). In contrast, we found that it is a potent and relatively efficacious partial agonist at both human and rat $\alpha 3\beta 4$ nAChRs. Its efficacy relative to ACh was 65-70% in whole-cell patch clamp measurements and 36-59 % relative to nicotine in $^{86}\text{Rb}^+$ efflux assays. The difference between the efficacies found in these two types of assays may be related

to receptor desensitization, which is more evident during the much longer stimulation period required in the $^{86}\text{Rb}^+$ efflux assay than in patch clamp measurements (see below). In our patch clamp measurements of human $\alpha 3\beta 4$ nAChRs expressed in HEK cells, AT-1001 displayed a potency of 0.4 μM , which is nearly identical to the potency found in a recent study in *Xenopus* oocytes expressing human $\alpha 3\beta 4$ nAChRs (Zaveri et al., 2015). The potency of AT-1001 was 3.5-fold higher at the human than the rat $\alpha 3\beta 4$ receptor in patch clamp measurements and 15-fold higher in $^{86}\text{Rb}^+$ efflux assays. These species differences in AT-1001 agonist potencies are consistent with, though somewhat lower, than its 21-fold difference in binding affinity at these $\alpha 3\beta 4$ receptors. AT-1001 also displayed partial agonist activity at the human $\alpha 4\beta 2$ nAChR in patch clamp studies, but its efficacy was only 18 percent of the ACh response and its potency was 10-times lower than at the human $\alpha 3\beta 4$ receptor.

The differences in binding and function of AT-1001 at the $\alpha 3\beta 4$ and $\alpha 4\beta 2$ nAChRs probably reflect the strong influence of both the α and β subunits on the pharmacology of nAChRs (Luetje and Patrick, 1991; Papke and Heinemann, 1994; Parker et al 1998); while the differences between the human and rat $\alpha 3\beta 4$ nAChRs may result from differences in two adjacent amino acids within the extracellular D loop of the $\beta 4$ subunit (Young et al., 2007). Several other drugs have now been found to display differences in affinity, potency, and/or efficacy between human and rat $\alpha 3\beta 4$ nAChRs (Young et al., 2007; Zwart et al., 2008; Stokes and Papke, 2012).

AT-1001 has one of the highest affinities for $\alpha 3\beta 4$ nAChRs that has been reported, similar to or only slightly lower than the affinity of epibatidine for these receptors. In contrast, its affinity for the $\alpha 4\beta 2$ subtype is 1 to 3 orders of magnitude lower than such ligands as nicotine, cytosine, A-85380 and epibatidine. Despite this difference in binding affinity at these two receptors, a 3 day

incubation of cells with AT-1001 at concentrations designed to occupy nearly all of the $\alpha 3\beta 4$ and most of the $\alpha 4\beta 2$ receptors increased both receptor subtypes. Both the lower (0.3 μM) and higher (10 μM) concentrations of AT-1001 increased human $\alpha 3\beta 4$ nAChRs by about 2-fold, which was similar to the increase induced by 10 μM nicotine measured here, as well as in rat $\alpha 3\beta 4$ receptors expressed in HEK cells in previous studies (Meyer et al., 2001; Xiao and Kellar, 2004). In contrast, while incubation with 0.3 μM AT-1001 increased human $\alpha 4\beta 2$ nAChRs ~2-fold, incubation with 10 μM AT-1001 increased these receptors ~6-fold, which was similar to the increase induced by 10 μM nicotine. Thus, despite its much higher affinity for $\alpha 3\beta 4$ nAChRs, AT-1001 can clearly affect the regulation of $\alpha 4\beta 2$ receptors as well. These results are consistent with the idea that occupancy of the receptor's orthosteric binding site, independent of channel function, is the trigger for up-regulation in these cells. However, since there appear to be differences between the mechanisms for up-regulation of nAChRs in heterologous systems and neurons (Lomazzo et al., 2011), it is not known whether AT-1001 would up-regulate either $\alpha 3\beta 4$ or $\alpha 4\beta 2$ nAChRs *in vivo*.

Nicotinic agonists desensitize nAChRs. Moreover, nicotine is usually 10 to 40 times more potent to desensitize than to activate nAChRs, both *in vivo* (Sharp and Beyer, 1986; Hulihan-Giblin et al., 1990) and *in vitro* (Grady et al., 1994; Marks et al., 1994; Lester and Dani, 1995; Meyer et al., 2001). In contrast, the potencies of AT-1001 to desensitize both $\alpha 3\beta 4$ and $\alpha 4\beta 2$ nAChRs are similar to its potencies to activate these receptors. Although this apparent difference between AT-1001 and other nicotinic ligands might be due to the time frame or other conditions of the measurements, it also suggests that the mechanisms triggering or underlying nAChR desensitization might differ among ligands. The Hill slopes for desensitization of human $\alpha 3\beta 4$ and $\alpha 4\beta 2$ nAChRs were significantly greater than 1, and that for rat $\alpha 3\beta 4$ nAChRs trended in that

direction, suggesting that desensitization might involve more than one step and include a cooperative process, such as binding of the ligand to a second orthosteric site or, less likely, to an allosteric site.

While the DC_{50} values of AT-1001 for desensitization by AT-1001 are similar to its EC_{50} values for activation at each of the three receptors, the time to recovery from desensitization follows the reverse order of its binding affinity; thus, the receptor with the highest affinity recovers slowest and the receptor with the lowest affinity recovers fastest. This is consistent with the time to recovery from desensitization being dependent on the dissociation of the ligand from the receptor. The $\alpha 4\beta 2$ nAChR subtype is usually found to exhibit the longest recovery times following desensitization, and our data here suggest this is probably a function of the slower dissociation rates of most nicotinic ligands, which usually have highest affinity at $\alpha 4\beta 2$ nAChRs. AT-1001 is clearly an exception.

In conclusion, we examined the binding affinities and functional effects of AT-1001 at human and rat $\alpha 3\beta 4$ and $\alpha 4\beta 2$ nAChRs, as well as its effects on the regulation of these receptors. We found AT-1001 to be a 65-70 percent partial agonist at $\alpha 3\beta 4$ nAChRs, with higher binding affinity and potency at the human than the rat version of these receptors. Moreover, it is highly selective in its binding affinity (989-fold), its functional potency (10-fold) and its desensitization potency (30-fold) for human $\alpha 3\beta 4$ over $\alpha 4\beta 2$ nAChRs.

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Footnotes

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Figure Legends

Figure 1. Representative competition binding curves of AT-1001 competing against 0.4 nM [³H]-EB at human and rat $\alpha 3\beta 4$ nAChRs and human and rat $\alpha 4\beta 2$ nAChRs heterologously expressed in HEK cells. See Table 1 for K_i values and selectivity ratios from 5 similar studies.

Figure 2. Representative competition binding curves of AT-1001 competing against 0.4 nM [³H]-EB at nAChRs in human and rat pineal gland and in human and rat cerebral cortex. See Table 2 for K_i values and selectivity ratios from 2 to 3 similar studies.

Figure 3. Effects of AT-1001 on [³H]-epibatidine binding saturation curves. [³H]-epibatidine saturation binding was measured in the absence and presence of AT-1001 in (A) human $\alpha 3\beta 4$, (B) rat $\alpha 3\beta 4$, (C) human $\alpha 4\beta 2$, and (D) rat $\alpha 4\beta 2$ nAChRs with a 4 hr incubation. These saturation curves are representative of 3-5 experiments.

Figure 4. AT-1001 induces up-regulation of human $\alpha 3\beta 4$ and human $\alpha 4\beta 2$ nAChRs. Cells were incubated for 3 days with either 0.3 μ M or 10 μ M AT-1001 or 10 μ M nicotine before membranes were prepared and nAChRs measured with 3 nM [³H]-EB. Each bar represents the mean \pm SEM from 3 - 5 experiments. Significantly different from control cells incubated without added drug: * $p < 0.05$; ** $p < 0.01$.

Figure 5. AT-1001 is a Partial agonist at human $\alpha 3\beta 4$, rat $\alpha 3\beta 4$ and human $\alpha 4\beta 2$ nAChRs as assessed with patch-clamp electrophysiology. A. Representative whole cell current traces elicited by AT-1001 from human and rat $\alpha 3\beta 4$ and human $\alpha 4\beta 2$ nAChRs expressed in HEK cells (the horizontal bar = 1 s drug application). B. Concentration-response curves of AT-1001 stimulated currents in the three nAChRs. The points in the curves were normalized to 1 mM ACh and are the mean \pm SEM of 6 - 9 experiments. The maximal responses to 1 mM ACh for human and rat $\alpha 3\beta 4$ and human $\alpha 4\beta 2$ nAChRs were, respectively: 1142 ± 246 pA, 1648 ± 382 pA and 312 ± 129 pA. See Table 3 for the EC_{50} values. (The maximal response of rat $\alpha 4\beta 2$ receptors to 1 mM ACh was 48 ± 7 pA, and the response to AT-1001 was only 7.2 pA, which was considered too small for a reliable calculation of an EC_{50} value for AT-1001.)

Figure 6. AT-1001 stimulates $^{86}Rb^+$ efflux at human and rat $\alpha 3\beta 4$ nAChRs. (A) Nicotine-stimulated $^{86}Rb^+$ efflux at human $\alpha 3\beta 4$, rat $\alpha 3\beta 4$ and human $\alpha 4\beta 2$ nAChRs. Efflux was measured in the absence (basal efflux) and presence (stimulated efflux) of 100 μ M nicotine. Values are the mean \pm SEM of 3 or 4 independent experiments. (B) Partial agonist response to AT-1001 at human and rat $\alpha 3\beta 4$ nAChRs measured in the $^{86}Rb^+$ efflux assay. Although nicotine reliably stimulates $^{86}Rb^+$ efflux at human $\alpha 4\beta 2$ nAChRs (see panel A), AT-1001 did not elicit a measurable response at these receptors in this assay. Curves are representative of 3 or 4 independent experiments. See Table 3 for EC_{50} and E_{max} values.

Figure 7. AT-1001 potently desensitizes human and rat $\alpha 3\beta 4$ and human $\alpha 4\beta 2$ nAChRs.

Desensitization was determined in $^{86}\text{Rb}^+$ -efflux measurements following a 10-minute exposure to AT-1001 at the concentrations shown. Values are the mean \pm SEM from 3 or 4 independent measurements. Where no SEM bar is evident, it is within the symbol. See Table 4 for the calculated parameters from these curves.

Figure 8. Recovery of function of nAChRs measured by patch clamp electrophysiology after desensitization.

Currents stimulated by 1 mM ACh were measured each min following a 10-min exposure to AT-1001 at a concentration 100-times the DC_{50} of the respective receptor (shown in the figure inset). Results were normalized to the maximal response to 1 mM ACh in the cells before exposure to AT-1001. Each curve fit to a one-phase exponential model with a $t_{1/2}$ of 9.7 min, 3.3 min and 0.68 min for human $\alpha 3\beta 4$, rat $\alpha 3\beta 4$ and human $\alpha 4\beta 2$ receptors, respectively. The points in the curves represent the mean \pm SEM of 3 – 5 experiments.

Table 1. The binding affinities of AT-1001 at human and rat $\alpha 3\beta 4$ and $\alpha 4\beta 2$ nAChRs expressed in HEK293 cells determined by competition binding with [^3H]-epibatidine. The K_i values were calculated from binding competition curves such as those shown in figure 1, using the Cheng-Prusoff equation and the K_d of [^3H]-epibatidine at each receptor. The Selectivity Ratio is the ratio of $K_i \alpha 4\beta 2/K_i \alpha 3\beta 4$ for each species. Values represent the mean \pm standard error of 5 experiments.

	K_i (nM)	Selectivity Ratio ($K_i \alpha 4\beta 2/K_i \alpha 3\beta 4$)
Human $\alpha 3\beta 4$	0.092 \pm 0.008	
Human $\alpha 4\beta 2$	91 \pm 8	989
Rat $\alpha 3\beta 4$	1.9 \pm 0.1 ***	
Rat $\alpha 4\beta 2$	78 \pm 10	41

***The K_i value of AT-1001 at the rat $\alpha 3\beta 4$ nAChR is significantly higher than at the human $\alpha 3\beta 4$ receptor ($p < 0.001$), while the K_i values at the $\alpha 4\beta 2$ nAChRs are not significantly different from each other.

Table 2. Binding affinities of AT-1001 at human and rat pineal gland (predominantly $\alpha 3\beta 4$ nAChRs) and human and rat cerebral cortex (predominantly $\alpha 4\beta 2$ nAChRs) determined by competition binding with [^3H]-epibatidine. The K_i values were calculated from the binding competition curves such as those shown in figure 2, using the Cheng-Prusoff equation and the K_d of [^3H]-epibatidine at each receptor. The Selectivity Ratio is the ratio of K_i cortex/ K_i pineal for each species. Values are the mean \pm standard error of 2 independent assays for pineal and 3 for cortex.

	K_i (nM)	Selectivity Ratio (K_i cortex/K_i pineal)
Human Pineal	1.2 \pm 0.6	917
Human Cortex	1100 \pm 300	
Rat Pineal	16 \pm 1 **	69
Rat Cortex	1100 \pm 90	

**The K_i value of AT-1001 at the rat pineal nAChR is significantly higher than at the human pineal nAChR ($p < 0.01$), while the K_i values at the human and rat cortex nAChRs do not differ.

Table 3. Functional parameters of AT-1001 at expressed human and rat $\alpha 3\beta 4$ nAChRs and human $\alpha 4\beta 2$ nAChRs measured in patch clamp and $^{86}\text{Rb}^+$ -efflux studies.

	Patch-clamp Electrophysiology			$^{86}\text{Rb}^+$ Efflux		
	EC ₅₀ (μM)	E _{Max} (%)	Hill slope	EC ₅₀ (μM)	E _{Max} (%)	Hill slope
Human $\alpha 3\beta 4$	0.4 \pm 0.1**	70 \pm 6**	1.8 \pm 0.2*	0.1 \pm .003**	59 \pm 0.3**	2.0 \pm 0.2*
Rat $\alpha 3\beta 4$	1.4 \pm 0.2**	65 \pm 4**	1.8 \pm 0.1*	1.5 \pm 0.3	36 \pm 3	2.3 \pm 0.4*
Human $\alpha 4\beta 2$	4.3 \pm 1.2**	18 \pm 3	1.3 \pm 0.1*	ND	ND	ND
Rat $\alpha 4\beta 2$	ND	16 \pm 9	ND	ND	ND	ND

** In patch clamp studies, the EC₅₀ values are statistically different from one another (p < 0.01); and the E_{max} values for both human and rat $\alpha 3\beta 4$ nAChRs are statistically greater than the values for the human $\alpha 4\beta 2$ receptors (p < 0.01). In the $^{86}\text{Rb}^+$ -efflux studies, both the EC₅₀ and the E_{max} values for human $\alpha 3\beta 4$ receptors are statistically different from the rat receptors (p < 0.01). * The Hill coefficients are statistically greater than 1 (p < 0.05). ND = not determined because responses to AT-1001 at rat $\alpha 4\beta 2$ receptors in patch clamp studies and at both rat and human $\alpha 4\beta 2$ receptors in $^{86}\text{Rb}^+$ -efflux studies were too small to calculate a reliable EC₅₀.

Table 4. Desensitization parameters of AT-1001 at $\alpha 3\beta 4$ and $\alpha 4\beta 2$ nAChRs as determined in $^{86}\text{Rb}^+$ -efflux assays. Desensitization of the receptor responses to 100 μM nicotine was determined after exposing cells to AT-1001 for 10 minutes at concentrations of 1 nM to 100 μM (see Fig. 7). The DC_{50} is the concentration of AT-1001 that decreases the response to nicotine by 50 percent.

	DC_{50} (μM)	Hill slope
Human $\alpha 3\beta 4$	$0.11 \pm 0.01^{**}$	$1.4 \pm 0.05^{**}$
Rat $\alpha 3\beta 4$	$1.2 \pm 0.1^{**}$	1.5 ± 0.2
Human $\alpha 4\beta 2$	$3.3 \pm 0.4^{**}$	$1.6 \pm 0.1^{**}$

**The differences between the DC_{50} values among the three receptors are all statistically significant, ($p < 0.01$). The Hill coefficients for human $\alpha 3\beta 4$ and $\alpha 4\beta 2$ receptors are greater than 1 ($p < 0.01$). The Hill coefficient for rat $\alpha 3\beta 4$ was not statistically different from 1, but trended in that direction ($p < 0.1$).

Figure 1

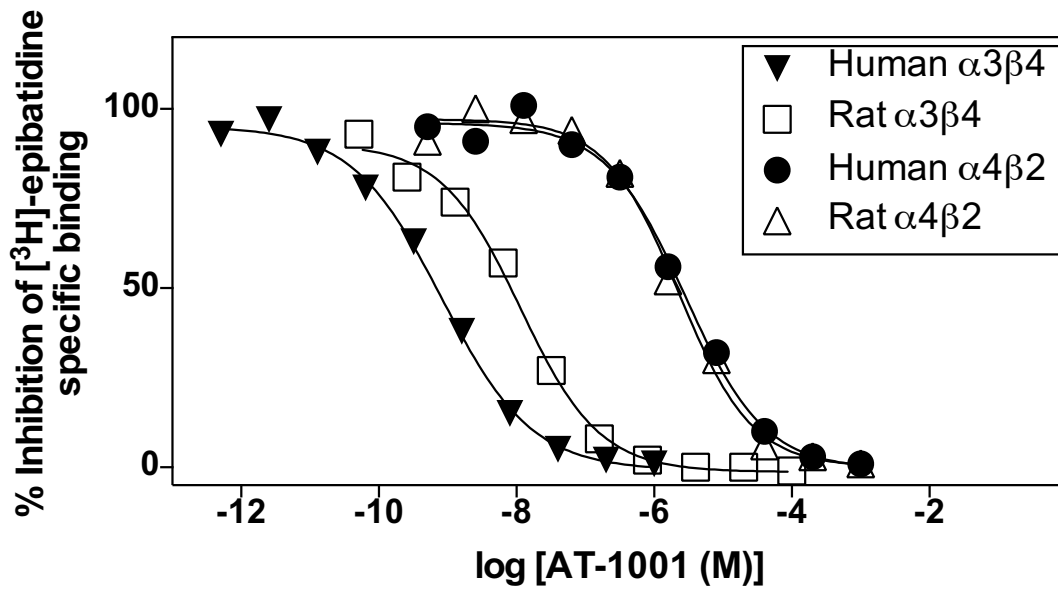


Figure 2

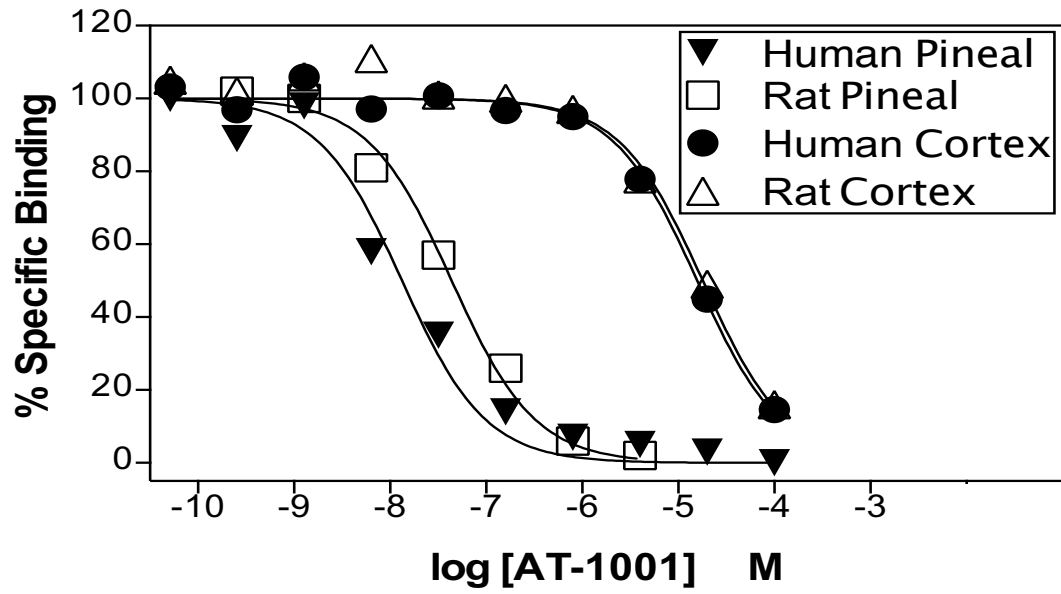


Figure 2

Figure 3

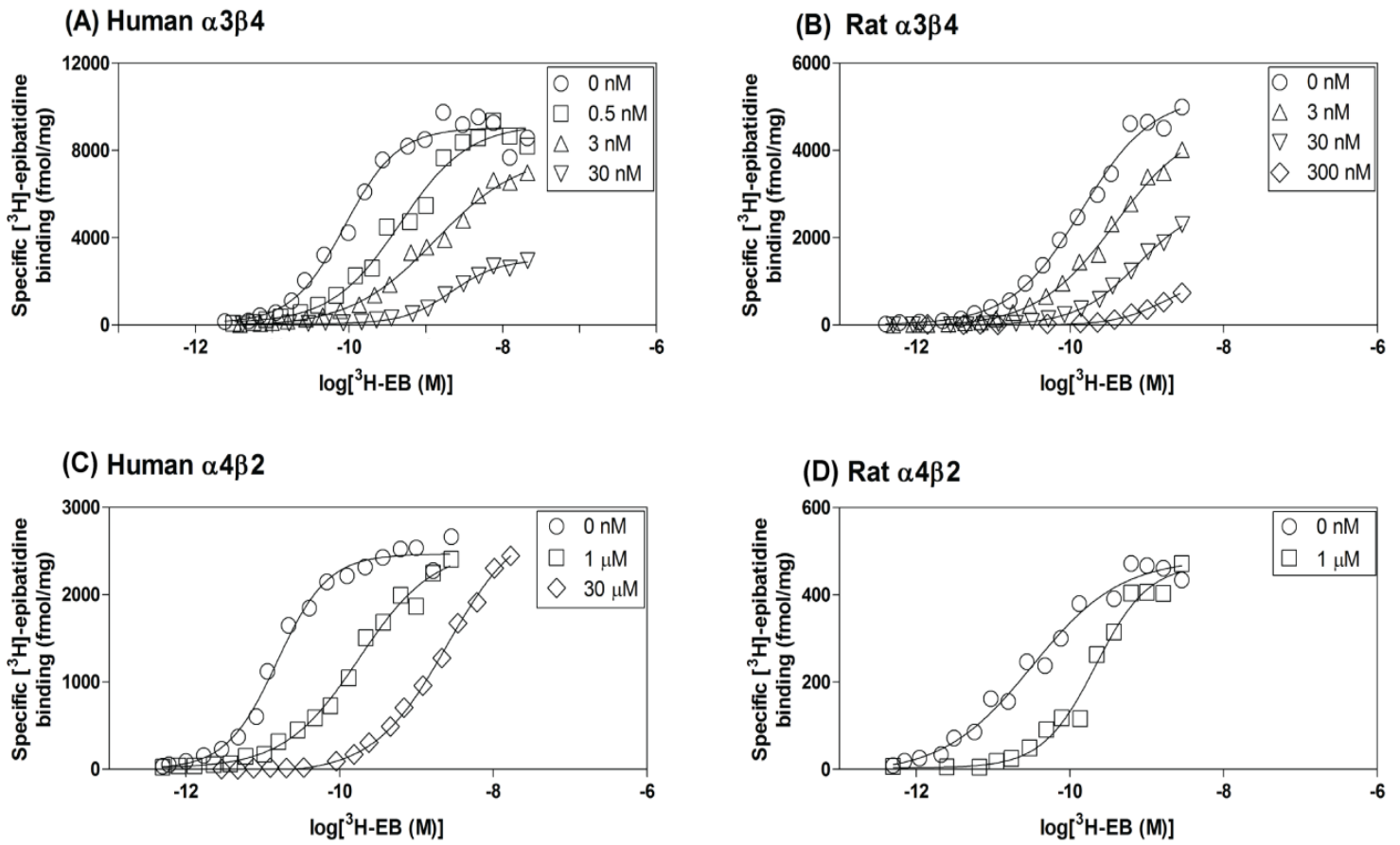


Figure 4

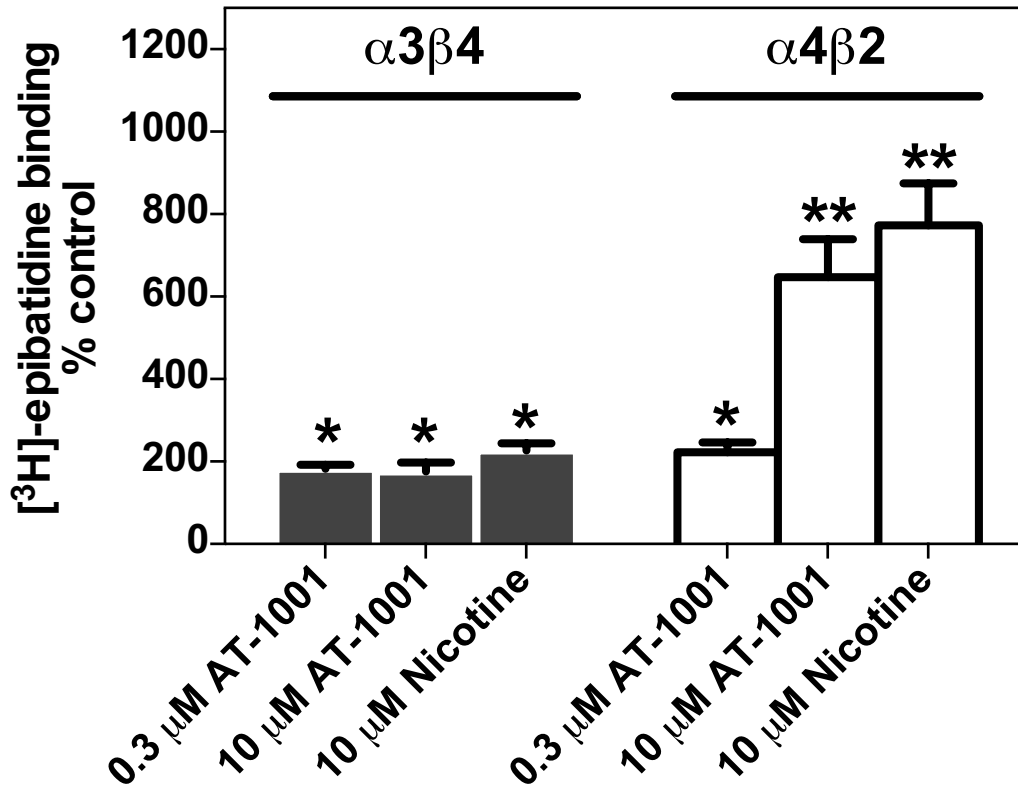


Figure 5

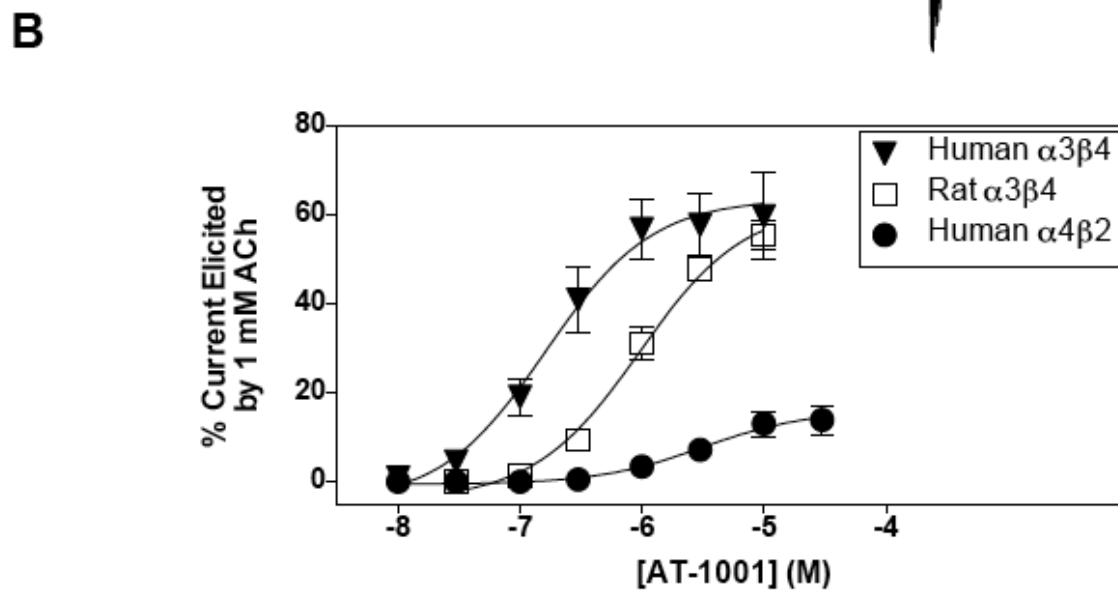
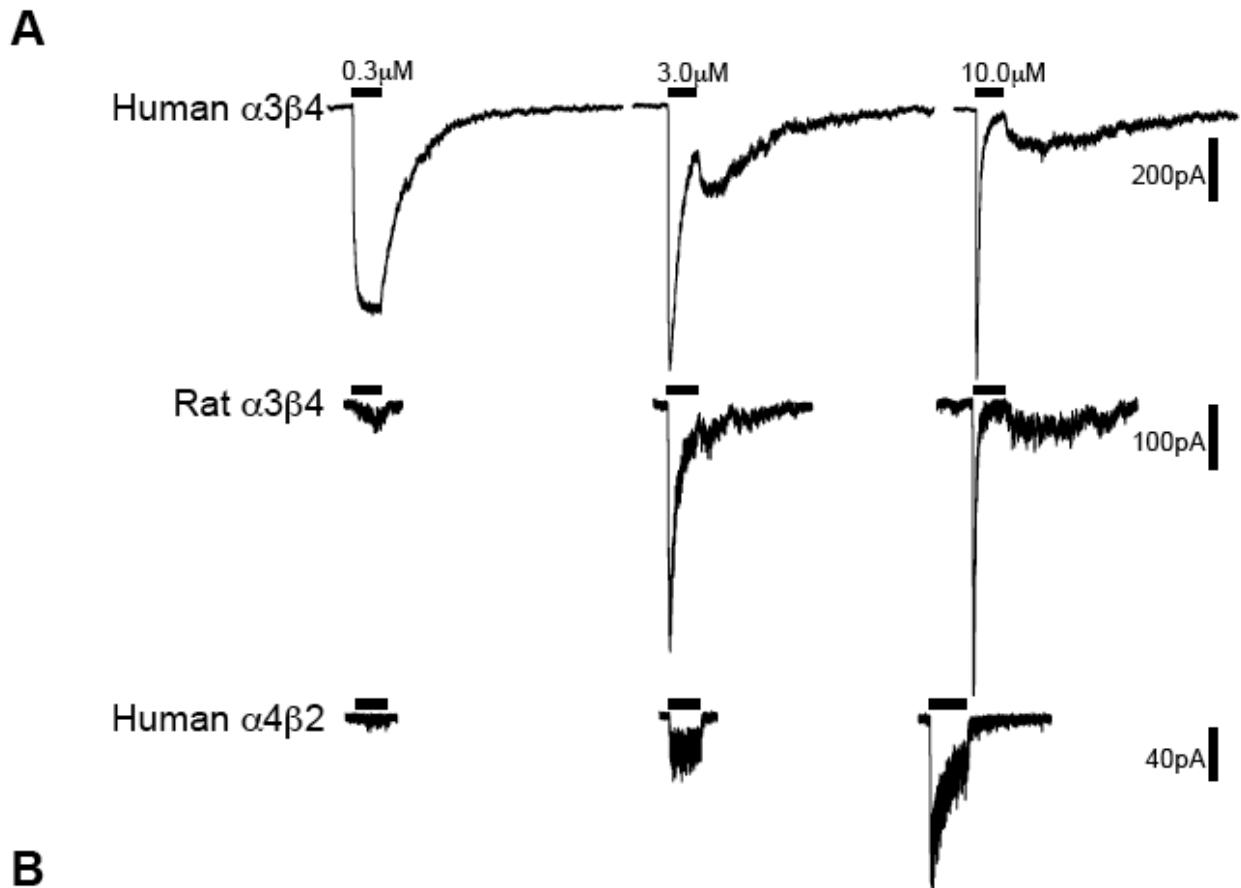


Figure 6

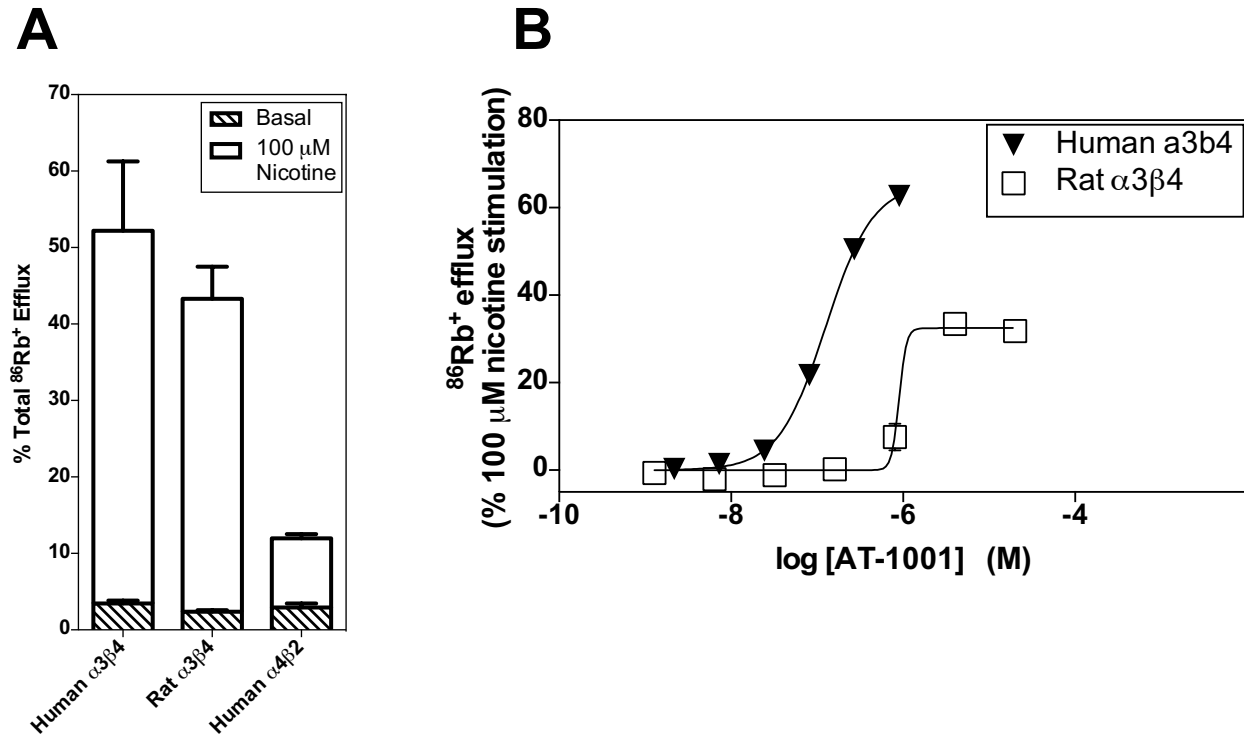


Figure 7

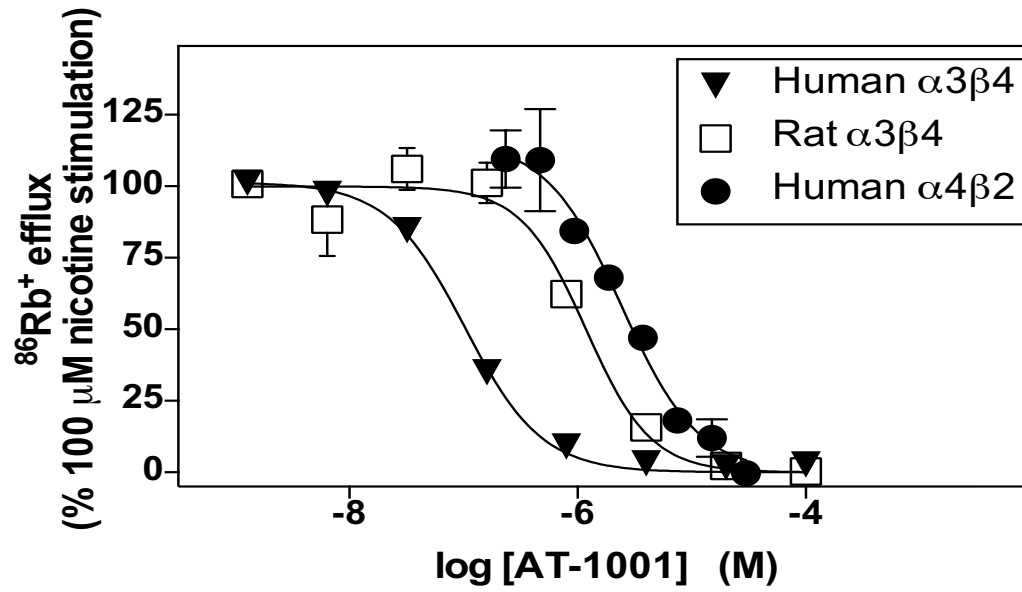


Figure 8

