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Evidence for alpha$_7$ nicotinic receptor activation during the cough suppressing effects induced by nicotine and identification of ATA-101 as a potential novel therapy for the treatment of chronic cough

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Running title: Antitussive effects of alpha$_7$ nicotinic receptor agonists

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Abbreviations used: BPM: beats/ min; eCig: electronic cigarette; IP: intraperitoneal; nAChR: nicotinic acetylcholine receptor; PIP: pulmonary inflation pressure; PO: per os (oral administration); TT: tracheal tension

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ABSTRACT

Studies performed in healthy smokers have documented a diminished responsiveness to tussive challenges and several lines of experimental evidence implicate nicotine as an antitussive component in both cigarette smoke and the vapors generated by electronic cigarettes. We set out to identify the nicotinic receptor subtype involved in the antitussive actions of nicotine and to further evaluate the potential of nicotinic receptor-selective agonists as cough suppressing therapeutics. We confirmed an antitussive effect of nicotine in guinea pigs. We additionally observed that the $\alpha_4\beta_2$-selective agonist Tc-6683 was without effect on evoked cough responses in guinea pigs, while the α_7-selective agonist PHA 543613 dose-dependently inhibited evoked coughing. We subsequently describe the preclinical evidence in support of ATA-101, a potent and highly selective α_7 selective nicotinic receptor agonist, as a potential candidate for antitussive therapy in humans. ATA-101, formerly known as Tc-5619, was orally bioavailable and moderately CNS penetrant and dose-dependently inhibited coughing in guinea pigs evoked by citric acid and bradykinin. Comparing the effects of airway targeted administration vs. systemic dosing and the effects of repeated dosing at various times prior to tussive challenge, our data suggest that the antitussive actions of ATA-101 require continued engagement of α_7 nicotinic receptors, likely in the CNS. Collectively, the data provide the preclinical rationale for α_7 nicotinic receptor engagement as a novel therapeutic strategy for cough suppression. The data also suggest that α_7 nAChR activation by nicotine may be permissive to nicotine delivery in a way that may promote addiction.

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Significance statement

This study documents the antitussive actions of nicotine and identifies the α_7 nicotinic receptor subtype as the target for nicotine during cough suppression described in humans. We additionally present evidence suggesting that ATA-101 and other α_7 nicotinic receptor selective agonists may be promising candidates for the treatment of chronic refractory cough.

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Cough is a prominent symptom emerging in patients with smoking related respiratory diseases and also manifests as part of the acute response to cigarette smoke exposure in nonsmokers. Although there are many irritating components contained in the particulate and vapor phases of burnt tobacco, nicotine may be a primary driver of airway irritation and cough in response to cigarette smoke and the vapors generated by electronic cigarettes (Lee et al., 2007). Molecular and electrophysiological analyses have documented nicotinic receptor (nAChR) expression by airway sensory nerves (Kuo et al., 1989; Lee et al., 1989; Kuo et al., 1990; Xu et al., 2007; Gu et al., 2008; Tao et al., 2019), and reflexes including cough are observed in human volunteers and in animals challenged with aerosolized nicotine (Burch et al., 1989; Hansson et al., 1994; Lee et al., 1985; Lee et al., 2007; McConnell et al., 2017; Soule et al., 2020).

In healthy human smokers studied before smoking related diseases emerge, the effects of cigarette smoking on responsiveness to tussive challenges has been varied. Belvisi and colleagues described a modest increase in responsiveness to some tussive challenges (Belvisi et al., 2016). But this has not been a consistent observation. We found responsiveness to capsaicin challenges was diminished in healthy smokers when compared to age matched nonsmoking controls (Dicpinigaitis et al., 2003). Comparable observations in healthy smokers were made by other research groups (Millqvist and Bende, 2001; Kanezaki et al., 2010) and a study by Ando and colleagues using functional Magnetic Resonance Imaging (fMRI) also observed a diminished sensitivity to tussive challenges in smokers, with evidence for an enhanced descending central inhibitory control (Ando et al., 2019). We followed up our study comparing smokers and nonsmokers with the observation of a rapidly emerging and sustained cough hypersensitivity in smokers over 8 weeks of abstinence from cigarettes (Dicpinigaitis et al., 2006). This result was in keeping with several previous studies that documented the

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development or worsening of cough in patients just starting a smoking abstinence intervention (Cummings et al., 1985; Kanner et al., 1999; Ussher et al., 2003).

In subsequent placebo-controlled studies performed in healthy nonsmokers, we observed that after an acute airways irritation resulting in cough, inhalation of vapors generated from electronic cigarettes containing nicotine markedly reduced cough responsiveness and urge to cough during capsaicin challenges (Dicpinigaitis et al., 2016a, 2016b). Comparable results had been reported previously in a study of young smokers using nicotine gum, and a study performed in cats showed that nicotine administered to the CNS could inhibit evoked cough responses (Davenport et al., 2009; Poliacek et al., 2015). From these results we concluded that nicotine, in addition to its irritating effects on the airways mucosa evoking cough, may be an antitussive component contained in cigarette smoke with a site of action in the central nervous system.

A goal of the present study was to identify the nicotinic receptor subtype(s) responsible for the antitussive effects of nicotine. We present evidence that α_7 nicotinic receptors may be a novel target for antitussive drugs. With the additional goal of translating our preclinical results into studies in patients with chronic cough, we used guinea pig models to demonstrate the antitussive effects of ATA-101, also known as Tc-5619 and bradanacline (Hauser et al., 2009; Mazurov et al., 2012a). This nAChR agonist with nanomolar potency and more than a 1000-fold selectivity for the human α_7 nAChR subtype displays little or no affinity for an additional 50 off target receptors and ion channels. ATA-101/ Tc-5619 was also found to be orally bioavailable in animals and in humans and was well tolerated in several clinical studies when administered daily for several weeks at doses as high as 125 mg/ day (Lieberman et al., 2013; Walling et al., 2016). The results of the present study should enable assessment of ATA-101 and other α_7 nAChR agonists as potential therapies for patients with chronic refractory cough.

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MATERIALS AND METHODS

All experiments described here were first approved by the Institutional Animal Care and Use Committee at Johns Hopkins. Male Hartley strain guinea pigs (200-400g; Charles River) were purchased pathogen free and housed in AAALAC accredited animal facilities. Animals were provided food and water ad libitum and were kept on 12-hour light: dark cycles.

To monitor coughing, guinea pigs were placed in a whole-body plethysmograph continuously filled with room air. Respiration and cough were monitored using a pressure transducer connected to the inside chamber of the plethysmograph (Canning et al., 2012; Hewitt et al., 2016). Coughing was evoked by aerosol challenges with citric acid (0.01-0.3M dissolved in water), bradykinin (1-10 mg/ mL dissolved in saline) or nicotine (3 mg/ mL dissolved in saline). Following a 5-minute equilibration period, concentration response curves were constructed, with each dose delivered for 5 minutes separated by 5-minute intervals in between ascending doses. The cumulative number of coughs were quantified as a median with intraquartile ranges.

In addition to counting the cumulative number of coughs evoked in each experiment, we also quantified the number of coughs evoked by threshold doses of citric acid (0.1M), and bradykinin (1 mg/ mL). We also made a measurement we called cough intensity, the highest number of coughs evoked by any dose of citric acid, bradykinin or nicotine in any 30 second recording epoch regardless of challenge agent concentration.

Nicotine and nicotinic receptor selective agonists were administered 30-60 minutes prior to cough challenges. The subtype selective nAChR agonists studied included ATA-101 (also known as Tc-5619 and bradanacline; Hauser et al., 2009; Mazurov et al., 2012a), choline (α_7 selective; Alkondon et al., 1997), PHA 543613 (α_7-selective; Wishka et al., 2006), Tc-6683 (also

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known as AZD-1446; $\alpha_4\beta_2$-selective; Mazurov et al., 2012b), and Tc-6987 (α_7-selective partial agonist; Bencharif et al., 2011; Hurst et al., 2013). The drugs were dissolved in saline and administered by intraperitoneal (IP) injection (0.1-100 mg/ kg), gavage (3-100 mg/ kg), or by aerosol (1-3 mg/ mL). Less than 1 mL of drug solution/ kg body weight was administered by gavage or by IP injection.

Unfortunately, few potent and selective antagonists of nAChR subtypes, including the α_7 subtype, have been characterized for their in vivo actions or CNS penetrance and essentially no such work has been done in guinea pigs. As an alternative to nAChR subtype-selective antagonists, we assessed the ability of mecamylamine to prevent the antitussive actions of nicotine. A suitable dose was first established using functional in vivo assays (see below). Guinea pigs were then dosed with either nicotine (10 mg/ kg), mecamylamine (1 mg/ kg) or their combination prior to citric acid challenges.

To quantify potential side effects of nicotinic receptor activation, we used an anesthetized, paralyzed mechanically ventilated preparation we had developed previously for studying airway autonomic reflexes (Mazzone and Canning, 2002). Guinea pigs were anesthetized with urethane (1.3-1.7 g/ kg IP, dosed to effect). Surgical preparation did not proceed until the animals had lost withdrawal responses to a sharp pinch of a hindlimb. The trachea was cannulated to facilitate mechanical ventilation (tidal volume=6 mL/ kg body weight, 60 breaths/ minute). Once ventilation had started the animals were paralyzed with succinylcholine (2.5 mg/ kg). The abdominal vena cava and abdominal aorta were visualized after a ventral incision in the abdomen and cannulated. Pressure transducers were connected to the cannulas in the aorta and trachea to monitor heart rate, blood pressure and pulmonary inflation pressure. The trachea was prepared for measurement of isometric smooth muscle

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tension, with the tracheal lumen continuously perfused with warmed, oxygenated Krebs buffer containing 3 μM indomethacin. When the surgeries were complete, nicotine and the nicotinic receptor-selective agonists were administered intravenously. We varied the order of ATA-101 and Tc-6683 administration but nicotine was always administered last. Using this same preparation, we established optimal dosing of mecamylamine following intravenous administration. In these experiments, bronchospasm and tracheal smooth muscle contraction were evoked by electrically stimulating the vagus nerves (16 Hz, 10 sec trains, 4-10V, bilateral). Muscarinic M_2 receptors were first blocked in these experiments by intravenous 5 mg/ kg gallamine administration to limit the bradycardia evoked by vagus nerve stimulation.

Pharmacokinetic and pharmacodynamic studies of ATA-101.

Pharmacokinetic and pharmacodynamic assessments of ATA-101 were performed in guinea pigs by researchers at PharmaLegacy (www.pharmalegacy.com). Male Hartley guinea pigs were dosed with ATA-101 by gavage (3-100 mg/ kg) or by intraperitoneal administration (30 mg/ kg). Cough was evoked by 0.4M citric acid (n=8/ group), immediately after which brain and plasma samples were collected from 4 animals in each group to measure ATA-101 concentrations. ATA-101 was also measured in plasma and brain samples in a second set of animals at various timepoints after oral administration. Brain samples were placed in saline (100mg tissue with 400 μL saline) followed by homogenization, with results reported as ng ATA-101/ mg tissue. ATA-101 was measured by LC-MS.

Reverse transcriptase polymerase chain reaction (RT-PCR) to detect α_7 nAChR mRNA.

After asphyxiation in a chamber filled with CO_2 and exsanguination, we extracted mRNA from the following guinea pig tissues: brain (cortex), brainstem/ medulla oblongata, bronchi,

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lung, nodose ganglia, jugular ganglia. These tissues were quickly homogenized in Trizole buffer followed by RNA extraction and quantification. The recovered mRNA was used for RT-PCR assessment of the presence of mRNA for the α_7 nicotinic receptor using the following primer: CAGTACCGCTGATAGCCCAG. Three repetitions of these experiments were completed

Statistical Analyses

The cough experiments were designed as nonpaired parallel group designs. The cough results are presented as a median and intraquartile ranges. In the anesthetized preparations, heart rate, blood pressure, pulmonary inflation pressure and tracheal smooth muscle tension were recorded as mean and standard deviation. Pharmacokinetic data were also reported as mean and standard deviation. Differences in group data for cough were compared by ANOVA and by Mann-Whitney U-tests. Group data for heart rate, blood pressure, pulmonary inflation pressure and airway smooth muscle tension were compared by t-test. P-values of less than 0.05 were considered statistically significant with Bonferoni corrections included for multiple comparisons.

Drugs and reagents.

Atropine, bradykinin, choline, citric acid, gallamine, indomethacin, mecamylamine, nicotine, succinylcholine and urethane were purchased from Sigma. Tc-5619, Tc-6683 and Tc-6987 were acquired from Targacept. Atropine (10 mM), citric acid (0.1-0.3M) and urethane (500 mg/ mL) were dissolved in water. Bradykinin (1-10 mg/ mL), choline (100 mg/ mL), gallamine (3 mg/ mL), succinylcholine (2.5 mg/ mL), mecamylamine (1-3 mg/ mL), nicotine (0.1-10 mg/ mL), Tc-5619 (1-100 mg/ mL), Tc-6683 (10-30 mg/ mL) and Tc-6987 (10-30 mg/ mL) were dissolved in saline. Indomethacin (30 mM) was dissolved in ethanol.

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RESULTS

The antitussive effects of nicotine: potential role of α_7 nAChR subtypes

Comparable to its acute effects on capsaicin-evoked coughing in humans (Dicpinigaitis et al., 2016a, 2016b), systemically administered nicotine (10 mg/ kg IP) inhibited coughing evoked by citric acid challenge in guinea pigs (figure 1A). Although no overtly quantifiable side effects of the naturally occurring alkaloid were noted in these experiments on conscious and uninstrumented guinea pigs, we suspected that, as dosed, nicotine would have undesirable systemic actions. Indeed, and consistent with previous studies (Hansson et al., 1994; Soria et al., 1996), when administered intravenously to anesthetized guinea pigs, nicotine (1 mg/kg) evoked airway smooth muscle contraction, airflow obstruction, and precipitous decreases in heart rate and blood pressure (Figure 1B). Based on these results and its known addictive properties, we concluded that nicotine, a modestly potent but nonselective and full agonist for all subtypes of nicotinic receptors (nAChRs), was not a viable option as an antitussive therapy.

It seemed plausible that the antitussive actions of nicotine are due to engagement of receptors distinct from those associated with neurotransmission in autonomic ganglia (e.g. $\alpha_3\beta_4$; Xu et al., 1999a, 1999b; Skok, 2002) and perhaps distinct from those nicotinic receptors capable of inducing reinforcing behaviors and addiction (e.g $\alpha_4\beta_2$; Nides et al., 2006; Smith et al., 2007; Pons et al., 2008; Rollema et al., 2009). It is unfortunate that few selective ligands for nicotinic receptor subtypes have been developed, and fewer still that are suitable for translational studies. But there are exceptions, and using available tools, we have evaluated the antitussive actions of $\alpha_4\beta_2$ and α_7 -selective nAChR agonists. We found that the $\alpha_4\beta_2$ selective agonist Tc-6683 (also known as AZD-1446; in vitro EC_{50} at human and rat $\alpha_4\beta_2$ nAChRs~30 nM) was without effect on citric acid evoked coughing in guinea pigs when administered at doses (10 and 30 mg/ kg)

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100-300 times a dose of the compound found efficacious in a cognitive assay performed in rats that is sensitive to $\alpha_4\beta_2$ nAChR agonism (Mazurov et al., 2012). By contrast, the potent (in vitro EC_{50}~9 nM; Wishka et al., 2006) and selective (>100-fold) α_7 nAChR agonist PHA 543613 (0.1-30 mg/ kg ip) dose-dependently inhibited the coughing evoked by citric acid. PHA 543613 (30 mg/ kg) also inhibited coughing following oral administration prior to citric acid challenge (Figure 2).

To facilitate translation of these preclinical observations into clinical assessments in patients, we sought an α_7 selective nAChR agonist suitable for human experimentation, with at least some evidence for oral bioavailability and CNS penetrance. ATA-101, formerly known as Tc-5619, seemed to be a suitable clinical candidate (Hauser et al., 2009; Mazurov et al., 2012a). After we had documented an antitussive action of ATA-101 in guinea pigs in our own laboratories (summarized below) we engaged a contract lab seeking independent confirmation of these results as well as pharmacokinetic and pharmacodynamic assessments. ATA-101 was orally bioavailable in guinea pigs and its antitussive actions were apparent 30-60 minutes after administration at doses ≥10 mg/ kg (Figure 3). Peak plasma concentrations at these antitussive doses of the drug ranged between 100 and 500 ng/ mL. Comparable plasma concentrations are observed in patients when dosed at 25-125 mg/ day (Lieberman et al., 2013; Walling et al., 2016). Importantly, and consistent with our hypothesis that α_7 nAChR agonists must be CNS penetrant to suppress evoked coughing, ATA-101 at doses of 10 and 30 mg/ kg appeared to be modestly CNS penetrant in guinea pigs, with brain concentrations reaching 10-20% of simultaneously measured plasma concentrations 1-2 hours after a single oral administration.

Mecamylamine is a CNS penetrant and relatively nonselective nAChR channel blocker that is considerably less potent (4-47-fold) at inhibiting α_7 channel function than that of other

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nAChR subtypes (Clark and Reuben, 1996; Chavez-Noriega et al., 1997; Fu et al., 2000; Mansbach et al., 2000; Mann and Greenfield, 2003; Endo et al., 2005; Papke et al., 2008). To further characterize the nAChRs involved in nicotine induced cough suppression we assessed the ability of mecamylamine to modulate the antitussive actions of nicotine. We first established a dose of mecamylamine that clearly engaged nAChRs as evidenced by reductions in heart rate and blood pressure (likely through actions in sympathetic ganglia) and with inhibition of vagally-induced airway smooth muscle contraction and bronchoconstriction (through actions in airway parasympathetic ganglia). At a dose (1 mg/ kg) that blocked nAChRs in autonomic ganglia (presumably $\alpha_3\beta_4$ nAChRs), mecamylamine failed on its own to modulate citric acid-evoked coughing and did not inhibit the antitussive actions of nicotine (Figure 4).

Mechanistic studies of α_7 nAChR-dependent cough suppression

Comparable to the results of our previous studies with PHA 543613 (Tao et al., 2019), we observed that ATA-101 was antitussive upon systemic administration (PO or IP; figs. 2a and 5) but failed to inhibit coughing when administered by aerosol (Figure 5). This argues for an extrapulmonary site of action (likely in the CNS). All of the nAChR agonists (nicotine, PHA 543613 and ATA-101) studied here that suppressed coughing are also full agonists at the α_7 subtype of nAChRs. Tc-6987 (10 and 30 mg/ kg), a moderately potent (EC_{50}=3 μM) but only partial (48%) α_7 nAChR agonist (Bencharif et al. 2011), did not inhibit citric acid evoked coughing. Choline, an endogenous low potency (EC_{50}~1 mM) α_7 selective agonist, also failed to inhibit citric acid evoked coughing in guinea pigs at an IP dose of 100 mg/ kg (see supplement).

While full agonism at α_7 nAChRs in the CNS may be required for cough suppression, activation-induced desensitization, a well-established in vitro attribute of α_7 nAChRs (Fenster et

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al., 1997; Olale et al., 1997; Hurst et al., 2013), may not be required for the antitussive actions of α_7 nAChR agonists. Rather, the continued engagement and activation of α_7 nAChRs may be necessary for cough suppression. We drew these conclusions based on the observations that an antitussive effect of 30 mg/ kg ATA-101 was measurable 30 minutes after administration (Figure 5) but was not apparent 4 hours after administration (when plasma concentrations are <10% of their peak in the first hour after IP administration; Figure 6). If ATA-101 was re-administered 30 minutes prior to challenge, however, coughing was suppressed.

A central site of action for α_7 nAChR agonists should make it more likely that drugs in this class would be antitussive regardless of the peripheral triggers for coughing in the airways and lungs. Consistent with this assertion, we found that in addition to inhibiting citric acid evoked coughing, ATA-101 also reduced cough responses evoked by bradykinin (Figure 7). Whether cough was evoked by either citric acid or bradykinin (or nicotine; see below), ATA-101 didn't obviously alter the threshold for evoking cough. ATA-101 did, however, reduce cough intensity, measured by counting the highest number of coughs observed during any 30 second epoch of cough challenge (see supplement). Comparable effects of nicotine on cough response intensity have been reported in healthy young smokers (Davenport et al., 2009).

ATA-101 inhibits coughing evoked by inhaled nicotine

The soothing, counterirritant effects of additives such as menthol and other flavorants combined with tobacco and included in electronic cigarette formulations may limit the airways irritation associated with cigarette smoke and electronic cigarette vapor inhalation, thus facilitating nicotine delivery in a way that is permissive to the emergence of nicotine addiction (Garten et al., 2004; Wise et al., 2012; Plevkova et al., 2013; Villanti et al., 2017; Wickham et

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al., 2018; DeVito et al., 2020; Odani et al., 2020). Our study suggests that α_7 nAChR engagement may also contribute to the permissive effects of nicotine inhalation. Consistent with that hypothesis, we observed that ATA-101 failed to evoke coughing upon inhalation or systemic administration but suppressed the cough responses evoked by nicotine inhalation (Figure 8).

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DISCUSSION

Evidence for the α_7 nAChR subtype as a target for antitussive therapy

Discovering the antitussive effects of nicotine in human volunteers was the unexpected result of studies designed to measure cough reflex sensitivity in smokers. In those studies, the surprising observation was made that otherwise healthy smokers are less responsive to capsaicin and citric acid challenges than age-matched nonsmokers (Millqvist and Bende, 2001; Dicpinigaitis et al., 2003; Kanezaki et al., 2010). In follow up studies we described a rapidly emerging and long-lasting cough hypersensitivity that manifested in previously heavy smokers (mean duration of smoking: 9.5 years) that were actively abstaining from cigarette use (Dicpinigaitis et al., 2006). The 2 most likely explanations for these results were that noxious components of smoke and its repetitive, irritating effects on the airways mucosa could desensitize or hinder responsiveness of airway sensory nerves and render them less capable of transducing cough reflexes, or that a cigarette smoke constituent actively suppressed coughing in smokers, and its withdrawal restored cough responsiveness. Studies using nicotine-containing gum and electronic cigarettes identified nicotine as an antitussive component of cigarette smoke (Davenport et al., 2009; Dicpinigaitis et al., 2016a, 2016b).

A cough suppressing effect of nicotine is counterintuitive to widely held assumptions about and personal experiences with smoking but are also difficult to reconcile with the well-documented pro-tussive acute effects of both cigarette smoke and nicotine inhalation in human volunteers and in animals (Burch et al., 1989; Karlsson et al., 1991; Hansson et al., 1994; Lee et al., 2007; McConnell et al., 2017; Soule et al., 2020). Even in the eCig study revealing the antitussive actions of nicotine, most of the smoking naive patients coughed immediately upon inhaling the nicotine-containing eCig vapors before the cough suppressing effects were apparent

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during subsequent capsaicin challenges (Dicpinigaitis et al., 2016a). And yet, despite these results and the reasonable counterintuition, antitussive effects of nicotine have now been documented in 3 species (humans, cats, guinea pigs), by 5 independent research groups and with 2 additional nAChR agonists (PHA 543613 and ATA-101) showing antitussive effects (present study; Davenport et al., 2009; Poliacek et al., 2015; Dicpinigaitis et al., 2016a, 2016b; Tao et al., 2019). Notably, just like with eCig vapor inhalation in humans, cigarette smoke inhalation is acutely pro-tussive in guinea pigs but in the first hour after cigarette smoke exposure, guinea pigs are nearly unresponsive to citric acid challenges, producing few if any coughs in the initial 30-60 minutes after cigarette smoke exposure had ceased (Karlsson et al., 1991). We can now speculate that nicotine was the cause of this cough suppressing effect of cigarette smoke.

In designing experiments to characterize the mechanisms by which nicotinic receptor agonists might suppress cough, we started with the assumption that an action at the sensory nerve terminals responsible for cough initiation seemed unlikely. This premise was based on the knowledge that all known nicotinic receptor subtypes are ligand-gated cation channels and would be excitatory in the majority if not all neurons (Hurst et al., 2013). Such excitation at the cough inducing sensory nerve terminals in the airways would not suppress cough but would instead evoke cough, as is seen acutely with nicotine and cigarette smoke inhalation. Rather, we hypothesized that the antitussive effects of nicotinic receptor agonists would require CNS penetrance and engagement of nAChRs in central locations that would then counteract the induction of cough. Nicotine is certainly highly CNS penetrant and after inhalation, this alkaloid rapidly and near maximally occupies nicotinic receptors in the CNS in humans (Brody et al., 2006). The elegant studies performed by Poliacek et al. in anesthetized cats provide direct evidence for a CNS site of antitussive action for nicotine (Poliacek et al., 2015).

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Our studies implicate α_7 nAChR engagement in the antitussive effects of nicotine. We observed a dose-dependent cough suppression with administration of the α_7-selective nAChR agonists PHA 543613 and ATA-101, recording peak inhibitory effects on cough (40-60% reductions) that approximated those achieved by nicotine. Both of the α_7-selective agonists were active upon oral or IP administration, but neither suppressed cough when administered by aerosol (present study, Tao et al., 2019). In contrast to the effects of the α_7 nAChR agonists, the $\alpha_4\beta_2$ agonist Tc-6683 was without effect on evoked coughing. In the absence of suitable tools to assess the involvement of other nAChR subtypes we cannot be dogmatic about which subtype ultimately accounts for the antitussive actions of nicotine and which other subtypes could be targeted to suppress cough. We also recognize that it is certainly possible that mechanisms delineated in guinea pigs may not fully translate to humans. But we are unaware of any evidence arguing against α_7 nAChR involvement in the antitussive effects of nicotine.

A reasonable working hypothesis to describe the antitussive actions of nicotinic receptor agonists would involve the activation of α_7 nAChR expressing inhibitory interneurons in midbrain and brainstem nuclei that regulate the encoding of cough. In this scenario the α_7 nAChR agonists would be actively recruiting an endogenous inhibitory pathway in cough regulation. Such a sensory gating mechanism may be relevant to the pathogenesis of cough, with clinical results implicating a loss of central inhibitory control in the emergence of cough hypersensitivity and chronic cough (Ando et al., 2016; Cho et al., 2019). Neurons in the midbrain periaqueductal gray have been implicated in the regulation of pain and cough, and a subset of these neurons express α_7 nAChRs (Ando et al., 2016; Umana et al., 2017). In several species including humans, brainstem α_7 nAChR binding sites and mRNA and functional responses in medullary neurons indicative of α_7 nAChR gating have also been described (present

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study; Falk et al., 2002; Smith and Uteshev, 2008). Immunohistochemical approaches have documented coexpression of α_7 nAChRs in medullary neurons that also express markers for GABA synthesis (Bitner et al., 2002; Dehkordi et al., 2007). We and others have documented GABA receptor dependent inhibition of coughing in multiple species including humans (Bolser et al., 1994; Dicpinigaitis et al., 1997; Dicpinigaitis et al., 1998; Mutolo et al., 2008; Canning and Mori, 2011; Canning et al., 2012; Cinelli et al., 2012; Kotmanova et al., 2018; Dong et al., 2019). In anesthetized cats, microinjections of nicotine in medullary locations inhibits evoked coughing (Poliacek et al., 2015). Which nAChR receptor subtype(s) were engaged by nicotine to inhibit coughing in cats was not established. Interestingly, however, the antitussive effects of the alkaloid in cats were not suppressed by a comparably administered pretreating dose of mecamylamine. Mecamylamine blocks nAChR channels somewhat nonselectively, but mecamylamine is considerably less effective at inhibiting inward currents induced by α_7 nAChR channel gating (Clark and Reuben, 1996; Chavez-Noriega et al., 1997; Fu et al., 2000; Mansbach et al., 2000; Mann and Greenfield, 2003; Endo et al., 2005; Papke et al., 2008). We also observed that mecamylamine failed to prevent the cough suppressing effects of nicotine.

Shared neurophysiological processes suggest that mechanistic studies of pain might inform efforts to delineate mechanisms in cough. Studies focused on nicotinic receptor pharmacology are no exception. Analgesic actions of α_7 nAChR agonists including PHA 543613 have been reported (Damaj et al., 2000; Freitas et al., 2013a; Freitas et al., 2013b; Bagdas et al., 2018). In these studies of pain, central sites of action for α_7 nAChR ligands have been implicated but the precise mechanisms of action are unclear, with evidence for analgesic effects reported in studies with both positive and negative allosteric modulators of α_7 nAChRs (Bagdas et al., 2018). Our data are most consistent with sustained receptor activation by full

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agonists being prerequisites for the antitussive effects we observed. At effective doses, ATA-101 concentrations in brain likely exceed the measured in vitro EC_{50} (~30 nM (12 ng/ mL)) for human α_7 activation (Hauser et al., 2009; Mazurov et al., 2012a). The partial α_7 nAChR agonist Tc-6987 failed to suppress cough and the actions of ATA-101 did not persist as the drug was cleared 4 hours after a single dose, but cough inhibition was rapidly restored when ATA-101 was re-administered 30 minutes prior to tussive challenge. It is hard to envision a scenario where this profile could fit with a mechanism reliant on receptor desensitization. In fact, when identified originally as Tc-5619, ATA-101 was described as unique for its inability to induce α_7 desensitization in vitro (Hauser et al., 2009). It is also worth noting that although α_7 receptor desensitization is a well-established phenomenon *in vitro* (Fenster et al., 1997; Olale et al., 1997; Hurst et al., 2013), it is at least debatable whether sustained desensitization of this subtype occurs *in vivo* (Thomsen et al., 2009; Christensen et al., 2010; Stevens et al., 2010).

Identification of ATA-101 as a candidate for antitussive therapy

Studies performed using nicotine-containing electronic cigarettes and nicotine gum clearly show that activating some nAChR subtype(s) can inhibit evoked cough responses in humans (Davenport et al., 2009; Dicpinigaitis et al., 2016a, 2016b). Our work implicates the α_7 nAChR subtype. The evidence summarized here showing that the α_7-selective agonist ATA-101 suppressed cough in guinea pigs, achieved modest but measurable CNS penetrance and has been administered safely to hundreds of patients previously made this drug a viable candidate for such translational studies.

A clinical trial has been completed with ATA-101 and is the subject of a separate manuscript in preparation (ClinicalTrials.gov Identifier: NCT03622216). Reasons to be

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optimistic about the prospects of ATA-101 as an antitussive therapy include the extensive evidence suggesting that nAChR agonists may suppress cough (3 species, 5 routes of administration), the predictive value of cough studies performed in guinea pigs (Canning, 2008; Canning and Chou, 2008; Adner et al., 2020), and the pharmacological evidence (3 structurally unrelated full agonists acting on a mecamylamine-insensitive nAChR subtype) identifying the α_7 nAChRs as the target for these therapies. Also encouraging is the established potency and selectivity of ATA-101 at human α_7 nAChRs studied in vitro, its oral bioavailability with a T1/2 of over 18 hours in humans, and its efficacy in guinea pigs (present study; Hauser et al., 2009; Lieberman et al., 2013; Walling et al., 2016). A potential anti-inflammatory effect of α_7 nAChR engagement might also be beneficial in some patients with chronic cough (Hoover, 2017; Bagdas et al., 2018).

Tempering our enthusiasm for the outcome of a clinical study with ATA-101 include the obvious, that documenting an antitussive effect in an otherwise healthy guinea pig studied in an evoked cough challenge model is not the same as treating a patient with chronic refractory cough. Also tempering our enthusiasm is the somewhat modest (~50%) inhibitory effects of all of the nAChR agonists on evoked cough and the reality that our studies in guinea pigs identifying the α_7 nAChR subtype as a therapeutic target doesn't rule out the possibility that the antitussive actions of nicotine in healthy volunteers may be the result of engaging nAChR subtype(s) other than the α_7 nAChRs. The cough suppressing effects of nicotine that have been documented in healthy human volunteers could even be lost as a result of the pathologies leading to chronic refractory cough (Ando et al., 2019; Cho et al., 2019). It is also worth noting that while ATA-101 is a reasonable clinical candidate, it may not be the ideal candidate. We still believe that CNS penetrance is essential to the antitussive actions of nAChR agonists and yet

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ATA-101 was only modestly CNS penetrant in our studies. Although there are many reasons a drug might fail in a clinical study, it is ominous that ATA-101 showed little or no efficacy in clinical studies in patients with CNS disorders where α_7 nAChRs had been implicated as potential therapeutic targets (Lieberman et al., 2013; Walling et al., 2016). A reasonable explanation for these disappointing results could be inadequate target engagement in the CNS by this modestly CNS penetrant drug. We are also aware of unpublished in vitro work showing that ATA-101 may be a substrate for human P-glycoprotein (PGP), and if so, this would limit the ability to sustain drug concentrations in the CNS of humans (Mahar et al., 2002; Kikuchi et al., 2013). With no widely available tools to document α_7 nAChR receptor occupancy (Horti, 2015) nor well-established biomarkers for monitoring target engagement (Hashimoto, 2015; Gee et al., 2017), only a positive result can permit assertions regarding the antitussive potential of nAChR agonists.

Finally, although we consider the broad efficacy of the α_7 agonist ATA-101 against multiple tussive stimuli a desirable attribute for an antitussive therapy, the suppression of nicotine-evoked coughing suggests that α_7 nAChR engagement may diminish the irritating effects of nicotine in a way that may facilitate nicotine delivery and hasten nicotine addiction. This has potential public health implications. It would be interesting to know whether α_7 nAChR blockade might aid smoking cessation efforts. Such an action of α_7 nAChRs in smokers notwithstanding, the results of the present study identify the α_7 nicotinic receptor subtype as a potentially viable therapeutic target for the treatment of chronic refractory cough.

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AUTHORSHIP CONTRIBUTIONS

Participated in research design: Canning, Liu, Tao, DeVita, Perelman, Hay, Dicpinigaitis, Lian

Conducted experiments: Canning, Liu, Tao

Contributed new reagents or analytic tools: DeVita, Liang

Performed data analysis: Canning, Liu, Tao

Wrote or contributed to the writing of the manuscript: Canning, Liu, Tao, Dicpinigaitis, Perelman, Hay

Secured funding for the research: Canning, Liang

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FOOTNOTES

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[2]Supplemental data related to this study is available on-line.

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Figure Legends

Figure 1. Nicotine inhibits citric acid evoked coughing in awake guinea pigs. A) Nicotine (10 mg/ kg) or its vehicle (saline) were administered intraperitoneally prior to citric acid challenges. The cumulative number of coughs evoked by the 3 doses of citric acid studied (0.01-0.3M) are displayed for each of 5-8 animals, with median and intraquartile ranges also indicated. An asterisk (*) indicates a statistically significant (by unpaired, 2-tailed t-test) decrease in the number of coughs recorded following nicotine administration ($p<0.05$). B) Although there were no overtly apparent side effects of nicotine recorded in our conscious cough challenge assays, when administered intravenously to anesthetized guinea pigs, nicotine but not α_7 (ATA-101) or $\alpha_4\beta_2$ (AZD 1446/ Tc-6683) selective nAChR agonists evoked airway smooth muscle contraction, bronchospasm, and precipitous decreases in heart rate and blood pressure. These traces are representative of 3 separate experiments. See supplement for additional results related to these studies.

Figure 2. An α_7-selective nAChR agonist inhibits citric acid evoked coughing in awake guinea pigs. Coughs were evoked by 3 doses of citric acid delivered sequentially in ascending concentrations and the median and intraquartile range for the cumulative number of coughs evoked are presented. A) The $\alpha_4\beta_2$-selective nAChR agonist Tc-6683 (10 and 30 mg/ kg IP; n=4-12) did not inhibit citric acid evoked coughing. By contrast, the α_7 selective nAChR agonist PHA543613 inhibited citric acid evoked coughing following administration B) by IP injection (0.1-30 mg/ kg; n=3-10) or C) by gavage (30 mg/ kg PO). An asterisk (*) indicates a statistically significant (by ANOVA or by unpaired, 2-tailed t-test) decrease in the number of coughs recorded after PHA 543613 treatment ($p<0.05$).

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Figure 3. ATA-101 inhibits citric acid evoked coughing in awake guinea pigs. A) ATA-101 (3-100 mg/ kg) or its vehicle (saline) was administered by gavage 60 minutes prior to citric acid challenge (0.4M). The cumulative number of coughs evoked are presented as a median with intraquartile ranges. An asterisk (*) indicates a statistically significant (by ANOVA) decrease in the number of coughs following ATA-101 administration ($p<0.05$; n=8/ treatment group). B) The pharmacokinetics of ATA-101 (3-30 mg/ kg po) were studied in parallel, with the data presented as a mean±SD of measurements made in 3 animals. The drug was orally bioavailable and moderately CNS penetrant, with dose-dependent increases in peak plasma and brain concentrations documented 1-2 hours after administration. The pharmacokinetics of a single IP dose (30 mg/ kg) of ATA-101 are also presented (n=3). See supplemental data for additional results related to these pharmacokinetic studies.

Figure 4. Nicotine inhibits citric acid evoked coughing by engaging nicotinic receptors less sensitive to mecamylamine blockade. A) Tracheal smooth muscle tension (grams), pulmonary inflation pressure (cmH_2O), blood pressure (mmHg) and heart rate (BPM) were recorded during bilateral vagus nerve stimulation (16 Hz, 10 sec) in anesthetized, paralyzed and mechanically-ventilated guinea pigs, before and after 1 mg/ kg mecamylamine administration. The effects of mecamylamine on B) heart rate and blood pressure and on C) pulmonary inflation pressure and tracheal tension are also shown, with data presented as a mean±SD of 3 experiments. D) Conscious guinea pigs were pretreated with 10 mg/ kg nicotine, 1 mg/ kg mecamylamine or their combination prior to evoking cough with citric acid (0.01-0.3M). The cumulative number of coughs evoked are presented as a median with intraquartile ranges (n=8/ treatment group). An asterisk (*) indicates a statistically significant (by ANOVA) decrease in the number of coughs

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recorded after nicotine treatment, an effect that was not prevented by mecamylamine ($p<0.05$). Mecamylamine alone was also unable to prevent citric acid evoked coughing.

Figure 5. ATA-101 inhibits citric acid evoked coughing following systemic administration (30 mg/ kg IP) but not when delivered selectively to the airways by aerosol (3 mg/ mL). Vehicle control experiments were carried out in parallel. After IP injections, cough was evoked by citric acid (0.01-0.3M). For aerosol delivery, ATA-101 was given simultaneously with 0.3M citric acid. The cumulative/ total number of coughs evoked are presented as medians and intraquartile ranges. We selected the concentration of ATA-101 used for aerosol delivery based on the observation that 3 mg/ mL nicotine delivered by aerosol evoked coughing (see figure 8), suggesting nicotinic receptor engagement (ATA-101 (EC_{50}=17 nM) is >1000 times more potent at activating α_7 nAChRs than nicotine (EC_{50}=23 μM); Mazurov et al., 2012a). An asterisk (*) indicates a statistically significant (by unpaired, 2-tailed t-test) decrease in the number of coughs recorded following IP ATA-101 administration ($p<0.05$).

Figure 6. The antitussive effects of ATA-101 do not depend upon receptor desensitization. Guinea pigs were pretreated with a single dose (30 mg/ kg IP) of ATA-101 4 hours prior to citric acid challenges or dosed twice with ATA-101, initially 4 hours prior to the citric acid challenge and with the second dose administered 30 minutes prior to the challenge (30 mg/ kg for both doses). An asterisk (*) indicates a statistically significant (by ANOVA) decrease in the number of coughs recorded following the 2 doses of ATA-101 ($p<0.05$; n=5/ treatment group).

Figure 7. ATA-101 inhibits bradykinin evoked coughing in awake guinea pigs. Thirty minutes prior to bradykinin challenge (1- 10mg/ mL), 30 mg/ kg ATA-101 or its vehicle (saline) were

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injected intraperitoneally (IP). Representative traces of bradykinin evoked coughing recorded in A) control animals and B) in animals pretreated with ATA-101 are shown. C) The cumulative number of coughs evoked by bradykinin in each animal are presented along with the median and intraquartile ranges of these results. An asterisk (*) indicates a statistically significant (by unpaired, 2-tailed t-test) decrease in the number of coughs evoked by bradykinin following ATA-101 administration ($p<0.05$; n=7/ treatment group).

Figure 8. ATA-101 inhibits nicotine evoked coughing in awake guinea pigs. ATA-101 (30 mg/ kg) or its vehicle (saline) were administered by intraperitoneal (IP) injection 30 minutes prior to challenge with aerosols of 3 mg/ mL nicotine. The results from each animal are shown, with median and intraquartile ranges also depicted. An asterisk (*) indicates a statistically significant (by unpaired, 2-tailed t-test) decrease in the number of coughs recorded following ATA-101 administration ($p<0.05$; n=8-9/ treatment group).

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Figure 1

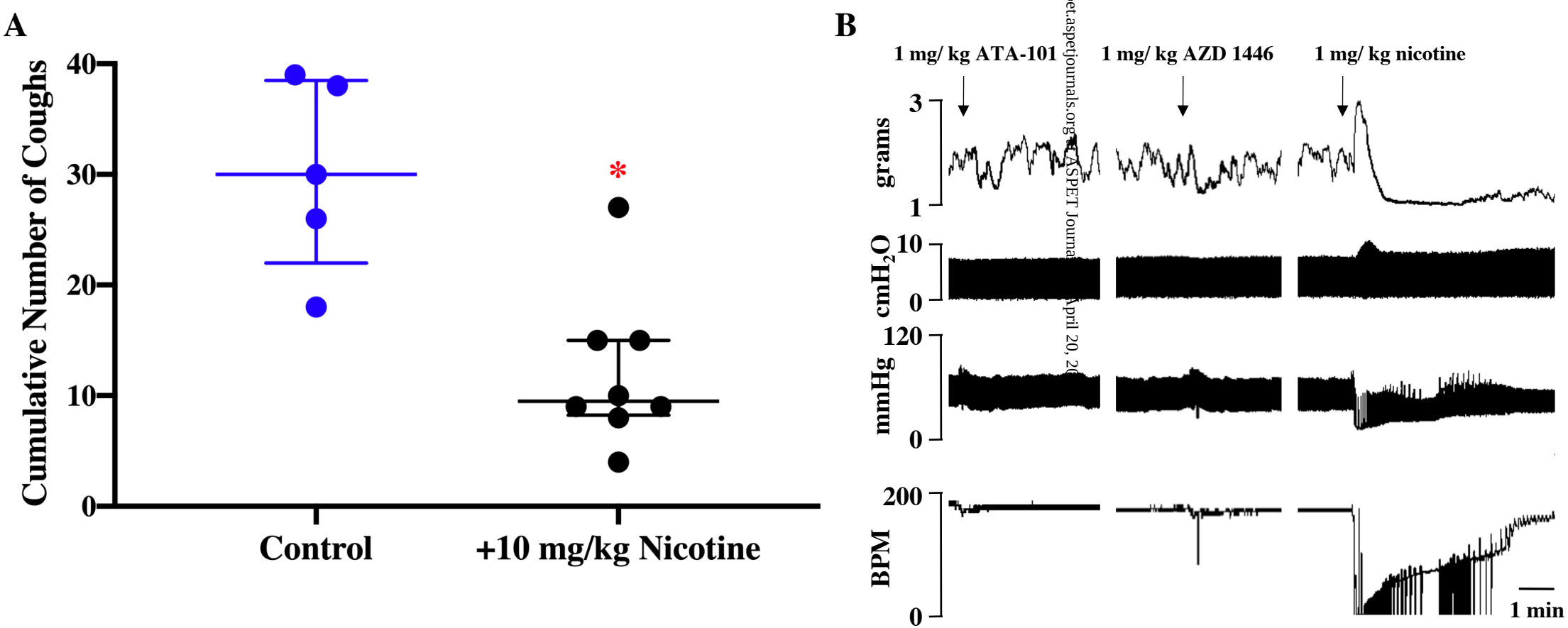

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Figure 2

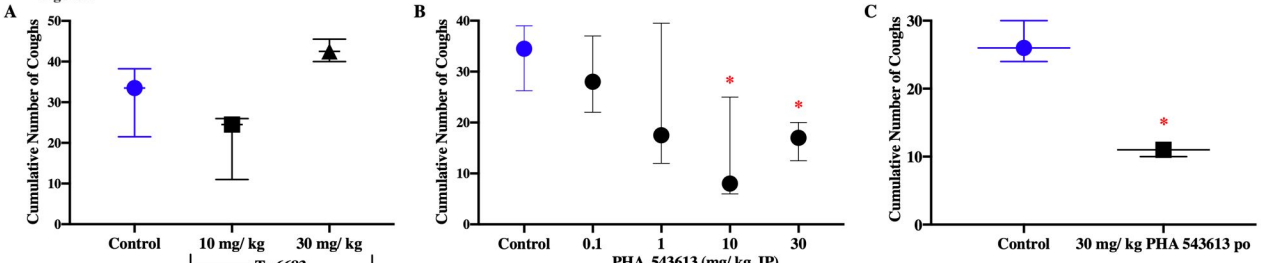

A
Cumulative Number of Coughs
50
40
30
20
10
0
Control
10 mg/ kg
30 mg/ kg
Tc-6683
B
Cumulative Number of Coughs
40
30
20
10
0
Control
0.1
1
10
30
*
*
PHA 543613 (mg/ kg, IP)
C
Cumulative Number of Coughs
30
20
10
0
*
Control
30 mg/ kg PHA 543613 po

Figure 3

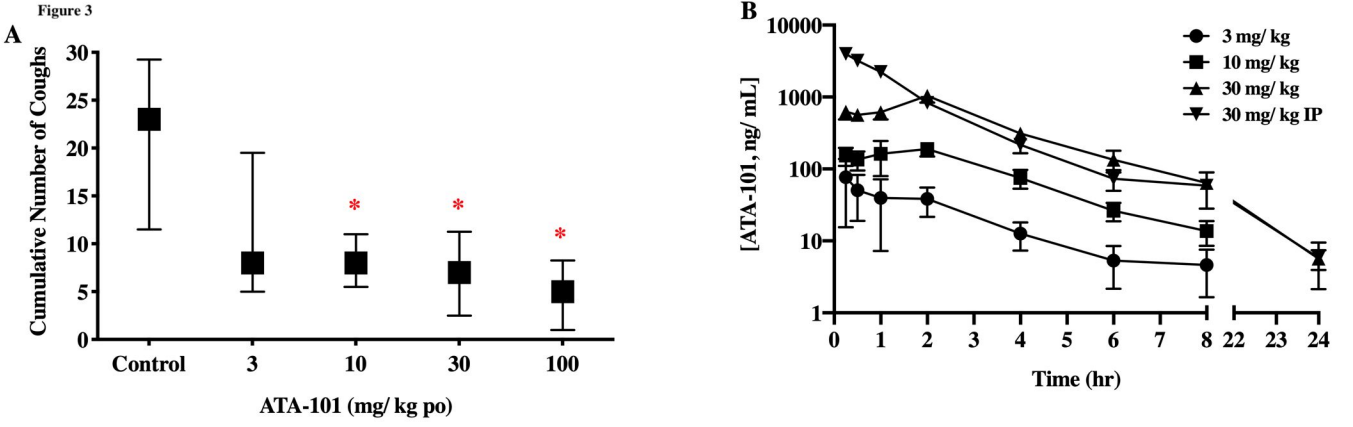

A
Cumulative Number of Coughs
30
25
20
15
10
5
0
Control
3
10
30
100
ATA-101 (mg/ kg po)
B
[ATA-101, ng/ mL]
10000
1000
100
10
1
3 mg/ kg
10 mg/ kg
30 mg/ kg
30 mg/ kg IP
0 1 2 3 4 5 6 7 8 22 23 24
Time (hr)

Figure 4

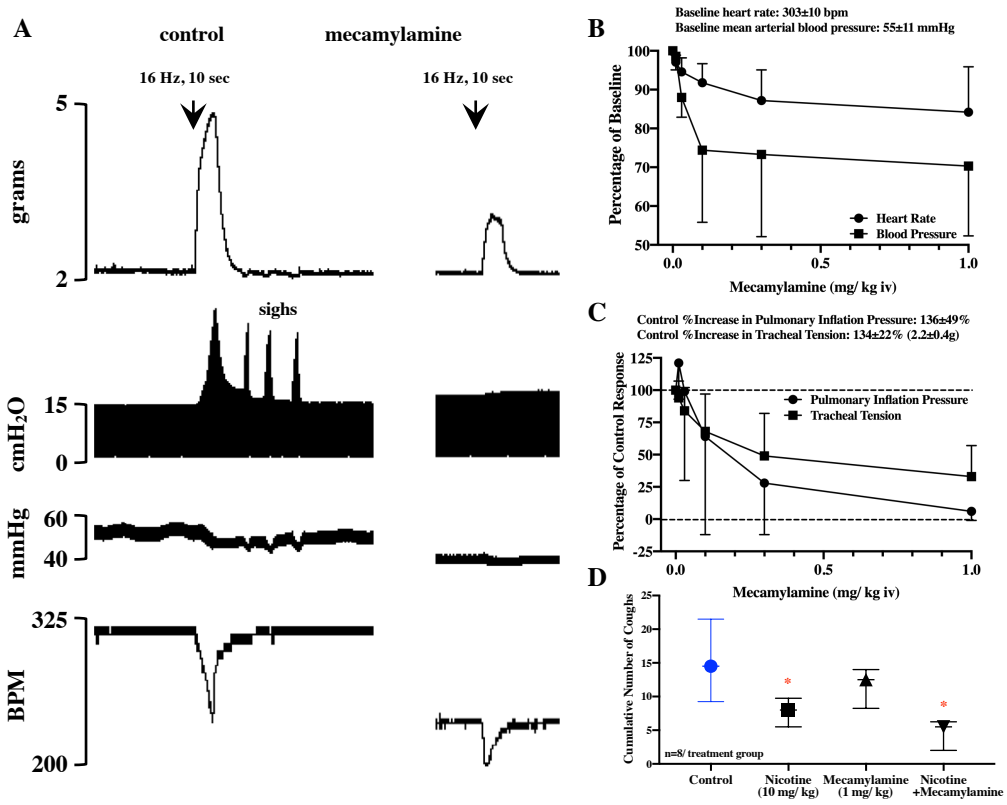

A
control
mecamylamine
16 Hz, 10 sec
16 Hz, 10 sec
grams
5
2
sighs
cmH2O
15
0
mmHg
60
40
BPM
325
200
B
Baseline heart rate: 303±10 bpm
Baseline mean arterial blood pressure: 55±11 mmHg
Percentage of Baseline
Heart Rate
Blood Pressure
Mecamylamine (mg/ kg iv)
C
Control %Increase in Pulmonary Inflation Pressure: 136±49%
Control %Increase in Tracheal Tension: 134±22% (2.2±0.4g)
Percentage of Control Response
Pulmonary Inflation Pressure
Tracheal Tension
Mecamylamine (mg/ kg iv)
D
Cumulative Number of Coughs
n=8/ treatment group
Control
Nicotine (10 mg/ kg)
Mecamylamine (1 mg/ kg)
Nicotine +Mecamylamine

Figure 5

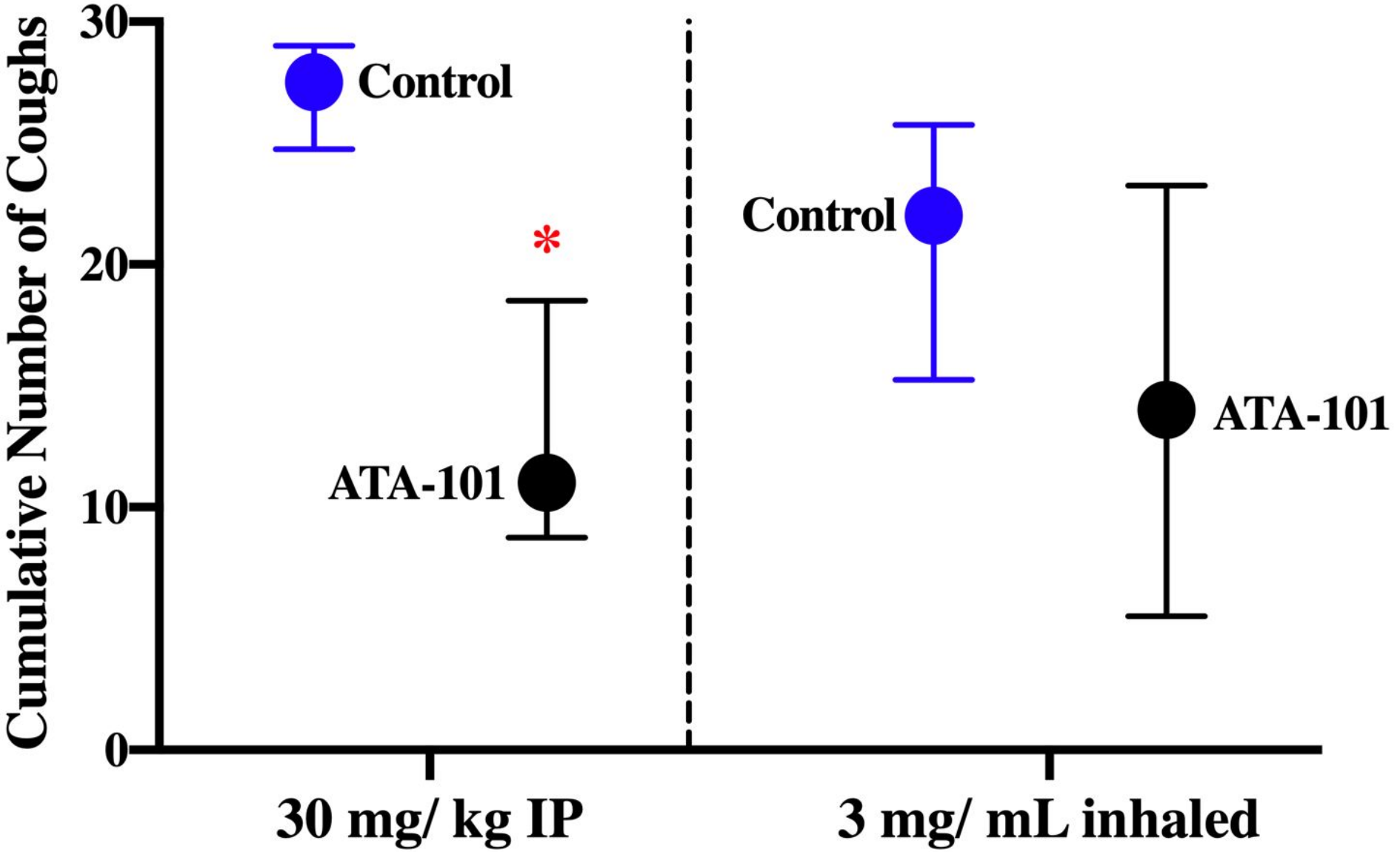

Cumulative Number of Coughs
30
20
10
0
Control
*
ATA-101
Control
ATA-101
30 mg/ kg IP
3 mg/ mL inhaled

Figure 6

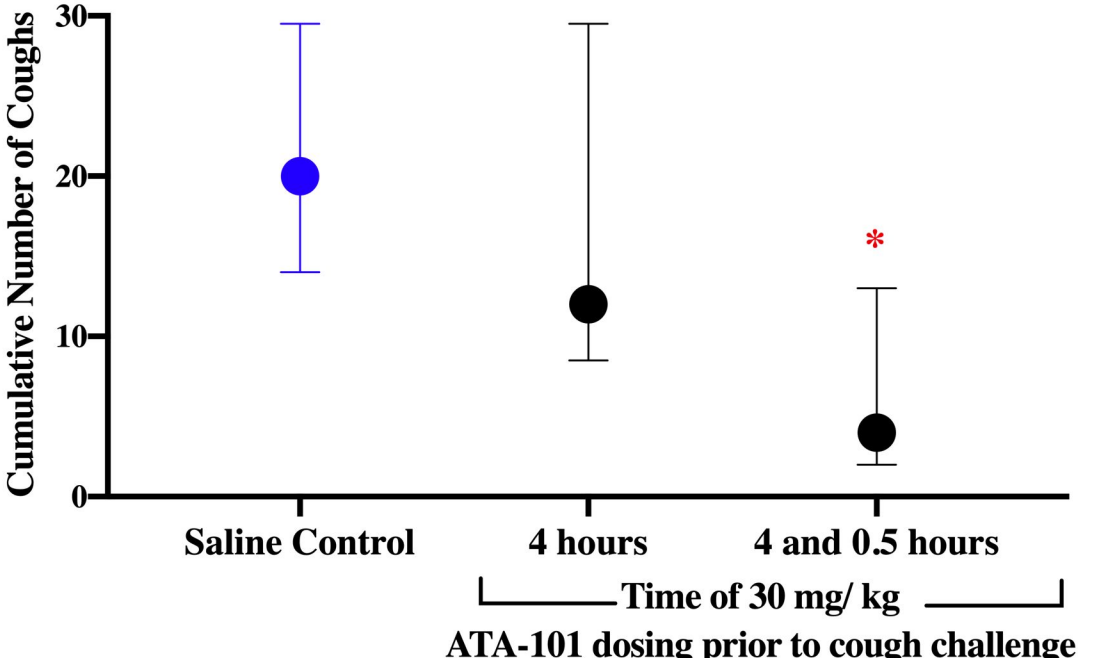
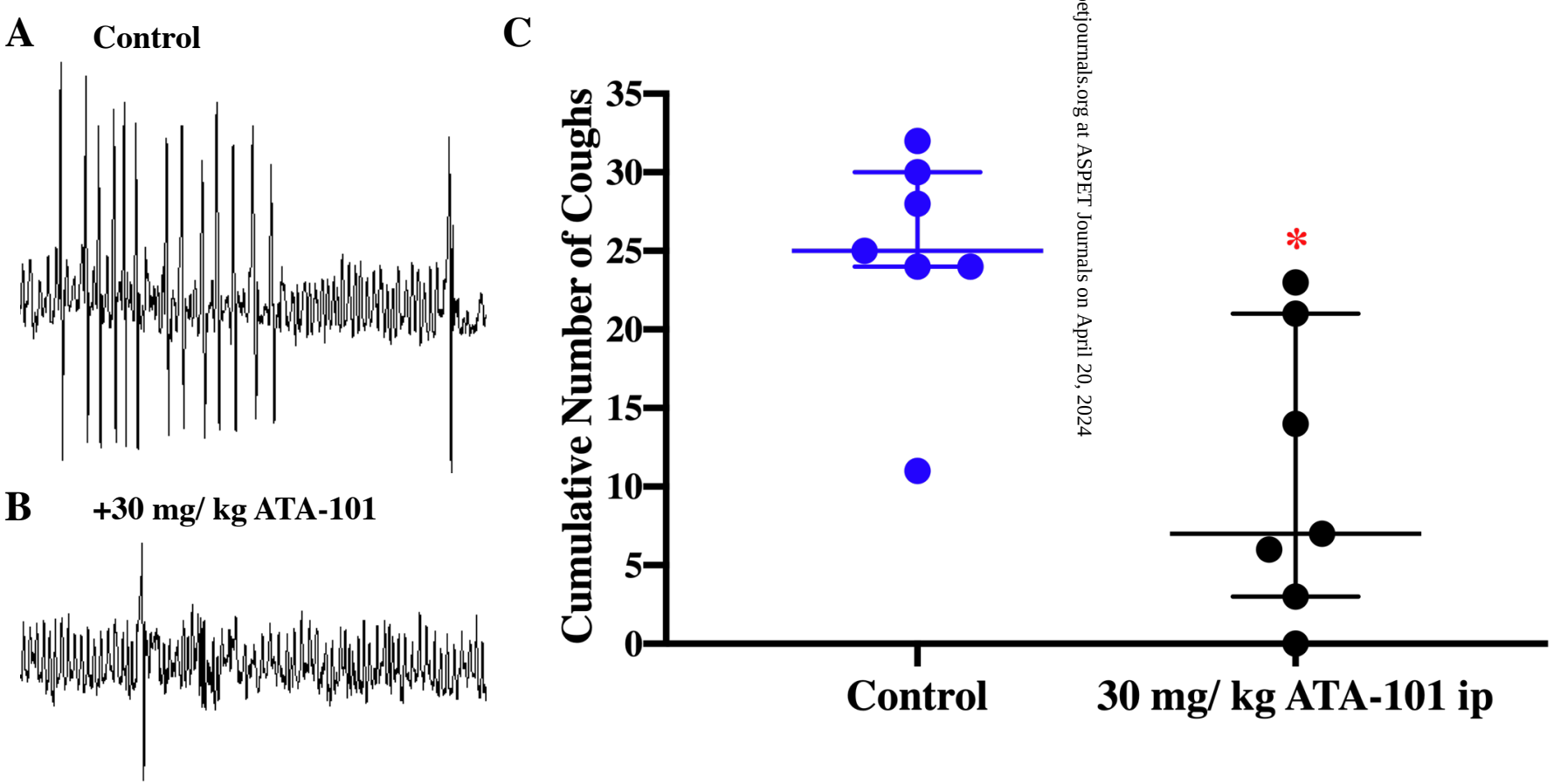

Cumulative Number of Coughs
30
20
10
0
*
Saline Control
4 hours
4 and 0.5 hours
Time of 30 mg/ kg
ATA-101 dosing prior to cough challenge

Figure 7



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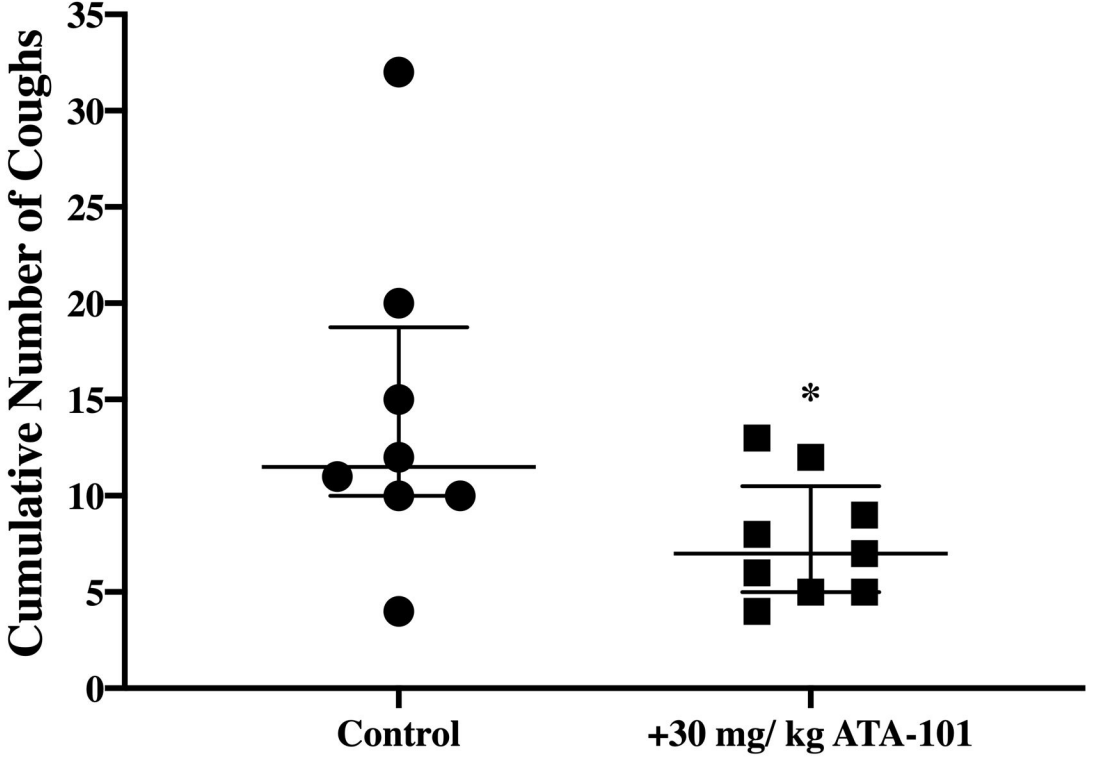
Cumulative Number of Coughs
35
30
25
20
15
10
5
0
*
Control
+30 mg/ kg ATA-101

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Figure legends for supplemental figures to Yu et al.

Evidence for alpha$_7$ nicotinic receptor activation during the cough suppressing effects induced by nicotine and identification of ATA-101 as a potential novel therapy for the treatment of chronic cough

Brendan J. Canning[1*], Qi Liu[1], Mayuko Tao[2], Robert DeVita[3], Michael Perelman[4], Douglas W Hay[5], Peter V. Dicpinigaitis[6] and Jing Liang[7]

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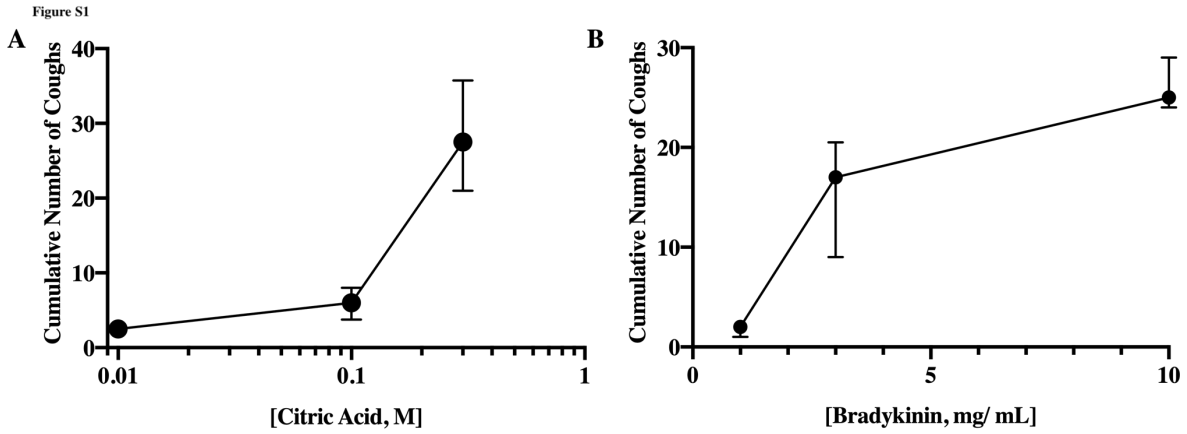

Figure S1. Dose-dependent coughing evoked by A) citric acid (n=32) and B) bradykinin (n=7) in awake guinea pigs treated with vehicle (saline). The cumulative number of coughs evoked by each tussive agent are presented as a median and intraquartile range.

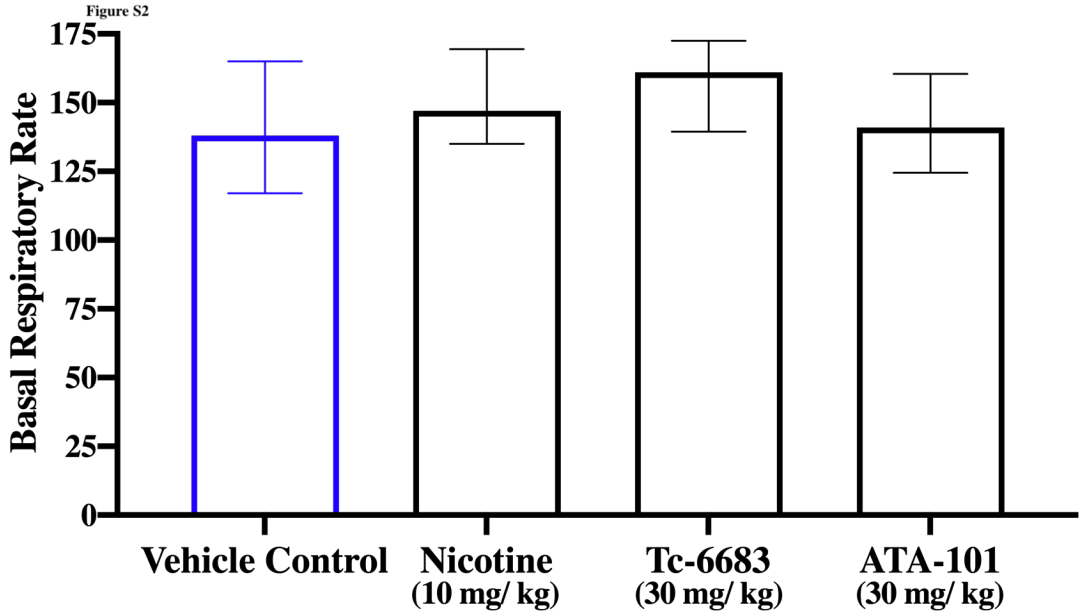

Figure S2. Nicotinic receptor agonists (administered by intraperitoneal injection) had no measurable effects on respiratory rate measured in awake guinea pigs. The results are presented as medians and intraquartile ranges for 4-15 experiments.

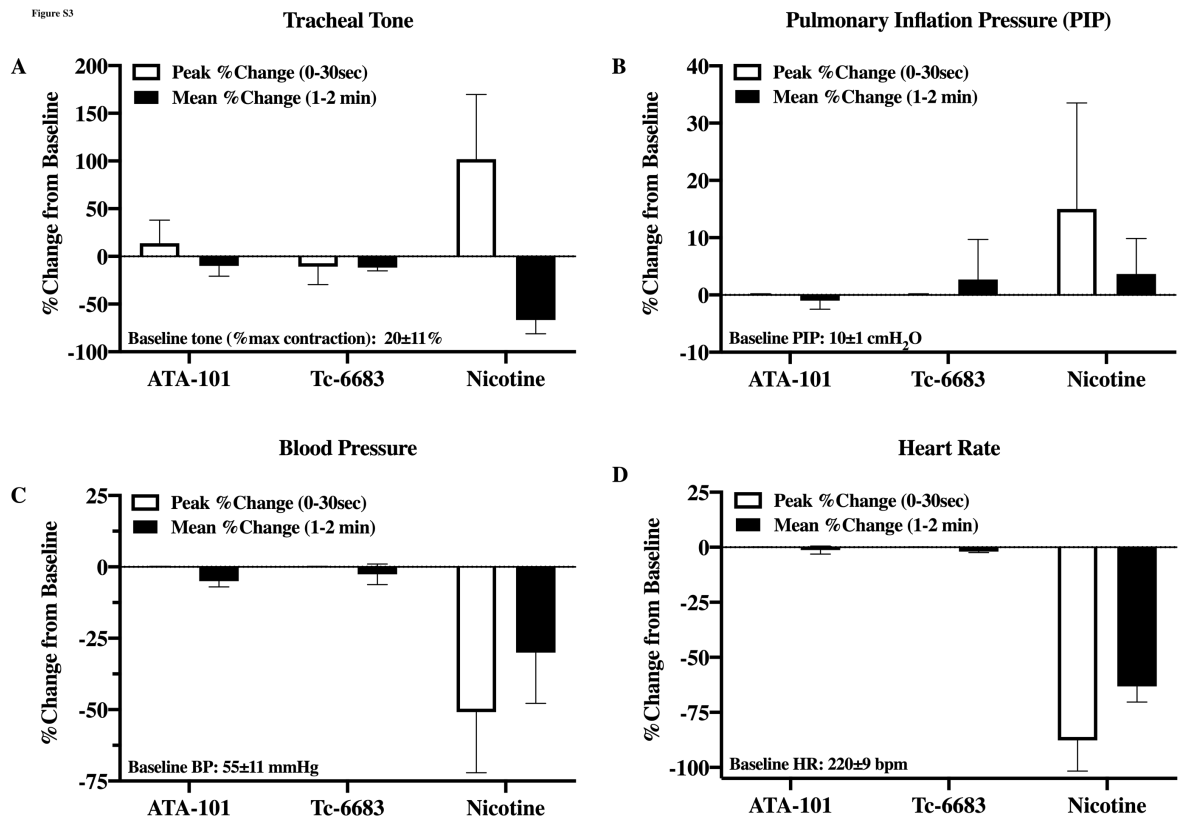

Figure S3. Nicotine but neither ATA-101 nor AZD 1446/ Tc-6683 evoked marked changes in tracheal tone, pulmonary inflation pressure, blood pressure or heart rate in anesthetized, paralyzed, mechanically-ventilated guinea pigs. Each drug was administered intravenously at a dose of 1 mg/ kg. Each of 3 animals was challenged with all 3 drugs. We alternated the order of ATA-101 and AZD 1446/ Tc-6683 delivery, but nicotine was always the last of the 3 drugs administered. Responses were measured during an acute peak effect in the first 30 seconds after administration and an average response over a 60 second timespan beginning 1 minute after administration (see figure 1 for a representative trace depicting the time course of the responses to these stimuli). The baseline values for each measurement are indicated within each graph. The results are presented as a mean±SD.

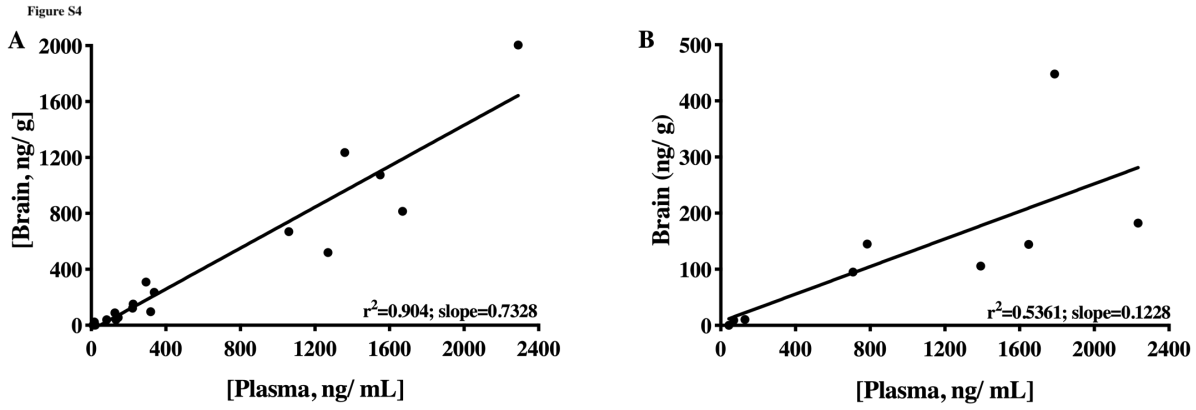

Figure S4. The relationship between ATA-101 plasma concentrations following oral administration and measured ATA-101 content in brain in 2 separate pharmacokinetic experiments performed in guinea pigs. See Methods for details of the experimental design. In the first experiment (left panel), brain and plasma were recovered for ATA-101 measurements immediately after evoking cough in animals first treated by gavage with 10, 30 or 100 mg/ kg ATA-101 (administered 60 minutes prior to cough challenges; see Figure 3). In the second experiment (right panel), brain and plasma were recovered 2-6 hours after oral administration of 30 mg/ kg ATA-101. Each data point represents the plasma and brain measurements for ATA-101 in a single animal.

Figure S5

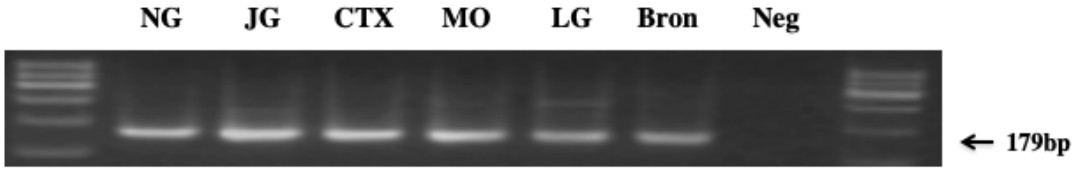

Figure S5. RT-PCR reveals expression of α_7 nAChR mRNA in nodose ganglia (NG), jugular ganglia (JG), brain/ cortex (CTX), medulla oblongata/ brainstem (MO), lung (LG) and bronchi (Bron). A negative control was also run. This gel is representative of 3 separate experiments.

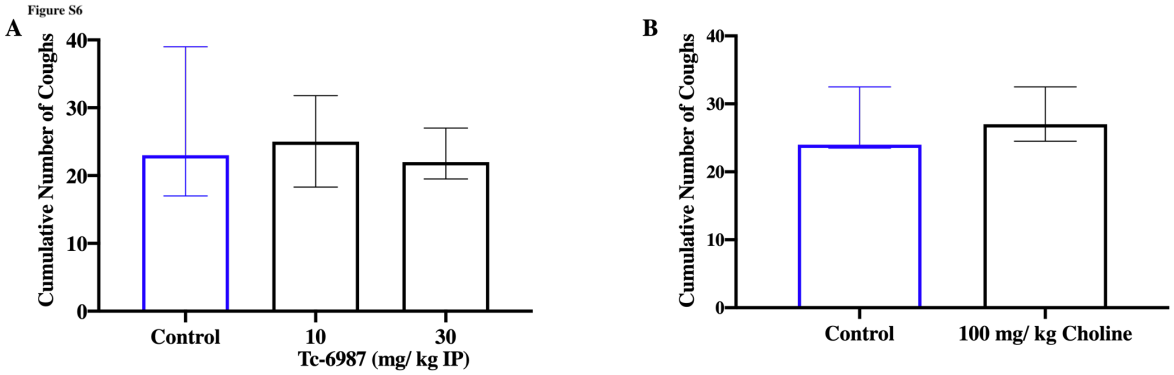

Figure S6. Citric acid (0.01-0.3M) evoked coughing was not inhibited by A) the partial α_7 nAChR agonist Tc-6987 (10 and 30 mg/ kg IP) or by B) the endogenous α_7-selective nAChR agonist choline (100 mg/ kg IP). The data are presented as the median and intraquartile ranges in each treatment group, with 3-9 animals per group.

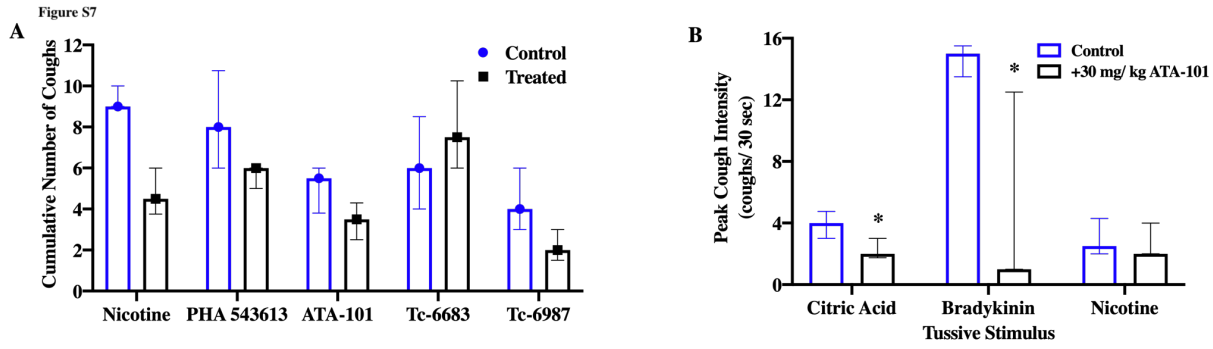

Figure S7. Nicotinic receptor agonists were A) without effect on the coughing evoked by threshold doses of citric acid (0.1M). ATA-101 (30 mg/ kg) also failed to modulate cough responses to a threshold concentration of bradykinin (1 mg/ mL bradykinin evoked 2±1 and 1±1 coughs in control animals and animals treated with 30 mg/ kg ATA-101, respectively; n=7/ treatment group). B) ATA-101 did, however, reduce the cough intensity associated with citric acid or bradykinin challenge, with cough intensity defined as the highest number of coughs recorded in any 30 second data epoch during challenge regardless of tussive agent concentration. Nicotine evoked comparatively few coughs and although ATA-101 reduced the total number of coughs evoked by nicotine (see Figure 8 of the parent manuscript file), the α_7-selective nAChR agonist did not measurably alter cough intensity to nicotine. All drugs were administered intraperitoneally. Nicotine was administered at a dose of 10 mg/ kg. All other drugs were administered at a dose of 30 mg/ kg. The data are presented as the median and intraquartile ranges of 3-12 experiments. An asterisk (*) indicates a statistically significant (by unpaired, 2-tailed t-test) decrease in cough intensity recorded following ATA-101 administration ($p<0.05$).

Supplemental Table 1 to Yu et al. (JPET MS#JPET-AR-2021-000641R1)

Evidence for alpha$_7$ nicotinic receptor activation during the cough suppressing effects induced by nicotine and identification of ATA-101 as a potential novel therapy for the treatment of chronic cough

Brendan J. Canning[1*], Qi Liu[1], Mayuko Tao[2], Robert DeVita[3], Michael Perelman[4], Douglas W Hay[5], Peter V. Dicpinigaitis[6] and Jing Liang[7]

Pharmacokinetics of ATA-101 following oral administration in 3 guinea pigs (mean±sd)

Dose	C_{max} (ng/ mL)	T_{max} (hour)	$T_{1/2}$ (hour)
3 mg/ kg PO	82±52	0.8±1	2.0±0.9
10 mg/ kg PO	222±31	1.1±0.9	1.6±0.4
30 mg/ kg PO	1041±204	2.0±0	4.1±0.5
30 mg/ kg IP	3971±116	0.3±0	3.9±0.2