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α4β2 neuronal nicotinic receptor positive allosteric modulation: An approach for improving the therapeutic index of α4β2 nAChR agonists in pain

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ABSTRACT

Nicotinic acetylcholine receptors (nAChRs) function as ligand-gated ion channels activated by the neurotransmitter acetylcholine. Gene knockout and antisense studies coupled with pharmacological studies with nAChR agonists have documented a role of α4β2 nAChR activation in analgesia. ABT-594, for the first time, provided clinical validation to the nAChR agonist pharmacology as a novel mechanism for treatment of pain. However, ABT-594 was poorly tolerated at the efficacious doses, particularly with respect to the side effects of nausea and emesis, which is thought to be mediated by activation of the ganglionic-type (α3-containing) nAChRs. An alternate approach is to selectively modulate the α4β2 nAChR via positive allosteric modulation. Positive allosteric modulators (PAMs) are compounds that do not interact with the agonist binding sites or possess intrinsic activity at the receptor per se, but potentiate the effects of the agonist. NS9283 (also known as A-969933), the first oxadiazole analog, was found to selectively enhance the potency of a range of nAChR agonists at α4β2, but not α3β4, nAChRs. Studies reported here, along with the accompanying manuscript [1] collectively point to the conclusion, based on preclinical models, that the analgesic efficacy of clinically well-tolerated doses of ABT-594 in humans can be significantly enhanced by co-administration with the α4β2 PAM. Additionally, studies in ferrets demonstrate no exaggeration of emetic effect when ABT-594 is co-dosed with NS9283. Cardiovascular studies in anesthetized dogs achieve supra-therapeutic plasma concentrations of ABT-594 (>20-fold) without hemodynamic or electrophysiological effects using the co-administration paradigm.

1. Introduction

Pain is one of the most prominent reasons for patient visits to physicians. Current treatments for pain are either refinements of opiates or nonsteroidal anti-inflammatory drugs (NSAIDs). Although these treatment approaches are available for more than a decade, several types of pain, particularly associated with nerve damage, are still poorly managed by these medications (for review [2]). Neuropathic pain is predominately treated by use of antidepressants and antiepileptics agents such as duloxetine, gabapentin and pregabalin. However, neuropathic pain is still poorly managed by these agents either due to limited efficacy across the patient population or due to the dose limited side effect/tolerability profiles. Given the need for novel analgesic agents with improved efficacy, interest continues in the identification and validation of novel molecular targets and approaches for pain.

Neuronal nicotinic receptors (nAChRs) are ligand-gated ion channels, each composed of five subunits surrounding a cation pore. Various neuronal nAChR subunits (α2–α10 and β2–β4) are differentially expressed throughout the nervous system and combine to form diverse subtypes with a wide range of physiological and pharmacological profiles. The two most abundant nAChRs in the CNS can be differentiated by their relative affinities for nicotine and α-bungarotoxin. nAChRs with high affinity for nicotine but low affinity for α-bungarotoxin largely contain combinations of α4 and β2 subunits, whereas nAChRs with low affinity for nicotine but high affinity for α-bungarotoxin are predominantly α7-containing. nAChRs derived from α3β4 subunits are in much higher abundance in the autonomic nervous system than in the CNS and are generally regarded to be responsible for many of the adverse effects of nicotine.

α4β2 nAChR agonists have the potential as broad-spectrum analgesics based on preclinical studies demonstrating their efficacy in diverse pain states including multiple forms of acute, chronic, inflammatory and neuropathic pain [2]. These ligands act

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at multiple locations throughout the pain pathway to relieve pain. For example, α4β2 nAChRs are found on primary sensory neurons (periphery) where nociceptive information is initiated, in the cell body regions of these neurons (i.e. the dorsal root ganglion or DRG), the dorsal spinal cord where the first pain synapse is located, in the brainstem cell body regions that control descending innervation, as well as in the higher brain regions that integrate and perceive sensory information such as the thalamus and the cortex. In addition to studies with nAChR agonists, evidence in support for a role in α4β2 nAChRs in pain has also emerged from antisense and knockout studies. Antisense knockdown of the α4 subunit has been found to decrease the analgesic effect of agonists [2]. Reduced antinociceptive responses to nicotine also are seen in α4 and β2 knockout animals [3,4]. Antinociceptive effects through α4β2 nAChRs have been attributed to enhancing descending inhibition, particularly in the raphe [5]. However, α4β2 nAChR stimulation of GABAergic and glycinergic inhibitory transmission in the spinal cord also may contribute [6,7].

ABT-594, the first nAChR agonist demonstrated clinical efficacy in the treatment of painful diabetic neuropathy [8]. With ABT-594, however the efficacious doses were associated with an undesirable side effect profile, particularly with respect to nausea and emesis. Although targeting the α4β2 subtype with nAChR agonists with enhanced subtype selectivity remains a viable approach, robust efficacy in pain may be limited by the range of side effects (nausea, emesis, etc.) arising from possible interactions with the ganglionic nAChR (α3β4*) at efficacious doses. In light of the significance of chronic pain and the limitations in current therapeutic agents, it would be beneficial to further exploit the nAChR platform toward developing new approaches for treating such disorders, particularly those aimed at reducing adverse effects.

One approach to selectively enhance activity at the α4β2 nAChR is via positive allosteric modulation. As reported previously, positive allosteric modulators (PAMs) can enhance the efficacy and potency of agonist(s) so as to selectively amplify effects at the α4β2 nAChR [9,10]. A positive allosteric modulator alone, in principle, does not exhibit intrinsic activity at the receptor, but can amplify the effects of agonists [11]. Because PAMs do not interact with agonist-binding site, they could, in principle, display a higher degree of selectivity at the target α4β2 subtype vs. other nAChR subtypes. Studies reported herein reveal that the analgesic efficacy of nAChR agonists such as ABT-594 in pain can be enhanced in combination with PAMs. Under these conditions, the overall preclinical side effect profiles (gastrointestinal, cardiovascular, etc.) are not affected. A portion of this study has been previously presented in an abstract form [12].

2. Materials and methods

2.1. FLIPR calcium imaging assay

Experiments were carried out according to the methods described previously [13,14]. HEK-293 cell lines stably expressing human α4β2, α3β4, or α4β4 nAChRs were established and maintained using standard procedures [13,14]. Agonist-evoked Ca^{2+} increases were measured using Fluo-4/AM to detect intracellular Ca^{2+} in conjunction with a fluorescence imaging plate reader (FLIPR) equipped with an argon laser and a CCD camera (Molecular Devices/Danaher Corp., Sunnyvale, CA). Black-walled 96-well plates were utilized to reduce light scattering. The cell permeant acetoxymethyl (AM) ester form of fluo-4 (Molecular Devices) was diluted in the assay buffer, and placed on the cells for 60–90 min at room temperature. When necessary, the unincorporated dye was removed from the cells by washing with assay buffer containing 140 mM N-methyl D-glucosamine (NMDG), 5 mM KCl, 1 mM $MgCl_2$, 10 mM $CaCl_2$, and 10 mM HEPES, pH 7.4. Two types of protocols were used in the studies. For determining the EC_{50} for NS9283, various concentrations of NS9283 (up to 30 μM) were added in the first addition followed by submaximum nicotine (300 nM) addition. In the second protocol, the effect of NS9283 (100 nM to 10 μM) was assessed in the presence of varying concentrations of ABT-594.

2.2. Animals and compounds

Adult male Sprague Dawley rats (200–250 g, Charles River Laboratories, Wilmington, MA) were housed five per cage. Animals were in quarantine for 5–7 days before entering the study. All animals were kept in a temperature-regulated environment under a controlled 12-h light-dark cycle with lights on at 6:00 AM. Food and water were provided *ad libitum*. All procedures were performed in an Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC) approved facility and approved by the Institutional Animal Care and Use Committee (IACUC) at Abbott Laboratories.

NS9283 [3-(3-(pyridine-3-yl)-1,2,4-oxadiazol-5-yl)benzonitrile], A-424274 3-((1S,5S)-3,6-diazabicyclo[3.2.0]heptan-3-yl)quinoline and ABT-594 [(R)-5-(2-azetidinylmethoxy)-2-chloropyridine] were synthesized at Abbott Laboratories as previously described [9] (molecular weight of 285, 370.08, respectively). Hydroxypropyl-β-cyclodextrin (HBC) and nicotine were obtained from Sigma Chemical Co. (St. Louis, MO). NS9283, ABT-594, and the combination of NS-9283 with ABT-594 were dissolved in a solution of HBC/physiological saline (30:70, v/v) and were intraperitoneally (i.p.) administered in a final injection volume of 4.0 mL/kg, in all behavioral experiments.

2.3. Spinal nerve ligation

Rats received unilateral ligation of the lumbar 5 (L5) and lumbar 6 (L6) spinal nerves as previously described [15]. The left L5 and L6 spinal nerves of the rat were isolated adjacent to the vertebral column and tightly ligated with a 5-0 silk suture distal to the dorsal root ganglia, and care was taken to avoid injury of the lumbar 4 (L4) spinal nerve. All animals were allowed to recover for at least 1 week and not more than 3 weeks prior to assessment of mechanical allodynia. Mechanical allodynia was measured using calibrated von Frey filaments (Stoelting, Wood Dale, IL) as previously described [16]. Rats were placed into inverted individual plastic containers (20 cm × 12.5 cm × 20 cm) on top of a suspended wire mesh grid, and acclimated to the test chambers for 20 min. The von Frey filaments were presented perpendicularly to the plantar surface of the selected hind paw, and then held in this position for approximately 8 s with enough force to cause a slight bend in the filament. Positive responses included an abrupt withdrawal of the hind paw from the stimulus, or flinching behavior immediately following removal of the stimulus. A 50% paw withdrawal threshold ($PWT_{von\ Frey}$) was determined using an up–down procedure [17]. Only rats with a $PWT_{von\ Frey} \leq 5.0$ g were considered allodynic and utilized to test the analgesic activity of the compound.

2.4. Ferret emesis studies

Fasted male ferrets (Marshall BioResources, North Rose, NY) weighing between 1.0 and 1.7 kg were used to determine the emetic effects of ABT-594 administered by itself and co-administered with the positive allosteric modulator NS9283. ABT-594 was dissolved in saline and NS9283 was dissolved in 10% hydroxypropyl-β-cyclodextrin in water. NS9283 was administered first, at 2.0 mL/kg, p.o., resulting in a dose of 1.0 mg base/kg. Thirty minutes later, ABT-594 was administered at 1.0 mL/kg, i.p., at doses of 10, 30, and 100 nmol/kg, respectively (n = 6/dose group). A

separate group of ferrets were dosed with the respective vehicles in the same order and the same routes of administration ($n = 6$). A blood sample was obtained from the caudal artery of each ferret 90 min after ABT-594 dosing for the determination of plasma concentrations. Following dosing ferrets were observed for emesis for a period of 90 min. The percentage of animals that experienced emesis at a given dose was recorded.

2.5. Dog cardiovascular studies

Male beagle dogs, weighing 9.4–11.5 kg, were anesthetized with pentobarbital (35.0 mg/kg, intravenously) and immediately placed on a constant intravenous infusion of pentobarbital (6.0 mg/kg/h). Once anesthetized, the dogs were incubated with a cuffed endotracheal tube and ventilated with room air by means of a mechanical respiration pump (Harvard Apparatus, Model 613). Expiratory CO_2 was monitored with an end-tidal CO_2 monitor (Criticare Systems; Model POET TE) and maintained at 4–5% CO_2. Electrocardiogram limb leads were attached to the animals, and a lead II ECG was recorded. A Swan-Ganz catheter (5.5F) was advanced into the pulmonary artery via the right jugular vein for measurement of cardiac output utilizing a cardiac output computer (Abbott Laboratories, Oximetrix 3). Central venous and pulmonary artery pressures were measured through the proximal and distal ports of the catheter, respectively. A dual tip micromanometer catheter (Millar, Model SPC-770, 7F) was advanced into the left ventricle of the heart via the right carotid artery for measurement of left ventricular and aortic blood pressure. Polyethylene catheters were inserted into the right femoral vein and artery for infusion of test agents and collection of blood samples, respectively. Systemic vascular resistance was calculated as [(mean arterial pressure − mean central venous pressure)/cardiac output]. Pulmonary vascular resistance was calculated as [(pulmonary arterial pressure − central venous pressure)/cardiac output]. Body temperature was monitored throughout the experiment.

The primary hemodynamic variables were computed using commercial software and a signal processing workstation (Ponemah, Gould Instrument Systems, Inc. The electronic Lead II ECG record was assessed for changes in QT-interval (QTc, corrected for heart rate using Fridericia's and Van de Water's formulae); and the PR-interval via the Ponemah system with manual over-reads conducted at 15-min intervals. NS9283 ($n = 6$, vehicle: PEG-400) was infused at 0.008 mg/kg/min. ABT-594 ($n = 6$, vehicle: D5W) was infused at 3 escalating doses of 0.25, 0.75, and 2.25 μg/kg/30 min. Animals were monitored for 1 h following administration of the third dose. Blood samples were collected into standard blood tubes containing heparin, beginning at time 0 and 15-min intervals throughout the experimental protocol and stored on ice. Hematocrit was determined at 30-min intervals using microcapillary tubes. Note that whole blood samples were used in the high dose series for drug concentration measurements, while plasma was used for the low dose series.

2.6. Data analysis

For FLIPR-studies, raw fluorescence data were corrected by subtracting fluorescence values from wells with buffer only added. Peak fluorescent responses were determined over the range of drug exposure using FLIPR software and expressed as a percentage of the reference peak response for 100 μM nicotine). Data were fitted using GraphPad Prism (San Diego, CA); p value of <0.05 was considered statistically significant.

Behavioral data are presented as mean ± S.E.M., statistical significance was evaluated using one-way analysis of variance (ANOVA) followed by Bonferroni's multiple comparison. Two-way repeated-measure ANOVA (GraphPad Prism, SanDiego, CA) was performed for the induction of locomotor sensitization, and locomotor activity on drug challenge days was analyzed by one- or two-way ANOVA where appropriate. Bonferroni post hoc testing was done for significant main effects. $p < 0.05$ was considered to be significant, and ED_{50} values were defined as the dose of drug producing 50% of the maximum possible effect (MPE). All experiments were performed by experimenters unaware of the treatment received by the animals, and rats were randomly distributed into different experimental groups.

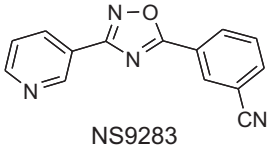
Dog cardiovascular data are expressed as the group mean ± SEM. Statistical analysis was performed by comparison of values from NS9283 co-administered with ABT-594 and vehicle-treated dogs at each time point for each variable, using two-sided t-tests. In addition, for each parameter at each treatment and post-treatment time point, two-sample, two-sided t-tests were used to compare changes from baseline for drug-treated vs. vehicle-treated dogs. All statistical tests and comparisons were assessed at a 0.05 level of significance.

3. Results

3.1. In-vitro pharmacology

As previously reported, NS9283 (also known as A-969933, Table 1) was found to increase agonist-evoked response amplitude of α4β2 nAChRs in Ca^{2+}-imaging and electrophysiology paradigms but did not itself produce receptor activation [9,10]. In this study, NS9283 was initially tested for in vitro activity as positive allosteric modulator in HEK-293 cells expressing the human α4β2 nAChRs using ABT-594 as the agonist. As shown in Fig. 1A, NS9283 left-shifted the concentration response profile of ABT-594 at α4β2 nAChR in a concentration-dependent manner. The EC_{50} value for NS9283 for shifting agonist response was estimated to be 451 nM (maximal efficacy 304%; $n = 6$; Fig. 1A inset). Under similar

Table 1
Effect of NS9283 on the functional responses of ABT-594 in α4β2, α3β4 and α4β4 nAChRs expressed in HEK-293 cells.



NS9283

nAChR subtype	α4β2		α3β4		α4β4	
	EC_{50} (nM)	E_{max} (%)	EC_{50} (nM)	E_{max} (%)	EC_{50} (nM)	E_{max} (%)
+0.1 μM NS9283	87 ± 13	109	374 ± 81	105	28 ± 8	89
+1 μM NS9283	26 ± 15	140	277 ± 51	121	23 ± 5	105
+10 μM NS9283	13 ± 4	169	284 ± 111	111	19 ± 11	103

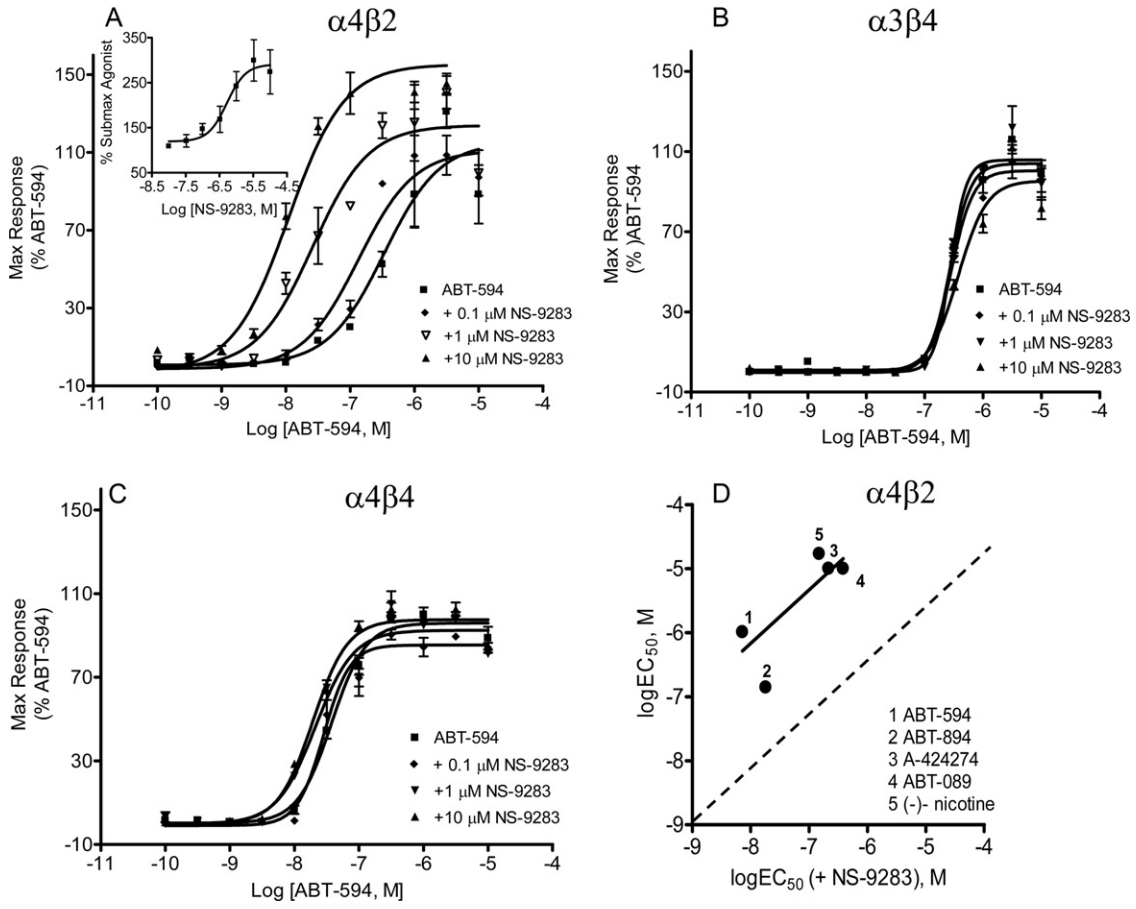


Fig. 1. Modulation of nAChR subtypes by NS9283. (A) Concentration dependent activation of human α4β2 nAChRs expressed in HEK-293 cells measured by changes in intracellular Ca^{2+} responses in absence and presence of varying concentration of NS9283 (0.1, 1, and 10 μM). (B) Concentration-dependent activation of human α3β4 nAChRs expressed in HEK-293 cells in absence and presence of varying concentration of NS9283 (0.1, 1, and 10 μM). (C) Concentration-dependent activation of human α4β4 nAChRs expressed in HEK-293 cells in absence and presence of varying concentration of NS9283 (0.1, 1, and 10 μM). (D) Correlation plot of the −log EC_{50} values of α4β2 nAChR activation by diverse nAChR ligands in the absence and presence of NS9283. The dashed line depicts the 1:1 correlation.

conditions, no shift in the concentration-response of ABT-594 was observed at α3β4 or α4β4 expressing cell lines under similar conditions (Fig. 1B and C). This phenomenon was not unique to ABT-594. NS9283 also caused the potency of diverse nAChR agonists including (−)-nicotine, ABT-894, A-424274 to be left-shifted some 10-fold at the α4β2, but not at α3β4, nAChRs (Fig. 1D).

As previously reported, NS9283 alone (at least up to 10 μM) did not displace [^{3}H]cytisine (α4β2) binding to cortical membranes and was inactive across a radioligand binding panel of GPCRs, ion channels and transporters (CEREP profile). In addition, no potentiation of functional responses at α7 nAChRs or $5HT_3$ receptors expressed in oocytes was observed with NS9283 (data not shown).

3.2. Co-administration of α4β2 nAChR PAM potentiates the antiallodynic effects of ABT-594

In vivo, NS9283 alone did not affect mechanical allodynia (up to 35 μmol/kg, i.p.) in the spinal nerve ligation model of neuropathic pain, examined 15 or 30 min post-drug administration (Fig. 2A). However, co-administration of NS9283 (35 μmol/kg, i.p., immediately prior to ABT-594) revealed a left-shift in the dose–response curve of ABT-594 such that previously non-efficacious doses of ABT-594 now evoked significant analgesic response. Measurements were done at the 30 min time point where the plasma levels of ABT-594 ranged 0.5–1 ng/mL; No changes in plasma levels of ABT-594 was observed with NS9283 suggesting that the improved efficacy was not due to alterations in the plasma concentration of ABT-594 itself.

The effects of NS9283 are dose-dependent, As shown in Fig. 2B, when ABT-594, at a non effective dose (1 nmol/kg) was co-administered with increasing doses of NS9283, doses dependent analgesic effects were realized with maximal efficacy, comparable to that gabapentin in the study.

3.3. Efficacy of α4β2 nAChR PAM depend on ABT-594 exposure levels

To determine the pharmacodynamic–pharmacokinetic relationship of PAM–agonist interactions, a time course study was conducted after dosing NS9283 in animals with spinal nerve ligation where a steady-state plasma concentration of ABT-594 was maintained. After maintaining a steady plasma level of ABT-594 (2.5 ng/mL) by continuous osmotic minipump infusion, animals were challenged with a single dose of NS9283 (1 μmol/kg) and efficacy was measured at various time points (0.5–24 h). As shown in Fig. 3A, robust efficacy was realized until 6 h post dose, but not at 24 h which may be related to, and consistent with, the half life of NS9283.

In another experiment (Fig. 3B) steady state levels of ABT-594 (∼0.7 ng/mL) that corresponded to a clinically well tolerated dose of ABT-594 (75 μg [9]), we measured efficacy of varying doses of NS9283 (measured at 30 min post dosing). As depicted, the response of NS9283 was dose-dependent with an EC_{50} of ∼650 ng/mL, at this plasma concentration of ABT-594.

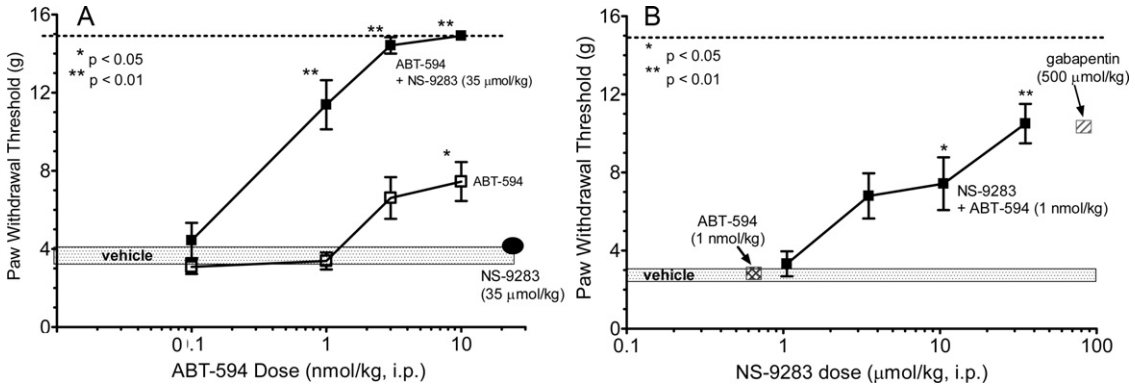


Fig. 2. Modulation of in vivo efficacy of ABT-594 by NS9283 in the rat model of spinal nerve ligation. (A) Dose response effects on pain threshold of ABT-594 in the absence and presence of NS9283 (35 μmol/kg). NS9283 alone at 35 μmol/kg did not alter pain threshold under similar conditions. (B) Dose response effects of NS9283 in the presence of a no-effect dose of ABT-594 (1 nmol/kg; ~0.06 ng/mL). The maximal efficacy realized by NS9283 is similar to the effects of gabapentin in this study.

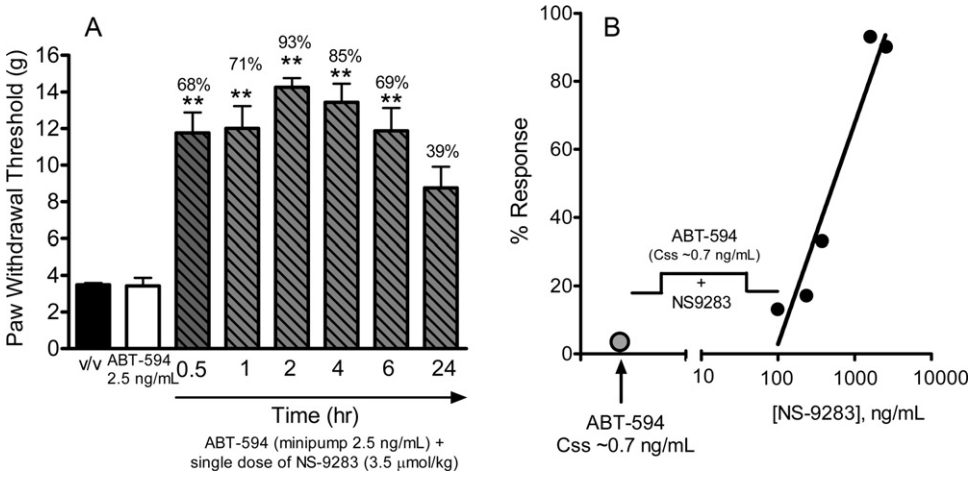


Fig. 3. Time course and pharmacokinetic–pharmacodynamic relationships. (A) Rats with spinal nerve ligation were administered ABT-594 via osmotic minipumps that maintained plasma levels of 2.5 ng/mL that alone were ineffective. However, when animals were then doses with NS9283, significant analgesic effects were realized that lasted at least till 6 h post dosing. (B). In this study, ABT-594 was maintained to achieve 0.7 ng/mL steady stage, and analgesic responses triggered by increasing doses of NS9283 was measured at the 40 min time point.

3.4. Partial agonists when combined with an α4β2 PAM evoke significant analgesic effects

Although many nAChR agonists have antinociceptive activity, some nAChR agonists such as GTS-21 and ABT-089 are not antinociceptive [2]. It has generally been observed that α4β2 nAChR agonist full efficacy (for example, as measured by ion flux in vitro) is necessary for translating to antinociceptive efficacy in rodents. For example ABT-089 binds to α4β2 nAChRs labeled with [^{3}H]cytisine (Ki = 17 nM) in rodent brain, but is inactive in pain models. A-424274 is a high affinity α4β2 ligand with an in vitro profile similar to ABT-089 (Ki = 7 nM) with very low agonist efficacy at α4β2 nAChRs as measured in FLIPR-based fluorescence assays (<5%; EC_{50} > 100 μM; Fig. 4A). However, when A-424274 was tested in presence of NS9283, agonist responses were observed (EC_{50} = 1.31 ± 0.54 μM; efficacy = 52%; n = 4).

In vivo, like ABT-089, A-424274 failed to produce any effects in the spinal nerve ligation model of neuropathic pain to the highest dose tested (Fig. 4B). The combination of A-424474 with NS9283, however, elicited robust efficacy, comparable to that observed with 100 nmol/kg ABT-594 (positive control) in the study. The dose response for A-424274, in the presence of NS9283 was somewhat dose dependent (estimated ED_{50} = 3 μmol/kg; n = 6 per group) while in its absence, no significant effects were observed (at least up to 30 μmol/kg). These studies demonstrate that combination of α4β2 ligands with low intrinsic efficacy, with PAM can be efficacious in pain, offering the potential to explore such drug combinations for the treatment of pain.

3.5. α4β2 nAChR PAM-mediated enhancement of pain efficacy is not accompanied by alterations in CV responses of ABT-594

To assess whether cardiovascular effects of ABT-594 were affected in presence of PAM, studies were conducted in anesthetized dog. NS9283 alone did not show any significant physiologically relevant effects in cardiovascular parameters (hemodynamic or electrophysiological) including heart rate and mean arterial pressure up to at least 4.3 ± 0.18 μg/mL (Fig. 5A). To assess interactions of PAM with ABT-594, the effects of ABT-594 was assessed in the presence of a steady state level of NS9283 (182 ng/mL; Fig. 5B). Peak plasma concentrations of 1.3 ± 0.0, 4.6 ± 0.2, and 15.2 ± 0.7 ng/mL for ABT-594 were achieved at the end of each infusion period while maintaining plasma concentrations of NS9283 between 94.6 ± 4.3 and 182 ± 10.1 ng/mL (Fig. 5B). When infused in the presence of NS9283, no physiologically relevant effects on any hemodynamic parameter measured including mean arterial pressure (Fig. 5B) as well as systolic arterial pressure, diastolic arterial pressure, heart rate, myocardial contractility (left ventricular dP/dt),

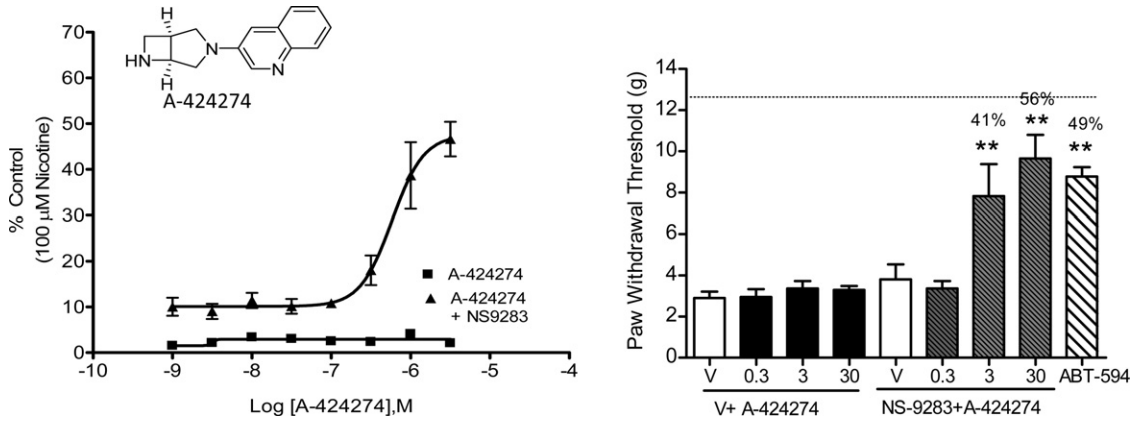


Fig. 4. Modulation of the efficacy of partial agonists by NS9283. (A) Concentration dependent activation of human α4β2 nAChRs expressed in HEK-293 cells measured by changes in intracellular Ca^{2+} responses in absence (solid square) and presence (solid triangle) of 10 μM of NS9283. Note significant agonist response in the presence of NS9283. (B) Paw withdrawal threshold responses in rats with spinal nerve ligation, measured without NS9283 and in presence of NS9283, the latter showing significant analgesic responses, comparable to that observed with 100 nmol/kg ABT-594 (positive control).

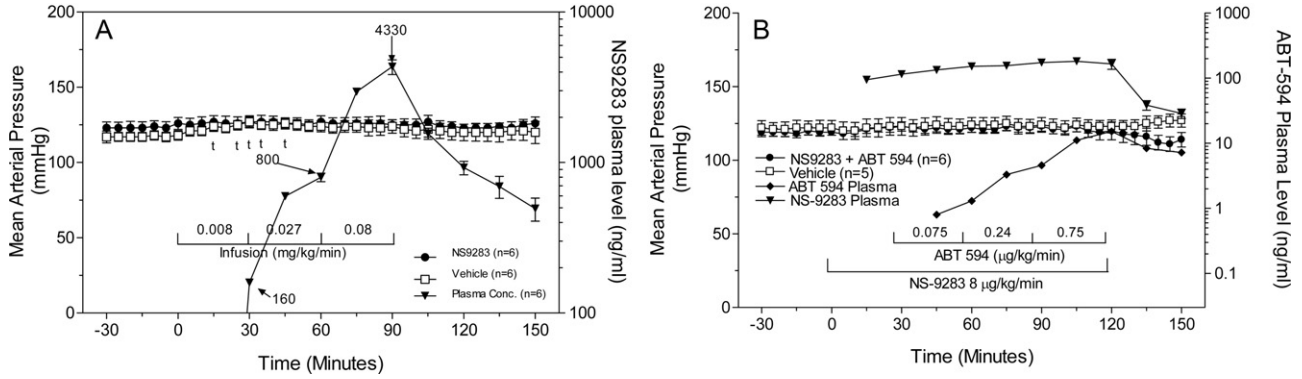


Fig. 5. Analysis of agonist–PAM interactions in the anesthetized dog model. (A) NS9283 alone revealed no physiologically relevant CV effects (C_{max} = 4330 ng/mL) in the anesthetized dog cardiovascular. (B) When NS9283 was maintained at a steady state (Css = 182 ng/mL) and dose dependent effect of ABT-594 (1.3–15.2 ng/mL) were assessed, no change in any hemodynamic or electrocardiographic effects were observed. Traces depicted are effects on mean arterial pressure.

cardiac output, pulmonary arterial pressure, pulmonary vascular resistance, and systemic vascular resistance, central venous pressure, left ventricular end-diastolic pressure, or hematocrit. The combination also produced no statistically significant changes in the QT-interval corrected for heart rate (Van de Water correction).

3.6. α4β2 nAChR PAM-mediated enhancement of pain efficacy is not accompanied by shift in emesis

Nausea and emesis have been identified as dose-limited adverse effects of multiple experimental nAChR ligands. Next, we assessed whether administration of NS9283 along with ABT-594 alters emesis threshold in the ferret model of emesis. When dosed alone, the NS9283 was essentially devoid of emetic effects (up to 35 μmol/kg i.p.). ABT-594 alone was tested at 10, 30 and 100 nmol/kg (i.p.) showed respectively 0%, 0% and 66% incidence of emesis (n = 6). When ABT-594 was co-dosed with NS9283 (3.5 μmol/kg), animals showed respectively 0%, 0% and 33% (n = 6) incidence of emesis. These studies shows that incidence of emesis in the ferret model is not exaggerated by co-dosing ABT-594 with PAM.

3.7. ABT-594: relating preclinical efficacy/tolerability profile to clinical findings

Since improved tolerability of nAChR agents remains a major goal in the development of nAChR-based therapeutics, it was important to assess whether substantial separation of analgesic vs. emetic effects could be realized, and to relate our preclinical observations with available clinical data with ABT-594. This is illustrated in Fig. 6. Fig. 6A shows the left-ward shift in efficacy of ABT-594 in the rat spinal nerve ligation model in the presence and absence of NS9283 (3.5 μmol/kg) where as Fig. 6B shows lack of shift in the emesis incidence in the ferret model. The bottom panel summarizes preclinical data of ABT-594 (both efficacy data and emesis data) and revealing very little separation of efficacy vs. emesis. The arrows indicated represent the plasma concentrations achieved clinically in a phase 2, randomized, multicenter, double-blind, placebo-controlled study in patients with diabetic peripheral neuropathic pain with ABT-594 [9]. Fig. 6D is preclinical data (efficacy and emesis) for ABT-594 in the presence of NS9283 showing left-ward shift in efficacy vs. emesis threshold. Should the preclinical separation in efficacy–tolerability profile translate to human, substantial pain relief may be realized with low doses of ABT-594. For example, it may be extrapolated that a well tolerated dose of ABT-594 (e.g. 75 μg dose), robust (>50%) pain efficacy may be realized following co-dosing with PAM.

4. Discussion

Discovery of highly selective and positive allosteric modulators of nicotinic receptor provides an unprecedented opportunity for further interrogation of the physiological roles of receptor

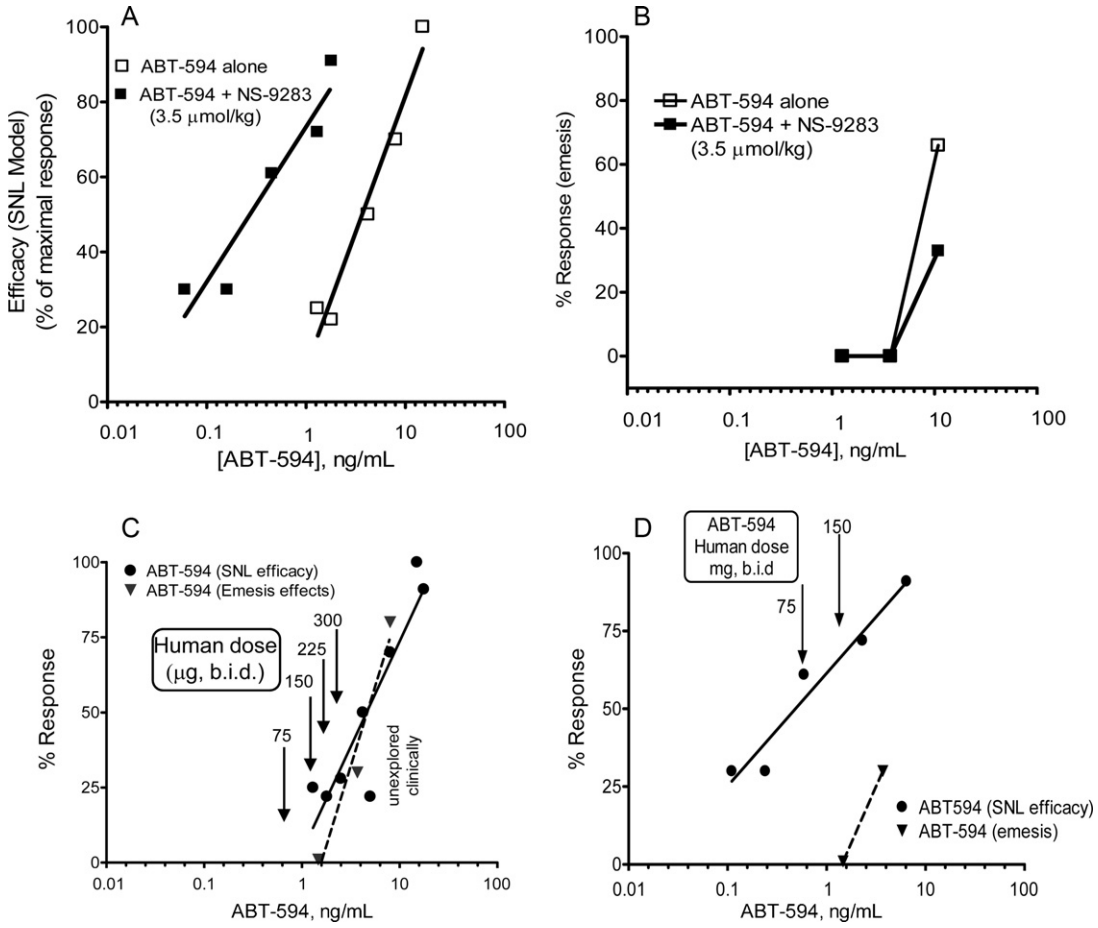


Fig. 6. NS9283 enhanced the efficacy of ABT-594 in neuropathic pain without altering its emesis profile. (A) Leftward shift of the analgesic efficacy of ABT-594 in the presence of NS9283 in the rat model of spinal nerve ligation. (B) Lack of shift of emesis threshold in the presence of NS9283. (C) Overlap of concentration–response relationships of analgesic efficacy and emesis responses of ABT-594 in preclinical models. Also depicted are doses of ABT-594 evaluated in the phase 2 study in patients, with arrows point to estimated plasma concentrations. (D) In the presence of NS9283, the efficacy of ABT-594 is left shifted such that, now at tolerated doses of ABT-594 ($\leq$75 μg, b.i.d.), robust efficacy can be realized in the spinal nerve ligation model of neuropathic pain.

subtypes, as in this study, dissecting pain efficacy vs. adverse effects of nAChR ligands using an α4β2 selective PAM. α4β2 nAChR agonism is a clinically validated approach for treatment of pain as revealed with ABT-594, the first nAChR agonist demonstrating efficacy (phase 2) in the treatment of diabetic neuropathy [9]. Compared to placebo, all three ABT-594 treatment groups showed significantly improved average pain rating scale (PRS) score in humans. However, efficacious doses of ABT-594 in humans were associated with an undesirable side effect profile, particularly with respect to nausea and emesis. The hypothesis tested in this study is that the therapeutic window of nAChR agonists in pain can be enhanced by co-administering agonists with a α4β2 selective positive allosteric modulator (PAM). Our preclinical data demonstrate that selective positive allosteric modulation of the α4β2 nAChR can enhance the efficacy of ABT-594 in neuropathic pain, without altering adverse effects that are predominantly α3* mediated.

The observation that allosteric potentiation in vitro is only observed at the α4β2, but not at the α4β4, nAChR combination suggests that the interaction site of PAM is located either at the β2 subunit or the interface of the heteromeric complex. Evidence of specific binding of PAM to native receptors were evidenced by radiolabled studies of a closely related analog, [^{3}H]A-998679. [^{3}H]A-998679 bound to both rat and human brain membranes with K_D values of 107 nM and 76 nM respectively indicating no species differences in PAM binding affinities [18]. To assess the in vivo relevance of the in vitro functional observations, studies were conducted in the rat model of neuropathic pain to establish whether the efficacy of α4β2 nAChR agonists (in particular, ABT-594) could be enhanced in combination with PAMs without affecting the tolerability profile (gastrointestinal, cardiovascular, CNS, etc.). Consistent with in vitro findings, PAM alone was found to be ineffective in vivo following acute administration in the rat model of spinal nerve ligation. When ABT-594 was co-administered with NS9283 (3.5 μmol/kg i.p.), the dose response curve of ABT-594 was left-shifted demonstrating that co-administration of NS9283 with ABT-594 enhanced the anti-allodynic effects of ABT-594. Likewise, when a low steady state level of ABT-594 (0.7 ng/mL equivalent to the 75 μg human dose) was maintained by a minipump, no efficacy was observed with ABT-594 alone, but dose-dependent efficacy was evoked by NS9283. In addition, we have examined the analgesic effect following co-administration of NS9283 and ABT-594 in a variety of preclinical models in rats to show that this benefit can be extended beyond neuropathic pain (see [1]; accompanying manuscript). Co-administration of NS9283 caused a 5–6-fold leftward shift of the dose response of ABT-594 in the carrageenan-induced thermal hyperalgesia, in the paw skin incision model of post-operative pain and in monoiodo-acetate induced knee joint pain. Although mechanisms remain to be fully elucidated, enhanced activation of descending inhibitory processes and spinal pathways, attributable to enhanced channel activation in the

presence of PAM, could contribute. For example, studies in spinal slices have shown that ABT-594 evoked inhibitory postsynaptic currents are robustly enhanced in presence of a PAM (unpublished observations).

As described earlier, the most prominent side effects of ABT-594 activation that is seen preclinically and confirmed in human clinical studies are nausea and emesis. Although, it is possible, in principle, that $\alpha4\beta2$ and $\alpha3^*$ containing subtypes may be responsible for the in vivo side effects, the lack of highly subtype-selective ligands for specific $\alpha4\beta2$ and $\alpha3\beta4$ subtypes have made it impossible to precisely definitively determine which nAChR subtypes mediate these effects. Our studies with the $\alpha4\beta2$ PAM reveal that the selective enhancement of $\alpha4\beta2$ effect of ABT-594 can translate to enhanced in vivo pain efficacy, without altering the emesis threshold, likely mediated via interactions at the $\alpha3^*$ containing nAChRs. This is consistent with results from pharmacological MRI studies showing that co-administration of NS9283 led to a leftward shift of ABT-594 dose–response for cortical activation, without activating the brainstem emetic center at efficacious analgesic doses examined ([1]; accompanying manuscript). Should the preclinically observed separation in analgesic efficacy vs. emesis translate to human, substantial pain relief may be realized with low doses of ABT-594. Importantly, co-administration of NS9283 at preclinical supra-therapeutic doses produced no hemodynamic or electrophysiological effects in the anesthetized dog cardiovascular model at plasma concentrations >20-fold those achieved using ABT-594 at the well-tolerated human dose of 75 μg.

As shown in the accompanying paper [1], ABT-594 induced CNS-related adverse effects were not exacerbated in presence of an efficacious dose of NS9283 (3.5 μmol/kg). Furthermore, acute challenge of NS9283 produced no cross sensitization in nicotine-conditioned animals. These results demonstrate that selective positive allosteric modulation at the $\alpha4\beta2$ nAChR can enhance nAChR agonist-induced analgesic activity across neuropathic and nociceptive preclinical pain models without potentiating ABT-594 mediated adverse effects, suggesting that selective positive modulation of $\alpha4\beta2$ nAChR subtype by PAM may represent a novel combination approach. It should be pointed out that although we have demonstrated a novel analgesic combination approach, it remains unclear whether preclinical improvements in therapeutic index (pain efficacy vs. emesis) will translate to meaningful differences in patients.

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