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Defining the Putative Inhibitory Site for a Selective Negative Allosteric Modulator of Human α4β2 Neuronal Nicotinic Receptors

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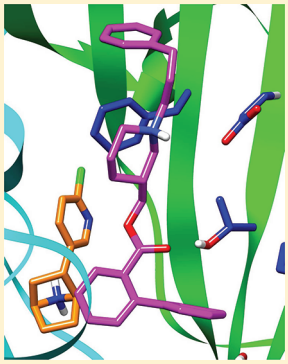
Supporting Information

ABSTRACT: Neuronal nicotinic receptors (nAChRs) have been implicated in several diseases and disorders such as autism spectrum disorders, Alzheimer's disease, Parkinson's disease, epilepsy, and nicotine addiction. To understand the role of nAChRs in these conditions, it would be beneficial to have selective molecules that target specific nAChRs *in vitro* and *in vivo*. Our laboratory has previously identified a novel allosteric site on human α4β2 nAChRs using a series of computational and *in vitro* approaches. At this site, we have identified negative allosteric modulators that selectively inhibit human α4β2 nAChRs, a subtype implicated in nicotine addiction. This study characterizes the allosteric site via site-directed mutagenesis. Three amino acids (Phe118, Glu60, and Thr58) on the β2 subunit were shown to participate in the inhibitory properties of the selective antagonist KAB-18 and provided insights into its antagonism of human α4β2 nAChRs. SAR studies with KAB-18 analogues and various mutant α4β2 nAChRs also provided information concerning how different physiochemical features influence the inhibition of nAChRs through this allosteric site. Together, these studies identify the amino acids that contribute to the selective antagonism of human α4β2 nAChRs at this allosteric site. Finally, these studies define the physiochemical features of ligands that influence interaction with specific amino acids in this allosteric site.



KEYWORDS: *Negative allosteric modulator (NAM), neuronal nicotinic acetylcholine receptors (nAChRs), α4β2, site-directed mutagenesis, structure−activity relationships, nicotine*

Neuronal nicotinic acetylcholine receptors (nAChRs) are associated with many physiological mechanisms (i.e., cognition, arousal, and pain sensation) as well as a number of neurological diseases and disorders (depression, schizophrenia, Alzheimer's disease, Parkinson's disease, lung cancer, Tourette's syndrome, autism spectrum disorders, and addiction).[1−4] nAChRs are pentameric, ligand-gated ion channels that can be expressed as multiple subtypes as determined by the assembled subunit composition (combination of α2-α10 and β2-β4).[5] In the CNS, the three most prominent subtype compositions are α4β2, α3β4, and α7 nAChRs. The specific nAChR subtypes involved with most of the above-mentioned physiological processes or diseases are not known, and their study is challenging due to the complex composition of some nAChRs (i.e., α4α6β2β3 and α3α5β4).[6,7] Despite this lack of understanding, it is widely accepted that nicotine addiction is mediated primarily by α4β2* (* denotes possible additional subunits) nAChRs.[8,9] The discovery of novel, selective molecules that target specific subtypes of nAChRs will contribute significantly to our understanding of the role nAChR subtypes play in normal and pathophysiological states and prove beneficial for the clinical treatment of several neuropathologies.

nAChRs are notable for their role in the addictive properties of nicotine, the primary addictive component of tobacco products.[10] Nicotine addiction is a significant health problem, with smoking ranking as the primary cause of preventable death worldwide. Roughly 90% of the people who attempt to quit are unable to do so.[11] FDA approved treatments for tobacco addiction are nicotine replacement, buproprion (Zyban), and varenicline (Chantix). Each of these therapies has a modest success of 20%−30% abstinence one year after quitting.[12−14] The target site of many nAChR drug discovery programs is the orthosteric (agonist binding) site of nAChRs,[6,15] and some laboratories have had some success in identifying molecules

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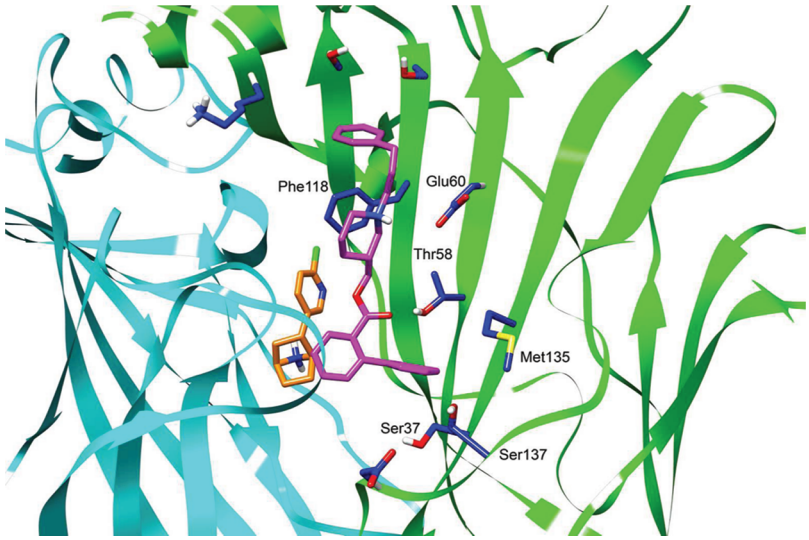

Figure 1. Docking of KAB-18 in the proposed allosteric site on H$\alpha4\beta2$ nAChRs. A snapshot showing the induced computational binding mode of KAB-18 (magenta) to the epibatidine-bound (orange) interface of the H$\alpha4$ (cyan ribbon) and H$\beta2$ (green ribbon) subunits, as described previously.[25] Amino acids proposed to contribute to the binding of KAB-18 are shown in stick representation. Amino acids chosen for site-directed mutagenesis are labeled with amino acid name and sequence number.

that show some selectivity using this approach.[16,17] One difficulty with this strategy is the high degree of conservation of the orthosteric site among the various nAChR subtypes, making it difficult to develop subtype-selective drugs.[6] For this reason, drugs targeting "non-orthosteric" sites of nAChRs (e.g., allosteric, noncompetitive sites) have been used by many laboratories.[18−20]

Mecamylamine, a nonselective and noncompetitive nAChR antagonist, was shown to promote 40% abstinence at the year mark when used as an agonist−antagonist therapy in combination with the nicotine patch.[21] In addition, antagonists of $\alpha4\beta2$ nAChRs block nicotine self-administration on fixed and progressive ratio schedules in rats.[18,22,23] These data support the use of antagonists as nicotine cessation therapies; however, to produce new therapeutic molecules, it is believed that nAChR subtype selectivity must be pursued.[6] Our laboratory has identified a novel class of allosteric modulators that target nAChRs. These allosteric modulators have been shown to not compete with the agonist at the orthosteric site. Instead, they enhance the binding of the agonist to the orthosteric site.[24] These allosteric modulators also act as antagonists of nAChRs, and some have been shown to be selective for human $\alpha4\beta2$ (H$\alpha4\beta2$) nAChRs.[25,26] Since these compounds act as antagonists, do not occupy the orthosteric site, and enhance the binding of agonists, we have classified these compounds as negative allosteric modulators (NAMs) according to the guidelines established by Neurig et al.[27] Additionally, our laboratory has identified a putative allosteric site for these NAMs on human $\alpha4\beta2$ (H$\alpha4\beta2$) nAChRs that is approximately 10 Å from the orthosteric site, at the interface of the $\alpha4$ and $\beta2$ subunits. This site was identified through a combination of homology modeling, blind docking, and molecular dynamics studies and was confirmed through the use of site-directed mutagenesis and structure-based virtual screening.[24,25,28,29] At this site, NAMs have been shown to selectively antagonize H$\alpha4\beta2$ nAChRs over H$\alpha3\beta4$ nAChRs.[25,26,29,30] To develop more potent and selective NAMs for nAChRs, the putative binding site and binding mode of KAB-18 must be refined. Also, the binding properties of other NAMs at this novel allosteric site should be defined in addition to KAB-18. Using the allosteric site that was identified through computational modeling,[25] specific amino acids were selected and targeted for site-directed mutagenesis to identify those that contribute to the inhibitory properties of our lead molecule, KAB-18, and additional NAMs. Finally, SAR studies were conducted using mutant H$\alpha4\beta2$ nAChRs to determine what physiochemical features are important for preserving the KAB-18 functional interactions at this novel allosteric site.

RESULTS

KAB-18 has been established previously as an allosteric modulator of nAChRs through several assays: (1) absence of competition with agonists in binding assays[24] (Supporting Information, Figure 1), (2) enhancement of the binding affinity of agonists,[24] and (3) by reducing the efficacy of agonists through noncompetitive mechanisms.[25] The identification of KAB-18's allosteric site has been described previously using docking studies and mutagenesis.[25] This putative allosteric site was found to be approximately 10 Å from the orthosteric site, with many potential stable interactions between KAB-18 and the $\beta2$ subunit (Figure 1). Two stable hydrogen bond interactions were predicted through molecular dynamics simulations: (1) a hydrogen bond between KAB-18's carbonyl group and Thr58 on the $\beta2$ subunit and (2) a hydrogen bond between KAB-18's protonated piperidine and Glu60 on the $\beta2$ subunit.[25] In addition to these two interactions, there is a potential cation−π interaction between the positively charged piperidine of KAB-18 and Phe118. Phe118 may also participate in $\pi-\pi$ interactions with the phenylpropyl of KAB-18. We have previously confirmed the interactions of Thr58 using the calcium selective probe, Fluo-4;[25,28] however, this mutation was coexpressed with an additional mutation in the $\alpha4$ subunit (R187I). It is possible that Arg187 on the $\alpha4$ subunit contributed to the observed change in potency observed with this mutation. Therefore, it is unclear whether both or only one of these mutated amino acids were involved in KAB-18's interaction with the allosteric site. Here, we answer this question and present a series of SAR studies using new $\beta2$

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subunit mutations, additional NAMs, and a new calcium selective probe (Calcium 5), which provided an improved signal-to-noise ratio when compared to that of Fluo-4, to identify the physiochemical features that are important for the interactions between the NAMs and the different amino acids in this allosteric site on H$\alpha4\beta2$ nAChRs. Additionally, this provides insight into the NAMs' selectivity for H$\alpha4\beta2$ nAChRs over H$\alpha3\beta4$ nAChRs. To clearly describe the SAR and site-directed mutagenesis studies with KAB-18 and KAB-18 analogues, regions of the KAB-18 scaffold (Figure 2) will be used to describe the results presented herein.

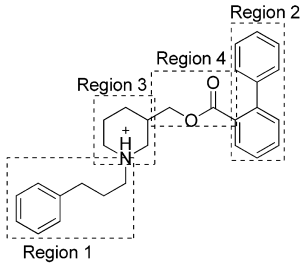

Figure 2. Regions of KAB-18 scaffold. KAB-18 has four regions as designated by a previously generated pharmacophore.[25] The phenylpropyl region is designated as Region 1, the biphenyl is designated as Region 2, the piperidine ring is designated Region 3, and the ester is designated as Region 4.

The proposed computational binding mode of KAB-18 has led to the identification of amino acids that potentially interact with the four regions of the KAB-18 scaffold (Figures 1 and 2): (1) Phe118 is proposed to contact the phenylpropyl of Region 1 and the piperidine of Region 3 (potentially through $\pi-\pi$ or cation$-\pi$ interactions, respectively), (2) Glu60 potentially forms an ionic bond with the positive nitrogen of Region 3, and (3) Thr58 is proposed to form a hydrogen bond with the ester carbonyl of Region 4. To determine the importance of the proposed target amino acids in the allosteric interactions of KAB-18, mutant H$\alpha4\beta2$ nAChRs were created (Tables 1 and 2). The importance of each target amino acid was assessed by the change of potency between the mutant and wild type (WT) H$\alpha4\beta2$ nAChRs. Fold differences in potency were calculated by the quotient of the functional IC_{50} of the drug (e.g., KAB-18) on mutant H$\alpha4\beta2$ nAChRs and the functional IC_{50} of the same drug on H$\alpha4\beta2$WT nAChRs. The WT and mutant H$\alpha4\beta2$ nAChRs were expressed through transient transfections in HEK tsa201 cells (see Methods).

To determine if the mutations in the H$\alpha4\beta2$ nAChRs affected the pharmacology at known sites (i.e., orthosteric and known allosteric sites), several control drugs were tested (Table 1 and Figure 3A–C). Both epibatidine (agonist) and *d*-tubocurarine (competitive antagonist), which bind at the orthosteric site, showed no difference in potency between H$\alpha4\beta2$ WT nAChRs and mutant H$\alpha4\beta2$ nAChRs (Table 1). The noncompetitive antagonist, mecamylamine, binds to the central luminal site[31,32] and showed no difference in potency between H$\alpha4\beta2$ WT nAChRs and mutant H$\alpha4\beta2$ nAChRs (Table 1). Thus, targeted mutations within the KAB-18 site did not nonspecifically alter the structure of the receptor.

Following the tests with control drugs, KAB-18 was tested for changes in potency with the mutant H$\alpha4\beta2$ nAChRs to provide new information regarding its interactions within the allosteric site (Table 2, Figure 3D, and Supporting Information, Figure 2). As mentioned previously, Glu60 was proposed to have a stable hydrogen bond with the piperidine nitrogen's hydrogen atom, and this interaction was shown to be stable over the course of an MD simulation of KAB-18 in the allosteric site.[25] Glu60 is conserved between $\beta2$ and $\beta4$ subunits; thus, it was mutated to the relatively inert residue, alanine (E60A). The E60A mutation had no effect on the functional activity of control agonists and antagonists (Table 1). On H$\alpha4\beta2$WT nAChRs, KAB-18 displayed an IC_{50} of 6.8 μM (Table 2). When KAB-18 was tested on H$\alpha4\beta2$M·E60A nAChRs, it displayed a 4-fold decrease in its potency (IC_{50} value, 25.3 μM, Table 2) when compared to that of H$\alpha4\beta2$WT nAChRs. Two additional amino acids in close proximity to KAB-18, Ser37, and Ser137, potentially interact with Region 2 of the KAB-18 scaffold (Figure 1). Like Glu60, these residues are also conserved among the $\beta2$ and $\beta4$ subunits and were therefore mutated to alanine to assess their effect on NAM binding. The S37A and S137A mutations, like the E60A mutant, showed no difference in the functional activity of

Table 2. Inhibition of Wild Type H$\alpha4\beta2$ and Mutant H$\alpha4\beta2$ nAChRs with the Lead Molecule KAB-18

	IC_{50} value (μM)[a]	n_h[b]	F_m[c]
H$\alpha4\beta2$WT	6.8 (3.6–12.9)	−1.4	
H$\alpha4\beta2$M·F118L	42.6 (24.3–72.6)[d]	−1.1	6.3
H$\alpha4\beta2$M·T58K	57.6 (40.2–82.5)[d]	−1.0	8.5
H$\alpha4\beta2$M·T58A	104.0 (62.4–173)[e]	−0.6	15.3
H$\alpha4\beta2$M·S137A	8.6 (5.5–13.5)	−0.8	1.3
H$\alpha4\beta2$M·S37A	6.4 (4.9–8.3)	−0.8	0.9
H$\alpha4\beta2$M·E60A	25.3 (16.6–38.5)[d]	−1.0	3.7

[a]Values represent geometric means (confidence interval), n = 4–10. [b]Hill coefficient. [c]Fold change of IC_{50} mutant/IC_{50}-wild-type. [d]Statistically significant ($p < 0.005$). [e]Statistically significant ($p < 0.001$).

Table 1. Pharmacological Effects of Control Drugs on Wild Type and Mutant H$\alpha4\beta2$ nAChRs

	epibatidine		*d*-tubocurarine		mecamylamine	
	EC_{50} value (nM)[a]	n_h[b]	IC_{50} value (μM)[a]	n_h[b]	IC_{50} value (μM)[a]	n_h[b]
H$\alpha4\beta2$WT	21.1 (16.3–27.3)	0.9	4.3 (2.9–6.3)	−1.0	0.19 (0.17–0.21)	−1.4
H$\alpha4\beta2$M· F118L	24.3 (13.3–44.4)	0.8	5.7 (3.9–8.2)	−0.9	0.21 (0.07–0.59)	−1.1
H$\alpha4\beta2$M· T58K	26.3 (9.6–77.6)	0.7	4.0 (1.0–17.0)	−0.6	0.37 (0.26–0.61)	−0.6
H$\alpha4\beta2$M· T58A	24.0 (11.5–50.2)	1.0	7.0 (2.7–18.0)	−1.4	0.18 (0.06–0.49)	−1.1
H$\alpha4\beta2$M· S137A	18.2 (16.2–20.5)	1.6	3.3 (2.3–4.6)	−1.0	0.14 (0.10–0.19)	−0.8
H$\alpha4\beta2$M· S37A	17.4 (12.8–23.6)	0.8	5.0 (2.2–11.1)	−1.4	0.16 (0.08–0.30)	−0.8
H$\alpha4\beta2$M· E60A	20.6 (15.7–27.1)	1.1	5.4 (2.3–12.7)	−0.9	0.15 (0.08–0.30)	−0.9

[a]Values represent geometric means (confidence interval), n = 4–7. [b]Hill coefficient.

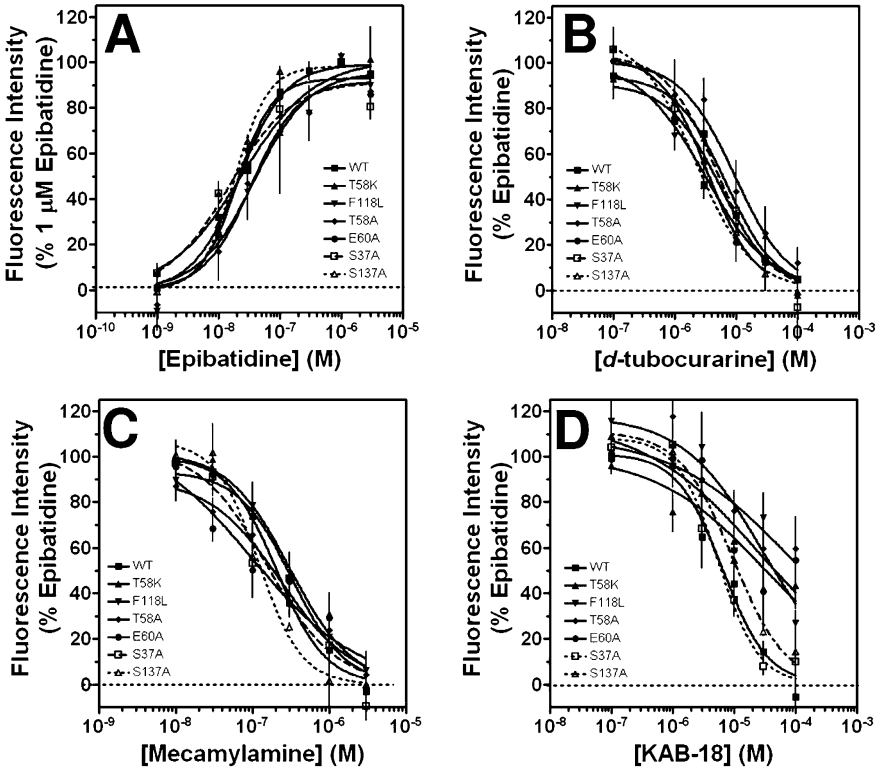

Figure 3. Concentration−response effects of agonists, antagonists, and KAB-18 on WT and mutant Hα4β2 nAChRs. Concentration−response effects of epibatidine (panel A), *d*-tubocurarine (panel B), mecamylamine (panel C), and KAB-18 (panel D) were investigated using WT and mutant Hα4β2 nAChRs. Data are expressed as a percentage of peak fluorescence responses for 1 μM epibatidine. The dotted horizontal lines show the 0% control response. Values represent means ± SEMs (n = 4 − 110).

control drugs (Table 1). However, both S137A and S37A mutations showed no significant difference in the potency of KAB-18 when compared to that of the Hα4β2WT nAChRs (Table 2).

Thr58 is proposed to maintain a stable hydrogen bond with the ester carbonyl of KAB-18 in Region 4 (Figure 1, [25]). Like Glu60, this interaction was shown to be stable in an MD simulation.[25] As opposed to the Glu60, Ser37, and Ser137 residues of the β2 nAChR subunit, Thr58 is not conserved between β2 and β4 sequences. In consideration of the fact that KAB-18 has no activity on Hα3β4 nAChRs,[25] the cognate amino acid residue found at position 58 (Lys) in the β4 subunit was selected for the mutation. Mutation of Thr58 to a lysine (T58K) resulted in an 8.5-fold decrease in potency of KAB-18 (IC_{50} value = 57.6 μM, Table 2). We must note that threonine and lysine residues have the potential for similar modes of hydrogen bonding, despite being different residues. Although threonine has a shorter side chain than lysine, there is still the potential for the NAMs to have similar hydrogen binding with both residues. To account for this, an additional Thr58 mutation was made using alanine (T58A). Here, KAB-18 had a 15-fold decrease in potency (IC_{50} value, 104.0 μM, Table 2).

Similar to Thr58, Phe118 is not conserved between the β2 and β4 sequences, and the amino acid at position 118 in the β4 subunit is a leucine residue. Similar to our approach for the T58K mutant, a leucine (the cognate amino acid found on β4 nAChR subunits) was chosen as the mutant residue for Phe118. Leucine cannot participate in cation−π or π−stacking interactions. Therefore, this mutation provides an opportunity to determine whether the amino acid at position 118 interacts with KAB-18 through these types of interactions. Mutation of Phe118 (F118L) resulted in KAB-18 having a 6-fold decrease in potency (IC_{50} value 42.6 μM, Table 2) on Hα4β2M·F118L nAChRs.

Potential Interactions of Other NAMs with Phe118. At this point, the putative site for KAB-18's interaction on Hα4β2 nAChRs as well as the amino acids that contribute to KAB-18 activity have been identified. However, it is not known how this information translates to the other 100+ molecules that exist in our KAB-18-like molecular library, some of which have selectivity for Hα4β2 nAChRs.[25] It can be hypothesized that since most of the NAMs have very similar chemical structures that all the molecules interact with the allosteric site in a similar manner (i.e., conserved binding to key residues and/or sharing a conserved binding bode). However, this assumes that small changes in NAM structure cause little perturbation on the interactions with this allosteric site.

To study how the mode of allosteric interactions may change, additional NAMs sharing similar chemical features with KAB-18 have been selected to gain additional insight into the physiochemical features that influence interactions with amino acids in this allosteric site. These molecules were selected based on chemical differences in the four regions of the KAB-18 scaffold (Figure 2). First, NAMs with Region 1 differences were studied with Hα4β2M·F118L nAChRs. DDR-13 and APB-8 resulted in a 2-fold difference in potency when Hα4β2M·F118L nAChRs were compared to Hα4β2WT nAChRs (Table 3). DDR-18 resulted in a 3-fold change in potency with Hα4β2M·F118L nAChRs when compared to that of Hα4β2WT nAChRs (Table 3). COB-1, COB-2, and COB-3, molecules with small substitutions in Region 1, showed no difference in potency when their effects on Hα4β2M·F118L

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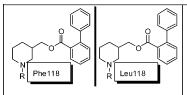
Table 3. Effects of Piperidine Substituted NAMs (Region 1) on Wild Type and Mutant Human $\alpha4\beta2$ (F118L) nAChRs

		Hα4β2 WT		Hα4β2M.F118L		
Compound	R group	IC_{50} Value (μM)[a]	n_h[b]	IC_{50} Value (μM)[a]	n_h[b]	F_m[c]
KAB-18		6.8 (3.6-12.9)	-1.2	42.6 (24.3-72.6)[d]	-1.1	6.3
DDR-13		10.1 (6.4-16.0)	-1.0	23.6 (18.3-30.4)[e]	-0.6	2.3
APB-8		15.4 (8.0-29.7)	-1.4	34.0 (21.2-54.6)	-1.3	2.2
DDR-18		7.5 (5.2-10.8)	-1.4	24.3 (16.0-36.8)[e]	-0.9	3.2
COB-1	-H	2.1 (1.1-4.2)	-1.1	4.1 (1.8-9.2)	-1.2	1.9
COB-2	$-CH_3$	1.7 (1.3-2.3)	-0.9	2.0 (1.4-3.0)	-1.2	1.2
COB-3	$-(CH_3)_2$	0.4 (0.2-0.8)	-1.1	0.4 (0.2-0.8)	-1.0	1.0
DDR-15		5.3 (2.8-5.4)	-1.0	3.1 (1.8-5.2)	-0.8	0.6

[a]Values represent geometric means (confidence limits), $n = 4-10$. [b]Hill coefficient. [c]Fold change of IC_{50} mutant/IC_{50}-wild-type. [d]Statistically significant ($p < 0.005$). [e]Statistically significant ($p < 0.05$).

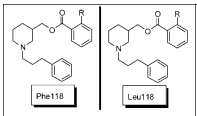
nAChRs were compared to those of H$\alpha4\beta2$WT nAChRs (Table 3). DDR-15, which contains an azidopropyl in Region 1, showed no difference in potency when H$\alpha4\beta2$M·F118L and H$\alpha4\beta2$WT nAChRs were compared (Table 3). Interestingly, all of the aromatic containing analogues (i.e., DDR-13, APB-8, and DDR-18) showed a change in potency, while the nonaromatic containing analogues did not. This suggests that the aromatic features influence the interaction with Phe118. This finding agrees with previously collected SAR showing that aromatic features in Region 1 conferred selectivity to H$\alpha4\beta2$ nAChRs.[25]

Although COB-3 did not show a change in potency with the F118L mutant, there was a significant increase in potency for H$\alpha4\beta2$WT nAChRs in comparison to that of KAB-18 (17-fold, $p < 0.005$). This suggests that COB-3 may bind in a different orientation or may bind more tightly to this allosteric site but does not involve interactions at Phe118 since the mutation did not change potency.

Several NAMs were chosen for their Region 2 physiochemical features and were tested on H$\alpha4\beta2$M·F118L nAChRs (Table 4). IB-2, KAB-30, and KAB-31 all have succinimide substitutions in Region 2 and showed no difference in potency when compared between H$\alpha4\beta2$M·F118L and H$\alpha4\beta2$WT nAChRs (Table 4). FFB-1, which contains a napthyl in Region 2, resulted in a 3-fold decrease in potency on H$\alpha4\beta2$M·F118L nAChRs when compared to H$\alpha4\beta2$WT nAChRs (Table 4). This study also suggests that aromatic features are important in this region. Although not directly involved in the interaction with Phe118, these features may preserve the ligands' physical orientation so that the NAMs interact with Phe118.

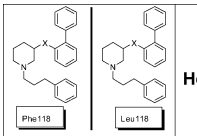
Several NAMs with differences in Region 4 were also selected for studies with H$\alpha4\beta2$M·F118L nAChRs. DDR-5, which contains an amide in Region 4, showed a 7-fold decrease in potency when compared between H$\alpha4\beta2$M·F118L and H$\alpha4\beta2$WT nAChRs (Table 5). JHB-9, DDR-3 (the reverse ester and reverse amide of KAB-18, respectively), and DDR14 showed negligible change in potency when H$\alpha4\beta2$M·F118L nAChRs were compared to H$\alpha4\beta2$WT nAChRs (Table 5).

Table 4. Effects of Anthranilic Acid Substituted NAMs (Region 2) on Wild Type and Mutant Human $\alpha4\beta2$ (F118L) nAChRs

		Hα4β2 WT		Hα4β2M.F118L		
Compound	R	IC_{50} Value (μM)[a]	n_h[b]	IC_{50} Value (μM)[a]	n_h[b]	F_m[c]
KAB-18		6.8 (3.6-12.9)	-1.4	42.6 (24.3.-72.6)[d]	-1.1	6.3
FFB-1		7.5 (5.4-10.5)	-0.9	22.9 (14.6-35.8)[e]	-0.8	3.1
IB-2		3.9 (2.8-5.4)	-1.2	4.9 (3.9-6.0)	-1.5	1.3
KAB-31		2.9 (1.0-8.4)	-2.4	2.7 (1.2-6.0)	-1.3	0.9
KAB-30		2.0 (1.4-2.9)	-1.0	3.7 (2.8-5.0)	-1.3	1.9

[a]Values represent geometric means (confidence limits), $n = 4-10$. [b]Hill coefficient. [c]Fold change of IC_{50} mutant/IC_{50}-wild-type. [d]Statistically significant ($p < 0.005$). [e]Statistically significant ($p < 0.05$).

Table 5. Effects of Ester Region Substituted NAMs (Region 4) on Wild Type and Mutant Human $\alpha4\beta2$ (F118L) nAChRs

		Hα4β2 WT		Hα4β2M.F118L		
Compound	X	IC_{50} Value (μM)[a]	n_h[b]	IC_{50} Value (μM)[a]	n_h[b]	F_m[c]
KAB-18		6.8 (3.6-12.9)	-1.4	42.6 (24.3-72.6)[d]	-1.1	6.3
DDR-5		4.1 (2.1-8.2)	-1.9	30.8 (15.0-63.4)[e]	-0.7	7.5
JHB-9		13.2 (9.1-19.3)	-1.3	13.9 (10.6-19.5)	-1.0	1.1
DDR-3		3.7 (2.1-6.4)	-1.0	5.7 (4.0-8.2)	-1.2	1.5
DDR-14		7.9 (4.0-15.5)	-1.4	15.5 (5.1-47.0)	-0.7	2.0

[a]Values represent geometric means (confidence limits), $n = 4-10$. [b]Hill coefficient. [c]Fold change of IC_{50} mutant/IC_{50}-wild-type. [d]Statistically significant ($p < 0.005$). [e]Statistically significant ($p < 0.05$).

These data suggest that the placement of the carbonyl in Region 4 influences the interaction with Phe118. In this case, the carbonyl of Region 4 is proposed to interact with Thr58. Therefore, the specific placement of the Region 4 carbonyl influences the interaction with Phe118 through a potential hydrogen bond with Thr58 (as proposed by computational modeling; Figure 1). This finding also agrees with previous SAR where it was determined that the placement of the carbonyl influenced selectivity on H$\alpha4\beta2$ nAChRs.[25]

Potential Interactions of Other NAMs with Thr58. Several Region 4 NAMs were selected for testing with the H$\alpha4\beta2$M·T58K nAChRs to confirm our hypothesis that the carbonyl placement influences the proposed hydrogen bond with Thr58 (Table 6). DDR-5, the amide analogue of KAB-18,

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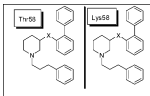
Table 6. Effects of Ester Substituted NAMs (Region 4) on Wild Type and Mutant Human $\alpha4\beta2$ (T58K) nAChRs and Human $\alpha4\beta2$ (T58A) nAChRs

		Hα4β2 WT		Hα4β2M.T58K			Hα4β2M.T58A		
Compound	X	IC_{50} Value (μM)[a]	n_h[b]	IC_{50} Value (μM)[a]	n_h[b]	F_m[c]	IC_{50} Value (μM)[a]	n_h[b]	F_m[c]
KAB-18		6.8 (3.6-12.9)	-1.4	57.6 (40.2-82.5)[d]	-1.0	8.5	104 (62.4-173)[e]	-1.0	15.3
DDR-5		5.1 (3.9-8.3)	-1.3	15.7 (7.7-32.0)	-1.0	3.1	2.9 (2.0-4.2)	-0.9	0.6
JHB-9		13.2 (9.1-19.3)	-1.3	27.6 (16.8-45.5)	-1.1	2.1	10.1 (3.7-27.3)	-2.1	0.8
DDR-3		3.7 (2.1-6.4)	-1.2	14.2 (6.3-37.0)	-0.8	3.8	6.0 (3.8-9.4)	-1.0	1.6
DDR-14		7.9 (4.0-15.5)	-1.0	40.8 (32.6-50.9)[e]	-1.3	5.2	50.0 (25.9-96.6)[e]	-3.4	6.3

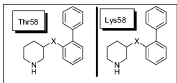
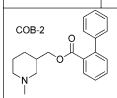
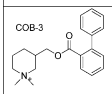
[a]Values represent geometric means (confidence limits), n = 4−10. [b]n_H, Hill coefficient. [c]Fold change of IC_{50} mutant/IC_{50}-wild-type. [d]Statistically significant (p < 0.005). [e]Statistically significant (p < 0.001).

showed a 3-fold decrease in potency when Hα4β2M·T58K nAChRs (IC_{50} value, 15.7 μM, Table 6) are compared to Hα4β2WT nAChRs (IC_{50} value, 5.1 μM, Table 6). JHB-9, the reverse ester of KAB-18, produced a 2-fold decrease in potency between Hα4β2M·T58K (IC_{50} value, 27.6 μM, Table 6) and Hα4β2WT nAChRs (IC_{50} value, 13.2 μM, Table 6). DDR-3, the reverse amide of DDR-5, showed a 4-fold decrease in potency when Hα4β2M·T58K nAChRs (IC_{50} value, 14.2 μM, Table 6) are compared to Hα4β2WT nAChRs (IC_{50} value, 3.7 μM, Table 6). Finally, DDR-14 showed a 5-fold decrease in potency when Hα4β2M·T58K nAChRs (IC_{50} value, 40.8 μM, Table 6) are compared to Hα4β2WT nAChRs (IC_{50} value, 7.9 μM, Table 6).

This same series of Region 4 NAMs were tested on Hα4β2M·T58A nAChRs (Table 6). DDR-5 and JHB-9 showed no difference in potency between Hα4β2M·T58A nAChRs and Hα4β2WT nAChRs (Table 6). DDR-3 produced a 2-fold decrease in potency between Hα4β2M·T58A nAChRs (IC_{50} value, 6.0 μM, Table 6) and Hα4β2WT nAChRs (IC_{50} value 3.7 μM, Table 6). DDR-14 produced a 6-fold decrease in potency between Hα4β2M·T58A nAChRs (IC_{50} value, 50.0 μM, Table 6) and Hα4β2WT nAChRs (IC_{50} value, 7.9 μM, Table 6). These data suggest that the carbonyl placement is important as the esters (with the exception of DDR-5 on the T58A mutation) showed a change in potency, while the reverse esters showed no change in potency.

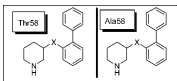
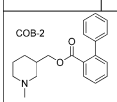
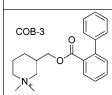
To further study the interactions of NAMs containing small Region 1 features with Thr58, COB analogues were tested with Hα4β2M·T58K nAChRs (Table 7) and Hα4β2M·T58A nAChRs (Table 8). COB-2, COB-10, COB-8, and NEB-2 showed no change in potency between Hα4β2M·T58K and Hα4β2WT nAChRs (Table 7). COB-9 showed nearly a 2-fold decrease in its potency (IC_{50} value, 35.3 μM, Table 7) between Hα4β2M·T58K nAChRs and Hα4β2WT nAChRs; however, the confidence limits for these values nearly overlap and suggest that there is no statistical difference (Table 7). COB-1 had a 3-fold decrease in potency on Hα4β2M·T58K nAChRs (IC_{50} value, 5.6 μM, Table 7) when compared to that of Hα4β2WT nAChRs (IC_{50} value, 2.1 μM, Table 7). The largest change in potency of all the NAMs was observed with COB-3 (172-fold change, Table 7).

Table 7. Effects of Ester Region Substituted NAMs (Region 4) on Wild Type and Mutated Human $\alpha4\beta2$ (T58K) nAChRs

		Hα4β2 WT		Hα4β2M.T58K		
Compound	X	IC_{50} Value (μM)[a]	n_h[b]	IC_{50} Value (μM)[a]	n_h[b]	F_m[c]
COB-1		2.1 (1.1-4.2)	-1.1	5.6 (2.4-12.9)	-1.3	2.7
		1.7 (1.3-2.3)	-0.9	2.8 (2.1-3.7)	-1.4	1.6
		0.4 (0.2-0.8)	-1.1	68.6 (48.2-97.6)[d]	-1.5	172
COB-10		13.6 (5.9-31.5)	-1.0	20.8 (8.2-52.6)	-0.9	1.5
COB-9		19.8 (6.3-62.6)	-0.7	35.3 (19.5-63.9)	-0.9	1.8
COB-8		1.1 (0.5-2.4)	-1.0	1.0 (0.7-1.3)	-1.4	0.9
NEB-2		1.0 (0.5-2.0)	-1.3	1.2 (0.4-4.2)	-1.0	1.2

[a]Values represent geometric means (confidence limits), n = 3−6. [b]n_H, Hill coefficient. [c]Fold change of IC_{50} mutant/IC_{50}-wild-type. [d]Statistically significant (p < 0.001).

Table 8. Effects of Ester Region Substituted NAMs (Region 4) on Wild Type and Mutated Human $\alpha4\beta2$ (T58A) nAChRs

		Hα4β2 WT		Hα4β2M.T58A		
Compound	X	IC_{50} Value (μM)[a]	n_h[b]	IC_{50} Value (μM)[a]	n_h[b]	F_m[c]
		1.7 (1.3-2.3)	-0.9	0.27 (0.1-1.0)[d]	-0.6	0.2
		0.4 (0.2-0.8)	-1.1	1.2 (0.4-4.0)[d]	-1.8	3.0
COB-10		13.6 (5.9-31.5)	-1.0	5.8 (1.7-20.2)	-0.6	0.4
COB-9		19.8 (6.3-62.6)	-0.7	17.1 (10.2-28.7)	-1.3	0.9
COB-8		1.1 (0.5-2.4)	-1.0	16.0 (9.9-26.0)[d]	-1.4	14.5
NEB-2		1.0 (0.5-2.0)	-1.3	5.8 (3.5-9.6)[d]	-4.2	5.8

[a]Values represent geometric means (confidence limits), n = 3−6. [b]n_H, Hill coefficient. [c]Fold change of IC_{50} mutant/IC_{50}-wild-type. [d]Statistically significant (p < 0.05).

COB analogues were also tested on Hα4β2M·T58A nAChRs. COB-9 showed no difference in potency between Hα4β2M·T58A nAChRs or Hα4β2WT nAChRs (Table 8). COB-2 showed a 5-fold increase in potency on Hα4β2M·T58A nAChRs (IC_{50} value, 0.27 μM, Table 8) when compared to that

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of Hα4β2WT nAChRs. COB-3 resulted in a 3-fold decrease in potency on Hα4β2M·T58A nAChRs (IC_{50} value, 1.2 μM, Table 8) when compared to that of Hα4β2WT. COB-10 resulted in a 2-fold increase in potency on Hα4β2M·T58A nAChRs (IC_{50} value, 5.8 μM, Table 8) when compared to that of Hα4β2WT nAChRs. COB-8 resulted in a 14-fold decrease in potency for Hα4β2M·T58A nAChRs (IC_{50} value, 16.0 μM, Table 8) when compared to that of Hα4β2WT nAChRs. NEB-2 resulted in a 6-fold decrease in potency on Hα4β2M·T58A nAChRs (IC_{50} value, 5.8 μM, Table 8) when compared to that of Hα4β2WT nAChRs.

DISCUSSION

Here, we have confirmed that KAB-18's activity depends on 3 target amino acids (Glu60, Phe118, and Thr58), which are all found on the β2 nAChR subunit. Two of these amino acids (Phe118 and Thr58) are not conserved among Hα4β2 and Hα3β4 nAChRs and may influence KAB-18's selectivity for Hα4β2 nAChRs. Therefore, better selective antagonists may be discovered through designing small molecules to selectively interact with Thr58 and Phe118 on the β2 subunit.

These studies support the location of KAB-18's putative binding site on Hα4β2 nAChRs but reveal little regarding other NAMs' interactions within this site. For this reason, SAR was conducted with the Hα4β2 nAChR mutants to gain insight into the NAMs' interactions with this allosteric site. We have previously described that aromatic features in Region 1 affect selectivity in Hα4β2 nAChRs.[25] Thus, potential interacting amino acids would be of aromatic or polar nature. Given the computational docking mode of KAB-18, Phe118 was implicated in the interaction with KAB-18's Region 1. The initial studies with control agonist and antagonists (Table 1) suggest that Phe118 does not contribute to functional activity at the orthosteric site as shown when the mutation of Phe118 did not change the potency of epibatidine or *d*-tubocurarine. For this reason, we count Phe118 among the contributing residues that form that allosteric binding site of KAB-18. Although Phe118 is not conserved among different β nAChR subunits, it has been suggested to play a role in the binding of two agonists, epibatidine and A-85380,[33] a finding that contradicts our control data. This residue is found on Loop E of the orthosteric binding site, and previous studies have shown that Loop E residues (e.g., β2 L119) stabilize a water molecule that facilitates hydrogen binding with the agonist, nicotine, at the orthosteric site.[34] Concerning Phe118, Hamouda et al. distinguished that this particular residue was important for mediating selectivity between β2 and β4 subunits.[33] This may be quite similar to the interactions we present between KAB-18 and Phe118. Despite the fact that there are reports showing Phe118 and other Loop E residues contributing to the binding of agonists at the othersteric site, we have previously shown that KAB-18 does not interact with the agonist, epibatidine, on Hα4β2 nAChRs through a competitive mechanism via functional assays[25] and binding assays.[24] It is important to emphasize here that epibatidine was one of the agonists used by Hamouda et al. in the affinity labeling assays which highlighted Phe118 as a contributor to agonist binding; but in our studies of NAMs,[25] there was no parallel shift in epibatidine's agonist-response curve. Instead, there was an observed reduction in epibatidine's maximum efficacy which suggests a noncompetitive interaction between KAB-18 and epibatidine on Hα4β2 nAChRs. We have also shown previously that KAB-18 does not compete with the agonist for the orthosteric site using binding studies in native bovine and recombinant nAChRs.[24] With these results, three amino acids have been identified to be important for the inhibitory effects of KAB-18 at the allosteric site (Phe118, Thr58, and Glu60).

In mutating Phe118, we demonstrated that compounds containing aromatic features in Region 1 have a shift in potency, while compounds without aromatic features in Region 1 show no changes in potency. These data suggest that the interaction with Phe118 is mediated through an aromatic ring in Region 1. Considering the fact that this residue is not conserved, this also suggests that Phe118 may be an important residue that mediates selectivity for Hα4β2 nAChRs.

Previous SAR suggested that the aromatic ring on the benzoate ester (i.e., the top benzene ring) in Region 2 affects selectivity on Hα4β2 nAChRs.[25] Considering the findings with Ser37 and Ser137 mutations, we hypothesized that Region 2 may not interact with residues in this allosteric site but that it may contribute to locking KAB-18 and other NAMs into an orientation that contacts Phe118, Thr58, and Glu60. In addition to KAB-18, FFB-1 was tested to determine whether aromatic rings compared to cyclic, nonaromatic features (IB-2, KAB-30, and KAB-31) in Region 2 alter the NAMs' allosteric interactions (Table 4). Here, a change in potency was observed for aromatic-containing compounds while the cyclic, non-aromatic containing NAMs showed no change. This suggests that aromatic features in Region 2 may be important for preserving KAB-18's physical orientation in the allosteric site.

Previous SARs supports the importance of the carbonyl group placement in Region 4. When adjacent to the biphenyl, it results in selective compounds. When reversed, it results in nonselective compounds.[25] Compounds where the carbonyl group is adjacent to the biphenyl all showed changes in potency on F118L nAChRs (Table 5). Compounds with the carbonyl group reversed (JHB-9 and DDR-3) showed no change in potency. This suggests that the placement of the carbonyl in Region 4 affects the NAMs' interactions with Phe118. This is most likely due to the proposed hydrogen bond between Region 4's carbonyl group and Thr58.

To follow-up on this, studies were conducted with Thr58 mutations to confirm the proposed interaction between the carbonyl in Region 4 and Thr58 (see Tables 6–8). First, Region 4 NAMs were tested with Hα4β2M·T58K nAChRs. DDR-5, DDR-14, and KAB-18 (compounds with the carbonyl adjacent to the biphenyl) all showed a change in potency with the T58K mutation. JHB-9 (the reverse ester of KAB-18) showed no change in potency on the T58K mutation. Surprisingly, the reverse amide (DDR-3) showed a 3-fold change in potency on the T58K mutation. With the T58A mutation, the reverse ester (JHB-9) and reverse amide (DDR-3) showed no change in potency with the T58A mutation. In agreement with the findings on the T58K mutation, KAB-18, DDR-5, and DDR-14 all displayed a change in potency. This result supports the hypothesis that placement of Region 4's carbonyl group is important for an interaction with Thr58. Additionally, since Thr58 is not conserved, the placement of the carbonyl may play a role in the selective antagonism of Hα4β2 nAChRs.

In addition to KAB-18, another set of compounds, designated COB analogues, have also been established as NAMs.[24] Previous SAR established that our COB analogues are the most potent NAMs on Hα4β2 and Hα3β4 nAChRs.[25] Although they are not selective, we decided to gain insight concerning their interactions in this allosteric site. We

confirmed that the COB analogues do not interact with Phe118 (Table 3), and several COB analogues were tested with the Thr58 mutations. On Hα4β2M·T58K nAChRs, only COB-3 showed a significant change in potency. We concluded that COB-3 may bind Thr58; however, the results with the other NAMs may have been obscured by the potential to interact with Lys58. Therefore, they were tested on Hα4β2M·T58A nAChRs (Table 8).

On the T58A mutant, 3 of the NAMs resulted in a decrease in potency: NEB-2, COB-8, and COB-3. COB-2 and COB-10 resulted in an increase in potency, while COB-9 showed no change in potency. COB-8 and NEB-2 showed no difference between Thr58 and Lys58 residues but showed a decrease in potency with Ala58. This suggests two possibilities: (1) these compounds do not bind Thr58 or Lys58, or (2) they bind both residues favorably. Considering their results with the T58A mutation (decrease in potency), we hypothesize that the latter is true. The other COB analogues resulted in no change or an increase in potency with the T58A mutation but a decrease in potency with the T58K mutation. This suggests that they do not bind Thr58, but the lysine at position 58 provides unfavorable sterics, resulting in a decrease in potency. This suggests that the COB analogues bind in an orientation that is different than KAB-18. Therefore, the elucidation of the precise binding mode of these analogues will require more extensive studies, as have been applied to KAB-18 (i.e., blind docking, MD simulations). In the case of COB-3, we do observe the largest change in potency of all the NAMs (172 fold change with T58K mutation, Table 7). This molecule is unique in our library due to the dialkyl addition to its piperidine ring. At physiological pH, lysine has a net positive charge; therefore, we hypothesize that the positive charge of COB-3's piperidine ring may interact unfavorably with the positive electrostatics of the lysine at position 58. Thus, this interaction drastically reduces COB-3's potency.

The hypothesized interaction between KAB-18 and Glu60 is a stable hydrogen bond between the piperidine ring's hydrogen and the carboxyl of Glu60. In this position of the ligand, there is no diversity in our molecular library to pursue SAR as all of our compounds are protonated at the piperidine's N1 atom. The one exception to this is DDR-13 (see compound structure in Table 3). This molecule has an amide at the interface of Region 1 and Region 3, and therefore, the piperidine nitrogen is not protonated. DDR-13 shows no change in potency between Hα4β2WT and Hα4β2M.E60A nAChRs (data not shown). This suggests that the protonated hydrogen plays a role in the proposed bond with Glu60, but more studies need to be conducted using a series of molecules that have more diversity. Given the nature of the assay (fluorimetric assay) used in this work, we cannot at this time determine if these NAMs' effect is due primarily to binding or a modulation of the nAChR's signal transduction. Overall, we admit that due to KAB-18's flexibility, the physical interactions within this allosteric site are clearly complex and warrant further investigation using additional techniques (i.e., electrophysiology and unnatural amino acid manipulation). Despite this complexity, we believe that the mechanism of noncompetitive inhibition does involve binding and affects the transduction of signal through a perturbation of nAChRs' biophysical mechanisms. Through molecular dynamics simulations with KAB-18 and homology models of Hα4β2 and Hα3β4 nAChRs, we have hypothesized that NAM binding to the allosteric site prevents closure of the C loop which in turn prevents opening of the channel.[28] These studies show that even though epibatidine is forming a stable hydrogen bond with the carbonyl oxygen of Trp148, the C-loop is obstructed from closing to an agonist-bound state due to the presence of KAB-18. Typically, the C-loop adopts a Cα−Cα distance of ∼7.4 Å with the binding of agonist to the orthosteric site. In the case of KAB-18, the C-loop adopts a Cα−Cα distance of ∼10.5−12.974 Å for Hα4β2 nAChRs, which is similar to what is found for partial agonists (Cα−Cα 9.75−12.3 Å) and antagonists (Cα−Cα 12.88−16.05 Å).[28] It could be possible that mutations presented in this work do not effect binding affinity but reposition the ligand such that it allows more or less C-loop closure, thereby modulating the amount of transduction observed. However, based on the close proximity between this allosteric site and the orthosteric site (∼10 Å), we suspect that NAM binding and ability to inhibit transduction are closely linked at this site.

In closing, these studies provide insight into the interactions of a class of NAMs at a novel allosteric site. Three amino acids have been identified (Thr58, Glu60, and Phe118) and confirmed to contribute to the inhibitory effects of KAB-18, a molecule that selectively antagonizes Hα4β2 nAChRs. The SAR studies with mutant nAChRs have revealed valuable insight into what physiochemical features influence KAB-18's selective inhibition of Hα4β2 nAChRs at this allosteric site. These studies will contribute to the understanding of selective antagonism of Hα4β2 nAChRs and may help facilitate the design of novel therapeutics for nAChR related diseases, such as nicotine addiction.

METHODS

Materials. The calcium sensitive fluorescent probe, Calcium 5, was obtained from Molecular Devices (Sunyvale, CA). Dulbecco's modified Eagle's medium (DMEM), penicillin, streptomycin, and L-glutamine were obtained from Invitrogen Corporation (Grand Island, NY). nAChR agonists and antagonists (Table 1, with exception of KAB-18) were purchased from Sigma-Aldrich (St. Louis, MO). All other reagents, other than our novel NAMs, were purchased from Fisher Scientific (Pittsburgh, PA). In general, NAMs (see Tables 2−8) were prepared by reaction of hydroxymethyl piperidine with the appropriate alkyl halide to provide the N-alkyl hydroxymethyl piperidine, as previously reported by our laboratory.[35−37] This molecule was then coupled to the appropriate carboxylic acid to provide the target molecule.[36] All molecules were >98% pure as shown by ^{1}H NMR, ^{13}C NMR, and HRMS. All compounds were converted to their hydrochloride or oxalate salts. For pharmacological evaluation, all compounds were initially dissolved in 100% DMSO (0.01 M stocks) due to solubility. Further dilutions of compounds were made in double distilled H_2O or HEPES-buffered Krebs (HBK) solution (≤100 μM).

Calcium 5 Assay (Calcium Accumulation Assay). The calcium 5 procedure was used with minor modifications of a previously published procedure using Fluo-4.[24,25] For this calcium accumulation assay, HEK tsA201 cells transiently expressing either Hα4β2 nAChRs or mutant Hα4β2 nAChRs were used (see below for transfection methods). Cells were plated at a density of 1.5−2.0 × 10^5 cells per well in clear 96-well culture plates previously coated with poly-L-ornithine. On the day of the experiment, cells were washed (100 μL) with HBK solution[25] and incubated (protected from light) for 1 h at 24 °C with 50% Calcium 5 NW dye (Molecular Devices). The plates were then placed into a fluid handling integrated fluorescence plate reader (FlexStation II, Molecular Devices, Sunnyvale, CA), and fluorescence was read at an excitation of 485 nm and an emission of 525 nm from the bottom of the plate with changes in fluorescence monitored at ∼1.5 s intervals. The experimental design involved three treatment periods (20 s, 40 s, and 60 s) and three treatment groups (control−sham treated, control−agonist treated, and antagonist treated). All

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groups were initially incubated in HBK, and fluorescence was monitored for 20 s. For the control–agonist treated groups, HBK (40 μL) was then added, and the fluorescence was monitored for 40 s, followed by the addition of epibatidine (40 μL of a 3 μM solution) to achieve a final concentration of 1 μM. The effects following agonist addition were monitored for 60 s. For the antagonist treated groups, the antagonist (40 μL of a 3× solution) was added, and fluorescence was monitored for 40 s, followed by the addition of epibatidine with the antagonist (at 60 s). The control–sham groups were treated with HBK during each of the three treatment periods, with fluorescence being monitored continuously to establish resting (background) levels of cell fluorescence.

Calculations. Functional responses were quantified by first calculating the net fluorescence (the difference between control–sham treated and control–agonist treated groups). Results were expressed as a percentage of control (1 μM epibatidine). For each drug, six concentrations were used in a series of concentration–response studies. Following transformation to log values, sigmoidal-varied slope curves were fit to data using Prism 4.0 (Graphpad, San Diego, CA). All antagonists that did not exhibit 100% inhibition at concentrations of 100 μM were constrained for maximal inhibition (bottom of curve was set to 0). From these curves, EC_{50} and IC_{50} values were determined for each drug. Functional data were calculated from the number of observations (n) performed in triplicate. Because of the use of log values in calculating the EC_{50} or IC_{50} values, geometric as opposed to arithmetic means were calculated for agonists and antagonists in these studies. All EC_{50} and IC_{50} values are expressed as geometric means (95% confidence limits). Because of solubility problems, compound concentrations greater than 100 μM were not used in our concentration–response studies. The DMSO concentration at this compound concentration was 1% and had no effects on basal or agonist-induced increases in fluorescence intensity. When statistical significance was calculated, the t test was used. To calculate the fold difference (F_m), the IC_{50} value of mutant nAChRs were divided by the IC_{50} value of WT nAChRs. The Hill coefficients (n_h) represented in the table were calculated by taking the mean of each Hill coefficient from individual experiments. For the mean curves in Figure 3 and Supporting Information, Figure 2, the mean Hill coefficients found in the tables were used to fit concentration–response curves.

Site Directed Mutagenesis and Transient Expression of α4β2 nAChRs. Human nAChR α4 and β2 full length cDNAs in the vector pSP64 (poly A) were obtained from Professor Jon Lindstrom (University of Pennsylvania, Philadelphia, PA). The α4 cDNA was subcloned without mutagenesis into the vector pcDNA 3.1+ (Invitrogen) for transfection. The β2 cDNA was subcloned into the vector pcDNA 3.1+Zeo (Invitrogen) and subsequently used for mutagenesis and transfection or unmutated for wild type expression. Single mutations were made in the β2 subunit using the QuikChange II XL Kit or the Lightning Multi Site-Directed Mutagenesis Kit (T58K) following the manufacturer's instructions to produce the mutant Hα4β2 nAChRs. Primers were designed using the QuikChange Primer Design Program (Stratagene) and Oligo 4.0 (National Biosciences). Primers were synthesized by Invitrogen. The following primer was designed to change the Thr (ACC) at position 58 in the β2 subunit to a Lys (AAG): β2 mutant 5′-CCACCAATGTCTGGCTGA**AAG**CAGGAGTGGGAAGATTATCG-3′. The following primer pair was designed to change the Thr (ACC) at position 58 in the β2 subunit to an Ala (GCT): β2 mutant 5′-CCACCAATGTCTGGCTG**GCT**CAGGAGTGGGAAGATTATCG-3′ and 5′- CGATAATCTTCCCACTCCTG**AGC**CAGCCAGA-CATTGGTGG-3′. The following primer pair was designed to change the phenylalanine (TTC) at position 118 in the β2 subunit to leucine (TTG): 5′-TCTCCTATGATGG**T**AGCATC**TTG**TGGCTG-CCGCCTGC-3′ and 5′- GCAGGCGGCAGCCA**C**AAGAT-GCT**A**CCATCATAGGAGA-3′. It should be noted that for the F118L mutation, an additional mutation (T) was introduced, which did not change the coding sequence but relaxed a potential loop in the primer in order to allow for the generation of this mutation. The following primer pair was used to change the Glu (GAG) at position 60 to an Ala (GCG): β2 mutant 5′- CCAATGTCTGGCTGAC**T**-CAG**GCG**TGGGAAGATTATCGC-3′ and 5′- GCGATAATCTTCC-CA**CGC**CTG**A**GTCAGCCAGACATTGG-3′. An additional mutation (T) was introduced, which did not change the coding sequence but relaxed a potential loop in the primer in order to allow for the generation of this mutation.

The following primer pair was used to change the Ser (TCA) at position 37 to an Ala (GCA): β2 mutant 5′-GTACAGCT-TATGGTG**GCA**CTGGCCCAGCTCA-3′ and 5′-TGAGCTGGGC-CAG**TGC**CACCATAAGCTGTAC-3′. The following primer pair was used to change the Ser (AGC) at position 167 to an Ala (GCC): β2 mutant 5′- AAGAGTGAGGTGGCC**GCC**CTGGACGACTTCAC-3′ and 5′-GTGAAGTCGTCCAG**GGC**GGCCACCTCACTCTT-3′. All cDNA clones were completely sequenced using a 3730 DNA Analyzer (Applied Biosystems, Foster City, CA) at the Ohio State University Plant-Microbe Genomics Facility. DNAs used for transfection were purified using PureLink High Pure Mini, Midi, or Maxi Kits (Invitrogen). HEK tsA201 cells (obtained from Dr. Rene Anand, the Ohio State University, Columbus, OH) were transiently transfected with 3 μg of mutant Hα4β2 or wild-type Hα4β2 cDNAs using Lipofectamine 2000 (Invitrogen, Grand Island, NY) in 60 mm dishes. After 7–8 h, the cells were seeded into clear-bottomed 96 well plates for the intracellular calcium accumulation assays.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Effects of KAB-18 on [^{3}H]epibatidine to nAChRs; concentration–response effects of KAB-18 on WT and mutant transiently transfected Hα4β2 nAChRs; and the method used for [^{3}H]epibatidine binding to native and recombinant nAChRs. This material is available free of charge via the Internet at http://pubs.acs.org.

■ AUTHOR INFORMATION

Author Contributions

H.B.J., B.R.T., and M.D.B. participated in research design; H.B.J., G.-C.T.F., Y.B., and P.R.E. conducted experiments; B.S.C., B.R.T., and L.C. contributed new reagents or analytic tools; H.B.J., G.-C.T.F., Y.B., and P.R.E performed data analysis; H.B.J., G.-C.T.F., B.R.T., and M.D.B. wrote or contributed to the writing of the manuscript.

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Notes

The authors declare no competing financial interest.

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ABBREVIATIONS

NAM negative allosteric modulator; nAChRs neuronal nicotinic acetylcholine receptors; LBD, ligand binding domain; HBK HEPES-buffered Krebs; n_h Hill's coefficient

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