Discovery of Novel Negative Allosteric Modulators of Neuronal Nicotinic Acetylcholine Receptors: Implications in the Treatment of Addiction and other Neurological Disorders

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1. INTRODUCTION

1.1 Nicotinic Receptors

Two natural phenomena were responsible for the first identification of nicotinic receptors. The fish genus *Torpedo* was found to use an electric impulse to stun its prey which was later found to be possible through elevated expression of nicotinic receptors in its predatory organ. Secondly, α-bungarotoxin found in Taiwanese krait venom was discovered to bind to muscle-type nicotinic receptors with high affinity resulting in debilitating paralysis. These two discoveries led to further investigation into the physiological relevance of nicotinic receptors [1].

Nicotinic receptors are physiologically important for many bodily processes. These include but are not limited to muscle contraction, synaptic transmission, catecholamine release, and autonomic nervous system signaling [1, 9, 12, 16]. For these reasons, nicotinic receptors have been implicated in various neurological diseases including but not limited to addiction, schizophrenia, Alzheimer's Disease, epilepsy, Attention Deficit Disorder (ADD/ADHD), and autism [1,9,12,14,16].

Neuronal nicotinic acetylcholine receptors (nAChRs) represent an important family of neurotransmitter receptors with diverse expression throughout the central and peripheral nervous system. In the central nervous system, nAChRs are expressed in many neurotransmitter pathways throughout the brain. Peripherally, nAChRs are expressed on autonomic ganglia and play an important role in the regulation of the sympathetic and parasympathetic nervous systems. Also, nAChRs expressed in the adrenal medulla play an important role in
regulating catecholamine release. Further, nAChR stimulation has important
effects on neuronal transmission throughout the brain and modulation of nAChRs
can be profoundly influential on brain function [1,9,12,14,16].

1.2 Structure and Function

nAChRs are markedly diverse in structure and function as demonstrated
by their widespread expression throughout the body. Structurally, nAChRs are
pentameric ligand gated ion channels composed of five protein subunits. The
five subunits are transcribed and translated independently and then assembled in
the endoplasmic reticulum before trafficking to the cellular membrane. The
resulting conformation is a cylindrical receptor with a non-selective cationic pore
[1,9,16]. Endogenously, nAChRs are modulated by the neurotransmitter
acetylcholine which binds to the extracellular domain of nAChRs. This results in
a conformational change in the extracellular domain of the receptor. This change
allows for the opening of the pore in the transmembrane region of the receptor,
and results in the influx of cations, namely calcium and sodium, into the cell. The
influx of cations depolarizes the cell membrane and creates an action potential
and subsequent neurotransmitter release [1,9,16].

It is important to consider the structural diversity of nAChRs. As
discussed above, nAChRs are assembled from five basic subunits. These
subunits are of two types, α(2-10) and β(2-4). Alpha and beta subunits assemble
in a 3:2 or 2:3 stoichiometry which plays an important role in receptor physiology
[13,19]. Generally, ligands bind at the interface between α and β subunits on
heteromeric receptors and between α subunits on homomeric receptors. The
composition and stoichiometry influence whether the receptor has high or low affinity for a particular ligand. Furthermore, postmortem brain studies have demonstrated that patients addicted to nicotine show elevated expression of high affinity α4β2 nicotinic receptors. [10,12,13,19]. In addition, homopentameric nAChRs exist, most notably α7 receptors, which are also expressed in the central and peripheral nervous systems. Pre- and post-synaptic expression of nicotinic receptors also influences their role in synaptic transmission. The varied synaptic expression of nicotinic receptors influences whether particular subtypes are excitatory or inhibitory in certain neuronal pathways which must be considered when utilizing exogenous agents to modulate nicotinic receptors.

nAChRs demonstrate marked conformational instability. Instability allows various ligands to open the cationic pore making the receptor functional. However, shortly following agonist binding, receptors adopt a new quaternary structure that desensitizes the receptor to further stimulation. This is important physiologically because without the phenomenon of desensitization, receptors would be constitutively stimulated by endogenous neurotransmitters resulting in sustained signaling [1,9,16]. Sustained signaling is also prevented through other mechanisms such as neurotransmitter reuptake, receptor down regulation, and degradation of acetylcholine.

To date, pharmacological agents targeting nAChRs primarily bind to the orthosteric or primary binding site [1,9,16]. This is the same site at which acetylcholine binds to nAChRs. Importantly, this site is highly conserved across all nAChR subtypes making it possible for acetylcholine to modulate any nAChR
when released into the necessary synaptic cleft. However, when an exogenous agent is introduced into the body that targets this site, it is more likely to affect all nAChRs that are available for binding. This has proven problematic in this clinic with the development of adverse side effects following treatment with drugs such as Chantix®, which will be discussed later. Therefore, it may be advantageous to target a site other than the orthosteric site on the extracellular domain of the receptor [9,16].

Targeting an allosteric site can possibly introduce selectivity to pharmacological agents targeting nAChRs [9,16]. Since there is much structural diversity in the composition of nAChRs, there must be unique binding domains outside the orthosteric site on various receptor subtypes. A novel binding domain has been previously described by our laboratory [9,16]. Within these novel binding domains, there exist amino acid residues that are specific for a particular receptor subtype. Ligands binding to an allosteric site may induce a conformational change that limits the activation of nAChRs following agonist stimulation [9,16,21]. Such a compound would desensitize the receptor without interfering with agonist binding. For example, it is known that agonist binding results in C-loop closure in the alpha-subunit. Therefore, an antagonist that induces a conformational change that prevents C-loop closure would inhibit the activity of an agonist. Such a ligand would be considered a non-competitive antagonist since it does not interfere with agonist binding but still inhibits the function of the receptor. Furthermore, non-competitive antagonists have the
potential to be designed for various receptor subtypes allowing for selective modulation of nAChRs.

1.4 Implications in Disease

The widespread expression of various nAChRs throughout the brain has implicated them in a variety of neurological brain disorders. These disorders include but are not limited to addiction, schizophrenia, Alzheimer’s Disease, Parkinson’s Disease, epilepsy, Attention Deficit Disorder (ADD/ADHD), and autism [1,9,12,14,16] However, since many of these disorders are still not fully understood, the process by which nAChRs affect them is not well known. Furthermore, the lack of subtype selective pharmacological agents limits our ability to implicate any particular subtype to a given disease.

Nicotine addiction is the leading cause of preventable death in the world with a total health care cost burden of over $92 billion annually [24]. Further, nAChRs have been implicated in the development of addiction as they are the principal mediator of nicotine’s psychoactive properties. nAChRs are expressed in the mesolimbic dopamine pathway in the midbrain. Specifically, α4β2 nAChRs are expressed in the ventral tegmental area (VTA). Expression in the VTA suggests that α4β2 nAChRs are involved in mediating dopamine release into the nucleus accumbens following stimulation by acetylcholine and other agonists [8]. The nucleus accumbens is responsible for dopamine signaling to the prefrontal cortex which is the decision making center of the brain. Therefore, altered signaling from the nucleus accumbens can profoundly affect behavior [8]. This is demonstrated by people who use addictive drugs that affect levels of dopamine
in the brain. This is confirmed by postmortem brain studies providing further evidence of the importance of α4β2 nAChRs in the development of addiction [12].

Autism is a psychobiological brain disease that affects the development of normal cognitive, social, and behavioral traits in affected children. The mechanism by which the disease elicits these symptoms is still poorly understood leading to conflicting and complicated methods of treatment. In addition, environmental factors play a large role in an affected individual’s unique disease profile. These variables have led to a diverse array of therapies including and not limited to communication therapy, social integration, cognitive development, and functional independence [14,15]. However, pharmacological therapies were not utilized until the 1950’s. Furthermore, due to the poorly understood mechanisms of autism, pharmacological treatment has been limited in its ability to effectively alleviate the various symptoms of the disease. To date, antidepressants, antipsychotics, stimulants, benzodiazepines, opiate antagonists, adrenergic antagonists, and lithium salts have been used among many others to treat symptoms of the disease [14,15]. However, the therapy resulting from these treatments remains limited to a particular symptom of the disease, and is rendered ineffective following development of tolerance and side effects or following discontinuation of the drug. These factors demonstrate a need for novel pharmacological agents that can effectively treat multiple symptoms of the disease while minimizing collateral side effects of treatment.
Since autism is a developmental disorder, neurotransmitter systems in the brain have been implicated in the disease because of their crucial role in brain development. Multiple neurotransmitter systems have been studied regarding autism including serotonin, dopamine, adrenaline, GABA, glutamate, and neuropeptides [14, 15]. However, more recently, the cholinergic system has been identified as a crucial influence in the disease with particular emphasis on nicotinic acetylcholine receptors. Interestingly, α4β2 nAChRs have been implicated in addiction and pain sensation. This is particularly striking when considering altered dopaminergic signaling and reduced pain sensation phenotype in autistic patients [14, 15].

Alzheimer’s disease is a major challenge to modern medicine. There are no current therapies that can effectively delay cognitive decline for an extended period of time. By delaying cognitive decline, treatment could push the onset of debilitating disease back, introducing the possibility of another cause of death. This would improve the quality of life of Alzheimer’s patients in their last years of life. Acetylcholinesterase inhibitors (AChEI) are currently used in the clinic as a means of slowing decline. AChEIs increase synaptic acetylcholine by slowing its degradation [18]. Therefore, cholinergic neurons affected by AD will maintain a higher level of signaling despite their degeneration. The clinical efficacy of AChEIs suggests that therapies targeting nicotinic receptors located in neurons degenerated in AD may provide a target for increased cognition and memory.

As stated above, nicotinic receptors are implicated in many neurological diseases. It is important to note that the molecular mechanisms underlying these
disorders are not well understood. Therefore, it is difficult to elucidate the primary role of nicotinic receptors within the pathology of various disease states. The development of selective nicotinic receptor agents will significantly benefit treatment and understanding of neurological disease.

1.5 Current Nicotinic Receptor Agents

To date, numerous pharmacological agents have been identified that target various nicotinic receptors. The majority of these compounds have their origin from natural products. The most common nAChR agonist is nicotine from the genus *Nicotiana* (tobacco). In addition, epibatidine has been identified and isolated from *Epipedobates tricolor*, a poisonous tropical frog. Cytisine is a naturally occurring agonist found in the plant subfamily *Faboideae* [5]. Since there has been a large push for identifying nAChR antagonists for therapy, there are many natural nAChR antagonists that have been identified. These include but are not limited to methyllycaconitine (MLA) from the plant genus *Consolida*, curare from poison dart frogs of the genus *Dendrobatidae*, α-bungarotoxin from the Taiwanese snake *Bungarus multicinctus*, and conotoxins from marine cone snails of the genus *Conus* [1]. Importantly, α-bungarotoxins and some conotoxins demonstrate selectivity for particular nAChR subtypes. However, since they are natural peptides, endogenous proteases would readily degrade them and render them ineffective in the clinic and introduce the possibility of adverse side effects caused by metabolites. Information gleaned from natural products will aid in the discovery of novel nAChR agents.
The advantage of identifying natural products is rooted in the ability to alter their structures in order to make more effective compounds. This was effectively done recently with the development of Chantix®, which was approved by the FDA in 2006 as a smoking cessation aid. Varenicline, the active compound in Chantix, is a derivative of the naturally occurring compound cytisine which is an agonist at nicotinic receptors. The chemical modification resulted in varenicline being a partial agonist at α4β2 nAChRs. As discussed earlier, α4β2 nAChRs are implicated in the development of nicotine addiction and by partially stimulating them, varenicline competitively blocks the binding of nicotine but does not reproduce a full response [8, 17, 20, 25]. Attenuating α4β2 nAChR signaling results in a gradual decline of nicotine craving. However, varenicline has been shown to have activity at other nAChRs subtypes including α3β4, α3β2, and α7 receptors [21,26]. These off target effects have associated multiple neurological side effects with varenicline treatment including behavior changes, agitation, depression, mood swings, and suicidal tendencies [26]. Chantix has been effective as smoking cessation but a selective antagonist would have similar therapeutic effects without the development of consequential side effects.

1.6 Discovery of Novel Negative Allosteric Modulators of the α4β2 nicotinic receptor

As discussed earlier, the α4β2 nicotinic receptor has been implicated in numerous neurological diseases, many of which are believed to be caused by its overstimulation. Therefore, a therapeutic effect can be achieved by attenuating the stimulation of these receptors. It is the goal of our laboratory to identify
potential compounds that are potent and selective antagonists for the \(\alpha 4\beta 2\)
receptor. Importantly, our laboratory is focusing on identifying allosteric
modulators of the \(\alpha 4\beta 2\) receptor, which will allow for targeting unique sites
present only on this receptor subtype.

To date, the laboratory has built a library of molecules that have shown
promise as negative allosteric modulators of nAChRs. These molecules were
generated by taking the naturally occurring compound MLA and synthesizing an
array of analogues that may have different pharmacological profiles. Since MLA
is a selective, competitive antagonist of \(\alpha 7\) nicotinic receptors, synthesizing
analogues of MLA may be advantageous to the discovery of new compounds
that are selective to different receptor subtypes [2,3,4,9,11,16]. Specifically, our
collaborators were able to take a specific section of the MLA molecule, known as
Ring E, and introduce novel chemical properties to its structure [3,4]. This
resulted in molecules that demonstrate a preference for \(\alpha 4\beta 2\) nAChRs as
compared to \(\alpha 3\beta 4\) nAChRs, which are widely expressed in the brain. [Henderson
et al 2010; in review]

Using the new molecular scaffolds from the synthetic process, structure
activity relationship studies (SAR) are being employed to continue to improve the
pharmacological activity of our molecules. Structure activity relationships
examine the effect of chemical substitutions on the activity of small molecules.
[2,3,4,26] Additionally, computational approaches are being used to gather
spatial and biochemical information about our molecules and the putative binding
pocket within the \(\alpha 4\beta 2\) ligand binding domain. A collaborator of the McKay
Laboratory, Dr. Chenglong Li, is focused on developing a homology model of the human α3β4 and human α4β2 nAChRs. Homology is defined as the biochemical similarity between species. Therefore, homology modeling is focused on using information from characterized species and translating it to human structures. Development of this model has proven challenging due to the lack of a crystalline structure of human nicotinic receptors. Crystallization is difficult because of the pentameric nature of nicotinic receptors. However, a model was generated using structural information from crystallized molluskan nicotinic receptor subunits and sequence data from the α3β4 and α4β2 nAChRs. Using sophisticated molecular modeling software, the α3β4 and α4β2 nAChRs were superimposed on the defined structure of the molluskan subunits. The homology model reveals interactions between drugs and the amino acid residues within the binding site. Knowledge of these interactions will facilitate understanding of the biochemical properties necessary for potency and selectivity. Following construction and validation of the homology model, it can be utilized in site directed mutagenesis studies, rational drug design, and virtual screening.

Our goal is to utilize a multidisciplinary approach to continue to identify novel molecular scaffolds that will provide information regarding increasing potency while maintaining selectivity towards the α4β2 nAChR. This paradigm facilitates rational drug design and will allow for the discovery of a potent, selective antagonist of the α4β2 nAChR.
2. APPROACHES

2.1 Intracellular Calcium Accumulation Assay

Since activation of nicotinic receptors results in the flow of calcium ions into the cell, monitoring levels of intracellular calcium provides information about the activity of nicotinic receptors in real time. Our laboratory utilizes a fluorescent calcium bioassay to monitor calcium levels.

Fluo-4-acetoxymethyl ester (Fluo-4-AM) is a synthetic calcium indicator that fluoresces at a wavelength of 500-550 nm when bound to calcium. When loaded onto cells, the ester linkages of Fluo-4-AM are cleaved by cellular esterases allowing the probe to enter the cell. Once cleaved, Fluo-4-AM can bind calcium ions and fluoresce. Cells are also treated with Pluronic F-127 to help solubilize the Fluo-4-AM. Finally, cells are treated with Probenecid, a calcium channel blocker, to prevent any off-target calcium influx and leakage of Fluo-4-AM out of the cells.

HEK-293 cells stably expressing human α3β4 or human α4β2 nicotinic receptors are used for this assay. Cells are collected and seeded at a density of 230,000 cells per well (α3β4) or 200,000 cells per well (α4β2) into clear 96-well plates. After 48 hours, cells are washed with Hepes Buffered Krebs (HBK) solution and loaded with Fluo-4-acetoxymethyl ester (Fluo-4-AM), Probenecid, and Pluronic F-127 dissolved in HBK at concentrations of 2 μM, 2.5 mM, and <.1% respectively. The cells are incubated for 30 minutes at 37°C followed by 30 minutes at room temperature. After the second incubation period, the cells are washed with HBK with 2.5 mM Probenecid (HBKP) and loaded with 80 μL of
HBKP. The cells are then placed into a Flexstation (Molecular Devices, Sunnydale, CA) for the experiment. The treatment protocol is as follows.

<table>
<thead>
<tr>
<th>Table 1. Treatment groups and conditions.</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Group</strong></td>
</tr>
<tr>
<td>1  Sham Treatment Group</td>
</tr>
<tr>
<td>2  Agonist Treatment Group</td>
</tr>
<tr>
<td>3  Experimental Treatment Group</td>
</tr>
</tbody>
</table>

Group 1 is used to establish a baseline level of fluorescence for non-treated cells. Group 2 is treated with epibatidine (agonist) at time 2 to establish maximal fluorescence following stimulation. Finally, Group 3 is treated with varying concentrations of antagonist (drug) at time 1 as a pretreatment and with both epibatidine and antagonist at time 2. Fluorescence readings are taken every 1.7 seconds to monitor changes in intracellular calcium. Drug effects are presented at percentages of maximal stimulation at the various doses. Using both cell types, we are able to assess the differential ability of antagonists to affect different receptor subtypes.

2.2 Ligand Based Computational Modeling

Ligand Based Computational Modeling is focused on utilizing functional and structural information from various molecules to examine biochemical interactions that may be involved in binding. To do this, molecules of interest are aligned in three dimensional space using Genetic Algorithm Similarities Program (GASP). GASP alignment provides a three dimensional conformation of the molecules in their lowest energy state. Following alignment, the software can integrate structural information to elucidate chemical properties that are
conserved across the molecules that may be important in binding affinity. This biochemical information is then cross referenced to functional data to further examine the importance of the conserved features.

*CoMFA (Comparative Molecular Field Analysis) and CoMSIA (Comparative Molecular Similarities Indices)*

CoMFA and CoMSIA utilize structural and functional information to create a three dimensional contour map that provides biochemical information about the binding pocket accessed by the molecules used to generate the model. Specifically, CoMFA and CoMSIA examine steric, electrostatic, hydrogen bonding, and hydrophobic properties of the molecules and their proposed binding pocket. These models provide information regarding specific molecular properties that enhance potency and selectivity of the given molecules and promote the ability for rational drug design.

**2.3 Pharmacophore Development and Virtual Screening**

Following GASP alignment and CoMFA/CoMSIA, a pharmacophore is generated unique to the molecules of interest. A pharmacophore is defined as a molecular framework that contains biochemical properties necessary for a molecule’s biological activity. Once defined, the pharmacophore can be used to screen virtual libraries for molecules that possess the desired chemical features outlined by the pharmacophore. Using UNITY software, SYBYL can integrate structural information from molecules in virtual libraries and compare them to the features in the pharmacophore. Multiple parameters can be adjusted to increase or decrease the stringency of the comparison. The UNITY screen results in “hit” molecules that are potential candidates for further functional characterization.
3. RATIONALE

Using the medium throughput calcium accumulation assay previously published by our laboratory, we have the ability to assess the pharmacological activity of our library on human α4β2 and α3β4 receptors [9,16]. Since stimulation of nAChRs results in calcium influx, we aim to find molecules that inhibit calcium influx for specific subtypes of nAChRs. KAB-18, our lead compound, has been shown to have an inhibitory effect on the α4β2 nAChR (IC$_{50}$ =13.5 µM) while maintaining lower affinity for the α3β4 nAChR (IC$_{50}$ >100 µM). This data suggests that KAB-18 provides a scaffold for developing nAChR selective drugs.

To date, our laboratory has identified three molecular entities within KAB-18 that are necessary for selectivity. These areas were identified using data from compounds IB-2, COB-1, and APB-21.

A. ![Image of KAB-18 and its regions](image)

**Figure 1.** a) Illustration of three regions of importance in KAB-18, our lead molecule. The biphenyl (green-Region 2), the carbonyl (red-Region 4), and the phenylpropyl (blue-Region 1) regions were identified as important through investigating compounds IB-2, APB-21, and COB-1, respectively. b) Functional data generated from the fluorescent calcium accumulation assay. These data identify three regions in the KAB-18 scaffold that are important in activity for the α4β2 receptor.
<table>
<thead>
<tr>
<th>Compound</th>
<th>Structure</th>
<th>α4β2 (IC&lt;sub&gt;50&lt;/sub&gt;)</th>
<th>α3β4 (IC&lt;sub&gt;50&lt;/sub&gt;)</th>
</tr>
</thead>
<tbody>
<tr>
<td>KAB-18</td>
<td>(X = (\text{CH}_2)_3\text{Ph}) (Y = \text{O}) (Z = \text{Ph})</td>
<td>13.5 (9.7-18.5)</td>
<td>No Activity*</td>
</tr>
<tr>
<td>IB-2</td>
<td>(X = (\text{CH}_2)_3\text{Ph}) (Y = \text{O}) (Z = \text{Ph})</td>
<td>10.5 (7.4-14.8)</td>
<td>10.9 (5.1-23.3)</td>
</tr>
<tr>
<td>APB-21</td>
<td>(X = (\text{CH}_2)_3\text{Ph}) (Y = \text{H}) (Z = \text{Ph})</td>
<td>12.6 (3-53.8)</td>
<td>15.5 (8.4-29.0)</td>
</tr>
<tr>
<td>COB-1</td>
<td>(X = \text{H}) (Y = \text{O}) (Z = \text{Ph})</td>
<td>7.1 (5.4-9.4)</td>
<td>8.1 (4.2-15.7)</td>
</tr>
</tbody>
</table>

A primary area of concern for targeting the α4β2 nAChR is identifying molecules that are potent. To date, our laboratory has identified a group of molecules that demonstrate activity on the α4β2 nAChR while exhibiting little to no effect on the α3β4 nAChR. These molecules are KAB-18, DDR-5, DDR-13, DDR-18, and SMB-1. However, the fact that these molecules exhibit affinities in the low micromolar range brings into question the degree to which they are truly selective. The laboratory is now taking a direction to utilize in silico screening using novel pharmacophores to potentially identify new scaffolds that may be more potent. Once a nanomolar potent compound is identified, we can utilize...
information about molecular entities important in selectivity (Fig 1.b) to design a potent and selective drug.

3.2 Introduction of Chemical Modification

Within our library of molecules, we have identified three molecules that contain substitutions in the KAB-18 molecule that maintain selectivity for the α4β2 receptor. One of these molecules, DDR-5 substitutes an amide linkage for the ester linkage found in KAB-18. The rationale behind this substitution is the idea that an amide would be resistant to endogenous esterase activity that would rapidly cleave the KAB-18 molecule. This substitution has no significant effect on affinity for the α4β2 receptor and maintains little to no affinity for the α3β4 receptor. DDR-13 contains an additional carbonyl in Region 1 of KAB-18 which produces a statistically significant increase in affinity for the α4β2 receptor while preserving little to no effect on the α3β4 receptor. Similarly, DDR-18 introduces a triazarene ring into Region 1 also resulting in a statistically significant increase in affinity for α4β2 receptor while maintaining little to no affinity for the α3β4 receptor. These studies demonstrate that molecular substitutions can be made to KAB-18 without affecting apparent selectivity. Additionally, two substitutions were identified that increase potency, both of which were located in Region 1. These molecules are documented in Table 2.
Table 2.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Structure</th>
<th>Hα4β2 (IC₅₀ μM)</th>
<th>Hα3β4 (IC₅₀ μM)</th>
</tr>
</thead>
</table>
| KAB-18   | X = (CH₂)₃Ph  
Y = O        | 13.5 (9.7-18.5) | No Activity*    |
| DDR-5    | X = (CH₂)₃Ph  
Y = NH       | 19.8 (8.3-47.1) | No Activity*    |
| DDR-13   | X = CHO(CH₂)₂Ph  
Y = O        | 5.7 (3.6-9.0)  | No Activity*    |
| DDR-18   | R =           
Y = O        | 6.4 (3.7-11.1)  | No Activity*    |

Since preliminary experiments suggested that Region 1 may provide a template for molecular substitution that increases potency, we examined our library for other molecules that possess novel molecular entities in Region 1. One such molecule was found in KAB-32 that showed increased affinity for the α4β2 receptor as compared to KAB-10 which did not contain the novel substitution. These data are shown in Figure 2.
3.3 Hydrogen Bond Acceptors in Region 1

Following the identification of KAB-32, we sought to identify molecules with a similar substitution in Region 1. The oxygen in the ether linkage of KAB-32 contains two lone pairs of electrons that can accept a hydrogen bond from an amino acid residue in the binding pocket of the receptor. Therefore, any
substitution that has the potential to accept a hydrogen bond in this region may be advantageous. Figure 3 documents a group of molecules that contain acceptor atoms in Region 1.

A.

**Figure 3.** a) Structures of the Region 1 molecules. Highlighted in blue are the substitutions that may act as hydrogen bond acceptor atoms from amino acid residues in the binding pocket. b) Functional data showing that the Region 1 molecules maintain a general trend towards higher affinity for the α4β2 receptor as compared to KAB-18.
While the Region 1 molecules show promise for introducing higher affinity for the α4β2 receptor, they do not show selectivity. This is most likely because of the succinamide ring in Region 2 of these molecules. The effect of a succinamide is documented in Table 1. IB-2 contains the succinamide and increases affinity for the α3β4 receptor. Since IB-2 does not show a significant increase in potency compared to KAB-18, we can infer that the increased affinity of the Region 1 molecules is due to the hydrogen bond acceptor in Region 1 and not the succinamide. Unfortunately, many of the Region 1 molecules cannot provide all of the chemical substitutions that are desired. However, the hydrogen bond acceptor moiety may provide insight into a chemical substitution that increases potency.

3.4 Evidence from Homology Modeling

In addition to functional evidence for an increase in potency, structural information provided by computational modeling of the extracellular domain of the α4β2 receptor provides additional insight into why these molecules maintain higher affinity. Through an iterative process of blind docking, and molecular dynamics simulation, a putative binding pocket for KAB-18 has been identified. The computational model of the proposed binding pocket can reveal unique molecular interactions that provide information regarding the differential activity of various molecules.
Figure 4. a) Structural model of the extracellular domain of the α4β2 receptor. The highlighted region is at the interface of an alpha and beta subunit. The alpha/beta interface is the traditional location of agonist binding. b) View of the putative binding pocket of KAB-18. This view provides much information about biochemical interactions that are important for activity. Multiple amino acid residues are shown to be in proximity to the KAB-18 molecule suggesting that non-covalent interaction with these residues is paramount for KAB-18 activity. Eight residues hypothesized to be involved in binding are highlighted in green.

Site directed mutagenesis has been employed to validate the binding site of KAB-18. Since the carbonyl adjacent to the biphenyl of KAB-18 has been shown to be important in selectivity, it would be advantageous to identify the amino acid residue with which it interacts. The α4β2 receptor model reveals that
Thr58 is in close proximity to the carbonyl providing an environment for hydrogen bonding. Therefore, by changing the Thr59 residue to its corresponding Lys59 residue in the α3β4 receptor, KAB-18 affinity should decrease. Indeed, a loss of affinity is seen when this mutation is introduced suggesting that the putative binding pocket is in the right area [Henderson et al 2010; in review].

Blind docking of the Region 1 molecules reveals a unique binding mode as compared to KAB-18. An additional feature of the Region 1 molecules is the size of the Region 1 substitution as well as the presence of a hydrogen bond acceptor atom. Therefore, the increased size may allow them to access new residues for non-covalent interaction, specifically hydrogen bond donor residues. Four molecules were docked to the α4β2 receptor model to visualize their binding mode. These molecules are KAB-32, KAB-34, DDR-18, and SMB-1 and their binding modes are illustrated in Figure 5. To enhance the translational nature of the modeling, the succinamide ring was replaced with a phenyl group in KAB-32, KAB-34, SMB-1 so that they may more closely resemble KAB-18. Therefore, we can compare the difference in binding modes of the Region 1 molecules and KAB-18 and attribute the differences to the Region 1 substitution alone.
**Figure 5.** a) Putative binding modes of KAB-32, KAB-34, DDR-18, and SMB-1 are shown in pink. The binding modes reveal that all four molecules fit into a binding pocket not accessed by KAB-18 (green). b) A close up view of the binding pocket accessed by KAB-32. Three additional amino acid residues are present in this pocket that may provide additional points of non-covalent interaction.
Docking the Region 1 Molecules to the homology model reveals that they may be accessing an additional binding pocket that does not interact with KAB-18 providing further evidence for investigation of substitutions in Region 1.
4. RESULTS

4.1 Pharmacophore Development

A novel pharmacophore was developed using KAB-32, KAB-34, KAB-37, and JHB-11. All four of these compounds contain an ether oxygen in the same position of the molecule that acts as a hydrogen bond acceptor. Further, the succinamide rings were substituted for phenyl rings to make the model more closely resemble our selective molecules.
Figure 6. a) GASP alignment of KAB-32, KAB-34, KAB-37 and JHB-11 demonstrating the conserved nature of an acceptor atom in Region 1. b) Pharmacophore defined by the Region 1 Molecules. (biphenyl substituted; AA= acceptor atom, HYD=hydrophobe). The novel acceptor atom is located in Region 1 of the pharmacophore.

4.2 Chembridge Hits and Screening Results

The pharmacophore outlined above was utilized in a UNITY Flex Search using SYBYL software. The UNITY Flex Search screens virtual molecular libraries for resemblance to the pharmacophore. The flexible nature of the screen allows the molecules of interest to be molded to best fit the pharmacophore. Therefore, when a molecule contains the molecular entities of interest, and can be conformed to resemble their arrangement in three dimensional space as defined by the pharmacophore, it will be identified as a hit. Hits are also scored and ranked based on the “closeness of fit” to the pharmacophore.

ChemBridge™ is a proprietary chemical company that contains over 700,000 compounds for drug discovery and development. Additionally, they have organized subsets of molecules that have specific qualities. Of interest to our laboratory is the CNS Set which is a collection of 10,000 molecules that can cross the blood brain barrier and have activity in the CNS. Since the blood brain barrier is a major obstacle in the oral bioavailability of compounds, the CNS Set provides a reservoir of compounds that are potentially clinically relevant. The CNS Set was screened in a UNITY Flex Search with the novel pharmacophore and revealed 109 “hits”. The top 20 hits were further characterized using the fluorescent calcium assay.
Figure 7. Single concentration (50 μM) screening of the top 20 Chembridge compound hits. The large majority of the 20 compounds demonstrate some affinity for the Ha4b2 receptor as well as the Ha3b4 receptor. However, compounds 9011677, 9010616, 9010615, 9002758, and 9055881 seem to have a significant effect on Ha4b2 receptors.
Figure 8. Structures of five scaffolds identified by virtual screening. These structures have no direct similarity to the compounds that have been characterized functionally in our laboratory. Therefore, they may be potential new scaffolds that possess high affinity for the α4β2 receptor.
The five molecules showing the highest degree of inhibition (Figure 8) were further characterized with full dose response curves using the calcium accumulation assay (Figure 9). Of the five molecules, two demonstrated low micromolar potency on α4β2 receptors. However, compound 9010616 demonstrated ~5 fold selectivity for α4β2 receptors (IC_{50} = 8.2 µM) as compared with α3β4 receptors (IC_{50} = 39.6 µM). These data suggest that 9010616 is a potential candidate for further structure activity relationships that may enhance potency while maintaining selectivity.
**Figure 9.** Inhibition curves documenting the activity of the five novel scaffolds. In the case of 9010616, 9010615, and 9055881 a rightward shift is seen in the α3β4 curve suggesting some degree of selectivity for α4β2 receptors.

### 4.3 Structure Activity Relationship Results

Three compounds that have similar structural properties to 9010616 were selected for additional Structure Activity Relationship Studies. These molecules are 9030037, 9021097, and 9029167. Compound 9030037 introduces a fluorine group and a pyridine ring, compound 9029167 contains an indole ring, and compound 9021097 introduces a fluorine group and a 3-chloropyridine ring to the 9010616 scaffold. Structures and functional activity of these molecules are shown in Figure 10.

**Figure 10.** a) Structures of 9010616, 9030037, 9029167, and 9021097. Novel substitutions are highlighted in green. b) Inhibition curves showing activity of 9030037, 9029167, and 9021097. c) Tabulated IC$_{50}$ values for the three novel molecules on α4β2 and α3β4 receptors.
A.  

9010616  

9030037  

9029167  

9021097  

B.  

![Graphs showing fluorescence intensity vs. [9030037] M](image)  

![Graphs showing fluorescence intensity vs. [9029167] M](image)  

![Graphs showing fluorescence intensity vs. [9021097] M](image)  

C.  

<table>
<thead>
<tr>
<th></th>
<th>Ha4β2 IC50 (μM)</th>
<th>Ha3β4 IC50 (μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>9010616</td>
<td>8.2 (4.3-15.5)</td>
<td>39.6 (17.4-90.1)</td>
</tr>
<tr>
<td>9030037</td>
<td>9.5 (1.37-66.2)</td>
<td>&gt;100</td>
</tr>
<tr>
<td>9029167</td>
<td>8.6 (3.8-19.8)</td>
<td>12.8 (6.6-24.8)</td>
</tr>
<tr>
<td>9021097</td>
<td>6.86 (1.81-26.0)</td>
<td>26.9 (12.3-58.8)</td>
</tr>
</tbody>
</table>
Compounds 9030037, 9029167, and 9021097 do not show a significant increase in potency as compared to 9010616. 9030037 shows activity at α4β2 receptors (IC₅₀ = 9.5 μM) while having little to no activity at α3β4 receptors (IC₅₀ >100 μM) suggesting that the fluorine and pyridine substitution are advantageous for selectivity. Both 9029167 and 9021097 showed increases in apparent affinity for α3β4 receptors suggesting that an indole and chloropyridine substitutions are not advantageous. These SAR data suggest that further chemical modification of the current molecular scaffolds may introduce more desirable pharmacological activity.
5. Discussion

5.1 Discussion of Chembridge Compounds

The Chembridge compounds that were identified through ligand based virtual screening demonstrate a moderate effect on α4β2 nAChRs. Specifically, 9010615 and 9010616 showed low micromolar potency while maintaining a 2- and 4-fold degree of selectivity respectively. It is important to note that while the activity of these molecules does not show a significant increase in potency or selectivity, their chemical structures are more bioavailable than the MLA analogs such as KAB-18. This means that these scaffolds may be used in additional structure activity relationship (SAR) studies. Within such a study, chemical modifications can be made to increase the potency of these compounds while maintaining their desirable pharmacokinetic properties.

SAR studies of compound 9010616 suggest that chemical modifications have an effect on selectivity. Compounds 9030037, 9029167, and 9021097 showed no significant change in potency when compared to 9010616. However, introducing a fluorine and pyridine ring, as seen in compound 9030037, results in a loss of affinity for α3β4 receptors thereby increasing the selectivity of the scaffold. While these modifications do not improve the primary goal of potency, the data provides further evidence for continuing SAR studies to alter the activity of our compounds.

In this study, ligand based virtual screening resulted in the identification of novel scaffolds that possess activity at nicotinic receptors. This is evidence that the paradigm of pharmacophore development and virtual screening has the
potential to identify compounds of direct clinical relevance. Through further SAR studies, chemical entities that are important for potency and selectivity will be identified and the incorporation of such entities into the pharmacophore will yield increasingly relevant compounds. Furthermore, utilizing homology modeling, we will be able to predict chemical modifications to introduce based on amino acid residues in the binding pocket. Our multidisciplinary approach to rational drug design will aid in the development of a potent and selective antagonist to the α4β2 receptor.

5.2 Future Directions

The continued development of the homology model will accelerate the drug discovery process. Using site directed mutagenesis to validate the model allows for the identification of specific amino acid residues within the binding pocket that interact with our compounds. This is advantageous because we can rationally design a compound that has a stronger interaction with particular residues within the desired α4β2 receptor pocket and weaker interaction with residues in an undesired pocket from another subtype.

Structure activity relationships continue to be utilized with the ChemBridge™ molecules. Dr. Rob Coleman, a medicinal chemist, is collaborating with the lab to synthesize rationally designed compounds based on knowledge gained from the ChemBridge™ molecules. This has allowed the laboratory to employ functional assays, ligand based computational modeling, homology modeling, and medicinal chemistry in the rational design of our drugs.
The multidisciplinary approach will aid in the development of a clinically relevant, potent, and selective modulator of the α4β2 nAChR.

5.3 Alternative Directions

While the laboratory’s primary goal is identifying drugs targeting the α4β2 receptor, we also have the potential to investigate compounds targeting the α3β4 receptor. Since our calcium accumulation assay screens for both receptors, we can identify drugs with activity on α3β4 receptors. In a similar paradigm as described above, we have the ability to rationally design a drug that is potent and selective to the α3β4 receptor which would have applications distinct from an α4β2 receptor drug. Further, this approach could be applied to any nAChR subtype and has the potential to influence the discovery of novel therapeutics as well as establishing the physiological roles of various subtypes in neurological disease.
6. Selected References


