Elaboration and Design of α7 nAChR Negative Allosteric Modulators

Osama I. Alwassil

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ELABORATION AND DESIGN OF α7 nAChR NEGATIVE ALLOSTERIC MODULATORS

A dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy at Virginia Commonwealth University

By

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Acknowledgment

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<tr>
<td>3D</td>
<td>Three-dimensional</td>
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<tr>
<td>5-HT</td>
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Abstract

ELABORATION AND DESIGN OF α7 nAChR NEGATIVE ALLOSTERIC MODULATORS

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α7 Neuronal nicotinic acetylcholine receptors are one of two major classes of receptors responsible for cholinergic neurotransmission in the central nervous system. The existence of α7 neuronal nAChRs in different regions of the nervous system suggests their involvement in certain essential physiological functions as well as in disorders such as Alzheimer’s disease (AD), drug dependence, and depression. This project was aimed toward the discovery and development of small–molecule arylguanidines that modulate α7 nAChR function with improved subtype-selectivity through an allosteric approach. Identifying the required structural features of these small molecules allowed optimization of their negative allosteric modulator (NAM) actions at α7 neuronal nAChRs. MD-354 (3-chlorophenylguanidine) was the first small–molecule NAM at α7 nAChRs; however, it also binds at 5-HT3 receptors. The N-methyl analog of MD-354
appeared to be more selective toward α7 nAChRs than 5-HT₃ receptors. Comparative studies using two series of novel compounds based on MD-354 and its N-methyl analog explored the aryl 3-position and investigated whether or not the MD-354 series and the N-methyl series bind in the same manner. Biological potencies of the MD-354 series and the N-methyl series of compounds, obtained from electrophysiological assays with *Xenopus laevis* oocytes expressing human α7 nAChRs in two-electrode voltage-clamp assays, showed that *N*-(3-iodophenyl)-*N*-methylguanidine (28) is the most potent analog at α7 nAChRs. Our comparative study and Hansch analyses indicated different binding modes of the two series.

In addition, we investigated: i) the length/size of the aliphatic side chain at the anilinic nitrogen, ii) the effect of alkylating the guanidine nitrogen atoms, and iii) the necessity of the presence of these nitrogen atoms for the inhibitory effects of arylguanidines at α7 nAChRs.

In efforts to explain the varied functional activity of these arylguanidines, homology models of the extracellular domain and the transmembrane domain of human α7 nAChRs were developed, allosteric sites identified, and docking studies and hydropathic analysis conducted. The 3D quantitative structure-activity relationships for our compounds were also analyzed using CoMFA. A pharmacophore for arylguanidines as α7 nAChR NAMs was identified.

Together, these data should be useful for the subsequent design of novel arylguanidine analogs for their potential treatment of neurological disorders.
I. Introduction

In the human body, neurotransmitter receptors play a central role in cellular communication within various kinds of organs and tissues. They are vital proteins with a wide range of recognition capacity allowing them to process precise signal transduction, specifically in neurons. The localization and density of all neurotransmitter receptors on neuronal cells in the central nervous system (CNS) and the peripheral nervous system (PNS) are highly regulated.\textsuperscript{1,2} Generally, there are numerous types of receptors and, among them, only one superfamily of receptors, the ligand-gated ion channel (LGIC) superfamily, exhibits a significantly rapid role in the processing of chemical-to-electrical transduction during neuronal signaling.\textsuperscript{2} Cholinergic neurons are characterized by the storage, release, and stimulation of the neurotransmitter acetylcholine (ACh) in the CNS and PNS.\textsuperscript{2} At intercellular connections (i.e., synapses), the release of ACh from presynaptic neurons into the synaptic cleft initiates channel opening of nicotinic acetylcholine receptors (nAChRs), one of the most widely studied families of the LGIC receptors, in the postsynaptic region. This initiation of the open channel leads to the transfer of ions across the cell membrane and to depolarization at the motor endplate and subsequent stimulation of the targeted tissue. The family of nAChRs are part of the Cys-
loop family of receptors, which were named because of the presence of a well-known disulfide bridge in their N-terminal domain within the superfamily of LGIC receptors.

Neuronal nAChRs are the center of many extensive scientific investigations because of their involvement in numerous important neurophysiological pathways such as cognitive learning and memory, synaptic plasticity, and neuroprotection.\(^3\)\(^4\) In addition, nAChRs exist in discrete locations and are involved in distinct processes such as arousal, cerebral blood flow and metabolism, and inflammation. Also, nAChRs can be located presynaptically for the regulation of neurotransmitter release, and both pre- and postsynaptic nAChR expression is altered by numerous pathophysiological disorders. Among these disorders are two critical worldwide health problems of current interest that are believed to be mediated through nAChRs: i) dementia and ii) certain aspects of drug abuse. At the molecular level, however, there is little information about these receptors, and their gating mechanism (i.e., ligand affinity and selectivity, dynamics of channel activation/inactivation, ion specificity and conductance, and desensitization properties) is still poorly understood.

i) Alzheimer’s disease (AD) is one of the most costly neuropsychiatric disorders, even when compared to other diseases such as stroke and heart disease. AD is a common form of dementia estimated to cause in all ages 5.3 million cases in 2015 in the US.\(^5\) Among those, approximately 5.1 million Americans are age of 65 or older, and around 200,000 Americans experienced early onset AD (i.e., under age 65). It is also estimated that an additional 3.5 million health care personnel will be needed by 2030 to preserve the present ratio of health care personnel to elderly Americans.\(^5\) Consequently, health care, extended term care and clinical expenses, are anticipated to drastically rise from
$203$ billion in 2013 to an estimated $1.2$ trillion by the year 2050. The available current therapies on the market are associated with severe side effects and do not effectively cover the problem of disease progression or improving patients’ quality of life. An optimal treatment should provide sufficient effectiveness to halt the progression of AD symptoms or even reverse the phases of this disease.

ii) Drug of abuse (specifically marijuana abuse) is a problem that is estimated to cause around 1,243,000 cases seeking treatment per year in the United States. This number is more than the number of patients looking for treatment for cocaine or heroin use.

There are indications that agents that can antagonize the effects of ACh are of therapeutic value. There are two types of such agents: i) direct-acting antagonists (i.e., those that block the effect of ACh at orthosteric binding site), and ii) indirect-acting antagonists (i.e., those that act via an allosteric mechanism, such as negative allosteric modulators). Arylguanidines were identified in our laboratory as the first novel class of small–molecule negative allosteric modulators (NAMs) of $\alpha7$ nAChRs (e.g., $m$-chlorophenylguanidine mCPG; MD-354; $IC_{50} = 7.98 \, \mu$M). Because of a selectivity problem associated with MD-354 (i.e., it also binds at 5-HT$_3$ receptor), we previously investigated arylguanidine structural components that are required for $\alpha7$ nAChRs and that detact from 5-HT$_3$ receptor binding. Here, we continue our previous investigation by examining different structural aspects of arylguanidines that are required for $\alpha7$ nAChR action using various medicinal chemistry approaches in order to develop novel analogs with an improved selectivity and inhibitory potency for $\alpha7$ nAChRs.
As a powerful tool in medicinal chemistry, molecular modeling has proven to provide significant molecular insight into various bimolecular interactions. It would be instructive to examine the possible interaction site(s) and mechanisms by which small-molecule α7 nAChR NAMs work. Since a crystal structure of the human α7 nACh receptor has not yet been determined, three-dimensional models of the extracellular domain (ECD) and the transmembrane domain (TMD) of human α7 nAChRs will be constructed in this work based on available nAChR crystal structures from different species. The results should allow, with certain caveats, for detailed insight about α7 nAChR allosteric sites and enhance the effort to develop novel small–molecule NAMs.

The present investigation is aimed at understanding why and how arylguanidines work as NAMs of ACh action at α7 nACh receptors.
II. Background

A. Cholinergic Receptors

In the CNS, cholinergic neuronal networks might represent the most important neuromodulatory neurotransmitter system.\textsuperscript{2,10} This cholinergic system is widely distributed and exists in both projection neurons and interneurons.\textsuperscript{11,12} The primary function of these neurons is to control activities that depend on selective attention, and to regulate higher cognitive functions such as memory and learning.\textsuperscript{2} Two major pathways that are considered the sources of the cholinergic system are the basal-forebrain cholinergic neurons and the pedunculopontine–lateral dorsal tegmental neurons where they project by widespread innervation to almost every area of the brain (Figure 1).\textsuperscript{13,14} In addition, cholinergic interneurons exist in multiple brain regions such as the striatum and neocortex where they provide innervation for nearby neurons.\textsuperscript{15,16} Both types of cholinergic neurons originate from the laterodorsal tegmental nucleus (LDT) or pedunculopontine tegmental nucleus (PPT), and cholinergic interneurons in the striatum regulate the mesolimbic dopamine system through acetylcholine receptors (AChRs) expressed on multiple neuronal populations within the system (e.g., dopaminergic neurons).\textsuperscript{15,16}
Nicotinic acetylcholine receptors (nAChRs) are one of two major classes of receptors responsible for cholinergic neurotransmission in the central nervous system (CNS).\textsuperscript{18} Cholinergic neurotransmission first requires the assembly of the neurochemical mediator, acetylcholine (ACh), by the synthetic effect of choline acetyltransferase (CAT) in presynaptic cholinergic neurons. The neurotransmitter ACh interacts with nAChRs as well as with the other class of receptors in the cholinergic system, muscarinic acetylcholine receptors (mAChRs).\textsuperscript{18} While mAChRs are metabotropic G-protein-
coupled receptors (GPCRs) that produce excitatory or inhibitory responses, nAChRs are ligand-gated ion channel receptors (LGICRs) in which the excitatory response is achieved by increased cellular sodium (Na\(^+\)) and calcium (Ca\(^{2+}\)) levels.\(^{18}\)

Nicotinic AChRs are highly expressed in human brain in the synaptic region and other extrasynaptic locations.\(^{19}\) In the synapse, presynaptic nAChRs regulate neurotransmitter release whereas postsynaptic nAChRs allow the excitatory transmission process.\(^{19}\) Extrasynaptic nAChRs influence many neurotransmitter systems by mediating intracellular signals as well as neuronal excitability.\(^{19}\)

Nicotinic AChRs are members of the Cys-loop family of LGICRs.\(^{20}\) Members of the Cys-loop receptor family are formed by the assembly of five identical (homomeric) or different (heteromeric) subunits (i.e., pentamers).\(^{20,21}\) Each subunit consists of three main domains (Figure 2): a large extracellular N-terminal domain (ECD), four transmembrane-spanning helices (i.e., the transmembrane domain (TMD), M1–M4), and an intracellular domain (ICD).\(^{20,21}\) The ECD consists of 10 β-strands and the loops connecting them. The sixth and the seventh β-strands are constrained by a disulfide bond connecting two highly conserved cysteine residues. This highly conserved Cys-Cys bridge is what gives the family its name.\(^{20}\)
Figure 2. Structural representation of a nAChR model. (A) A whole receptor representation including the three main domains obtained from PDB (PDB ID: 2BG9); the ECD, the TMD domain, and the ICD. (B) A top view of five different colored subunits forming the receptor. (C) The orthosteric binding site formed by loops located at the interface between the two subunits; the principal subunit ribbon colors: red, loop A; blue, loop B; yellow, loop C; the complementary subunit ribbon colors: green, loop D; orange, loop E; purple, loop F.
Generally, nAChRs can be divided, based on subunit composition, into two general types, neuronal nAChRs (NnAChRs) and muscle-type nAChRs (MnAChRs). Considering the seventeen nAChR subunits of vertebrate species that have been identified (i.e., α1-α10, β1-β4, γ, δ and ε), various families of nAChR subtypes can be formed by different subunit combinations. All of these subunits are present in humans as well as in other mammalian species except α8 that exists only in avian species. The muscle-type nAChRs are composed of two α1 subunits as well as β1, γ, and δ subunits. The latter is replaced by ε subunits in the adult-neuromuscular junction of vertebrates. Conversely, isoforms that form the neuronal-type of nAChRs are composed of certain subunits (i.e., α2-7, 9, 10; β2-4) and can exist as heteropentamers (e.g., α4β2) or, sometimes, as homopentamers (e.g., α7, Figure 2). The α4β2 receptors are the major population of nAChRs in mammalian brain; the α7 receptor subtype is considered the second most abundant nAChR in the central nervous system. On the other hand, a common heteromeric nAChR (i.e., α3β4) represents the most abundant type of nAChR in autonomic ganglia, adrenal medulla, and in the dorsal medulla, pineal gland, medial habenula, nucleus interpeduncularis, and retina.

Although α7 nACh receptors are well-known to be cation-permeable (Na⁺ and Ca²⁺) homopentamers, there is also emerging evidence that α7 nAChRs can form functionally-active heteropentamers with β-subunits. The binding site of the endogenous ligand ACh (i.e., the orthosteric site) is located at the interface of ECDs of every two adjacent subunits in homomeric receptors. Upon agonist binding, α7 nAChRs are characterized by extremely fast activation (<5 milliseconds (ms)) and rapid desensitization (<100 ms). The α7 nAChRs are also known, among other nAChR
subtypes, by their high permeability to calcium, thus triggering a series of intracellular calcium-dependent systems.\textsuperscript{19}

As will be discussed in a subsequent section, $\alpha 7$ nAChR ligands are viable therapeutic agents, however, there is a lack of commonly available selective $\alpha 7$ nAChR competitive agonists and classical antagonists (i.e., competitive antagonists).\textsuperscript{24} However, current attempts in the development of $\alpha 7$ nAChR-selective ligands are confronted with a considerably high sequence homology of the orthosteric binding sites among nAChR subtypes.\textsuperscript{28,29} The lack of subtype-selectivity for nAChR drugs often leads to multiple cholinergic side effects and thus prevents them from being used as therapeutic agents. Furthermore, the limited availability of nAChR-selective ligands has hindered progress toward a complete understanding of the essential functions of different nAChR subtypes.\textsuperscript{29}

Competitive antagonists, by definition, maintain the inactive state of receptors. To understand the deactivation process caused by $\alpha 7$ nAChR competitive antagonists, Monod, Wyman, and Changeux proposed a model (i.e., the MWC model) explaining the nature of protein allosteric transitions.\textsuperscript{30,31} Receptors are dynamic structures that can exist in different states (i.e., distinct functional conformations). For $\alpha 7$ nAChRs, four different states are possible under normal conditions (Figure 3). The binding of $\alpha 7$ nAChR competitive antagonists shifts the equilibrium and stabilizes the receptor's conformation to the inhibition/desensitization state.\textsuperscript{32}
Figure 3. Different nAChR conformations according to the MWC model that explain the allosteric nature of the receptor: the resting state (R), the active state (A), the fast-onset desensitized state (I), and the slow-onset desensitized state (D). Adapted from a review by Jensen et al.\textsuperscript{24}
B. α7 nAChR Agonists

Naturally-occurring alkaloids represent an old class of nonselective and high-affinity agonists for nAChRs. Compounds such as (-)-nicotine (1), (-)-lobeline (LOB; 2) and (±)-epibatidine (EPI; 3) (Figure 4) are some members of this class that have been extensively investigated to understand their molecular role in activating nAChRs.\textsuperscript{24,33,34} The selectivity of agonists for α4β2 and α7 nAChRs was found to be highly dependent on the local binding forces and the long-range electrostatic interactions between the protonated portion or the cationic part of the agonist structures and the receptors.\textsuperscript{35} For example, a conformationally restricted analog of ACh (i.e., 4; Figure 4), is a highly selective full agonist at the α7 nAChRs ($K_i = 92 \text{ nM}$) over α4β2 nAChRs ($K_i = 16,000 \text{ nM}$).\textsuperscript{35} Detailed information of ligand-receptor attractive forces could guide the design for novel, highly selective agonists targeting nAChR subtypes.\textsuperscript{34}

An important focus of medicinal chemistry studies in the field of nAChRs was on a series of novel compounds derived from the nicotine (1) and epibatidine (3) skeletons. For example, substitution of the pyrrolidine ring of nicotine with an azabicyclo[3.2.2]nonane ring (i.e., TC-1698; 5) (Figure 4) resulted in a compound with full agonist activity at α7 nAChRs (EC\textsubscript{50} = 0.44 μM) with insignificant activities at other nAChR subtypes.\textsuperscript{24} However, the synthesis of novel α7 nAChR agonists still faces a selectivity challenge due to the well-known cross-activity with 5-HT\textsubscript{3} receptors.\textsuperscript{24} This cross-activity results from the high homology in the orthosteric site between the two receptor types. The 5-HT\textsubscript{3} receptor antagonist tropisetron (6) (Figure 4), for instance,
Figure 4. The chemical structures of selective and nonselective α7 nAChR agonists: (-)nicotine (1), (-)lobeline (LOB; 2), (±)epibatidine (EPI; 3), ACh-restricted analog (4), TC-1698 (5), tropisetron (6), PNU-282987 (7), and GTS-21 (8).
acts as a partial agonist at α7 nAChRs (EC₅₀ = 0.38 µM). Tropisetron has higher binding affinities (more than 1000-fold) at 5-HT₃ and at α7 nACh receptors than at α4β2 and muscle-type nAChRs; however, tropisetron is not selective for the α7 nAChRs since it antagonizes α3β4 nAChR signaling. Nevertheless, a new generation of quinuclidine analags, such as the p-chlorobenzamide analog PNU-282987 (7), are highly selective α7 nAChR agonists (EC₅₀ = ~3 µM). Moreover, the anabaseine analog GTS-21 (8) is considered the prototypical selective partial agonist of the α7 nAChRs (EC₅₀ = 6 µM). The ACh precursor and metabolic product, choline, produces selective-full agonist activity at α7 nAChRs (EC₅₀ = ~1000 µM).

C. α7 nAChR Antagonists

Antagonists of α7 nAChRs are represented by a large group of structurally heterogeneous compounds, and most have been obtained from natural sources. The need for α7 nAChR antagonists is highly appreciated in animal studies and tissue cultures. Potent and selective α7 nAChR antagonists can provide optimal settings to precise detection of different receptor-effects in multiple receptor systems. Several naturally occurring toxins represent ideal examples of compounds antagonizing ACh at α7 nAChRs. Furthermore, the inhibitory activity of some drugs on α7 nAChRs was identified as an adjuvant effect of their main pharmacological effect.
1. Competitive Antagonists

1. Peptides

Although several peptides were identified as antagonists for nAChRs, the majority of these toxins show inhibition in all subtypes of neuronal nAChRs. For example, neosurugatoxin and lophotoxin are two peptides recognized as non-selective nAChR inhibitors. There is, however, a minor group of peptides that show selectivity toward α7 nAChR binding. The two particular peptides best-known to selectively antagonize α7 nAChRs are: α-Conotoxin, a peptide that belongs to the conotoxin group that is known to be a cysteine-rich peptide extracted from cone snails (*Conus pennaceus*), and α-bungarotoxin, a venom that is extracted from the Taiwanese krait *Bungarus multicinctus*. Although these two groups of peptides are highly specific to nAChRs, their interactions with the receptors are irreversible. This significant binding property has been exploited in the standardization of experimental procedures to isolate nAChRs from the electric organs of the marine ray *Torpedo*. Consequently, extensive knowledge of the diversity and molecular properties of receptors have been identified due to developments in molecular biology techniques.

Due to their size and physicochemical properties, both α-conotoxin and α-bungarotoxin do not pass biological barriers such as the gastrointestinal (GI) tract and blood–brain barrier (BBB). The structures of these peptides lack drug-like properties, and this limits their use as therapeutic agents. The disadvantages of peptides has led to an effort to identify alternative small molecules that have better pharmacokinetic features (i.e., absorption, distribution, metabolism and elimination (ADME)) in humans.
2. Methyllycaconitine

Methyllycaconitine (MLA; 9) (Figure 5) is a naturally occurring norditerpenoid alkaloid isolated from the Delphinium and Consolida species. MLA is a competitive antagonist for α7 nAChRs (IC$_{50}$ = 0.0017 µM) and is the standard α7 nAChR antagonist in pharmacological studies. The binding of MLA is relatively selective for α7 nAChRs ($K_i$ value of 5-10 nM), although it possesses moderate binding affinity for α3β4 and α4β2 nAChRs ($K_i$ value of around 1,300 nM).

The therapeutic utility of MLA is limited because of its large molecular mass and narrow therapeutic index. Thus, the typical use of MLA is as an experimental tool for investigating the pharmacological properties and the heterogeneity of nAChRs. In binding studies, the sensitivity to low nanomolar concentrations of [³H]MLA has been interpreted as an indication for the presence of α7 nAChRs. Other pharmacological experiments indicate that, when MLA potently inhibits presynaptic nAChRs, it blocks the mediation of [³H]dopamine release from striatal synaptosome sites. Furthermore, MLA has been utilized as a lead compound for the identification of novel nAChR antagonists, considering the structural components of MLA that might produce favorable features for the newly proposed nAChR ligands.
Memantine (10; Figure 5) is a low molecular-weight drug, presently approved by the Food and Drug Administration (FDA) for symptomatic treatment of moderate-to-severe AD. The approval of this drug is based on its ability to noncompetitively antagonize NMDA receptors.\textsuperscript{38,50} Beside this pharmacological action, memantine was shown to be an $\alpha_7$ nAChR antagonist ($IC_{50} = 0.34$ $\mu$M). While some\textsuperscript{50} consider the effect on $\alpha_7$ nAChRs as an undesired property that might worsen the condition of AD patients, Banerjee et al.\textsuperscript{51} considered this inhibitory activity as an advantageous action. However,
more experiments are needed to clarify the significance of memantine’s inhibitory activity on α7 nAChRs for AD. In any event, memantine certainly cannot be considered as an α7-selective agent.

2. Non-Competitive Antagonists

Non-competitive antagonists (NCAs) are a class of compounds that inhibit the activity of receptors by interacting with sites distinct from the agonist’s binding site, and therefore do not compete with agonists for binding. Since the action of NCAs is not surmountable by agonist, there are two possible mechanisms by which NCAs can exert their activity on nAChRs. The first possible mechanism is through direct blockade of the receptor’s channel (i.e., channel blockers). Channel blockers are characterized, pharmacologically, by blocking the receptor’s currents in a voltage-dependent manner. On the other hand, NCAs can inhibit the receptor activity in a voltage-independent manner (i.e., negative allosteric modulators (NAMs); discussed in the next section). NAMs exert their activity on nAChRs without blocking the receptor’s channel.

A typical example of an NCA for neuronal nAChRs is the synthetic compound mecamylamine (11; Figure 6). Mecamylamine blocks the ion-channel of most neuronal nAChRs with more sensitivity toward α/β heteromers than α7 nAChRs, which requires 10 µM for full blockade. Another example of channel blockers of α7 nAChRs is naltrexone (12; Figure 6) (IC_{50} ~ 25 µM). In addition to its opioid antagonist activity, naltrexone shows a voltage-dependence effect on the α7 nACh receptor currents.
Allosteric modulators are those ligands that interact with the receptor’s allosteric sites and modify either or both the binding and the signaling of the orthosteric ligand. Allosteric modulators have no intrinsic channel activation properties and, thus, their pharmacological function is to modify normal receptor mechanisms.\textsuperscript{55} Both type of allosteric modulators (i.e., positive allosteric modulators or PAMs, and negative allosteric modulators or NAMs) are believed to alter the energy barriers for state transitions and/or stabilize the relative energy levels of particular receptor states.\textsuperscript{55,56} Consequently, modulation of receptor activity involves not only the stabilization of channel opening/closing states but also the kinetics of activation, deactivation, desensitization, or resensitization (Figure 3).

Figure 6. The chemical structures of two channel blockers of α7 nAChRs: mecamylamine (11) and naltrexone (12).
Once an agonist associates with the receptor, it induces a conformational transition (Figure 3) from a resting state (channel closed) to an activated state (channel open).\textsuperscript{57} For \(\alpha_7\) nAChRs, a conformational transition to a desensitized state (channel closed) is expected in less than one second, if the agonist remains bound. In order for the receptor to return to the resting state, the agonist must dissociate.\textsuperscript{57} The variety of structures of \(\alpha_7\) nAChR allosteric modulators alter this system with mechanistically distinguishable modes that, eventually, can be observed in the different forms of the agonist-evoked inward currents.\textsuperscript{57,58}

Based on the pharmacological profiles of structurally distinct \(\alpha_7\) nAChR modulators, two types are recognized.\textsuperscript{59} Type I PAMs predominantly increase agonist response amplitude without significant effect on response decay rate. Type II PAMs enhance response amplitude and also reduce receptor desensitization, allowing fast reactivation from the desensitized state. Given the intricacies of LGIC states and kinetics, it should be anticipated that more than one mechanism could underlie the broad modulator categories identified to date.\textsuperscript{57} In addition to these two types, some identified PAMs display exceptional properties that are intermediate between the type I and type II classes.\textsuperscript{57-59}

The definition of an allosteric modulator for \(\alpha_7\) nAChRs is also applicable to polypeptides that have been found to significantly enhance the ACh-evoked currents. The Ly-6/uPAR related protein 1 SLURP-1 (secreted by human keratinocytes) and the C terminus peptide fragment of acetylcholinesterase (AChE) are found to be highly selective PAMs at the human \(\alpha_7\) nAChRs.\textsuperscript{60,61} However, because of their size and physicochemical properties, these agents cannot cross the blood brain barrier and are not useful as for exploring their pharmacological actions for in vivo studies.\textsuperscript{62} This issue
signifies that the development of small molecule modulators is a promising approach in medicinal chemistry.

1. Extracellular Domain (ECD) PAMs

   i. Galantamine

   Galantamine (Gal; 13) (Figure 7), an alkaloid originally isolated from snowdrop flowers, is an FDA-approved drug for symptomatic treatment of mild-to-moderate AD and has been also tested as an adjuvant therapy to improve cognitive function in schizophrenia.\textsuperscript{63-65} The approval of this drug is based on its ability to inhibit the cholinesterase enzyme, which is responsible for the breakdown of ACh. Besides this pharmacological action, galantamine was shown to have a synergistic effect at α7 nAChRs through an allosteric-modulating mechanism.\textsuperscript{66,67} Galantamine is considered a type I PAM since it does not affect the desensitization time of α7 nAChRs.\textsuperscript{24,55}

   The location at which galantamine binds on α7 nAChRs has been extensively investigated using photoaffinity labeling, epitope mapping studies with the monoclonal antibody (mAb) FK1, and site-directed mutagenesis studies.\textsuperscript{68-70} These studies identified the location of the binding site to be at the outer surface of the ECD, in an area that overlapped with the ACh site. Three amino acid residues (i.e., Val196, Thr197, and Phe198) located on β-strand 10 of the α7 subunit can weaken galantamine binding after
mutation. From these three residues, only Thr197 appeared to be an essential attachment point since its mutation influenced binding the most.\textsuperscript{70}

ii. **Physostigmine**

Like galantamine, physostigmine (14; Figure 7) shows a dual mode of action, as a cholinesterase inhibitor (AChEI) and PAM.\textsuperscript{68,69} Historically, physostigmine showed the ability to enhance memory in normal individuals as well as in patients suffering dementia.\textsuperscript{71} However, the very short half-life of physostigmine (t\textsubscript{1/2} ~ 30 min) limited its use as a memory protective agent.\textsuperscript{72} Currently, the FDA-approved use of physostigmine is to reverse the anticholinergic effects of toxic doses of cholinergic drugs.

The binding site location of physostigmine at \(\alpha7\) nAChRs was identified using epitope mapping studies with the FK1 monoclonal antibody and photoaffinity labeling studies with physostigmine.\textsuperscript{68-70} Furthermore, physostigmine binding was blocked by the mAb FK1 which indicates the same general area as the galantamine binding site.\textsuperscript{69}
Figure 7. The chemical structures of PAMs of \( \alpha_7 \) nAChRs: galantamine (13), physostigmine (14), PNU-120596 (15), and ivermectin (16).
2. Transmembrane Domain (TMD) PAMs

a. PNU-120596

1-(5-Chloro-2,4-dimethoxyphenyl)-3-(5-methylisoxazol-3-yl)urea or PNU-120596 (15; Figure 7) is an α7 nAChR PAM that was originally discovered by high-throughput screening. PNU-120596 is considered a type II PAM since it has the ability to enhance peak ACh-evoked currents and slow the response decay rates in cells expressing α7 nACh receptors. Moreover, there is a remarkable selectivity of PNU-120596 for α7 nAChRs with no significant effect on most other subtypes of nACh receptors.

Many efforts targeting the identification of the exact binding site for PNU-120596 at α7 nAChRs have been reported. Evidence shows that the intrasubunit cavity in the TMD of α7 nAChRs is the potential location for the PNU-120596 interaction. Furthermore, mutagenesis studies indicate that the amino acids Ser222 and Ala225 from M1, Met253 from M2, and Phe455 and Cys459 from M4 have essential attachment points with PNU-120596.

b. Ivermectin

Ivermectin (16; Figure 7) is a macrocyclic lactone derivative of a natural compound that can be isolated by fermentation of the soil microorganism Streptomyces avermitilis. Ivermectin is available in the market as an antiparasitic agent in both human and veterinary medicine. The antiparasitic effect of ivermectin is believed to result from its
agonist activity on glutamate-gated chloride channels as the major target. Additional biological targets of ivermectin include the modulation of multiple LGICCR actions such as its positive modulatory activity on human α7 nAChRs. Unlike PNU-120596, ivermectin modulation is considered as a type I PAM.

Ivermectin, however, shares a similarity with PNU-120596, in that mutagenesis studies indicated the potential location for ivermectin interaction at α7 nAChRs to be at the large intrasubunit cavity in the TMD. Moreover, the amino acids Ala225 from M1, Gln272 from M3, and Thr456 and Cys459 from M4 have essential attachment points with ivermectin. Interestingly, mutations of three amino acids (S222M, M253L and S276V) were able to convert ivermectin from a PAM into a NAM.

E. Negative Allosteric Modulators

Another approach to inhibiting the action of ACh at α7 nAChRs is to identify an allosteric modulator that selectively deactivates the receptors (i.e., negative allosteric modulators (NAMs)). As described previously, allosteric modulators are receptor ligands that interact with sites distinct (i.e., allosteric sites) from the orthosteric site and mediate either or both the binding and the signaling of the orthosteric ligand. The mechanism of allosteric modulations has been extensively studied and was clinically initiated by the binding of benzodiazepines at the allosteric binding site of GABA_A receptors. For α7 nAChRs, allosteric modulators tune up (i.e., PAMs) or tune down (i.e., NAMs) receptor activity when the endogenous neurotransmitter ACh is present. NAMs are different
from classical antagonists where direct binding to the orthosteric site and a continuous effect occurs, regardless of normal neuronal activity. Allosteric modulators have unique advantages, such as the saturability of the allosteric site, which provides better control over the administered agent.\textsuperscript{82,83} While classical agonists and antagonists can cause extreme stimulation or inhibition of the receptor, an allosteric modulator has a maximal ceiling.\textsuperscript{83} This advantage reduces any undesired effects (e.g., toxic effects) that might interfere with the desired effect.\textsuperscript{82,83} Another advantage is that an allosteric modulator is expected to work only at a time when an endogenous agonist is present at the orthosteric site.\textsuperscript{83} By this feature, the allosteric modulator effect would vary depending on the neuronal tone and the tissue state, which apparently differs from that of the orthosteric ligand that works in a continuous fashion irrespective to neuronal activity.\textsuperscript{83,84} A third advantage is the receptor subtype selectivity of allosteric ligands over classical orthosteric ligands and this is because the orthosteric sites of nAChRs are the most conserved region among this receptors subtype.\textsuperscript{20,83,84}

The main problem in the development of a drug discovery program toward noncompetitive inhibitors of nAChRs is the difficulty of identifying and quantifying allosteric modulators. Allosteric modulators are impossible to design \textit{a priori} and serendipity is the common route of their identification.\textsuperscript{8,24,55,85,86} The current standard experimental approach for the determination of a noncompetitive inhibitor’s activity at nAChRs involves the use of concentration-dependent methods on whole cell currents\textsuperscript{87} or the nicotine-induced $^{86}\text{Rb}^+$ cellular efflux model system.\textsuperscript{88} These approaches do not satisfy the requirement of efficient drug discovery practices as they are expensive, time-consuming, and cannot be generalized to all nACh receptor subtypes.\textsuperscript{89} Even the use of
an efficient approach, such as displacement chromatographic techniques, assumes the existence of known competitive displacers.\textsuperscript{90} To date, several allosteric sites on nAChRs have been identified, including three major sites that are present on all nAChR subtypes.\textsuperscript{89} Moreover, a wide variety of structurally diverse allosteric modulators of nAChRs have been reported over the last decade.\textsuperscript{8,24,55,85}

Allosteric ligand NAMs have gained great attention lately for their potential clinical applications. The primary effect of NAMs is to elevate the energy barrier between the receptor’s active and resting states.\textsuperscript{56} Medicinal chemistry efforts to identify novel allosteric ligands that selectively antagonize nACh receptor function are required.

Kynurenic acid (KYNA; 17) (Figure 8) is a tryptophan metabolite, produced mainly in astrocytes, that has long been recognized as an NMDA receptor antagonist.\textsuperscript{91} It was not until recently that evidence showed KYNA at nanomolar concentrations, the normal level in the brain, to be the first endogenous negative allosteric modulator at $\alpha_7$ nAChRs, and that this can cause a marked reduction in striatal DA.\textsuperscript{92} These concentration limits are not enough to exert activity at the NMDA receptor. Another NAM of $\alpha_7$ nAChRs reported by Yoshimura et al.,\textsuperscript{81} UCI-30002 (18; Figure 8), was discovered through a library screening of subtype A of the $\gamma$-aminobutyric acid (GABA\textsubscript{A}) receptor’s modulators for nAChRs. However, this compound lacks selectivity, and no evidence of its action as a NAM at $\alpha_7$ nAChRs was provided. Abdrakhmanova et al.\textsuperscript{93} reported in 2010 that 1,2,3,3\textsubscript{a},4,8b-hexahydro-2-benzyl-6-$N,N$-dimethylamino-1-methylindeno[1,2-b]pyrrole (HDMP; 19) (Figure 8) is a potent and selective NAM for $\alpha_7$ over $\alpha_4\beta_2$ and $\alpha_3\beta_4$ nACh receptors ($IC_{50} = 0.07 \mu\text{M}$).
meta-Chlorophenylguanidine (MD-354; 20) (Figure 8) was reported by our laboratory\textsuperscript{8,94} as the first subtype-selective small molecule to function as a NAM at α7 nAChRs. MD-354 (20) does not bind at the orthosteric site of α4β2 ($K_i > 10,000 \text{ nM}$) or α7 nACh receptors ($K_i > 100,000 \text{ nM}$) but blocks the antinociceptive effect of the agonist

**Figure 8.** The chemical structures of NAMs of α7 nAChRs: kynurenic acid (KYNA; 17), and UCI-30002 (18), HDMP (19), and meta-chlorophenylguanidine (MD-354; 20).
nicotine in the mouse tail-flick assay. Electrophysiological studies, using the whole-cell configuration of the patch-clamp technique to obtain functional data at stably transfected human embryonic kidney (HEK) 293 cells expressing rat \( \alpha_7 \) nAChRs, showed that MD-354 (20) is a noncompetitive \( \alpha_7 \) nACh receptor antagonist (IC\(_{50} \) = 7.98 \( \mu \)M). The NAM activity of MD-354 (20) was confirmed through the elimination of possible competitive antagonism activity since competitive antagonists do not produce inhibition at currents evoked by an ACh-saturated concentration (Figure 9A). Also, the elimination of possible channel blocking activity was achieved by showing that MD-354 (20) produces a voltage-independent effect at various holding potentials (Figure 9B). However, MD-354 (20) has been found to act as a partial agonist (in an \textit{in vivo} model) at 5-HT\(_3\) receptors (\( K_i \) = 35 nM), in addition to its NAM activity at \( \alpha_7 \) nAChRs. The origin of the cross-reactivity of many \( \alpha_7 \) nAChR and 5-HT\(_3\) receptor ligands is due to the high homology (~30\%) between the two receptor types, which is considered as the highest similarity within the LGIC superfamily.
Figure 9. The inhibitory effects of MD-354 (20) at 10 µM at α7 nAChRs. (A) The inhibitory effects of MD-354 on α7 nAChRs at an EC$_{50}$ concentration of ACh (i.e., 280 µM) and at a saturated concentration of ACh (i.e., 1 mM). (B) The inhibitory effects at various holding potentials in the range from -100 to +30 mV. The upper part represents superimposed traces of ACh-induced currents in the absence and presence of 20. In the lower part, squares represent the maximal amplitude in the absence of 20 plotted versus the corresponding holding potential, whereas circles represent the maximal amplitude in the presence of MD-354 (20) at a concentration around its IC$_{50}$ value plotted versus the corresponding holding potential.$^{8,9}$
Arylguanidine SAR for 5-HT₃ receptor binding affinity showed that introduction of certain aryl substituents reduces the 5-HT₃ receptor affinity of MD-354 (20) \((K_i = 35 \text{ nM})\) (i.e., 21, 22, 23 (Figure 10); \(K_i = 2,340, 2,440, 1,600 \text{ nM}\); respectively) and that N-methylation of the aniline nitrogen atom (i.e., 24; Figure 10) resulted in a significant reduction in affinity \((K_i = 6,200 \text{ nM})\) as well. Although these structural alterations of MD-354 (20) are detrimental to 5-HT₃ receptor binding, there is no reason to believe that the inhibitory activity (i.e., NAM action) at α7 nACh receptors will be affected. Based on this assumption, the inhibitory activity (i.e., IC₅₀ values) at α7 nACh receptors of compounds 20 - 24, were obtained (Table 1). The resulting IC₅₀ values showed that when the chloro group of MD-354 (20) is removed, potency is decreased (i.e., 21, IC₅₀ = 34.84 µM). In the same manner, inhibitory potency is reduced when the chloro group of 20 is replaced with the more electron-withdrawing –CF₃ group (i.e., 22; IC₅₀ = 18.46 µM).
**Figure 10.** Chemical structures representing different modifications of the MD-354 (20) structure. 5-HT<sub>3</sub> receptor affinity is provided [in brackets] as \( K_i \) values.\textsuperscript{94,95}
Table 1. The IC\textsubscript{50} values of MD-354 (20) and analogs for \(\alpha_7\) nACh receptors, and their affinities at 5-HT\textsubscript{3} serotonin receptors.

<table>
<thead>
<tr>
<th>Compound</th>
<th>X</th>
<th>R</th>
<th>(\alpha_7) nAChR IC\textsubscript{50} ± SEM (µM)</th>
<th>5-HT\textsubscript{3} R (K_i) (nM) **</th>
</tr>
</thead>
<tbody>
<tr>
<td>20</td>
<td>H</td>
<td>Cl</td>
<td>7.98*</td>
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<td>21</td>
<td>H</td>
<td>H</td>
<td>34.84 ± 6.85</td>
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<tr>
<td>22</td>
<td>H</td>
<td>CF\textsubscript{3}</td>
<td>18.46 ± 0.42</td>
<td>2,440</td>
</tr>
<tr>
<td>23</td>
<td>H</td>
<td>OCH\textsubscript{3}</td>
<td>7.54 ± 0.74</td>
<td>1,600</td>
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<td>24</td>
<td>CH\textsubscript{3}</td>
<td>Cl</td>
<td>1.26 ± 0.26</td>
<td>6,200</td>
</tr>
</tbody>
</table>

* \(\alpha_7\) nAChR IC\textsubscript{50} value of 20 reported by Dukat et al.\textsuperscript{8}

** 5-HT\textsubscript{3} receptor \(K_i\) values reported by Dukat et al.\textsuperscript{94,95}

Compound 23 (IC\textsubscript{50} = 7.54 µM), where –OCH\textsubscript{3} is an electron donating group at the 3-position of the phenyl ring, showed a potency comparable to that of MD-354 (20) at \(\alpha_7\) nACh receptors; however, its low affinity at 5-HT\textsubscript{3} receptors makes 23 a more selective \(\alpha_7\) nACh receptor inhibitor than 20. A major increase in potency at \(\alpha_7\) nACh receptors is shown by the N-methyl analog of MD-354 (i.e., 24; IC\textsubscript{50} = 1.26 µM) (Figure 11).\textsuperscript{9} Compound 24 exhibits a more than 1,000-fold shift in selectivity (5-HT\textsubscript{3} receptor \(K_i\) = 6,200 nM) and higher potency at \(\alpha_7\) nACh receptors compared to 20 (5-HT\textsubscript{3} receptor \(K_i\) = 35 nM).\textsuperscript{9} Moreover, the NAM activity of 24 was confirmed through the elimination of possible competitive antagonism activity (Figure 12A) and the elimination of possible channel blocking activity (Figure 12B).\textsuperscript{9} Thus, it was demonstrated that the structural
requirements of the arylguanidines for 5-HT₃ receptor binding, and actions as α7 nAChR NAMs, are not inextricably linked.

**Figure 11.** Functional description of the N-methyl analog of MD-354 (i.e., 24) activity at α7 neuronal nAChRs.⁹ (A) A concentration–response relationship for different concentrations of 24 at α7 nAChRs where peak amplitude due to exposure to ACh (EC₅₀)-evoked currents was normalized. The Hill equation was applied to the curve, and the symbols and bars represent the mean ± SEM. (B) The inhibitory effect of 24 at a concentration of 1 µM. The recorded results in (A) and (B) were performed at a holding potential of −80 mV.
Figure 12. The inhibitory effects of 24 at 1 µM at α7 nAChRs.9 (A) The inhibitory effects of 24 on α7 nAChRs at an EC$_{50}$ concentration of ACh (i.e., 280 µM) and at a saturated concentration of ACh (i.e., 1 mM). (B) The inhibitory effects at various holding potentials in the range from -100 to +60 mV. This represents superimposed traces of the ACh-induced currents in the absence and presence of 24. (C) The squares represent the maximal amplitude in the absence of 24 plotted versus the corresponding holding potential, whereas circles represent the maximal amplitude in the presence of 24 at a concentration around its IC$_{50}$ value plotted versus the corresponding holding potential.
The activity of 20 - 24 at a fixed concentration of 10 µM at α3β4 nACh receptors was investigated to test the selectivity of these compounds among nAChR subtypes (Figure 13). MD-354 (20) showed a lack of activity at 10 µM concentration on ACh at α3β4 nAChRs relative to ACh at its EC\(_{50}\) concentration (EC\(_{50}\) = 100 µM) (Figure 14), whereas 21 and 24 slightly attenuated the ACh (EC\(_{50}\) = 100 µM)-evoked currents. Compounds 22 and 23 reduced the current of ACh to a different extent.

![Figure 13](image)

**Figure 13.** Effect ± SEM of 20, 21, 22, 23 and 24 at 10 µM concentration on acetylcholine (ACh) function at α3β4 nAChRs relative to ACh EC\(_{50}\) = 100 µM (normalized current = 1).
Figure 14. A) The effect of ACh (EC$_{50} = 100$ µM)-evoked current at α3β4 nAChRs. B) The effect of MD-354 (20) at 10 µM concentration on ACh (EC$_{50} = 100$ µM)-induced current at α3β4 nAChRs. Holding potential for these recording was -80 mV.

MD-354 (20) neither displayed affinity (K$_i > 10,000$ nM) nor possessed functional activity at α4β2 nAChRs. The activity of 20, as well as 24, at α4β2 receptors was investigated as part of the selectivity test of these compounds among nAChR subtypes (Figure 15). Both compounds at concentrations comparable to the IC$_{50}$ for α7 neuronal nACh receptors showed no activity on ACh (EC$_{50} = 20$ µM) at α4β2 nACh receptors. In addition, both compounds were examined for their agonist activity at α4β2 nACh receptors at their respective IC$_{50}$ concentrations and failed to mimic ACh actions.
Figure 15. Effect of MD-354 (20) and 24 on α4β2 nAChRs. (A) Effect of ACh at EC$_{50}$ = 20 µM and saturated concentrations = 1 mM. (B) Effect of MD-354 (20) on ACh (EC$_{50}$ = 20 µM)-evoked current and by itself at α4β2 nAChRs. (C) Effect of ACh (EC$_{50}$ = 20 µM) alone and effect of ACh at EC$_{50}$ = 20 µM accompanied by 10 µM of 24. (D) Effect of 24 at 1 µM on ACh (EC$_{50}$ = 20 µM)-evoked current and by itself at α4β2 nAChRs. Holding potential for these recording was -80 mV.
Further investigations for the required structural features of these small-molecule were applied to determine and optimize their NAM activity at α7 neuronal nAChRs. Considering the functional data from MD-354 (20) and its N-methyl analog 24, since the N-methyl group is known to be detrimental to 5-HT3 receptor binding, a new series of compounds was synthesized. The chloro group at the 3-position of N-(3-chlorophenyl)-N-methylguanidine (24) was replaced with a number of substituents considering the electronic, the lipophilic, and the steric nature of the new substituent (Table 2). In addition, the activity of any new modification at α7 nACh receptors was compared to that determined for the same modification in the MD-354 series to test if parallel structural modification would result in parallel change in activity, which might suggest whether or not the two series bind at the same manner (Table 2).

The functional data on two compounds of each series (i.e., 20 and 21 of MD-354 series, and 24 and 25 of the new N-methyl series) revealed the inhibitory activity of these compounds at α7 nACh receptors. Removal of the chloro group from 20 (i.e., 21) resulted in around a four-fold reduction in the current (Table 1). In contrast, removing the chloro group from 24 (i.e., 25; IC\textsubscript{50} = 30.81 ± 2.13 µM) results in around a thirty-fold reduction of its effect. However, the number of compounds tested here might not be sufficient to confirm that the N-methyl series binds in the same manner with α7 nAChRs as MD-354 series. The possibilities that the N-methyl series might bind to a different allosteric site than that of the MD-354 series, or that the N-methyl series could bind to the same allosteric site as that of the MD-354 series, remain to be considered.
Table 2. Structural representation and substituent constants of the N-methyl series and the MD-354 series of arylguanidines.

<table>
<thead>
<tr>
<th>Compound</th>
<th>R</th>
<th>$\pi^a$</th>
<th>$\sigma_m^a$</th>
<th>Compound</th>
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<tbody>
<tr>
<td>20</td>
<td>Cl</td>
<td>0.71</td>
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<td>H</td>
<td>0.00</td>
<td>0.00</td>
<td>25</td>
</tr>
<tr>
<td>31</td>
<td>Br</td>
<td>0.86</td>
<td>0.39</td>
<td>26</td>
</tr>
<tr>
<td>32</td>
<td>F</td>
<td>0.14</td>
<td>0.34</td>
<td>27</td>
</tr>
<tr>
<td>33</td>
<td>I</td>
<td>1.12</td>
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<td>28</td>
</tr>
<tr>
<td>34</td>
<td>CH$_3$</td>
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<td>-0.07</td>
<td>29</td>
</tr>
<tr>
<td>23</td>
<td>OCH$_3$</td>
<td>-0.02</td>
<td>0.12</td>
<td>30</td>
</tr>
</tbody>
</table>

$^a$ $\pi$ and $\sigma_m$ aromatic R substituent constants reported by Hansch et al.$^{97}$
F. Clinical Implications of $\alpha_7$ nAChR Ligands

The discovery of small molecules that influence $\alpha_7$ nAChR activity would be of great importance. As research tools, they would enhance our understanding of the roles that nAChRs play in physiological and pathological states. Furthermore, they hold clinical promise as therapeutic agents for disorders associated with nAChRs.

1. Alzheimer’s Disease

Alzheimer’s disease (AD) is a neurodegenerative disorder associated with a slow and progressive loss of memory and cognition. The neuropathology of AD is characterized by the presence of two types of abnormal deposits, cortical senile (amyloid) plaques (SP) and neurofibrillary tangles (NFT) that, consequently, cause extensive neuronal loss. Evidence showed that $\alpha_7$ nAChRs mediate the pathology that leads to the formation of these AD hallmarks.

Controversially, both agonists and antagonists of $\alpha_7$ nAChRs can offer benefits not only for symptomatic therapy, but also for decreasing the progression of AD. Apparent explanation of the desired effects of agonists might be related to the fact that $\alpha_7$ nAChRs desensitize (i.e., reducing cholinergic transmission) rapidly after exposure to agonists such as nicotine. Conversely, antagonists directly attenuate nAChR-mediated transmission. Another explanation suggests that $\alpha_7$ nAChR ligands prevent the toxic effect of the high-affinity binding of $\beta$-amyloid peptide 1-42 (A$\beta_{1-42}$) to the $\alpha_7$ nAChRs and, consequently, prevent A$\beta_{1-42}$ cellular internalization.
Current clinically approved drugs to treat mild-to-moderate AD cause inhibition of acetylcholinesterase, the enzyme responsible for the hydrolysis of the neurotransmitter ACh. These drugs include galantamine (structures not shown). These drugs, when co-administered with other therapies, show a reduction in cognitive impairment in small samples of patients with schizophrenia. Although these results were derived from open, uncontrolled studies, only galantamine showed positive outcomes when applied as an add-on therapy for a small randomized sample in a double-blind trial, and no positive results were detected with donepezil or rivastigmine.

2. Marijuana Addiction

The nucleus accumbens (NAc) and ventral tegmental area (VTA) are two major compartments of the mesolimbic dopamine system that are responsible for addictive properties of abused drugs (Figure 1). Both the NAc shell and the VTA express α7 nAChRs. The activation of these receptors causes the release of glutamate and the activation of ionotropic glutamate receptors, which, consequently, stimulates dopamine release at dopaminergic terminals. There is evidence that animals treated with Δ⁹-tetrahydrocannabinol (THC), the primary constituent responsible for marijuana abuse and dependence, experience elevation in mesolimbic dopamine transmission, whereas a reduction in mesolimbic dopamine transmission is observed during drug withdrawal. Furthermore, methyllycaconitine (structures not shown), a relatively selective α7 nAChR antagonist, blocks the behavioral rewarding properties and neurochemical actions of
THC. This implies a possible role of modulation by α7 nAChR activity and indicates the pharmacological potential of α7 nAChR antagonists for treating marijuana dependence.

Inhibition of presynaptic α7 nAChRs was recently investigated as a useful approach to treat the withdrawal symptoms of marijuana. Kynurenic acid (KYNA; 17) (Figure 8) is an endogenous noncompetitive antagonist at α7 nAChRs. Studies showed that KYNA (17) was capable of lowering extracellular glutamate and, consequently, reduced the release of dopamine in the striatum and prefrontal cortex. Therefore, targeting presynaptic α7 nAChRs with a potent antagonist might provide a valuable tool for the treatment of drug dependence and other dopamine-related disorders.

3. Depression

Ketamine (Ket; 35) (Figure 16) is a chiral drug known to act as an N-methyl-D-aspartate (NMDA) receptor antagonist, but it also acts at numerous other targets including nAChRs. Ket (35) is known clinically for its anesthetic properties; however, it is associated with several other advantageous properties such as potentiation of the opioid analgesic effect, an anti-inflammatory effect, anti-tumor effect and antidepressant effect. In an effort to study the pharmacological activity of Ket (35) and its metabolite norketamine (norKet), Moaddel et al. investigated their antidepressant properties using sub-anesthetic dosing of Ket in treatment-resistant patients. Data indicated that there are strong links between the antidepressant effect and the plasma
levels of diastereomeric hydroxynorketamine (HNK) and some of the HNK metabolites that are normally found in circulating blood obtained from patients. This study also showed that only one particular isomer (i.e., (2S,6S)-HNK; 36) (Figure 16), out of the other major metabolites, was the most potent in *in vitro* studies. The pharmacological activity of this metabolite was tested, later, at NMDA receptors, α7 nAChRs, and α3β4 nAChRs and results showed that (2S,6S)-HNK is a potent and selective NAM at α7 nAChRs with no detection of anesthetic activity in rat.

![Figure 16. The chemical structures of NAMs of α7 nAChRs: ketamine (Ket; 35), and (2S,6S)-HNK (36).](image)

Activity at presynaptic α7 nAChRs results in a potential consequence on the activity of the enzyme serine racemase (SR). SR is a Ca\(^{2+}\)-dependent enzyme that works to increase the intracellular level of D-serine (D-Ser). Endogenous D-Ser is a known co-agonist of NMDA receptors, and a long-term potentiator that is associated with NMDA-
induced neurotoxicity.\textsuperscript{111,112} The inhibition of $\alpha 7$ nAChRs lowers intracellular $\text{Ca}^{2+}$ levels that, in turn, decreases SR activity and, consequently, endogenous D-Ser levels. Thus, potent and selective antagonists of $\alpha 7$ nAChRs might represent promising antidepressant agents through the indirect modulation of NMDA receptor activity.\textsuperscript{109,110,112}

4. Lung Cancer

During the period between 1989 and 1994, several publications described the presence and the potential importance of nicotinic receptors in lung cancer cells.\textsuperscript{113-116} In addition, the presence of ACh and all the required components for its synthesis and activity such as CAT and nAChRs in human airway epithelial cells widened the possibility of different pathological roles in the periphery.\textsuperscript{113-116} Exposure to nicotine through tobacco smoke or cigarette substitutes can initiate the proliferation of a variety of small-cell lung carcinoma cell lines and endothelial cells.\textsuperscript{117,118} This effect is also seen in structurally-related carcinogens such as 4-(methylnitrosoamino)-1-(3-pyridyl)-1-butanone (NNK) and $N'$-nitrosonornicotine (NNN) which also can reach non-neuronal tissues and increase the production of growth factors (e.g., TGF-$\alpha$, and VEGF). These factors eventually lead to protein upregulation and activation of the Raf/MEK/ERK pathway that is associated with tumor development and progression.\textsuperscript{117,118}

Numerous laboratories have reported the observed transformations of pharmacologic doses of tobacco and other carcinogenic nitrosamines on different cell lines.\textsuperscript{118} In an in vitro study analyzing the level of gene expression of nAChR subtypes in smokers’ and nonsmokers’ non-small-cell lung cancer (NSCLC) tissues after 72 hours exposure to
nicotine (0.1 µM) indicated a significantly reversible upregulation of α1, α5, and α7 subunits in bronchial epithelial cells.\textsuperscript{119} Other studies reported a major role for α7 nAChRs over other nicotinic receptor subtypes in mediating nicotine’s proliferative properties in lung tumor cells.\textsuperscript{120-122} Although there are several studies investigating the possible mechanisms and the contributing factors for carcinogenesis in different types of lung tumors, the involvement of nicotinic receptors, in particular α7 nAChRs, represents a separate and unique therapeutic approach that might provide an unprecedented opportunity for progress.\textsuperscript{118}

G. Molecular Characteristics of Human α7 nAChRs

The cDNA of the human α7 receptor subunit encodes a polypeptide of 502 amino acid residues.\textsuperscript{98,123} The human α7 protein subunit is about 56 kDa and is composed of 222 amino acids forming the ECD, 87 amino acids forming the TMD, and 152 amino acids forming the ICD.\textsuperscript{123} Important regions on the primary sequence of α7 nAChR subunit were identified, including the orthosteric binding site (i.e., loops A, B, C, D, E, and F) (Figure 17).\textsuperscript{123} The essential amino acids involved in orthosteric ligand recognition are Trp171, Tyr115, Tyr217, and the adjacent two cysteine residues (Cys212 and Cys213) forming the disulfide bond at the C loop.\textsuperscript{123} There are also the well-known two cysteine residues (i.e., Cys150 and Cys164) that form the disulfide bond characterizing the Cys-loop family of receptors (Figure 17).\textsuperscript{123}

The level of sequence identity between human α7 subunits and the different subunit classes of the nACh receptors is approximately 41%.\textsuperscript{124} For interspecies comparison, the
The human α7 subunit is highly conserved, exhibiting 94% and 92% amino acid sequence identity to rat and chicken receptors (Figure 17), respectively. This high sequence identity implies that there are very few sequence differences among human, chicken, and rat α7 receptors to account for pharmacological differences between species.

The transmembrane sequences are identical in all three species. The M2 sequence is the transmembrane portion responsible for lining the ion channel and, as expected, shows an identical amino acid sequence to those of homomers of α7 from chickens and rats. The most sequence diversity is in the large intracellular loop connecting M3 and M4, as is typical for interspecies sequence variation of nAChR subunits.
Figure 17. Multiple sequence alignment of three amino acid sequences (i.e., human, rat, and chicken α7 nAChR monomers) showing a high degree of identity. Amino acid residues forming the orthosteric site are: red, loop A; blue, loop B; yellow, loop C (for the principal subunit ribbon); and green, loop D; orange, loop E; purple, loop F (for the complementary subunit ribbon).
H. Previously Reported Molecular Models of Human α7 nAChRs

The significance of molecular modeling, as an important computational tool in drug design, is widely appreciated.125 The level of structural information to be gained from these models might provide tremendous insight for interpreting pharmacological data. Among several interesting biological targets, molecular modeling studies on α7 nAChRs gained much attention and were reported in a number of scientific papers.74,80,126-129 The motivation for these studies is to explore and learn more about the structural basis of the molecular effect on α7 nAChRs through homology modeling and ligand docking. In addition, molecular information was also obtained from the publication of crystal structures of similar protein targets that share acceptable sequence identity with α7 nAChRs.129

In 2006, two models of α7 nAChRs were introduced. The first one was by Iorga et al.126 where they docked three allosteric modulators seeking the identification of their potential binding site(s) at the ECD (see Table 3). The second model was by Cheng et al.127 where they studied the gating motion of the receptor. The differences between the two models was the crystal structure template used for homology modeling.126,127
Table 3. Summary of previously reported models of α7 nAChRs for identifying possible allosteric binding sites.

<table>
<thead>
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<th></th>
<th>Iorga et al.\textsuperscript{126}</th>
<th>Brannigan et al.\textsuperscript{129}</th>
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<td>AChBP (1UV6)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Cavity Search Software</td>
<td>Alpha spheres (MOE)</td>
</tr>
<tr>
<td></td>
<td>Blind Docking Software</td>
<td>AutoDock</td>
</tr>
<tr>
<td><strong>TM</strong></td>
<td>Template</td>
<td>Torpedo m. (2BG9)</td>
</tr>
<tr>
<td></td>
<td>Cavity Search Software</td>
<td>Molecular dynamic simulation “flooding”</td>
</tr>
<tr>
<td></td>
<td>Blind Docking Software</td>
<td></td>
</tr>
</tbody>
</table>

Young et al.\textsuperscript{74} constructed a homology model of human α7 nAChRs in 2008, based on the 4 Å crystal structure of the *Torpedo* nAChR. Their aim was to predict the binding modes of two PAMs (i.e., PNU-120596 and LY-2087101) at the intrasubunit cavity. The following year, Luttmann et al.\textsuperscript{128} presented a model for the extracellular portion of the human α7 nAChR. The model was used to identify possible binding sites for allosterically-potentiating ligands (APLs) by searching for cavities and blind-docking experiments with several APLs. Collins et al.\textsuperscript{80} also used a homology model of the human
α7 nAChR TMD to investigate the binding modes of ivermectin in 2010. The binding site was proposed to be in the intrasubunit cavity located between the four transmembrane α-helices.\textsuperscript{80}

Brannigan et al.\textsuperscript{129} investigated the binding sites of the general anesthetic isoflurane in the TMD of nAChRs. The nAChR from \textit{Torpedo} was subjected to computation-based “flooding” (i.e., simulating a high concentration of anesthetic in the surroundings) of the receptor and was allowed to partition in the system over the course of an MD simulation trajectory (Table 3). Their aim was to reduce potential errors that might arise from homology models.

All the published models mentioned above investigated different aspects of structural information. Only two models, out of them, were generated to explore the possible binding site of galantamine (13) and isoflurane in the α7 nAChR ECD and TMD, respectively, and these are shown in Table 3.\textsuperscript{126,129}
III. Specific Aims

Within the nAChR family as attractive therapeutic targets for drug discovery, considerable effort has been aimed toward the identification and development of drugs that target α7 nAChRs. The binding sites of the endogenous neurotransmitter ACh (i.e., orthosteric binding sites) have been the major concern of many drug discovery efforts. The main objective of research projects targeting orthosteric sites is to identify drugs that bind to these sites of α7 nAChRs and produce better activation or inhibition of the endogenous ligand ACh. Nevertheless, one conceivable problem that attenuates the value of this approach is the difficulty of identifying drugs with adequate subtype-selectivity due to the high level of sequence homology within the orthosteric binding sites of different nAChR subtypes.\(^{25}\) The lack of subtype-selective drugs for α7 nAChRs can result in several undesirable side effects and, thus, limits their clinical use as therapeutic agents. For example, activation of α4β2 nAChRs can be accompanied by nausea and vomiting, whereas activation of α3β4 nAChRs at autonomic ganglia and in the CNS is associated with constipation and weight loss, respectively.\(^ {130,131}\) Accordingly, projecting efforts toward the regulatory binding sites (i.e., the allosteric binding sites) that are at distinct locations from the highly conserved ACh-binding site (i.e., the orthosteric site) has
gained increasing attention as a promising approach to developing α7 nAChR drugs. Allosteric binding sites are topologically distinct from the orthosteric binding site and can represent promising targets for the development of subtype-selective α7 nAChR drugs.20,25

This dissertation focuses on the discovery and development of small-molecule arylguanidines that modulate α7 nAChR function with improved subtype-selectivity that might be achieved through the allosteric approach. Identifying the required structural features of these small molecules will allow for optimizing their NAM activity at α7 neuronal nAChRs. The two previously synthesized series of compounds identified by our laboratory, based on MD-354 (20) and its N-methyl analog 24 (Table 2),9 were used as a starting point to determine the effect of modifications on NAM activity of both series. Furthermore, the effect of these parallel structural modifications might suggest whether or not the two series bind at the same allosteric site and, if so, if they bind in a similar manner (Table 2).9

The present study was aimed toward identifying novel chemical classes of agents that bind to allosteric binding sites and antagonize α7 nAChR function (i.e., negative allosteric modulators; NAMs). The primary goals of this research are as follows:

A. QSAR

The identification of the physicochemical properties essential for α7 nAChR NAM activity is a key approach in current drug discovery and development methods. This step
can be addressed through quantitative structure-activity relationship (QSAR) studies on closely related analogs possessing the same core structure. QSAR studies are based on the Hansch assumption of activity–modification of a parent compound by a binding site that recognizes chemical substituents by specified lipophilic (π), electronic (σ), and other properties.\textsuperscript{132}

Previous molecular modeling studies at the ECD of α7 nAChRs\textsuperscript{9} were analyzed using SYBYL 8.1. Docking of both 20 and 24, performed by the GOLD Suite 5.0 program, showed plausible binding modes in an allosteric site (characterized by a Thr221) and revealed two main clusters (Figure 18).\textsuperscript{9} The rotamers of 20 appeared to utilize the same binding mode (N\textsubscript{1} and N\textsubscript{2} form ionic hydrogen bonds with Asp219; hydrophobic interactions exist between the phenyl ring and Thr221) with alternate locations of the chloro group (possible hydrophobic interaction with the aliphatic chain of Lys204). In contrast, N\textsubscript{2} and N\textsubscript{3} of 24 form ionic hydrogen bonds with Asp219 as the major difference detected at this allosteric site. N\textsubscript{3} shows further a hydrogen bond interaction with Thr221. The introduction of an N\textsubscript{1}-methyl group to MD-354 (i.e., 24) appears as the key difference in activity since the methyl group on N\textsubscript{1} precludes ionic hydrogen bonding with Asp219 (Figure 18).\textsuperscript{9}
The two possible binding modes add to the necessity of investigating the structural requirements for the binding of arylguanidines at α7 nAChRs. The purpose of this study is to analyze the structural requirements of these compounds that act as NAMs using a Hansch analysis. Analogs of the MD-354 series and the N-methyl series with parallel structural modifications will be synthesized and investigated. The physicochemical properties associated with other halogens (i.e., -F, -Br, and -I) in place of the chloro group will allow for testing mainly the effect of substituent size variation over a relatively fixed electron-withdrawing nature (Table 4). Replacement of the chloro group with a -OCH₃ or -CH₃ group as electron donating groups will explore the effect of such modifications on activity (Table 4). For the -CF₃ group, there is a greater electron

**Figure 18.** Binding modes of 20 and 24 at the ECD allosteric site (characterized by a Thr221) of α7 nAChRs as suggested by preliminary molecular modeling studies. D = Asp219.
withdrawing nature than -Cl and other halogens. However, the -CF₃ group has a lower relative electron affinity (EA) than -Cl, a property that provided an explanation for the lower binding affinity of -CF₃-substituted arylguanidines than -Cl-substituted arylguanidines to 5-HT₃ receptors.¹³³

**Table 4.** Structural representation of different modifications at the 3-position of the MD-354 (i.e., 20) series and the N-methyl (i.e., 24) series of arylguanidines.

<table>
<thead>
<tr>
<th>Compound</th>
<th>X</th>
<th>Compound</th>
</tr>
</thead>
<tbody>
<tr>
<td>21</td>
<td>H</td>
<td>25</td>
</tr>
<tr>
<td>20</td>
<td>Cl</td>
<td>24</td>
</tr>
<tr>
<td>31</td>
<td>Br</td>
<td>26</td>
</tr>
<tr>
<td>32</td>
<td>F</td>
<td>27</td>
</tr>
<tr>
<td>33</td>
<td>I</td>
<td>28</td>
</tr>
<tr>
<td>22</td>
<td>CF₃</td>
<td>37</td>
</tr>
<tr>
<td>34</td>
<td>CH₃</td>
<td>29</td>
</tr>
<tr>
<td>23</td>
<td>OCH₃</td>
<td>30</td>
</tr>
</tbody>
</table>
Hansch-type QSAR studies will be conducted using several physicochemical properties such as $\pi$ (hydrophobic constant), $\sigma_m$ (Hammett electronic constant), $L$ (Verloop length), $B_1$ (Verloop minimum width), $B_5$ (Verloop maximum width), MR (Molar Refraction), and solvent accessible volume (Vol) (Table 5). This study also involved application of a widely accepted concept proposed by Portoghese\textsuperscript{134} in his study on analgesics, which suggests that “If identically substituted compounds in two different series are interacting with receptors in a similar manner, then the quantitative contribution of various substituents to the … effect should produce, under steady-state conditions, proportionate variations of activity in both series”.

**Table 5.** Description of various physicochemical parameters.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>$\pi$</td>
<td>Hydrophobic constant\textsuperscript{132}</td>
</tr>
<tr>
<td>$\sigma_m$</td>
<td>Hammett electronic constant\textsuperscript{132}</td>
</tr>
<tr>
<td>$L$</td>
<td>Verloop length of the substituent\textsuperscript{159}</td>
</tr>
<tr>
<td>$B_1$</td>
<td>Verloop minimum width of the substituent\textsuperscript{159}</td>
</tr>
<tr>
<td>$B_5$</td>
<td>Verloop maximum width of the substituent\textsuperscript{159}</td>
</tr>
<tr>
<td>MR</td>
<td>Molar refraction\textsuperscript{160}</td>
</tr>
<tr>
<td>Vol</td>
<td>Solvent accessibility volume\textsuperscript{a}</td>
</tr>
<tr>
<td>NVE</td>
<td>Number of valence electron (Verma et al.)</td>
</tr>
<tr>
<td>CMR</td>
<td>Complete molar refraction\textsuperscript{b}</td>
</tr>
<tr>
<td>MV</td>
<td>Molar volume\textsuperscript{c}</td>
</tr>
<tr>
<td>Pc</td>
<td>Parachor based on surface tension and MV\textsuperscript{c}</td>
</tr>
<tr>
<td>Polarizability</td>
<td>Polarizability of the compound\textsuperscript{c}</td>
</tr>
</tbody>
</table>

\textsuperscript{a} Calculated by Chimera  
\textsuperscript{b} Calculated by ChemDraw  
\textsuperscript{c} Calculated by ChemSketch
**Hypothesis 1** is that 3-position substituents control the potency of arylguanidines at α7 nAChRs, and that this is related to the physicochemical attributes of these substituents. This can be evaluated by conducting QSAR studies.

**Hypothesis 2** is that arylguanidines and their N-methyl counterparts bind in a dissimilar manner at α7 nAChRs (as shown in Figure 18). This can be addressed by examining two related series of compounds to determine if parallel structural changes result in parallel or non-parallel shifts in NAM potency in accordance with the Portoghese Hypothesis.

**B. Topliss Tree**

The Topliss Tree approach is a non-mathematical guide for the use of the Hansch principle.\(^{135}\) The Topliss Tree, usually with the guidance of the Craig Plot,\(^{136}\) allows for an efficient optimization of the potency of a lead compound with minimal number of compounds needed to be synthesized. The Topliss operational scheme for the aliphatic side chain (Figure 19) will be used to optimize pharmacological activities associated with the alkyl group at the anilinic nitrogen (i.e., N₁) of the arylguanidines at α7 nAChRs. Furthermore, we hope to develop a better understanding of how small-molecule NAMs interact at α7 nAChRs.
Accordingly, replacement of the N$_1$ methyl group in the N-methyl guanidine series (i.e., 24, 25) with an isopropyl (i-Pr) group (i.e., 38, 39; Table 6) should increase its π effect. The isopropyl group not only provides more lipophilic character, but also adds more steric bulk than the methyl group. By examining this structural feature, we can determine how much bulk can be tolerated by α7 nAChRs for optimal NAM activity. A total of four pairs of compounds will be examined through which the necessity of the chloro group will be also investigated with deschloro compounds (Table 6).

**Figure 19.** Schematic representation of the Topliss decision-tree for aliphatic side chain substituents (M, more potent; E, equipotent; L, less potent). Adopted from Topliss.$^{135}$
Table 6. Structural representation of proposed compounds with different modifications at the N₁ and 3-position.

<table>
<thead>
<tr>
<th>Compound</th>
<th>R</th>
<th>X</th>
</tr>
</thead>
<tbody>
<tr>
<td>21</td>
<td>H</td>
<td>H</td>
</tr>
<tr>
<td>20</td>
<td>H</td>
<td>Cl</td>
</tr>
<tr>
<td>25</td>
<td>CH₃</td>
<td>H</td>
</tr>
<tr>
<td>24</td>
<td>CH₃</td>
<td>Cl</td>
</tr>
<tr>
<td>38</td>
<td>CH(CH₃)₂</td>
<td>H</td>
</tr>
<tr>
<td>39</td>
<td>CH(CH₃)₂</td>
<td>Cl</td>
</tr>
</tbody>
</table>

Hypothesis 3 is that application of the Topliss scheme should allow optimization of the N₁ substituent of arylguanidines as α7 nAChR NAMs, if the size and/or lipophilic nature of the substituent contributes to activity.
C. Role of the Guanidine Nitrogen Atoms

To gain an understanding of the role of the guanidine nitrogen atoms in the inhibitory effects of arylguanidines on α7 nACh receptor function, a series of compounds will be tested (Figure 20). The compounds in this series retain the 3-chlorophenyl moiety, whereas the differences appear at the guanidine part. With the first compound in this series, we will test the effect of substitution at a guanidine terminal nitrogen atom (i.e., 40; Figure 20) on NAM activity. In the remaining compounds of this series, the guanidine is replaced with modified forms of substructures representing the guanidines missing one or two nitrogen atoms (i.e., 41, 42, and 43). Our purpose here is to understand the minimum structural requirement of the guanidine moiety needed for the NAM activity at α7 nAChRs. In addition, this can provide further insight on the specific molecular interactions of arylguanidines NAMs at α7 nAChRs.

Hypothesis 4 is that not all of the three guanidine nitrogen atoms (or NH functions) are required for α7 nAChR NAM actions. This can be examined by investigating compounds where the NH moiety is replaced by an N-methyl group (i.e., 40), where the NH moiety is replaced by a methylene group (i.e., 41), or eliminated altogether (i.e., 42). Compound 43 represents an arylguanidine where basic properties have been eliminated (i.e., 43 is an amide).
Figure 20. The chemical structures of modified forms of arylguanidines.
D. Molecular Modeling

1. Homology Modeling and Docking Studies

The primary goal of our molecular modeling studies is to construct and validate a homology model of the active α7 nAChR. The constructed model will be used to identify the putative allosteric binding site(s) and to determine the interaction mode(s) of the studied compounds with α7 nAChRs. Since the α7 nAChR is a homomeric structure, the molecular modeling of only two attached subunits (i.e., a dimer) is justified, and should be sufficient to mimic the essential ligand binding domain. The construction will undergo a number of steps and different software will be employed. Aligning the amino acid sequence of the targeted portion of the protein with the matching portion of the selected template amino-acid sequence will be performed using ClustalX 2.0.\textsuperscript{137} The generation of multiple models lacking validation will be constructed using Modeller 9.12. Different docking programs (e.g., GOLD Suite 5.2 and AutoDock 4.2), as well as accurate residue orientation methods, should allow for model validation by comparing the results with previously published biochemical or crystallographic data. Therefore, the blind docking feature of AutoDock 4.2 using Gal (13) and the Connolly surface feature of SYBYL-X 2.1 will be used to explore the possible interaction site(s) at the ECD and the TMD of α7 nAChRs. GOLD Suite 5.2 will be used for model validation through the docking of α7 nAChR agonists (i.e., ACh, nicotine (1), EPI (3)) and the antagonist MLA (9) and comparing the results with reported crystal structures (PDB ID: 2XZ5, 1UW6, 2BYQ, and 2BYR, respectively).\textsuperscript{138-140} Further validation will be performed by docking of α7
nACh receptor modulators (i.e., Gal (13) for ECD, PNU-120596 (15) and ivermectin (16) for TMD). Prior to docking of the molecules, we will select the favorable low-energy conformations that were previously reported as a result of a systematic search investigation. The validated ECD and TMD models of α7 nAChRs will then be used for the new docking studies after specifying the targeted allosteric cavity. The resulting complexes of compounds with the candidate ECD and TMD models of α7 nAChRs will be rescored with the HINT (Hydropathic INTeractions) program, an empirical scoring function based on the experimental free energy information derived from log $P_{o/w}$ (the solvent partition coefficient for 1-octanol/water). The obtained candidate complexes of docked compounds at the ECD models of α7 nAChRs will provide beneficial information that will help in the prediction of required structural modifications to the current series.

2. Crystal Structures of Guanidines

For any bimolecular interaction in biological systems, the existence of specific attractive forces controls their molecular recognition. Sufficient knowledge about the nature of these specific interactions and their geometries, usually through their crystal structures, can offer valuable insight for medicinal chemists seeking innovative ways for drug development. When a lead compound has been identified, the optimization process of its specific attractive interactions becomes a promising approach.

Through reviewing the literature for molecular interactions and analyzing the specific interactions for a molecule of interest, we hope to reach a deeper understanding of how guanidines, as a key structural moiety in our series of compounds, might interact with α7
nAChRs. The approach of optimizing geometry of binding of guanidine compounds with their protein targets was used, previously, in a number of literature studies for the development of new guanidine analogs.\textsuperscript{142,143} As a valued resource for the nature of molecular interactions, we investigated the PDB database for molecular interactions of guanidine-containing ligands. We found eight available crystal structures (i.e., PDB IDs: 1S6F, 1S5S, 2VNT, 1ZMJ, 1ZML, 1ZMN, 3PO1, and 3HPT) including guanidine-containing ligands interacting with different types of serine protease enzymes.\textsuperscript{144-148} Besides that, identifying these key interactions can be used as references for predicting the binding mode of our arylguanidine compounds.

Overall, our eventual aim is to rationally design α7 nAChR NAMs with desired physicochemical and biological properties using multiple medicinal chemistry approaches.

**Hypothesis 5** is that the continual flow of data obtained from different parts in this project (e.g., biological data and molecular modeling studies) can be integrated using medicinal chemistry approaches to identify and optimize arylguanidines as NAMs of α7 nAChRs.
Previous knowledge of the reported allostERIC binding sites on \(\alpha_7\) nACHRs as well as \(\alpha_7\) nACHR NAMs can also be utilized to provide a rationale for designing novel \(\alpha_7\) nACHR NAMs.

The specific aims of the current project are:

a) Determination of the similarity or dissimilarity of the binding modes of the MD-354 series and the N-methyl series of arylguanidines at \(\alpha_7\) nACHRs.

b) Identification of what structural features of the 3-position in the arylguanidine compounds contribute to the activity profile of \(\alpha_7\) nACHR NAMs.

c) Optimization of the alkyl substituent at the anilinic nitrogen atom of arylguanidine NAMs.

d) Investigation of the role of the nitrogen atoms in the guanidine moiety of arylguanidine compounds for the inhibition profile at \(\alpha_7\) nACHRs.

e) Identification of the potential allostERIC binding site and the mode of interactions of arylguanidine NAMs at \(\alpha_7\) nACHRs.

f) Identification of a pharmacophore for small-molecule arylguanidine inhibitors for \(\alpha_7\) nACH receptor action.
IV. Results and Discussion

A. Synthesis

The nitrate salt of $m$-chlorophenylguanidine (MD-354; 20), the hydrochloride salt of phenylguanidine (PG; 21), the nitrate salt of 3-trifluoromethylphenylguanidine (22), the hemisulfate salt of 3-methoxyphenylguanidine (23) and the hydrochloride salt of 3-methylphenylguanidine (34) were available from previous studies. The syntheses of the hydrochloride salt of $N$-(3-chlorophenyl)-$N$-methylguanidine (24), the hydrochloride salt of $N$-methyl-$N$-phenylguanidine (25), the hydrochloride salt of $N$-(3-bromophenyl)-$N$-methylguanidine (26), the hydrochloride salt of $N$-(3-fluorophenyl)-$N$-methylguanidine (27), the hydrochloride salt of $N$-(3-iodophenyl)-$N$-methylguanidine (28), the nitrate salt of $N$-(3-methylphenyl)-$N$-methylguanidine (29), and the hydrochloride salt of $N$-(3-methoxyphenyl)-$N$-methylguanidine (30) were reported in our previous work. $^9$

$N$-Methyl-3-trifluoromethylaniline nitrate (37) was prepared in three steps according to a previously published procedure described for similar compounds (Scheme 1). $^{149-151}$ A first unsuccessful attempt was made to synthesize the ethyl $N$-[3-(trifluoromethyl)phenyl] carbamate (46). In this attempt, ethyl chloroformate was added to a solution of 3-
trifluoromethylaniline (44) and triethylamine in Et₂O and the reaction mixture was heated at reflux for 1 h resulting in a major product of urea derivative 45 (i.e., dimeric product). An alternative condition to obtain the desired intermediate carbamate 46 was used; ethyl chloroformate was added in a dropwise-manner to a solution of 3-trifluoromethylaniline (44) and sodium hydride to yield the desired carbamate in 88% yield. The carbamate 46 was then reduced to N-methyl-3-trifluoromethylaniline using LiAlH₄ in THF and the HCl salt 47 was prepared. An ethanolic solution of 47 was heated at reflux with cyanamide to give 37. The structure of the compound was confirmed by IR, ¹H NMR, and elemental analysis for C, H, N.

Scheme 1.*

*Reagents and conditions: a. ethyl chloroformate, Et₃N, Et₂O, reflux; b. ethyl chloroformate, NaH, THF, reflux; c. LiAlH₄, THF, reflux; d. HCl/Et₂O; e. NH₂CN, absolute EtOH, reflux; f. NH₄NO₃, H₂O.
Scheme 2 describes the synthesis of \(N\)-isopropyl-\(N\)-phenylguanidine nitrate (38). The synthesis consisted of a one-step reaction according to a literature procedure for a similar compound.\(^{152}\) Cyanamide was reacted with \(N\)-isopropylaniline hydrochloride (48) in EtOH. The hydrochloride salt of \(N\)-(3-methylphenyl)-\(N\)-methylguanidine was hygroscopic and, because of that, was converted to the nitrate salt using ammonium nitrate. The structure of \(N\)-isopropyl-\(N\)-phenylguanidine nitrate (38) was confirmed by IR, \(^1\)H NMR, and elemental analysis for C, H, N.

**Scheme 2.**

\(^*\)Reagents and conditions: a) \(\text{NH}_2\text{CN} (50\% \text{ aqueous}), \text{EtOH}, \text{reflux} \); ii) \(\text{NH}_4\text{NO}_3, \text{H}_2\text{O} \).

\(N\)-(3-Chlorophenyl)-\(N\)-isopropylguanidine hydrochloride (39) was prepared as described in Scheme 3. This was an unknown compound at the time of synthesis, but was prepared according to a literature procedure for a similar compound.\(^{151,153}\) 3-Chloroaniline (49) was treated with 2-bromopropane (50) in the presence of an aqueous solution of NaOH, allowing for a nucleophilic substitution reaction to yield the 3-chloro-
\(N\)-isopropylaniline (51). The final step was performed by heating an ethanolic solution of the hydrochloride salt of 51 at reflux with cyanamide to give 39. The desired compound was confirmed by IR spectrometry, \(^1\)H NMR spectrometry, and elemental analysis for C, H, N.

**Scheme 3.**

\[
\begin{align*}
\text{NH}_2 + \text{H}_3\text{C} \text{CH}_3 \quad a, b & \rightarrow \quad \text{CH}_3 \text{NH} \text{HCl} \\
\text{Cl} & \rightarrow \quad \text{Cl} \\
49 & \quad 50 & \quad 51 & \quad 39
\end{align*}
\]

\(^a\)Reagents and conditions: a. NaOH (15\% aqueous), ZnCl\(_2\) (saturated aqueous), 150 °C; b. HCl/Et\(_2\)O; c. NH\(_2\)CN, EtOH, reflux.

Based on the initial biological results obtained for compounds 38 and 39, compounds 52 and 53 were synthesized (see discussion below). \(N\)-Ethyl-\(N\)-phenylguanidine hydrochloride (52) was synthesized according to a literature procedure in a one-step reaction as described in Scheme 4.\(^{154}\) The \(N\)-ethylaniline hydrochloride (54) was allowed to react with a cyanamide in 1-pentanol. The desired product obtained was confirmed by IR, \(^1\)H NMR, and melting point.
Scheme 4.$^a$

![Reaction Scheme]

$^a$Reagents and conditions: a. NH$_2$CN, 1-pentanol, reflux.

Scheme 5 summarizes the synthesis of $N$-(3-chlorophenyl)-$N$-ethylguanidine hydrochloride (53). This was an unknown compound at the time of synthesis, but was prepared according to a literature procedure for a similar compound.$^{151,155}$ 3-Chloroaniline (49) was alkylated through a reductive amination step using acetaldehyde and pyridine-borane to yield 3-chloro-$N$-ethylaniline (55). The final step was performed by heating the ethanolic solution of the hydrochloride salt (i.e., 55) at reflux with cyanamide to give 53. The desired compound was confirmed by IR spectrometry, $^1$H NMR spectrometry, and elemental analysis for C, H, N.
Scheme 5.\textsuperscript{a}

\begin{align*}
\text{NH}_2 & \quad \xrightarrow{\text{a,b}} \quad \text{CH}_3 \quad \xrightarrow{\text{c}} \quad \text{CH}_3 \text{NH} \quad \text{HCl} \\
\text{49} & \quad \xrightarrow{\text{a,b}} \quad \text{55} & \quad \xrightarrow{\text{c}} \quad \text{53}
\end{align*}

\textsuperscript{a}Reagents and conditions: a. acetaldehyde, pyridine-borane, MeOH, reflux; b. HCl/Et\textsubscript{2}O; c. NH\textsubscript{2}CN, EtOH, reflux.

Three additional compounds (i.e., 56-58), suggested by the obtained results (see discussion below), were synthesized. \(N\)-(3-Cyanophenyl)-\(N\)-methylguanidine nitrate (56) was synthesized according to a literature procedure for a similar compound in a one-step reaction as described in Scheme 6.\textsuperscript{154} 3-(Methylamino)benzonitrile hydrochloride (59) was allowed to react with cyanamide in ethanol. The desired product obtained was confirmed by IR, \(^1\text{H} \) NMR, MS, and elemental analysis for C, H, N.

Scheme 6.\textsuperscript{a}

\begin{align*}
\text{H}_3\text{C} \quad \text{NH} \quad \text{HCl} & \quad \xrightarrow{\text{a}} \quad \text{H}_3\text{C} \quad \xrightarrow{\text{HNO}_3} \\
\text{59} & \quad \xrightarrow{\text{a}} \quad \text{56}
\end{align*}

\textsuperscript{a}Reagents and conditions: a. NH\textsubscript{2}CN, EtOH, reflux.
Scheme 7 summarizes the synthesis of N-cyclopentyl-N-phenylguanidine hydrochloride (57) and N-(3-chlorophenyl)-N-cyclopentylguanidine nitrate (58). The first step in the synthesis of these two unknown compounds was a reductive amination reaction of aniline (60) and 3-chloroaniline (49), respectively, and cyclopentanone (61) in the presence of sodium triacetoxyborohydride (STAB). The hydrochloride salts of the obtained compound (i.e., 62 and 63, respectively) were then heated at reflux with cyanamide to give 57 and 58 (the former as its hydrochloride salt). An additional step was performed to convert the hygroscopic 58 hydrochloride salt to the nitrate salt using ammonium nitrate.

Scheme 7.\(^a\)

\[^{a}\text{Reagents and conditions: a. STAB, acetic acid, DCM, room temperature; b. HCl/Et}_2\text{O; c. NH}_2\text{CN, EtOH, reflux; d. NH}_4\text{NO}_3, \text{H}_2\text{O.}\]
B. Biological Data

Our previous findings for investigating and optimizing the inhibitory effect of the arylguanidines were obtained using the whole-cell configuration of the patch-clamp technique on rat α7 nAChRs expressed in stably transfected human embryonic kidney (HEK) 293 cells. The number of compounds tested were neither sufficient to identify the key physicochemical property for interaction with α7 nAChRs nor enough to decide if the N-methyl series binds in a similar or dissimilar manner at α7 nAChRs as MD-354 series.

The functional data in the current project were obtained by evaluating the biological potencies of the compounds in inhibiting ACh-induced responses in *Xenopus laevis* oocytes expressing human α7 nAChRs using an automated two-electrode voltage-clamp assay. Here, the automated system was introduced to overcome several drawbacks associated with conventional patch-clamping techniques such as time-consuming issues and being very laborious. However, recorded potencies of our compounds are expected to be lower compared to those from the previously used HEK 293 cells because oocyte surface components, such as the large number of invaginations in the membrane and the surrounding vitelline membrane and follicle cells, decrease the accessibility of the compounds. All inhibitory effects measured here were reported in the form of half-maximal inhibitory concentrations (i.e., IC₅₀ values). Compounds 20-34, 37 and 38 showed inhibitory action (Table 7) and potency varied over a 10-fold range.
Table 7. IC\textsubscript{50} values for \(\alpha7\) nAChRs of different structural modifications at the 3-position of the MD-354 series and the N-methyl series of arylguanidines.

![MD-354 (20) Series](image)

<table>
<thead>
<tr>
<th>IC\textsubscript{50} ± SEM ((\mu\text{M}))</th>
<th>Compound</th>
<th>X</th>
<th>Compound</th>
<th>IC\textsubscript{50} ± SEM ((\mu\text{M}))</th>
</tr>
</thead>
<tbody>
<tr>
<td>59.75 ± 1.21</td>
<td>21</td>
<td>H</td>
<td>25</td>
<td>125.40 ± 1.12</td>
</tr>
<tr>
<td>41.51 ± 1.24</td>
<td>20</td>
<td>Cl</td>
<td>24</td>
<td>31.45 ± 1.40</td>
</tr>
<tr>
<td>23.58 ± 1.08</td>
<td>31</td>
<td>Br</td>
<td>26</td>
<td>30.23 ± 1.27</td>
</tr>
<tr>
<td>53.60 ± 6.69</td>
<td>32</td>
<td>F</td>
<td>27</td>
<td>73.22 ± 1.41</td>
</tr>
<tr>
<td>21.81 ± 1.24</td>
<td>33</td>
<td>I</td>
<td>28</td>
<td>12.63 ± 1.41</td>
</tr>
<tr>
<td>21.29 ± 1.21</td>
<td>22</td>
<td>CF\textsubscript{3}</td>
<td>37</td>
<td>31.04 ± 2.82</td>
</tr>
<tr>
<td>118.40 ± 1.97</td>
<td>34</td>
<td>CH\textsubscript{3}</td>
<td>29</td>
<td>31.41 ± 1.30</td>
</tr>
<tr>
<td>40.00 ± 1.44</td>
<td>23</td>
<td>OCH\textsubscript{3}</td>
<td>30</td>
<td>57.09 ± 1.98</td>
</tr>
</tbody>
</table>
1. QSAR

The chloro group at the 3-position of both MD-354 (20) and 24 was replaced with a number of substituents considering the electronic, the lipophilic, and the steric nature of the new substituent (Table 7). The introduction of other halogen atoms (i.e., -F, -Br, and –I) at the 3-position allowed for testing mainly the effect of substituent-size variation over a relatively fixed range of electron-withdrawing effects. Furthermore, replacement of the chloro group with -CH₃ or -OCH₃ groups as electron-donating groups explored the effect of such modification on activity. By examining the inhibitory activity of these two series of arylguanidine analogs (n = 16) at α7 nACh receptors, we hoped to answer multiple questions regarding binding modes of arylguanidines and the impact of parallel substituent modifications at the aryl 3-position. The obtained IC₅₀ values showed a range of 21 – 118 µM and 12 – 125 µM, respectively (Table 7). Plots for representative compounds of these two series (i.e., MD-354 (20) and its N-methyl analog (24)) are shown in Figure 21; IC₅₀ of 41.51 ± 1.24 µM and 31.45 ± 1.40 µM, respectively were calculated. While removing the chloro group of MD-354 (20) caused a slight reduction in potency (i.e., 21, IC₅₀ = 59.75 µM; compared to MD-354 (20), IC₅₀ = 41.51 µM), a more pronounced effect of chloro group removal (more than four-fold reduction in potency) was seen in the N-methyl series (i.e., 25, IC₅₀ = 125.40 µM; compared to 24, IC₅₀ = 31.45 µM). Furthermore, the least potent inhibitor in the MD-354 series was the 3-methyl analog 34 (IC₅₀ = 118.40 µM) whereas the least potent in the N-methyl series is the deschloro analog 25 (IC₅₀ = 125.40 µM). Detailed graphs representing functional data of these compounds are shown in Appendix A.
To determine if the two series bind in similar manner, we investigate the impact of parallel substituent modifications at the aryl 3-position in the two series of analogs. That is, if the two series are binding in a common manner, there should be a relationship between their potencies. That is, it would be expected that their potencies would co-vary. Linear regressions and statistical analyses implemented in this project were conducted.
using GraphPad Prism® software (version 5.04), SPSS (Statistical Package for the Social Sciences; version 22.0), and JMP (John’s Macintosh Program; version 11.2). Correlations expressing a $p$ value of less than 0.05 were considered significant. A poor correlation ($r = 0.474; n = 8$) was found between pIC$_{50}$ values of the MD-354 (i.e., NH) series versus pIC$_{50}$ values of the N-methyl series (Figure 22). Data on the 3-CH$_3$ analog of the N-methyl series (i.e., 29) were replicated and no difference was observed in the IC$_{50}$ value. Follow-up statistical analysis was conducted to detect any potential outliers among the obtained inhibitory potency values using Cook’s $D$, and Z score methods, specifically for the 3-CH$_3$ compounds (Table 8). All IC$_{50}$ values of the tested compounds, including the 3-CH$_3$ analogs, appeared to be within the acceptable range and no single value exceeded the cutoffs. Therefore, it is unlikely that the two series are interacting in the same manner at $\alpha_7$ nAChRs. This finding supported our previously proposed binding modes identified by preliminary docking studies (i.e., as shown in Figure 18).

Figure 22. Plot of pIC$_{50}$ values of the MD-354 (20) series versus the N-methyl analog series ($r = 0.474, p = 0.235, n = 8$).
Table 8. Outlier detection tests and the calculated cut-off values.

<table>
<thead>
<tr>
<th>Test</th>
<th>Calculated Range</th>
<th>Cut-off</th>
<th>Software</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cook’s D</td>
<td>0.00092 – 0.4128</td>
<td>&gt; 0.5</td>
<td>SPSS\textsuperscript{a}</td>
</tr>
<tr>
<td>Zscore</td>
<td>-1.6549 – 1.6459</td>
<td>± 2.5</td>
<td>SPSS\textsuperscript{a}</td>
</tr>
<tr>
<td>Cook’s Influence</td>
<td>0.0182 – 0.4471</td>
<td>&gt; 1</td>
<td>JMP\textsuperscript{b}</td>
</tr>
</tbody>
</table>

\textsuperscript{a} The Statistical Package for the Social Sciences (SPSS Science, Chicago, IL).

\textsuperscript{b} John’s Macintosh Program (JMP, SAS Institute Inc., Belmont, Canada).

To analyze the influence of structural features at the 3-position of these compounds to act as inhibitors at $\alpha_7$ nACh receptors, Hansch-type QSAR studies were performed examining several physicochemical properties of the substituent at the 3-position; $\pi$ (hydrophobic constant), \textsuperscript{132} $\sigma_m$ (Hammett electronic constant), \textsuperscript{132} Verloop’s steric parameters ($L$, Verloop length; $B_1$, Verloop minimum width; $B_5$, Verloop maximum width), \textsuperscript{159} MR (Molar refraction), \textsuperscript{160} and solvent accessibility volume (Vol) as calculated using Chimera 1.10.1, \textsuperscript{161} Number of valence electron (NVE), \textsuperscript{162} Complete molar refraction (CMR) as calculated using ChemBioDraw (version 13.0), Molar Volume (MV), Parachor value (Pc) as calculated using ChemSketch (version 10.0), and polarizability. \textsuperscript{162} Detailed numerical values of all these physicochemical parameters for the currently used substituents are shown in Table 9. Data showed that the action of the MD-354 series seemed to be positively correlated with the electronic character ($\sigma_m$) of their meta substituent ($r = 0.820; n = 8$) (Figure 23) and no other correlations were found (Table 10). However, the narrow range of IC\textsubscript{50} values in this series indicated a need to consider additional compounds.
Table 9. Hansch-type QSAR analysis of the α7 nAChR inhibitory activity of 3-substituted arylguanidines.

| X  | IC<sub>50</sub> (µM)<sup>a</sup> | <pi><sup>b</sup></pi> | <sigma><sup>b</sup></sigma> | L<sup>b</sup> | B<sub>1</sub><sup>b</sup> | B<sub>5</sub><sup>b</sup> | MR<sup>b</sup> | Vol<sup>b</sup> | NVE<sup>b</sup> | CMR<sup>b</sup> | MV<sup>b</sup> | Pc<sup>b</sup> | Polariz.<sup>b</sup> | IC<sub>50</sub> (µM)<sup>a</sup> | X  |
|----|------------------|---|---|---|---|---|---|---|---|---|---|---|---|---|------------------|---|
| H  | 59.75 ± 1.21     | 0  | 0  | 2.06 | 1 | 1 | 0 | 122 | 52 | 4.08 | 115 | 304 | 15.51 | 125.40 ± 1.12 | H  |
| Cl | 41.51 ± 1.24     | 0.14 | 0.34 | 2.65 | 1.35 | 1.35 | 0.92 | 126 | 58 | 4.1 | 118 | 304 | 15.46 | 31.45 ± 1.40 | Cl |
| Br | 23.58 ± 1.08     | 0.71 | 0.37 | 3.52 | 1.8 | 1.8 | 6.03 | 145 | 58 | 4.57 | 124 | 333 | 17.33 | 30.23 ± 1.27 | Br |
| F  | 53.60 ± 6.69     | 0.86 | 0.39 | 3.82 | 1.95 | 1.95 | 8.88 | 166 | 58 | 4.86 | 127 | 347 | 18.51 | 73.22 ± 1.41 | F  |
| I  | 21.81 ± 1.24     | 1.12 | 0.35 | 4.23 | 2.15 | 2.15 | 13.94 | 198 | 58 | 5.39 | 132 | 367 | 20.53 | 12.63 ± 1.41 | I  |
| CF<sub>3</sub> | 21.29 ± 1.21     | 0.88 | 0.43 | 3.3 | 1.98 | 2.61 | 5.02 | 151 | 76 | 4.59 | 145 | 358 | 17.39 | 31.04 ± 2.82 | CF<sub>3</sub> |
| CH<sub>3</sub> | 118.40 ± 1.97    | -0.02 | 0.12 | 3.98 | 1.35 | 3.07 | 7.87 | 146 | 64 | 4.7 | 137 | 354 | 17.81 | 31.41 ± 1.30 | CH<sub>3</sub> |
| OCH<sub>3</sub> | 40.00 ± 1.44     | 0.56 | -0.07 | 2.87 | 1.52 | 2.04 | 5.65 | 138 | 58 | 4.54 | 130 | 335 | 17.26 | 57.09 ± 1.98 | OCH<sub>3</sub> |

<sup>a</sup> IC<sub>50</sub> data from Table 7.

<sup>b</sup> See Table 5 for the description of parameters.
Table 10. Summary of the linear regression analysis results for MD-354 series \((n = 8)\).

<table>
<thead>
<tr>
<th>Equation No.</th>
<th>QSAR Equation for pIC(_{50})</th>
<th>(r)</th>
<th>(F)</th>
<th>(p)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.9700 ((\pm 0.5705) \pi -3.732 (\pm 2.511))</td>
<td>0.570</td>
<td>2.89</td>
<td>0.140</td>
</tr>
<tr>
<td>2</td>
<td>0.6194 ((\pm 0.1763) \sigma_m -2.481 (\pm 0.7762))</td>
<td><strong>0.820</strong></td>
<td><strong>12.34</strong></td>
<td><strong>0.012</strong></td>
</tr>
<tr>
<td>3</td>
<td>1.851 ((\pm 0.8862) L -4.830 (\pm 3.901))</td>
<td>0.648</td>
<td>4.36</td>
<td>0.081</td>
</tr>
<tr>
<td>4</td>
<td>1.075 ((\pm 0.4440) B_1 -3.088 (\pm 1.954))</td>
<td>0.703</td>
<td>5.86</td>
<td>0.052</td>
</tr>
<tr>
<td>5</td>
<td>0.9364 ((\pm 0.9639) B_5 -2.119 (\pm 4.243))</td>
<td>0.368</td>
<td>0.94</td>
<td>0.369</td>
</tr>
<tr>
<td>6</td>
<td>9.317 ((\pm 5.899) MR -34.91 (\pm 25.97))</td>
<td>0.541</td>
<td>2.49</td>
<td>0.165</td>
</tr>
<tr>
<td>7</td>
<td>64.89 ((\pm 27.64) Vol -136.2 (\pm 121.7))</td>
<td>0.691</td>
<td>5.51</td>
<td>0.057</td>
</tr>
<tr>
<td>8</td>
<td>12.93 ((\pm 9.965) NVE +3.413 (\pm 43.86))</td>
<td>0.468</td>
<td>1.68</td>
<td>0.242</td>
</tr>
<tr>
<td>9</td>
<td>0.9616 ((\pm 0.5335) CMR +0.3771 (\pm 2.348))</td>
<td>0.592</td>
<td>3.25</td>
<td>0.121</td>
</tr>
<tr>
<td>10</td>
<td>17.49 ((\pm 13.79) MV +51.62 (\pm 60.69))</td>
<td>0.459</td>
<td>1.61</td>
<td>0.252</td>
</tr>
<tr>
<td>11</td>
<td>57.77 ((\pm 29.11) Pc +83.82 (\pm 128.1))</td>
<td>0.629</td>
<td>3.94</td>
<td>0.094</td>
</tr>
<tr>
<td>12</td>
<td>3.694 ((\pm 2.085) Polarizability +1.238 (\pm 9.178))</td>
<td>0.586</td>
<td>3.14</td>
<td>0.127</td>
</tr>
</tbody>
</table>

Figure 23. Plot of pIC\(_{50}\) values of the MD-354 \((20)\) series versus the electronic character \((\sigma_m)\) \((r = 0.821, p = 0.012, n = 8)\).
On the other hand, a significant positive correlation was found between pIC_{50} values of the N-methyl series compounds with their lipophilic nature ($\pi$) ($r = 0.912; n = 8$) and their minimum width (Verloop $B1$) ($r = 0.927; n = 8$) (Figure 24A and B, respectively) (Table 11). These two parameters gave the best-fitting models that are supported by the highest F values (Table 11). This suggests the importance of these physicochemical properties at the 3-position of the N-methyl series. However, a significant internal correlation exists between the two properties ($r = 0.956; n = 8$) (Figure 25). Detailed correlation plots of all these physicochemical parameters for the currently used substituents in the N-methyl series are shown in Appendix B.

Table 11. Summary of the linear regression analysis results for N-methyl (i.e., 24) series ($n = 8$).

<table>
<thead>
<tr>
<th>Equation No.</th>
<th>QSAR Equation for pIC_{50}</th>
<th>$r$</th>
<th>F</th>
<th>$p$</th>
</tr>
</thead>
<tbody>
<tr>
<td>13</td>
<td>1.326 (± 0.2431) $\pi - 5.303 \ (± 1.072)$</td>
<td><strong>0.912</strong></td>
<td>29.74</td>
<td><strong>0.001</strong></td>
</tr>
<tr>
<td>14</td>
<td>0.2923 (± 0.2348) $\sigma_m - 1.045 \ (± 1.035)$</td>
<td>0.453</td>
<td>1.55</td>
<td>0.260</td>
</tr>
<tr>
<td>15</td>
<td>1.860 (± 0.6427) $L - 4.883 \ (± 2.835)$</td>
<td>0.763</td>
<td>8.38</td>
<td>0.030</td>
</tr>
<tr>
<td>16</td>
<td>1.211 (± 0.2000) $B_1 - 3.693 \ (± 0.8819)$</td>
<td><strong>0.927</strong></td>
<td><strong>36.68</strong></td>
<td><strong>0.001</strong></td>
</tr>
<tr>
<td>17</td>
<td>0.9730 (± 0.7918) $B_5 - 2.286 \ (± 3.492)$</td>
<td>0.448</td>
<td>1.51</td>
<td>0.265</td>
</tr>
<tr>
<td>18</td>
<td>12.85 (± 2.902) $MR - 50.53 \ (± 12.80)$</td>
<td>0.875</td>
<td>18.61</td>
<td>0.021</td>
</tr>
<tr>
<td>19</td>
<td>70.71 (± 15.37) $Vol - 162.2 \ (± 67.81)$</td>
<td>0.882</td>
<td>12.16</td>
<td>0.013</td>
</tr>
<tr>
<td>20</td>
<td>6.216 (± 9.293) NVE + 32.89 (± 40.99)</td>
<td>0.263</td>
<td>0.44</td>
<td>0.528</td>
</tr>
<tr>
<td>21</td>
<td>1.238 (± 0.2545) $CMR - 0.8450 \ (± 1.123)$</td>
<td>0.893</td>
<td>3.24</td>
<td>0.122</td>
</tr>
<tr>
<td>22</td>
<td>17.57 (± 11.16) $MV + 51.17 \ (± 49.21)$</td>
<td>0.540</td>
<td>2.48</td>
<td>0.166</td>
</tr>
<tr>
<td>23</td>
<td>63.14 (± 18.97) $Pc + 59.83 \ (± 83.65)$</td>
<td>0.805</td>
<td>11.08</td>
<td>0.023</td>
</tr>
<tr>
<td>24</td>
<td>4.794 (± 1.001) Polarizability - 3.625 (± 4.416)</td>
<td>0.890</td>
<td>9.87</td>
<td>0.016</td>
</tr>
</tbody>
</table>
Figure 24. Plot of pIC$_{50}$ values of the N-methyl series versus A) the Verloop minimum width ($B_1$) ($r = 0.927$, $p = 0.001$, $n = 8$) and B) the hydrophobic constant $\pi$ ($r = 0.912$, $p = 0.001$, $n = 8$).
Based on the above QSAR studies, there appears to be dissimilar binding modes for the two series of compounds at α7 nAChRs (i.e., structure-activity results for one series cannot be applied to the other). Among the examined physicochemical properties, only electronic character appeared to play a role at the 3-position of the MD-354 series for α7 nAChR action; however, more compounds are required to substantiate this. For the N-methyl series, two major factors appear to be involved as important structural features at that position for α7 nAChR action (i.e., lipophilic nature and the minimum width of the substituent). Due to a significant intercorrelation between the two parameters examined for the current data set (i.e., between π and Verloop B1), it was not possible to identify a single parameter as being the more important. Consequently, it was of interest to synthesize an additional compound in this series that clarifies uncertainty associated

**Figure 25.** Plot describing the internal correlation between the hydrophobic constant (π) and Verloop minimum width (B1) ($r = 0.956, p = 0.0016, n = 8$).
with the importance between these two parameters. Thus, the next candidate compound to be synthesized and tested was the 3-CN analog (56) since it provides a high Verloop minimum width value \(B1 = 1.6\) and low lipophilic character \(\pi = -0.57\). According to Equation 13 (Table 11), if the hydrophobic character is the most important physicochemical property, the IC\(_{50}\) value will be 0.873 \(\mu\)M. Whereas, if the minimum width property is the most important physicochemical parameter, the IC\(_{50}\) value, according to Equation 16, will be 17,563 \(\mu\)M.

From this point, our efforts will only consider the N-methyl series for further investigation. The MD-354 series is not of particular interest due to selectivity reasons since these compounds bind reasonably well at 5-HT\(_3\) receptors.

2. Topliss Tree

The above QSAR analysis of the N-methyl series of aryl guanidines focused on investigating the effects of various substituents at the aryl 3-position. The Topliss-decision tree approach allows an investigation of the lipophilic effect at the N\(_1\) position. The necessity of introducing the N-isopropyl pairs was also to test whether or not the two series of compounds (i.e., the chloro and the deschloro compounds) interact with \(\alpha 7\) nACh receptors in a similar manner depending on the fold-difference in biological data obtained. The examination of inhibitory action of a fixed concentration (i.e., 10 \(\mu\)M) of compounds 20, 21, 24, 25, 38, and 39 at \(\alpha 7\) nACh receptors resulted in a different extent of inhibition compared to the normalized current evoked by ACh at its EC\(_{50}\) concentration (Figure 26). Incorporation of the N-isopropyl group as seen in 39 not only
increases lipophilicity but adds extra steric bulk that appears to be not as well tolerated as the N-methyl group in 24. Results also showed that the introduction of an isopropyl group in 38 seems to result in retention of inhibition potency at α7 nAChRs. The new isopropyl analogs appear to have less selectivity and can inhibit α3β4 nAChRs, especially the 3-chloro compound 39 (Figure 26). Furthermore, it seems that there is a non-parallel inhibitory effect between the chloro-deschloro pairs of compounds indicating either possible different binding modes or distinct binding sites for the two series. This suggests that the introduction of an ethyl group (i.e., 52, and 53 for the deschloro and chloro analogs, respectively) might optimize the effect, especially for the chloro series (20, 24, and 39).

![Chemical structures](image)

**Figure 26.** Effect ± SEM of 20, 21, 24, 25, 38, and 39 at 10 μM concentration on ACh function at α7 (ACh EC\textsubscript{50} = 280 μM; red bars) and α3β4 (ACh EC\textsubscript{50} = 100 μM; blue bars) nAChRs relative to ACh (normalized current = 1).
The IC<sub>50</sub> values obtained later showed different inhibition patterns of these compounds on α7 nAChR function (Table 12). Replacing the methyl group in 24 with isopropyl (i.e., 39) caused a slight improvement in potency. Removing the chloro group of 39 (i.e., 38) caused no effect on potency (Table 12). The most dramatic effect of chloro group removal (more than four-fold reduction in potency) was seen in the N-methyl series (i.e., 25, IC<sub>50</sub> = 125.40 µM; compared to 24, IC<sub>50</sub> = 31.45 µM). Furthermore, although it is perhaps premature to draw definitive conclusions, when comparing antagonist potency of the two pair of compounds 20, 21 in the guanidine series and 24, 25 in the N-methyl series, the data suggest that the two series of compounds might bind in a different manner because parallel structural modifications did not result in a parallel shift in activity (Figure 26). That is, removal of the 3-Cl group in the guanidine series resulted in a ca. 5-fold decreased potency whereas in the N-methyl series it was ca. 25-fold. If the compounds were binding in the same manner, the shift in activity might be expected to be of the same magnitude. However, additional compounds are needed to support such an assumption and the next candidate compounds, according to Topliss tree approach, are the deschloro and the 3-chloro analogs of N-cyclopentyl-N-phenylguanidine (i.e., 57 and 58, respectively).<sup>163</sup>
Table 12. IC<sub>50</sub> values at α7 nAChRs for arylguanidines with different structural modifications at the N<sub>1</sub> and 3-positions.

<table>
<thead>
<tr>
<th>Compound</th>
<th>R</th>
<th>X</th>
<th>IC&lt;sub&gt;50 ± SEM µM&lt;/sub&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>21</td>
<td>H</td>
<td>H</td>
<td>59.75 ± 1.21</td>
</tr>
<tr>
<td>20</td>
<td>H</td>
<td>Cl</td>
<td>41.51 ± 1.24</td>
</tr>
<tr>
<td>25</td>
<td>CH&lt;sub&gt;3&lt;/sub&gt;</td>
<td>H</td>
<td>125.40 ± 1.12</td>
</tr>
<tr>
<td>24</td>
<td>CH&lt;sub&gt;3&lt;/sub&gt;</td>
<td>Cl</td>
<td>31.45 ± 1.40</td>
</tr>
<tr>
<td>52</td>
<td>C&lt;sub&gt;2&lt;/sub&gt;H&lt;sub&gt;5&lt;/sub&gt;</td>
<td>H</td>
<td>39.84 ± 1.07</td>
</tr>
<tr>
<td>53</td>
<td>C&lt;sub&gt;2&lt;/sub&gt;H&lt;sub&gt;5&lt;/sub&gt;</td>
<td>Cl</td>
<td>26.92 ± 1.07</td>
</tr>
<tr>
<td>38</td>
<td>CH(CH&lt;sub&gt;3&lt;/sub&gt;)&lt;sub&gt;2&lt;/sub&gt;</td>
<td>H</td>
<td>19.78 ± 1.35</td>
</tr>
<tr>
<td>39</td>
<td>CH(CH&lt;sub&gt;3&lt;/sub&gt;)&lt;sub&gt;2&lt;/sub&gt;</td>
<td>Cl</td>
<td>21.89 ± 1.42</td>
</tr>
</tbody>
</table>

3. Role of Nitrogen Atoms

The purpose here was to develop an understanding of the role of the guanidine moiety in arylguanidine inhibitory actions at α7 nAChRs. We investigated the effect of alkylating one or more of the guanidine nitrogen atoms, and the necessity of the presence of these nitrogen atoms in our compounds for their inhibitory action. With respect to the arylguanidines, it is already known that the introduction of a methyl group at the aniline nitrogen of MD-354 (20) (i.e., 24)
improved both potency and selectivity at α7 nAChRs. The incorporation of a methyl group at the terminal nitrogen atom (i.e., 40; IC₅₀ = 22.07 µM) resulted in a slight improvement in potency compared to MD-354 (20, IC₅₀ = 41.51 µM); however, this modification is detrimental to the inhibition efficacy since the dose-response curve does not show a complete inhibition of the ACh response at high concentrations (Figure 27A). Furthermore, replacement of the aniline NH by a methylene group (i.e., 41), or removing one of the terminal nitrogen atoms of 41 (i.e., 3-chlorophenylethylamine; 42) resulted in retention of potency relative to MD-354 (20), respectively, at α7 nACh receptors (Figure 27B and C). The replacement of one of the two terminal nitrogen atoms with a carbonyl oxygen atom (i.e., 3-chlorophenylurea; 43; EC₅₀ = 365.9 µM) resulted in an activator molecule for α7 nAChRs (Figure 27D).

From the tested compounds reported in the above data, it would seem that not all of the nitrogen atoms are required for activity at α7 nAChRs. This observation, however, requires further testing to determine the mechanism of inhibition (i.e., competitive or non-competitive inhibition) and the mode of binding of these compounds at the molecular level. That is, these provide multiple means to block the actions of ACh. These analogs might produce their action by binding at the orthosteric site, a different allosteric site (other than that to be described in the next section) and/or as channel blockers. However, these findings open new avenues for future investigation.
Figure 27. Plot of IC$_{50}$ curves for 40 (A), 41 (B), and 42 (C) in µM at α7 nAChRs (ACh concentration is 100 µM). Compound 43 (D) showed weak agonist activity at α7 nAChRs.
C. Molecular Modeling

1. Sequence Alignment and Homology Modeling

Homology modeling studies were used to address a number of questions regarding the potential binding site(s) and the mode of interactions that explain the functional activity of our compounds at $\alpha_7$ nAChRs. All primary sequences used in our alignment were obtained from the Universal Protein Knowledgebase (UniProtKB). Multiple alignments of the human $\alpha_7$ nAChR sequence with several orthologs were performed using ClustalX 2.0 and key amino acid residues were determined (Figure 28 and 29). Furthermore, these alignments were established to identify and match conserved residues between the human $\alpha_7$ nAChR and the template, the X-ray crystal structure of a chimeric ECD of a human $\alpha_7$ nAChR and an *Lymnaea stagnalis* acetylcholine binding protein (AChBP) (PDB ID 3SQ6) that shares 64% sequence identity with the ECD of the human $\alpha_7$ nAChR ECD, the NMR structure of the TMD of the $\alpha_4$ nAChR subunit (PDB ID 2LLY) that shares 49% sequence identity with the TMD of the human $\alpha_7$ nAChR, and the cryo-electron microscopy structure of *Torpedo marmorata* nAChR (PDB ID 2BG9) that shares 53% sequence identity with the TMD of the human $\alpha_7$ nAChR.

Through aligning the primary sequence of the ECD of the human $\alpha_7$ nAChR with the template, important regions in the aligned sequences were identified including the loops A, B, C, D, E, and F that primarily formed the orthosteric binding sites in these receptors (Figure 28). Furthermore, the two cysteine residues (i.e., Cys150 and Cys164) forming the disulfide bond characterizing the Cys-loop, and the adjacent two cysteine residues (Cys212 and Cys213) forming the disulfide bond at the C loop were also matched. The advantage of using the primary
sequence of the chimeric ECD of the α7 nAChR (PDB ID 3SQ6) can be seen through the presence of many amino acids that are highly conserved with the human α7 nAChR sequence. Other important amino acids are Val220, Thr221, and Phe222 that are known to interact with α7 nAChR PAM galantamine (13).70

![Sequence alignment of the ECD portion from the human α7 nAChR and α7 nAChR chimera. The asterisks (*) indicate conserved amino acids, whereas the colons (:) and periods (.) indicate strongly and weakly conserved amino acids, respectively. The colored lines represent the main loops in the ECD. The red box indicates the galantamine binding site as shown by Ludwig et al.70](image)

**Figure 28.** Sequence alignment of the ECD portion from the human α7 nAChR and α7 nAChR chimera. The asterisks (*) indicate conserved amino acids, whereas the colons (:) and periods (.) indicate strongly and weakly conserved amino acids, respectively. The colored lines represent the main loops in the ECD. The red box indicates the galantamine binding site as shown by Ludwig et al.70

Sequence alignments of the TMDs of the α7 nAChRs and the templates used revealed a low degree of similarity compared to the ECDs alignments. Important regions in the aligned sequences were identified which included Glu237 and Glu258 at the ends of M2, the amino acids responsible in forming the upper and lower rings within the pore of the α7 nAChR (Figure 29).25 Other identified residues include Ser222 and Ala225 from M1, Met253 from M2, Gln272 from M3, and Phe455, Thr456 and Cys459 from M4 that are known to have essential interactions with two α7 nAChR PAMs, PNU-120596 (15) and ivermectin (16).80
Construction of 100 homology models was accomplished in the form of dimers of the human α7 nAChR ECD and in the form of monomers of the TMD using Modeller 9.12. Dimer formation provided the essential part of the ECD for the molecular modeling study since α7 nACh receptors are homomeric and the orthosteric binding site is located between each two subunits. The three-dimensional structures of 100 models of each domain were energetically evaluated based on DOPE (Discrete Optimized Protein Energy) scores. A subsequent validation step was performed by the docking of α7 nACh receptor modulators (i.e., galantamine (13) for the ECD, and PNU-120596 (15) and ivermectin (16) for the TMD).

Figure 29. Sequence alignment of the TMD portion of nAChRs from three species. The asterisks (*) indicate conserved amino acids, whereas the colons (:) and periods (.) indicate strongly and weakly conserved amino acids, respectively. The four main TMDs are represented by the turquoise lines. The red box indicates important amino acid residues interacting with either PNU-120596 or ivermectin as shown in Young et al. 74
2. Crystal Structures of Guanidines

In the PDB archive, we found that guanidine-containing ligands have common patterns in their interactions with macromolecules. The protonated form of guanidines (i.e., the guanidinium group) is the common form at physiological pH, which suggests their potential involvement in electrostatic interactions.\textsuperscript{144,145} In most cases, guanidinium groups were found to form bidentate interaction with the side-chain carboxylate of aspartate amino acids (i.e., 1 and 3-5; Table 13).\textsuperscript{144-146} The orientation of the guanidine-containing ligands, in some cases, restricted the bidentate form, so that only one ionic hydrogen bond instead of two was formed with the side-chain carboxylate of aspartate and glutamate amino acids (i.e., 2, 6, and 7; Table 13).\textsuperscript{146} Guanidinium groups, also, are able to form hydrogen bonds with the side-chain hydroxyl groups of serine residues (i.e., 1-3; Table 13) and with the backbone oxygen atoms of amino acids (i.e., 1-6, and 8; Table 13).\textsuperscript{144-148}
Table 13. Summary of interactions of guanidinium groups in guanidine-containing ligands from the PDB database.

<table>
<thead>
<tr>
<th>PDB ID</th>
<th>Interaction Diagram</th>
</tr>
</thead>
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</tr>
<tr>
<td>1S5S</td>
<td><img src="image2.png" alt="Interaction Diagram" /></td>
</tr>
<tr>
<td>2VNT</td>
<td><img src="image3.png" alt="Interaction Diagram" /></td>
</tr>
<tr>
<td>1ZMJ</td>
<td><img src="image4.png" alt="Interaction Diagram" /></td>
</tr>
</tbody>
</table>
5) PDB ID: 1ZML

6) PDB ID: 1ZMN

7) PDB ID: 3PO1

8) PDB ID: 3HPT
3. Allosteric Binding Site Exploration

Eight putative allosteric binding pockets for the small-molecule NAMs of α7 nAChRs were initially identified, through cavity-search studies conducted with the Connolly surface feature of SYBYL-X 2.1 and the blind docking feature of AutoDock 4.2 at the α7 nAChR model. Initial findings indicated the presence of five cavities at the ECD and three cavities at the TMD (I-VIII; Figure 30). The five cavities found in the ECD are in agreement with the previously reported work of Iorga et al.\(^\text{126}\) (ECD upper exterior site (I), the orthosteric site (II), ECD-lower exterior site (III), ECD-upper interior site (IV), and ECD-lower interior site (V); Figure 30A and B). Further evaluation of these potential binding pockets was performed to exclude cavities that violate logical standards. Prior knowledge that both MD-354 (20) and 24 are not competitive antagonists and not channel blockers led to the elimination of three cavities (i.e., the orthosteric site (II), ECD-upper interior site (IV), and ECD-lower interior site (V)). Also, the ECD-upper exterior site was eliminated from consideration due to limited cavity volume (i.e., 123 Å\(^3\) compared to 145 Å\(^3\) and 161 Å\(^3\), the molecular volume for 20 and 24, respectively). This suggested that the ECD-lower exterior site is the only ECD site candidate for our modeling and docking studies.
Figure 30. A and B represent side views of the modeled ECD of the human α7 nAChR dimer showing the location of allosteric cavities I-V at the exterior and interior surfaces, respectively. C represents top view of the TMD showing the location of allosteric cavities VI-VIII.
Similarly, the three TMD cavities (i.e., TMD intrasubunit site (VI), TMD exterior site (VII), TMD interior site (VIII); Figure 30C) were analyzed. These cavities were in agreement with reported computational work of Brannigan et al.\textsuperscript{129} on possible binding pockets of the anesthetic isoflurane in nicotinic receptors. Both the TMD exterior site and TMD interior site were eliminated due to limited cavity volume (i.e., 116 Å\textsuperscript{3}) and the channel blocking problem, respectively. This makes the TMD intrasubunit site the most likely TMD site candidate for our modeling and docking studies.

The two identified binding pockets (i.e., the ECD-lower exterior site (III) and the large TMD intrasubunit cavity (VI)) are known to be the binding site for α7 nAChR modulators. For example, galantamine (13) is an α7 nAChR PAM that interacts at the ECD-lower exterior site (III), whereas ivermectin and PNU-120596 are two α7 nAChR PAMs that interact in distinct locations at the general TMD intrasubunit cavity. This fact led us to target three different locations; one at the ECD characterized by amino acid residue Thr221 and two in the TMD intrasubunit cavity that are characterized by amino acid residues Met253 (i.e., the key amino acid for ivermectin binding) and Ser222 (i.e., the key amino acid for PNU-120596 binding). All these key residues were determined using the reported mutagenesis data\textsuperscript{70,74} that were discussed above.

4. Docking Studies and Hydropathic Analyses

Within SYBYL-X 2.1, four low-energy rotamers of arylguanidine analogs were computationally prepared for docking. Molecular docking was conducted using the CHEMPLP scoring function (within the genetic algorithm docking program GOLD 5.2) and the docking solutions were analyzed (SYBYL-X 2.1). The binding site was defined to include all atoms
within 10 Å of the α-carbon atom of the key amino acid residue for the candidate homology models. Based on the fitness scores and the binding orientation of each ligand within the binding cavity, the best-docked solution was selected and merged into the receptor. Energy minimization was carried out using the Tripos Force Field to optimize the interactions between ligand and receptor within the binding pocket, followed by PROCHECK and ProTable analyses to validate the candidate models.

The optimized model-ligand complexes were then rescored with the HINT program (Table 14) in order to compare and contrast different binding pockets. Further evaluation of HINT scores was performed using Boltzmann analysis (Table 14). Hydropathic analyses of the data indicated an energetic preference (the larger the positive number the more energetically preferable) of the interaction of 20 and 24 at the ECD (917 and 627, respectively) allosteric site over the TMD site (-390 and 251 , respectively, for TMD site characterized by Met253 and 129 and 273 , respectively, for TMD site characterized by Ser222). The Boltzmann-weighted score in the score population for 20 and 24 at the ECD are 417 and 645, respectively, whereas Boltzmann values for 20 and 24 at the TMD site characterized by Met253 are -639 and -386, respectively. Boltzmann values for 20 and 24 at the TMD site characterized by Ser222 are -174 and -62, respectively.
Table 14. Summary of HINT and Boltzmann scores for the interactions of MD-354 (20) and its N-methyl analog 24 at the two (i.e., the ECD and TMD) putative binding sites.

<table>
<thead>
<tr>
<th></th>
<th>Site 3</th>
<th>Site 6</th>
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<tr>
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<td>ECD (Thr221)</td>
<td>TMD (Ser222)</td>
</tr>
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<td>HINT</td>
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<td>273 [-0.53]</td>
</tr>
<tr>
<td>Boltzmann Av.</td>
<td>417 [-0.81]</td>
<td>-62 [0.12]</td>
</tr>
</tbody>
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<thead>
<tr>
<th></th>
<th>Site 3</th>
<th>Site 6</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>ECD (Thr221)</td>
<td>TMD (Ser222)</td>
</tr>
<tr>
<td>HINT</td>
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</tr>
<tr>
<td>Boltzmann Av.</td>
<td>645 [-1.25]</td>
<td>-174 [0.34]</td>
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</tbody>
</table>

Different possible binding modes were suggested in our docking studies for 20 and 24 as depicted in Figure 31. MD-354 (20) appears to utilize a binding mode at which N$_1$ and N$_2$ form an ionic bond with Asp219 and possible hydrogen bonds with Ser206 and Thr221 (Figure 31A). Furthermore, the same part of the guanidine moiety appeared to form a hydrogen bond with Lys204, Ser206 and Asp219 amino-acid backbone oxygen atoms. The chloro group of 20 displayed Van der Waals interactions with the side chains of Pro202 and Lys204, and the backbone of Gly203 (not shown) connecting these two amino acid residues from one side, and a possible hydrogen bond with Thr223. In contrast, the N$_1$-methyl analog 24 utilized the N$_2$ and N$_3$ atoms to form an ionic bond with Asp219 and possible hydrogen bonds with Ser206 and Thr221 (Figure 31B). In addition, the same part of the guanidine moiety appeared to form a hydrogen bond with Asp219 and Ser206 amino-acid backbone oxygen atoms. The terminal N of Lys165
seemed to form a possible cation-π interaction with the phenyl ring of 24. The chloro group of 24 displayed Van der Waals interactions with the side chains of Pro202 and Lys204, and the backbone of Gly203 (not shown) connecting these two amino acid residues from one side, and a possible hydrogen bond with the Thr223. The two distinct models (Figure 31) are consistent with the functional data and in agreement with the common patterns of interactions of guanidine-containing ligands in the PDB archive (Table 13).
**Figure 31.** A and B represent the binding modes of MD-354 (20) and 24 in the ECD allosteric site III of α7 nAChRs, respectively, and are depicted by the schematic representation in the upper right corner (where D = Asp, aspartate). The lower right corner represents the alignment of the orientation of both molecules.
HINT contour maps, which contain hydrophobic/polar fields and acid/base fields, were generated as another useful visualization of the above interactions of 20 and 24 with amino acids at the binding site (ECD site III; Figure 32). The obtained data define the extent of polar and hydrophobic intermolecular forces contained in the total scores obtained at each site (positive values imply favorable interactions while negative values denote disfavorable interactions). Both 20 and 24 show more polar interactions at the ECD site compared to both TMD sites.

<table>
<thead>
<tr>
<th>Location</th>
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<th>Hydrophobic</th>
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<td>(Thr221)</td>
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<tr>
<td>(Met253)</td>
<td>24</td>
<td>251</td>
<td>-27</td>
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</tr>
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</table>

**Figure 32.** HINT contour maps for the plausible binding site of MD-354 (20) and 24 showing a polar area (blue and red) and a hydrophobic area (green).
D. Pharmacophore for Arylguanidines as α7 nAChR NAMs

In comprehensive reviews, Glennon, and Dukat\textsuperscript{164-166} discussed development of nAChR pharmacophores from an historical perspective to the more recent α4β2 nAChR pharmacophores. Briefly, the first attempt to determine the ligand features necessary for nicotinic cholinergic activity was in 1952 when Hey defined two structural features, a quaternary amine separated by a suitable distance from a partial positively-charged atom.\textsuperscript{164-166} In 1962, Barlow and Hamilton estimated this distance to be 3.4 to 3.5 Å.\textsuperscript{164,165} A partial negatively-charged atom was also reported by Holland and co-workers to be required in addition to the quaternary amine.\textsuperscript{164,165} In 1968, Kier reported an optimal distance of 4.85 Å between the quaternary amine and the partial negatively-charged atom.\textsuperscript{164} A specific pharmacophore model for nAChR ligands was reported in 1970 by Beers and Reich.\textsuperscript{165-167} The model indicated that the specific binding of nicotinic agonists to nAChRs is mediated by an electrostatic interaction between a positively-charged nitrogen atom, and a hydrogen bond from an electronegative atom such as a carbonyl oxygen atom or a nitrogen atom. Since that time, several publications about the pharmacophore showed other details for the structural requirements of nAChR orthosteric ligands, such as a cationic center, an electronegative atom capable of forming a hydrogen bond, and the center of a lipophilic region (e.g., pyridine ring in (-)-nicotine (1)).\textsuperscript{168} Most of these studies were for peripheral nAChRs or for neuronal α4β2 nAChRs.\textsuperscript{168,169} And, since then, other types of nAChRs have been identified.

A pharmacophore model specifically for α7 nAChR orthosteric ligands, generally, fits the classical three nAChR pharmacophoric elements (i.e., a cationic center, an electronegative atom capable of forming a hydrogen bond, and a lipophilic center; Figure 33).\textsuperscript{170,171} However, some
refinements of this model were reported in order to achieve subtype selectivity using α7 nAChR-selective anabaseine analogs (e.g., GTS-21 (8)) and other α7 nAChR-selective N,N-dialkylpiperidine compounds. The refined model indicated a secondary (relatively small) hydrophobic pocket that exists in the binding site of α7 nACh receptors closer to the charged nitrogen pharmacophoric element (Figure 33).

On the other hand, there have been few previous attempts to determine a pharmacophore model specifically for α7 nAChR allosteric ligands. For example, a small set of phthalazinone, pyridazinone, and quinazoline compounds was identified through screening to possess high potency as α7 nAChR PAMs. Compelling molecular modeling studies suggested that all three classes of compounds display a common binding mode. Although some of the compounds tested in this study were known to bind at the TMD allosteric site of α7 nAChRs (i.e., TMD intrasubunit site (VI); Figure 30), no single compound has been reported to act at the galantamine binding site (i.e., the ECD-lower exterior site (III); Figure 30). The authors proposed a pharmacophore model consisting of a hydrogen bond donor, a hydrogen bond acceptor, and three hydrophobic regions for the molecule in order to fit the proposed model (Figure 34). To date, no NAM pharmacophore for α7 nAChR ligands has been reported.
Figure 33. A pharmacophore model that confers selectivity for the α7 nAChR orthosteric binding site. An analog of GTS-21 is embedded in the center of the model where the blue lines indicate the classical α7 nAChR ligand pharmacophore, and the red line represents the additional features for selective α7 nAChR ligands (adapted from Leonik et al.¹⁷² and Lightfoot et al.¹⁷⁴).

Figure 34. A pharmacophore model for α7 nAChR PAMs. A quinazoline compound is embedded in the center of the model where the blue lines indicate the pharmacophoric elements at α7 nAChR allosteric sites (adapted from Capelli et al.¹⁷³).
Here, we attempted to define a pharmacophore for small-molecule arylguanidine NAMs at α7 nAChRs using comparative molecular field analysis (CoMFA; an auxiliary set in SYBYL-X 2.1 used to assess 3D-QSAR). Our assumption here was that the compounds used in this study might or might not interact in a similar manner at the site that we proposed, according to our hydropathic analysis, to be most likely the ECD-lower exterior site (III) (Figure 30). CoMFA provides an assessment of the relationship between electrostatic and steric interactions, and biological activity, toward the determination of significant structural features within a group of compounds. This was accomplished in two steps. First, we examined the entire set of arylguanidines as a whole (n = 19). Next, we examined the two sets individually (i.e., the MD-354 (20) or the aniline NH series, and the N-alkyl series, n = 7 and 12, respectively).

In our initial CoMFA run, we used the molecular conformation of each molecule as observed in our docking solutions. Although CoMFA studies showed a high cross-validated $r^2$ ($q^2$) value of 0.88 for the aniline NH series (n = 7) (i.e., 20-23, and 31-33; Table 7), it showed a low predictive value of 0.02 and 0.01 (the predictability should be higher than 0.6 in order for the model to be valid) for the whole arylguanidines set of compounds (n = 19) (i.e., 20-33, 37-39, 52, and 53; Table 7 and 12) and the N-alkyl series (n = 12) (i.e., 24-30, and 37; Table 7), respectively. In a second run of CoMFA, all structures of the arylguanidines were manually aligned (i.e., three non-linear points: the aromatic centroid, the cationic carbon and the meta substituted carbon) to the most common conformations observed in our docking solutions. The highest cross-validated $r^2$ ($q^2$) obtained by our CoMFA studies for the whole arylguanidine set of compounds (n = 19) was 0.43, whereas the highest $q^2$ value was -0.40 for the N-alkyl series (n = 12). The $q^2$ value for the aniline NH series (n = 7) using manual alignment was similar to what was obtained previously in the first CoMFA run ($q^2 = 0.88$).
In a third CoMFA run, we applied GASP\textsuperscript{175} (genetic algorithm similarity program; an unbiased alignment algorithm implemented in SYBYL-X 2.1) to our compounds (i.e., 20-33, 37-39, 52, and 53; Table 7 and 12) in order to perform the alignment step (Figure 35A). GASP-guided alignments for all nineteen compounds were then used for our CoMFA studies. The significance of each CoMFA model was indicated by a cross-validated $r^2$ ($q^2$) value that showed a low predictive value of -0.36 for the nineteen arylguanidine compounds [seven compounds (20-23, and 31-33; Table 7) from the MD-354 (20) series (i.e., all the currently investigated analogs except the 3-CH$_3$ analog due to uncertainty of its experimental results), eight compounds from the N-methyl series (24-30, and 37; Table 7), and four compounds representing the chloro and deschloro analogs of the N-ethyl and N-isopropyl compounds (38, 39, 52, and 53; Table 12)]. The results above indicated the possible lack of a common pharmacophore model for the nineteen arylguanidine compounds due to the weak $q^2$ values (CoMFA map is not shown).

GASP-guided alignments were also performed for the two individual sets of compounds. The seven compounds (i.e., 20-23, and 31-33; Table 7) of the MD-354 (or NH) series were used for GASP alignment (Figure 35B) and the results showed the possibility of a hydrogen bond being formed (i.e., AS1) compared to the twelve compounds (i.e., 24-30, 37-39, 52, and 53; Table 7 and 12) representing the N-alkylated series (Figure 35C) where the AS1 hydrogen bond was absent.
Figure 35. Alignment of the molecules using GASP for the nineteen analogs (i.e., 20-33, 37-39, 52, and 53) (A), the seven analogs representing the MD-354 series (i.e., 20-23, and 31-33) (B), and the twelve analogs representing the N-alkylated series (i.e., 24-30, 37-39, 52, and 53) (C) showing hydrogen bond Acceptor Sites (AS), and HYdrophobic centers (HY) (yellow circles), and hydrogen bond Donor Atoms (DA).
CoMFA studies showed a cross-validated $r^2$ ($q^2$) value for the seven compounds in the NH series (i.e., 20-23, and 31-33; Table 7) of 0.78 ($r^2 = 0.98$, SE = 0.036, number of components is 2) (Figure 36A). These results suggested that the model for the MD-354 (i.e., aniline NH series) is dominated by a steric component, which explains about 91% of the variation in pIC$_{50}$ at $\alpha$7 nAChRs. The $r^2$ of 0.98 for the model indicated a good correlation with pIC$_{50}$ values.

The cross-validated $r^2$ ($q^2$) value for the twelve compounds (i.e., 24-30, 37-39, 52, and 53; Table 7 and 12) representing the N-alkylated series was 0.44 ($r^2 = 0.95$, SE = 0.07, number of components is 4). This marginal value of $q^2$ was improved to 0.60 when the number of components was increased to 5 ($r^2 = 0.98$, SE = 0.04) (Figure 36B). The model was dominated by a steric component, which explains about 84% of the variation in pIC$_{50}$ at $\alpha$7 nAChRs. The $r^2$ value of 0.98 for the model indicated a robust correlation with pIC$_{50}$ values.

These results support our previous conclusion of possible different binding modes between the two series (i.e., the MD-354 or NH series, and the N-alkylated series). That is, using an unbiased GASP alignment, CoMFA failed to account for the action of the entire series of nineteen compounds to behave as NAMs at $\alpha$7 nAChRs. However, when examined separately, the two series, that is, the MD-354 (or NH) series, and the aniline N-alkyl series, allowed the identification of pharmacophore features. Overall, these studies support our other findings that the NH and N-alkyl series bind in a different manner.
The N-alkyl arylguanidine series compounds appear to adopt a common binding mode, and this differs from that of the MD-354 (i.e., NH) series in that the latter possesses five hydrogen bonding sites, whereas the former only possesses four (see Figure 35B and 35C, respectively). The model indicates the need for four hydrogen bond components and a hydrophobic region for the molecule in order to fit the proposed pharmacophore model (Figure 37).

**Figure 36.** CoMFA maps for the seven analogs (i.e., 20-23, and 31-33) representing the MD-354 series (A) \((n = 7, q^2 = 0.78, r^2 = 0.98;\) number of components = 2; 90.7% steric and 9.3% electrostatic) and the twelve analogs (i.e., 24-30, 37-39, 52, and 53) representing the N-alkylated series (B) \((n = 12, q^2 = 0.60, r^2 = 0.98;\) number of components = 5; 84.6% steric and 15.4% electrostatic). Green regions representing bulky substituents are favored, whereas the yellow regions representing bulky substituents are disfavored. Blue regions represent positively charged substituents are favored.
There are some shortcomings associated with our CoMFA studies (i.e., the relatively small set of compounds and the narrow range of IC\textsubscript{50} values). It should be kept in mind that all the compounds were arylguanidines. Compounds such as the 3-chlorophenylethylamine (42), if it is ever shown to be a NAM, can influence the pharmacophore. Nevertheless, the results suggest new compounds that can be proposed, synthesized, and evaluated.\textsuperscript{176}

Figure 37 describes the first pharmacophore model for N-alkyl arylguanidine α7 nAChR NAMs. A generalized N-alkyl arylguanidine is embedded in the center of the model where the blue lines indicate the pharmacophoric requirements at α7 nAChR allosteric sites as suggested by our pharmacophore study.

There are some shortcomings associated with our CoMFA studies (i.e., the relatively small set of compounds and the narrow range of IC\textsubscript{50} values). It should be kept in mind that all the compounds were arylguanidines. Compounds such as the 3-chlorophenylethylamine (42), if it is ever shown to be a NAM, can influence the pharmacophore. Nevertheless, the results suggest new compounds that can be proposed, synthesized, and evaluated.\textsuperscript{176}

Figure 37 describes the first pharmacophore model for N-alkyl arylguanidines as α7 nAChR NAMs. It is rather interesting that the model lacks a hydrogen bond feature associated with the anilinic nitrogen atom, a feature found in the MD-354 (or anilinic NH) model (see Figure 35B). Because Dukat\textsuperscript{177} has previously proposed an anilinic NH as being important for binding of
arylguanidines at 5-HT₃ receptors, the lack of this feature might account for the greater \( \alpha_7 \)
nAChR selectivity of the N-alkyl arylguanidines over the NH arylguanidines.
V. Conclusions

A number of compounds based on MD-354 (20) and its N-methyl analog 24 were synthesized and evaluated for their activity as inhibitors at α7 nACh receptors. MD-354 was initially developed as a 5-HT_3 receptor agonist earlier in our laboratory and has been found to be the first example of a small-molecule NAM of α7 nAChRs. Our previous studies showed that the incorporation of an N_1-methyl substituent into arylguanidine led to compounds with enhanced selectivity as NAMs of α7 nACh receptors. This indicated that the two actions can be divorced and that removing structural features required for 5-HT_3 receptor binding also resulted in enhancement of α7 nACh receptor NAM potency. The N-methyl analog of MD-354 (i.e., 24) showed 177-fold lower affinity than 20 for 5-HT_3 receptors (i.e., 5-HT_3 receptor \( K_i = 6,200 \) nM) and higher potency at α7 nACh receptors (i.e., \( IC_{50} = 1.26 \) μM), examined using patch-clamp technique on rat α7 nAChRs expressed in HEK cells, compared to MD-354 (20) (i.e., 5-HT_3 receptor \( K_i = 35 \) nM; \( IC_{50} = 7.98 \) μM).

Previous molecular modeling studies\(^9\) indicated possible different binding modes between MD-354 (20) and its N-methyl analog 24, which prompted us to investigate the structural requirements for the action of arylguanidines at α7 nAChRs. One of the aims of this project was
to study the SAR and determine the optimal structural features of arylguanidines for inhibiting ACh responses at α7 nACh receptors. Figure 38 summarizes the three main structural regions that were investigated for optimizing arylguanidine inhibitory action.

![Figure 38](image)

**Figure 38.** Representation of the three main structural components of arylguanidines investigated in this study.

Two series of compounds were prepared, based on 20 and 24, to explore the aryl 3-position, to test the effect of substituent size, lipophilic nature, and a different range of electronic effects and to test a possible change in pharmacological profile of the new molecules. The structural modifications included H, F, Cl, Br, I, CF₃, OCH₃, and CH₃. Biological potencies of the MD-354 series and the N-methyl series of compounds, obtained from electrophysiological assays with *Xenopus laevis* oocytes expressing human α7 nAChRs using a two-electrode voltage-clamp assay, showed IC₅₀ values ranging from 12 – 125 µM. Among those compounds, *N*(3-iodophenyl)-*N*-methylguanidine (28) was the most potent inhibitor of ACh responses at α7 nACh
receptors. A poor correlation \((r = 0.474; n = 8)\) of pIC\(_{50}\) values of the 20 series versus pIC\(_{50}\) values of the 24 series supported our docking results suggesting that the two series bind differently. Hansch-type QSAR studies were conducted using several physicochemical properties to determine the effect of similar modifications in the two series on NAM activity. Data for the 24 (i.e., N-CH\(_3\)) series showed a significant correlation with the minimum width (Verloop \(B_1\)) \((r = 0.927; n = 8)\) and lipophilic nature (\(\pi\)) \((r = 0.912; n = 8)\) of the 3-position substituent. However, a significant internal correlation exists between the two properties \((r = 0.956; n = 8)\). Consequently, additional analogs will be required to resolve this issue.\(^{176}\) Nevertheless, the effect of the parallel structural modifications at \(\alpha_7\) nAChRs and Hansch analyses indicate different binding modes for the two series.

The Topliss operational scheme\(^{135}\) for the aliphatic side chain was used to optimize pharmacological activities associated with the alkyl group at the anilinic nitrogen atom of the arylguanidines at \(\alpha_7\) nAChRs. According to the approach, replacement of the N-methyl group in 24 with an N-isopropyl group should increase its \(\pi\) effect.\(^{135}\) A total of four pairs of compounds were initially examined at which the effect of the chloro group was also investigated through deschloro compounds. Introduction of isopropyl groups was examined. The findings indicate that the N\(_1\)-iPr group (i.e., increased lipophilicity) appears to increase NAM potency, especially with deschloro analogs where more pronounced effects are detected.

We also studied the role of the guanidine nitrogen atoms on the inhibitory effects of arylguanidines at \(\alpha_7\) nAChRs. The effect of alkylating one or more of the guanidine nitrogen atoms, and the necessity of the presence of these nitrogen atoms for their NAM action, and/or action as an \(\alpha_7\) nAChR antagonist was investigated, because these structural changes are known to be detrimental to 5-HT\(_3\) receptor binding. The results appear to support the concept that
alkylation of the nitrogen atoms is well tolerated and that not all of the nitrogen atoms are required for α7 nAChR antagonist action. However, more pharmacological studies are needed to confirm this observation.

Molecular modeling studies were conducted to identify the potential binding site(s) as well as the mode of interactions of arylguanidines at α7 nAChRs. Eight potential allosteric binding cavities were initially identified, but only two of them, one at the extracellular domain (ECD) and the other at the transmembrane domain (TMD), appeared to be reasonable binding sites for our α7 nAChR allosteric modulators. Docking studies resulted in different binding modes for 20 and 24. HINT analyses were performed on the two binding sites and an energetic preference was found for the interaction of 20 and 24 at the ECD allosteric site over the TMD site. These results indicated that small-molecule arylguanidine NAMs (although the 20 and 24 series could bind somewhat differently) might interact at the same binding site as PAMs at α7 nACh receptors.

A pharmacophore model for arylguanidine NAMs at α7 nACh receptors has not been investigated previously. We initiated the first attempt to identify a pharmacophore model of small-molecule arylguanidine NAMs of α7 nAChRs. Our pharmacophore studies showed a low likelihood of a common pharmacophore model for all arylguanidine NAMs which supported the possibility of different binding modes between the NH guanidines and the N-alkylated guanidines. The first pharmacophore model of N-alkyl arylguanidines at α7 nAChRs was proposed. Newly synthesized compounds will be subsequently used to evaluate the predictability of the model, however, there might be a need for more compounds and a wider range of IC₅₀ values in order to have better predictability.

Overall, three main regions in the arylguanidines were studied for their influence on NAM action at α7 nAChRs. Multiple medicinal chemistry approaches were employed to
rationally design novel $\alpha_7$ nAChR NAMs. We succeeded in improving the selectivity as well as the potency of the parent compound MD-354 (20) toward $\alpha_7$ nAChRs. In addition, we have constructed models of the ECD and the TMD of $\alpha_7$ nAChRs, energetically assessed binding at the potential allosteric sites, and studied the plausible binding modes that might explain the actions of our compounds. The study led to the 24 series of compounds and the N$_1$-isopropyl arylguanidine compounds that opened an investigation window to a number of compounds that could guide us to promising novel $\alpha_7$ nAChR small-molecule NAMs.

Marijuana addiction, especially with the increasing legalization for medicinal and non-medicinal use, increases the necessity to open a new approach for treatment of their abuse. For medicinal chemists as well as other researchers in the drug discovery field, it is required to investigate possible ways to fight diseases such as AD, depression and cognitive dysfunction. Here, $\alpha_7$ nAChR-selective antagonists might represent a valuable pathway for promising therapeutic strategy. Small-molecule NAMs (arylguanidines), because they offer an alternative to orthosteric $\alpha_7$ nAChR antagonists, might represent a novel class of compounds with potential therapeutic value.
VI. Experimental

A. Synthesis

Melting points (mp) were taken in glass capillary tubes using a Thomas-Hoover melting point apparatus and are uncorrected. Proton nuclear magnetic resonance ($^1$H NMR) spectra were obtained using a Bruker ARX 400 MHz spectrometer at which peak positions are given in parts per million ($\delta$) downfield from the internal standard tetramethylsilane (TMS), followed by the splitting pattern ($s =$ singlet, $d =$ doublet, $t =$ triplet, $q =$ quartet, $dd =$ doublet of doublets, $m =$ multiplet), coupling constant (Hz), and integration. Infrared spectra were obtained on a Thermo Nicolet iS10 FT-IR. Purity of compounds was determined by elemental analysis performed by Atlantic Microlab Inc. (Norcross, GA) for the indicated elements, and the obtained values are within 0.4% of theoretical values. Reactions were monitored by thin-layer chromatography (TLC) on silica gel GHLF plates (250 µm, 2.5 X 10 cm; Analtech Inc. Newark, DE), and Flash chromatography was performed on a CombiFlash Companion/TS (Teledyne Isco Inc. Lincoln, NE) using packed silica gel (Silica Gel 230-400 mesh) columns (RediSep Rf Normal-phase Silica Flash Column, Teledyne Isco Inc., Lincoln, NE). Electrospray ionization-mass spectroscopy (ESI-MS) profiles were recorded using a Waters Acquity TQD (tandem quadrupole) spectrometer in positive ion mode.
**N-Methyl-3-trifluoromethylaniline Nitrate (37).** N-Methyl-3-trifluoromethylaniline nitrate (37) was prepared according to a literature procedure for a similar compound.³⁵¹ Cyanamide (0.7 g, 17.9 mmol) was added to a solution of N-methyl-3-trifluoromethylaniline hydrochloride (47) (1.9 g, 8.9 mmol) in absolute EtOH (20 mL). The stirred reaction mixture was heated at reflux for 24 h. Upon cooling, the reaction mixture was concentrated under reduced pressure to yield a residue that was recrystallized from 1-butanol to give 4.8 g (21%) of the desired product as white crystals: mp 247-249 °C; IR (diamond, cm⁻¹): 3120 (NH), 3282 (NH₂); ¹H NMR (DMSO-d₆) δ 3.29 (s, 3H, CH₃), 7.72 (m, 2H, ArH), 7.79 (d, J = 7.68 Hz, 1H, ArH), 7.82 (s, 1H, ArH). Anal. Calcd (C₉H₁₀F₃N₃·HCl) C, 42.62; H, 4.37; N, 16.57. Found: C, 42.66; H, 4.30; N, 16.52.

**N-Isopropyl-N-phenylguanidine Nitrate (38).** N-Isopropyl-N-phenylguanidine nitrate (38) was prepared according to a literature procedure.³⁵² An aqueous solution of cyanamide (50%; 1.5 mL) was added to a solution of N-isopropylaniline hydrochloride (48) (100 mg, 0.58 mmol) in absolute EtOH (9 mL). The stirred reaction mixture was heated at reflux for 25 h, and then cooled to 0 °C (freezer) for 20 h. The solvent was removed under reduced pressure, the resulting oily residue was dissolved in H₂O (1 mL) followed by addition of NH₄NO₃ (108 mg, 1.34 mmol). The solution was concentrated under reduced pressure and the residue was dissolved in H₂O (5 mL), washed with Et₂O (3 x 20 mL), followed by evaporation of H₂O under reduced pressure. The resulting solid was recrystallized from H₂O and, then, from absolute EtOH to give 30 mg (21%) of the desired product as white crystals: mp 164-165 °C; IR (Diamond) cm⁻¹: 2159, 3172 (NH), 3319 (NH2); ¹H NMR (DMSO-d₆): δ 1.05 (d, J = 6.52 Hz, 6H, CH₃), 4.4 (m, 1H, CH), 7.3 (m, 2H, ArH), 7.6 (m, 3H, ArH). Anal. Calcd (C₁₀H₁₅N₃·HNO₃) C, 49.99; H, 6.71; N, 23.32. Found: C, 49.85; H, 6.64; N, 23.27.
N-(3-Chlorophenyl)-N-isopropylguanidin hydrochloride (39). N-(3-Chlorophenyl)-N-isopropylguanidine hydrochloride (39) was prepared using a literature procedure for a similar compound.\(^{151}\) Cyanamide (1.02 g, 24.26 mmol) was added to a solution of 3-chloro-N-isopropylaniline (51) (2.50 g, 12.13 mmol) in absolute EtOH (30 mL). The reaction mixture was allowed to stir at reflux for 24 h. The solution was concentrated under reduced pressure and anhydrous Et\(_2\)O (25 mL) was added. The white precipitate was collected by filtration and recrystallized from i-PrOH to give 0.51 g (17\%) of the desired product as white crystals: mp 272-274 °C; IR (diamond, cm\(^{-1}\)): 3108 (NH), 3269 (NH\(_2\)); \(^1\)H NMR (DMSO-d\(_6\)) \(\delta\): 1.04 (d, \(J = 6.56\) Hz, 6H, CH\(_3\)), 4.36 (m, 1H, CH), 7.28 (td, \(J = 7.72, 1.62\) Hz, 1H, ArH), 7.45 (t, \(J = 1.84\) Hz, 1H, ArH), 7.58 (m, 2H, ArH). Anal. Calcd (C\(_{10}\)H\(_{14}\)ClN\(_3\)·HCl) C, 48.40; H, 6.09; N, 16.93. Found: C, 48.68; H, 6.11; N, 17.06.

\(N,N'\)-Di(3-trifluoromethylphenyl)urea (45). \(N,N'\)-Di(3-trifluoromethylphenyl)urea (45) was accidently prepared using a patent procedure for the synthesis of carbamate compounds.\(^{178}\) Triethylamine was added to a solution of 3-trifluoromethylaniline (3.8 g, 23.6 mmol) in anhydrous Et\(_2\)O (10 mL). A solution of ethyl chloroformate (2.5 g, 23.6 mmol) in anhydrous Et\(_2\)O (5 mL) was added in a dropwise manner at 0 °C (ice-bath), and the stirred reaction mixture was allowed to stir for 1 h at room temperature. The solid was collected by filtration and washed with DCM to yield 0.7 g (18\%) of 45 as a white crystals: mp 190-192 °C (\(^{179}\)lit. mp 198 °C, DMF/H\(_2\)O); IR (diamond, cm\(^{-1}\)): 1702 (CO), 3302 (NH); \(^1\)H NMR (DMSO-d\(_6\)) \(\delta\) 7.34 (d, \(J = 7.64\) Hz, 2H, ArH), 7.53 (t, \(J = 7.80\) Hz, 2H, ArH), 7.62 (d, \(J = 8.52\) Hz, 2H, ArH), 8.01 (s, 2H, ArH).

Ethyl \(N\)-(3-(trifluoromethyl)phenyl] carbamate (46). Ethyl \(N\)-(3-(trifluoromethyl)phenyl] carbamate (46) was prepared according to a literature procedure.\(^{149}\) Sodium hydride 60\% in
mineral oil (0.5 g, 23.6 mmol) was washed with anhydrous toluene (2 \times 5 \text{ mL}) under an N\textsubscript{2} atmosphere to remove the oil. The obtained solid was dissolved in anhydrous THF (10 \text{ mL}) and a solution of 3-trifluoromethylaniline (3.8 g, 23.6 mmol) in anhydrous THF (10 \text{ mL}) was added. A solution of ethyl chloroformate (2.5 g, 23.6 mmol) in anhydrous THF (5 \text{ mL}) was added in a dropwise manner at 0 °C (ice-bath), and the stirred reaction mixture was allowed to stir for 1.5 h at room temperature. The mixture was filtered and the filtrate was concentrated under reduced pressure and dried under high vacuum to yield 4.8 g (88%) of 46 as a yellow oil: IR (diamond, cm\textsuperscript{-1}): 1707 (CO), 3381 (NH); \textsuperscript{1}H NMR (DMSO-\textit{d}_6) \delta 1.34 (t, J = 7.10 Hz, 3H, CH\textsubscript{3}), 2.71 (q, J = 7.12 Hz, 2H, CH2), 7.36 (d, J = 7.76 Hz, 1H, ArH), 7.44 (t, J = 8.0 Hz, 1H, ArH), 7.57 (d, J = 8.36 Hz, 1H, ArH), 7.72 (s, 1H, ArH). The product was used without further characterization in the preparation of 47.

\textbf{N-Methyl-3-trifluoromethylaniline Hydrochloride (47).} N-Methyl-3-trifluoromethylaniline hydrochloride (47) was prepared according to a literature procedure.\textsuperscript{150} A solution of ethyl \textit{N}-[3-(trifluoromethyl)phenyl] carbamate (46) (5.0 g, 31.0 mmol) in anhydrous THF (15 \text{ mL}) was added in a dropwise manner to a stirred suspension of LiAlH\textsubscript{4} (2.4 g, 64.3 mmol) in anhydrous THF (15 \text{ mL}) at 0 °C (ice-bath) under an N\textsubscript{2} atmosphere. The reaction mixture was heated at reflux overnight. Excess of LiAlH\textsubscript{4} was decomposed by addition of H\textsubscript{2}O (2.5 \text{ mL}), NaOH (2.5 \text{ mL}, 15%), and H\textsubscript{2}O (7.5 \text{ mL}), slowly and in a dropwise manner, and the mixture was allowed to stir for 0.5 h. The reaction mixture was filtered and the collected solid was washed with hot THF (10 \text{ mL}). The filtrate was dried (MgSO\textsubscript{4}), solvent was removed under reduced pressure and dried under high vacuum to yield 1.7 g of the free base of 47 as a yellow oil. The free base was dissolved in Et\textsubscript{2}O and a saturated solution of HCl in Et\textsubscript{2}O (50 \text{ mL}) was added. The precipitate was collected by filtration and recrystallized from a mixture of absolute EtOH/Et\textsubscript{2}O to give 1.9 g
(44%) of the product as white crystals: mp 108-110 °C; IR (diamond, cm\(^{-1}\)): 1453, 2638 (NH); \(^1\)H NMR (DMSO-\(d_6\)) \(\delta\) 2.73 (s, 3H, CH\(_3\)), 6.89 (m, 2H, ArH), 7.33 (m, 2H, ArH).

3-Chloro-\(N\)-isopropylaniline Hydrochloride (51). 3-Chloro-\(N\)-isopropylaniline hydrochloride (51) was prepared according to a literature procedure.\(^{153}\) A mixture of 3-chloroaniline (49) (1.00 g, 7.83 mmol) and 2-bromopropane (50) (0.48 g, 3.92 mmol) was heated to 150 °C. Then, the reaction mixture was allowed to cool to room temperature and washed with an aqueous solution of NaOH (15%, 10 mL). The oily residue was shaken with an aqueous solution of ZnCl\(_2\) (10%, 10 mL). Excess ZnCl\(_2\) solution was decanted and the obtained residue was extracted with petroleum ether (4×10 mL). The solvent was removed under reduced pressure and the residue was purified by flash chromatography (silica gel; hexane/EtOAc; 9:1). The obtained oil was dried under high vacuum for 6 h to yield 0.6 g (45%) of the free base of 51 as a yellow oil. The free base was dissolved in Et\(_2\)O and a saturated solution of HCl in anhydrous Et\(_2\)O (50 mL) was added. The precipitate was collected by filtration and recrystallized from a mixture of absolute EtOH/Et\(_2\)O to give 0.71 g of the desired product as white crystals: mp 238-239 °C; IR (diamond, cm\(^{-1}\)): 1594, 3044 (NH); \(^1\)H NMR (DMSO-\(d_6\)) \(\delta\): 1.5 (d, \(J = 6.28\) Hz, 6H, CH\(_3\)), 3.52 (septet, \(J = 6.35\) Hz, 1H, CH), 6.43 (d, \(J = 8.12\) Hz, 1H, ArH), 6.54 (s, 1H, ArH), 6.59 (d, \(J = 7.84\) Hz, 1H, ArH), 7.0 (t, \(J = 8.02\) Hz, 1H, ArH). The product was used without further characterization for synthesis of compound 39.

\(N\)-Ethyl-\(N\)-phenylguanidine Hydrochloride (52). \(N\)-Ethyl-\(N\)-phenylguanidine hydrochloride (52) was prepared according to a literature procedure.\(^{154}\) Cyanamide (299 mg, 7.10 mmol) was added to a solution of \(N\)-ethylaniline hydrochloride (54) (800 mg, 5.09 mmol) in 1-pentanol (15 mL). The stirred reaction mixture was heated at reflux for 24 h. Upon cooling, the reaction mixture was concentrated under reduced pressure to obtain an oily residue that was crystallized
from a mixture of absolute EtOH/Et₂O to give 209 mg (21%) of the desired product as off-white crystals: mp 175-177 °C (lit. mp 185 °C, EtOH/Et₂O); IR (diamond, cm⁻¹): 2977, 3189 (NH), 3303 (NH₂); ¹H NMR (DMSO-d₆) δ: 1.10 (t, J = 7.12 Hz, 3H, CH₃), 3.66 (q, J = 7.12, 7.14 Hz, 2H, CH₂), 7.35 (d, J = 7.08 Hz, 2H, ArH), 7.47 (t, J = 7.34 Hz, 1H, ArH), 7.54 (t, J = 7.36 Hz, 2H, ArH).

N-(3-Chlorophenyl)-N-ethylguanidine Hydrochloride (53). N-(3-Chlorophenyl)-N-ethylguanidine hydrochloride (53) was prepared using a literature procedure for a similar compound. Cyanamide (0.13 g, 3.12 mmol) was added to a solution of 3-chloro-N-ethylaniline hydrochloride (55) (0.3 g, 1.56 mmol) in absolute EtOH (10 mL). The stirred reaction mixture was heated at reflux for 24 h. Upon cooling, the reaction mixture was concentrated under reduced pressure to obtain a residue that was recrystallized from a mixture of absolute EtOH/Et₂O to give 0.048 g (13%) of the desired product as white crystals: mp 186-189 °C; IR (diamond, cm⁻¹): 3077, 3187 (NH), 3274 (NH₂); ¹H NMR (DMSO-d₆) δ: 1.07 (t, J = 7.16 Hz, 3H, CH₃), 3.66 (q, J = 7.12, 7.16 Hz, 2H, CH₂), 7.34 (m, 1H, ArH), 7.54 (m, 3H, ArH). Anal. Calcd (C₉H₁₂ClN₃·HCl) C, 46.17; H, 5.60; N, 17.95. Found: C, 45.93; H, 5.64; N, 17.72.

3-Chloro-N-ethylaniline Hydrochloride (55). 3-Chloro-N-ethylaniline hydrochloride (55) was prepared according to a literature procedure. Acetaldehyde (1.5 mL, 27.0 mmol), 3-chloroaniline (49) (3.4 g, 27.0 mmol), and pyridine-borane (1.9 mL, 22.5 mmol) were sequentially added to a methanol solution (120 mL) containing powdered 4 Å molecular sieves (2.4 g) under an N₂ atmosphere. The stirred reaction mixture was heated at reflux for 24 h. Upon cooling to room temperature, the mixture was filtered through a small bed of Celite (Hyflo Super-Cel®) and the filtrate was collected. This filtrate was concentrated under reduced pressure to give an oily residue. The residue was purified by flash chromatography (silica gel;
hexane/EtOAc; 9:1) to obtain a solution that was concentrated under reduced pressure and dried under vacuum for 14 h to yield 0.6 g of the free base of 55 as a yellow oil. The free base was dissolved in Et2O and a saturated solution of HCl in Et2O (50 mL) was added. The precipitate was collected by filtration and recrystallized from a mixture of absolute EtOH/Et2O to give 0.7 g (12%) of the product as white crystals: mp 144-146 °C; IR (diamond, cm⁻¹): 1587, 2650 (NH); N (3-Cyanophenyl)-N-methylguanidine Nitrate (56). N-(3-Cyanophenyl)-N-methylguanidine nitrate (56) was prepared using a literature procedure for a similar compound.¹⁵¹ Cyanamide (0.25 g, 6.0 mmol) was added to a solution of 3-(methylamino)benzonitrile hydrochloride (59) (0.5 g, 3.0 mmol) in absolute EtOH (15 mL). The stirred reaction mixture was heated at reflux for 24 h. Upon cooling the reaction mixture, the solvent was evaporated under reduced pressure and the obtained residue was dissolved in H₂O (8 mL), followed by addition of NH₄NO₃ (0.29 g, 3.62 mmol). The solvent was evaporated under reduced pressure and the resultant semisolid was recrystallized from EtOAc to give 0.13 g (19%) of the desired product as white crystals: mp 117-119 °C; IR (diamond, cm⁻¹): 2803, 3039 (NH), 3123 (NH₂); ¹H NMR (DMSO-d₆) δ: 3.28 (s, 3H, CH₃), 7.74 (m, 2H, ArH), 7.89 (d, J = 7.48 Hz, 1H, ArH), 7.98 (s, 1H, ArH). Anal. Calcd for C₉H₁₁N₄·HNO₃: C, 45.57; H, 4.67; N, 29.52. Found: C, 45.87; H, 4.68; N, 29.17; LRMS (ESI) calculated for C₉H₁₁N₄, [M + H]⁺, m/z 175.0905, found m/z 174.8022.

N-Cyclopentyl-N-phenylguanidine Hydrochloride (57). N-Cyclopentyl-N-phenylguanidine hydrochloride (57) was prepared using a literature procedure for a similar compound.¹⁵¹ Cyanamide (0.64 g, 15.17 mmol) was added to a solution of N-cyclopentylaniline hydrochloride
(62) (1.5 g, 7.58 mmol) in absolute EtOH (15 mL). The stirred reaction mixture was heated at reflux for 24 h. Upon cooling, the solvent was evaporated under reduced pressure to obtain a residue that was washed with cyclohexane and recrystallized from THF to give 0.15 g (9%) of

*the desired product as white crystals: mp 209-211 °C; IR (diamond, cm⁻¹): 2956, 3117 (NH), 3276 (NH₂); ¹H NMR (DMSO-d₆) δ: 1.20 (m, 2H, CH₂), 1.43 (m, 4H, CH₂), 1.92 (m, 2H, CH₂), 4.31 (m, 1H, CH), 7.28 (m, 2H, ArH), 7.53 (m, 3H, ArH). Anal. Calcd (C₉H₁₂ClN₃·HCl·0.1 C₆H₁₂) C, 65.94; H, 9.06; N, 13.44. Found: C, 65.98; H, 9.13; N, 13.15.*

**N-(3-Chlorophenyl)-N-cyclopentylguanidine Nitrate (58).** *N-(3-Chlorophenyl)-N-cyclopentylguanidine nitrate (58) was prepared using a literature procedure for a similar compound.¹⁵¹ Cyanamide (0.72 g, 17.23 mmol) was added to a solution of 3-chloro-N-cyclopentylaniline hydrochloride (63) (2.0 g, 8.61 mmol) in absolute EtOH (15 mL). The stirred reaction mixture was heated at reflux for 24 h. The solution was concentrated under reduced pressure and was dissolved in H₂O (8 mL). The solution was washed with Et₂O (3 x 20 mL), followed by evaporation of H₂O under reduced pressure. The resultant oily residue was dissolved in H₂O (8 mL), followed by addition of NH₄NO₃ (0.12 g, 1.44 mmol). The solvent was evaporated under reduced pressure and the resultant semisolid was recrystallized from EtOH to give 0.34 g (15%) of the desired product as white crystals: mp 225-227 °C; IR (diamond, cm⁻¹): 2969, 3175 (NH), 3346 (NH₂); ¹H NMR (DMSO-d₆) δ: 1.19 (m, 2H, CH₂), 1.45 (m, 4H, CH₂), 1.94 (m, 2H, CH₂), 4.27 (m, 1H, CH), 7.29 (dd, J = 1.64, 7.52 Hz, 1H, ArH), 7.48 (s, 1H, ArH), 7.55 (m, 2H, ArH). Anal. Calcd (C₁₂H₁₆ClN₃·HNO₃) C, 47.93; H, 5.70; N, 18.63. Found: C, 48.06; H, 5.59; N, 18.61.*

**N-Cyclopentylaniline Hydrochloride (62).** *N-Cyclopentylaniline was synthesized according to a patent procedure.¹⁸⁰ Cyclopentanone (61) (3.8 mL, 43.0 mmol), acetic acid (1.2 mL, 21.5
mmol), and sodium triacetoxyborohydride (9.1 g, 43.0 mmol) were added to a solution of aniline (60) (2.0 mL, 21.5 mmol) in dichloromethane (15 mL) and the reaction mixture was allowed to stir at room temperature for 1.5 h. The mixture was washed with saturated aqueous sodium bicarbonate solution (3 × 25 mL) and the solvent was evaporated under reduced pressure, dried under vacuum for 14 h to yield 4.0 g of the free base of 62 as yellow oil. The free base was dissolved in anhydrous Et₂O and a saturated solution of HCl in Et₂O (50 mL) was added. The precipitate was collected by filtration and recrystallized from EtOAc to give 3.1 g (89%) of the product as white crystals: mp 149-151 °C; IR (diamond, cm⁻¹): 1584, 2631 (NH); ¹H NMR (DMSO-d₆) δ: 1.55 (m, 2H, CH₂), 1.77 (m, 4H, CH₂), 1.85 (m, 2H, CH₂), 3.88 (m, 1H, CH), 7.41 (m, 1H, ArH), 7.52 (m, 4H, ArH). The product was used without further characterization for synthesis of compound 59.

3-Chloro-N-cyclopentylaniline Hydrochloride (63). 3-Chloro-N-cyclopentylaniline hydrochloride (63) was synthesized according to a patent procedure for a similar compound. Cyclopentanone (61) (2.8 mL, 31.35 mmol), acetic acid (0.6 mL, 15.68 mmol), and sodium triacetoxyborohydride (6.6 g, 31.35 mmol) were added to a solution of 3-chloroaniline (49) (1.6 mL, 15.68 mmol) in dichloromethane (15 mL) and the reaction mixture was allowed to stir at room temperature for 1.5 h. The mixture was washed with saturated aqueous sodium bicarbonate solution (3 × 25 mL) and the solvent was evaporated under reduced pressure and dried under vacuum for 7 h to yield 2.7 g of the free base of 63 as yellow oil. The free base was dissolved in Et₂O and a saturated solution of HCl in Et₂O (50 mL) was added. The precipitate was collected by filtration and recrystallized from EtOAc to give 3.0 g (98%) of the product as white crystals: mp 115-117 °C; IR (diamond, cm⁻¹): 1589, 2628 (NH); ¹H NMR (DMSO-d₆) δ: 1.58 (m, 4H, CH₂), 1.72 (m, 2H, CH₂), 1.88 (m, 2H, CH₂), 3.78 (m, 1H, CH), 7.01 (m, 3H, ArH), 7.27 (m, 1H,
ArH). The product was used without further characterization for synthesis of compound 58.

B. Electrophysiology

*Xenopus* oocytes: (studies were conducted by Shailesh Khatri, a graduate student in Dr. Schulte’s Laboratory)

“Arylguanidine analogs were evaluated functionally using two-electrode voltage clamp of human α7 nACh receptors expressed in *Xenopus* oocytes using previously published procedures. Four independent recording stations with integral autosampling and data collection were available for this study.

The chimeric DNAs (cDNA) for human α7 receptors were obtained from Dr. Jon Lindstrom’s Laboratory (Department of Neuroscience, School of medicine, University of Pennsylvania). cDNA was cloned into a pBud-CE4.1 (Invitrogen, CA) vector prior to RNA synthesis.

Ovarian lobes were surgically removed from *X. laevis* frogs and washed twice in Ca\(^{2+}\) free Barth’s buffer [82.5 mM NaCl/2.5mM KCl/1 mM MgCl2/5 mM HEPES, pH 7.4]. Lobes were gently shaken with 1.5mg/ml collagenase (Sigma type II, Sigma–Aldrich) for 20-30 minutes at room temperature. Stage IV oocytes were selected for microinjection. Synthetic cRNAs for wild type human α7 were prepared using the mMESSAGE mACHINE™ High Yield Capped RNA Transcription Kit (Ambion, TX). Each oocyte was injected with 50 nl cRNA at a concentration of 300 ng/µl. Oocytes were incubated at 19 °C for 36-48 hours before electrophysiological experiments. Electrical recordings were made using automated two-electrode voltage clamp at –60 mV employing an OC-725C oocyte clamp amplifier (Warner Instruments, CT, US) coupled to an online, computerized data acquisition system (pCLAMP, Molecular Devices, LLC, USA) along with auto injection system (Gilson).

Recording and current electrodes were filled with 3 M KCl and had resistances of 1–4 MΩ. Oocytes were held in a vertical flow chamber of 200-280µl volumes and perfused with ND-96 recording buffer (96 mM NaCl/2 mM KCl/1.8 mM CaCl2/1 mM MgCl2/5 mM HEPES, pH 7.4) at a rate of 20 ml/min. Details of chamber and methodology employed for two electrode voltage clamp recordings have been described earlier (Joshi et al., 2004). All agonists (Acetylcholine) and antagonists (Analogs in test) were prepared in ND-96 buffer. Antagonists were co-applied with 100µM acetylcholine (ACh) to record inhibition of ACh evoked currents by antagonist.

Dose–response curves obtained from electrophysiological data were fit using the equation I = Imax/(1 + EC50/[A])\(^n\), where I is the current at a given agonist concentration, Imax is the maximal current, EC50 is the agonist concentration that elicits a half-maximal current, and n is the Hill coefficient. Ki were calculated using Cheng–Prusoff equation. All the analysis of the data was done using GraphPad PRISM. Quantative data were expressed as mean ± S.E. Association between the variables, where need, were tested by using paired students t test. All statistical differences were deemed significant at the level of P<0.05.”
HEK 293 cells: (studies were conducted by Dr. Galya Abdrakhmanova)

“Stably transfected HEK 293 cells expressing rat α7 nAChRs were prepared as described previously. All three cell lines were maintained at 37 °C with 5% CO₂ in the incubator. Growth medium for HEK 293 cells was minimum essential medium supplemented with 10% fetal bovine serum, 100 U/ml penicillin, and 100 mg/ml streptomycin. The stably transfected cell line was raised in selective growth medium containing 0.7 mg/ml of geneticin (Invitrogen Corp, Carlsbad, CA). Growth medium for SH-EP1 cells was Dulbecco’s Modified Eagle’s medium with high glucose supplemented with 10% heat inactivated horse serum, 5% fetal bovine serum, 100 U/ml penicillin, 100 mg/ml streptomycin, 8 mM L-glutamine, 1 mM sodium pyruvate, and 0.25 mg/ml amphotericin (all from Invitrogen Corp, Carlsbad, CA). This stably transfected cell line was raised in selective medium containing 0.5 mg/ml zeocin (Invitrogen) and 0.4 mg/ml hygromycin B (Roche Diagnostics Corp, Indianapolis, IN).”

“In some cells, ~85% of electrode resistance was compensated electronically, so that the effective series resistance in the whole-cell configuration was accepted when less than 20 MΩ. Stably transfected HEK cells were studied for 2–3 days after plating the cells on 15-mm round plastic cover slips (Thermanox, Nalge Nunc, Naperville, IL, USA). Generation of voltage-clamp protocols and acquisition of the data were carried out using pCLAMP 9.0 software (Molecular Devices). Sampling frequency was 5 kHz and current signals were filtered at 5 or 10 kHz before digitization and storage. All experiments were performed at room temperature (22–25 °C).

Cells plated on cover slips were transferred to an experimental chamber mounted on the stage of an inverted microscope (Olympus IX50, Olympus Corporation, Tokyo, Japan) and were bathed in a solution containing 140 mM NaCl, 3 mM KCl, 2 mM MgCl₂, 25 mM D-glucose, 10 mM HEPES, and 2 mM CaCl₂ (pH adjusted to 7.4 with Tris base). The experimental chamber was constantly perfused with control bathing solution (1–2 ml/min). The high-speed solution exchange system, HSSE-2 (ALA Scientific Instruments, Westbury, NY, USA), was used to deliver control and test solutions. Under optimal conditions, the delay in switching between solutions was ~10 ms. Data presented herein were obtained through subtraction from the leak current.”
C. MolecularModeling

The primary sequence of the human $\alpha_7$ nACh receptor was acquired from the Universal Protein Resource (UniProt) (entry code: P36544; *Homo sapiens*). Multiple alignments of the $\alpha_7$ nAChR sequence were performed with several orthologs by ClustalX 2.0\(^{137}\) using the following UniProtKB accession codes: Q05941 (rat $\alpha_7$ nAChR; *Rattus norvegicus*), P22770 (chicken $\alpha_7$ nAChR; *Gallus gallus*). Homology models of the ECD and TMD of human $\alpha_7$ nAChRs were generated using Modeller 9.12 (Version 9.12; University of California San Francisco, San Francisco, CA) based on three templates: the X-ray crystal structure of a chimeric ECD of the $\alpha_7$ nACh receptor (PDB ID 3SQ9)\(^70\) that shares 64% sequence identity with the ECD of the human $\alpha_7$ nACh receptor subunit, the NMR structure for the TMD of the $\alpha_4$ nAChR subunit (PDB ID 2LLY)\(^74\) that shares 49% sequence identity with the TMD of the human $\alpha_7$ nAChR subunit, and the cryo-electron microscopic structure of the *Torpedo marmorata* nAChR (PDB ID 2BG9)\(^21\) that shares 53% sequence identity with the TMD of the human $\alpha_7$ nAChR subunit. All the template structures were retrieved from the PDB Data Bank at [http://www.rcsb.org](http://www.rcsb.org). The resulting models of each domain were evaluated based on the discrete optimized protein energy (DOPE) and the molecular probability density function (Molpdf) scores and validated by the docking of $\alpha_7$ nACh receptor modulators (i.e., galantamine for ECD, PNU-120596 and ivermectin for TMD). A cavity-search study was conducted based on the Connolly surface feature of SYBYL-X 2.1 (Tripos Inc. St. Louis, MO) and the blind docking approach within AutoDock (Version 4.2; Scripps Research Institute, La Jolla, CA). The empirical data from two $\alpha_7$ nACh receptor PAMs (i.e., ivermectin, PNU-120596) were used as a guiding tool in plausible binding site exploration.
Four low-energy rotamers of $N$-methyl $m$CPG were computationally prepared using SYBYL-X 2.1. Molecular docking was conducted using the CHEMPLP scoring function within the genetic algorithm docking program GOLD (Version 5.2; Cambridge Crystallographic Data Centre, Cambridge, UK), and the docking solutions were analyzed (SYBYL-X 2.1). The binding site was defined to include all atoms within 10 Å of the $\alpha$-carbon atom of the key amino acid residue for the candidate homology models. Based on the fitness scores and the binding orientation of each ligand within the binding cavity, the best-docked solution was selected and merged into the receptor. The initial models were energetically optimized using the Tripos Force Field (Gasteiger–Hückel charges, distance-dependent dielectric constant = 4.0) to optimize the interactions between ligand and receptor within the binding pocket, followed by PROCHECK and ProTable analyses to validate the candidate models. These optimized model-ligand complexes were then rescored with HINT (Hydropathic INTeractions) program; an empirical scoring function based on the experimental free energy information derived from log P<sub>o/w</sub> (the solvent partition coefficient for 1-octanol/water).

In the pharmacophore determination study, the compounds were aligned using GASP with modifications in the default parameter. The population size (i.e., alignment solutions conformations) was set to 125, the allele mutate weight (i.e., conformation diversity) to 96, and the fitness increment (i.e., superimposition criteria) to 0.02. CoMFA calculations were conducted using the QSAR module of SYBYL-X 2.1. The standard setting of 30 kcal/mol as energetic cut-off value was used. Regression analyses were performed using the SYBYL-X 2.1 tool of the partial least square (PLS) algorithm with cross-validation (leave-one-out) and optimum number of components.
Bibliography


158. GraphPad Software, San Diego California USA, [www.graphpad.com](http://www.graphpad.com)


163. These compounds were prepared, and their synthesis is described in the Experimental section, but biological data have not yet been obtained.


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176. Several additional compounds were synthesized to evaluate the proposed models; these include compounds **52, 53** and **56**. Although the synthesis of these compounds is reported herein, functional data have yet to be obtained.


182. Personnal communication “Shailesh Khatri; Dr. Shulte’s student”.


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Appendix A

Concentration/response curves for compounds included in this study.
Figure A1. Concentration/response curves for arylguanidines 21-23, 25-34, 37-39, 52, and 53 (A-R) inhibition of peak currents elicited by 100 μM ACh at α7 nAChRs.
Appendix B

Primary QSAR analyses for the N-methyl series compounds.

Figure B1. Plot of pIC$_{50}$ values of the N-methyl analog series versus the electronic character ($\sigma_m$) ($r = 0.453$, $p = 0.260$, $n = 8$).
**Figure B2.** Plot of pIC$_{50}$ values of the N-methyl analog series versus Verloop length ($L$) ($r = 0.763$, $p = 0.030$, $n = 8$).

**Figure B3.** Plot of pIC$_{50}$ values of the N-methyl analog series versus Verloop maximum width ($B_5$) ($r = 0.448$, $p = 0.265$, $n = 8$).
Figure B4. Plot of pIC$_{50}$ values of the N-methyl analog series versus the molar refraction (MR) ($r = 0.875$, $p = 0.021$, $n = 8$).

Figure B5. Plot of pIC$_{50}$ values of the N-methyl analog series versus the solvent accessibility volume (Vol) ($r = 0.882$, $p = 0.013$, $n = 8$).
Figure B6. Plot of pIC$_{50}$ values of the N-methyl analog series versus the number of valence electron (NVE) ($r = 0.263$, $p = 0.528$, $n = 8$).

Figure B7. Plot of pIC$_{50}$ values of the N-methyl analog series versus the complete molar refraction (CMR) ($r = 0.893$, $p = 0.122$, $n = 8$).
Figure B8. Plot of pIC$_{50}$ values of the N-methyl analog series versus the molar volume (MV) ($r = 0.540$, $p = 0.166$, $n = 8$).

Figure B9. Plot of pIC$_{50}$ values of the N-methyl analog series versus Parachor values (Pc) ($r = 0.805$, $p = 0.023$, $n = 8$).
Figure B10. Plot of pIC$_{50}$ values of the N-methyl analog series versus the polarizability of the compounds ($r = 0.890$, $p = 0.016$, $n = 8$).
Vita

Osama Ibrahim Alwassil was born on December 22, 1981, in Riyadh, Saudi Arabia. Osama received his Bachelors of Science in Pharmaceutical Sciences from the College of Pharmacy, King Saud University in Riyadh, Saudi Arabia in 2005. He worked as a teaching assistant in the College of Clinical Pharmacy, King Faisal University in Al-Ahsaa, Saudi Arabia from 2005 to 2008. In 2008, he was awarded a scholarship from King Faisal University towards a Master of Science degree with a concentration in Medicinal Chemistry at Virginia Commonwealth University and he received this degree by 2012. Subsequently, he was enrolled in the Virginia Commonwealth University’s School of Pharmacy, Pharmaceutical Sciences Ph.D. program with a concentration in Medicinal Chemistry in August 2012.