SYNTHETIC AROMATIC AGMATINE ANALOGS AS ALLOSTERIC MODULATORS OF THE N-METHYL-D-ASPARTATE (NMDA) RECEPTOR CHANNEL

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ABSTRACT OF DISSERTATION

Joshua Roderick Ring

The Graduate School
University of Kentucky
2006
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ABSTRACT OF DISSERTATION

A dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy in the College of Pharmacy at the University of Kentucky

By
Joshua Roderick Ring
Lexington, Kentucky

Director: Dr. Peter A. Crooks, Professor of Pharmaceutical Sciences
Lexington, Kentucky
2006

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The N-methyl-D-aspartate (NMDA) receptors are highly regulated ligand-gated ion channels, which are affected by many substrates. Overactivation of the NMDA receptor can lead to hyperexcitability and a number of neurotoxic effects and neurological diseases. Agmatine has been demonstrated to act allosterically as an inhibitory modulator at the polyamine recognition sites of the NMDA receptor complex. The present study synthesized and evaluated a library of agmatine analogs for their ability to displace tritiated MK-801 from NMDARs in P2 membrane preparations from rat brains at ligand concentrations of 1 mM and 50 uM. A full dose-response curve was generated for the most active compounds, in the presence and absence of a pathological level of spermidine (100 uM). A forty-five member subset of arylidenamino-guanidino compounds was synthesized and all were demonstrated to be NMDA receptor inhibitory modulators in the above assay. Three of these compounds generated biphasic curves, indicating activity at two binding sites: the postulated high-affinity agmatine binding site, and a low-affinity site (perhaps the channel itself). (4-Chlorobenzylidenamino)-guanidine hydrochloride demonstrated an IC50 of 3.6 uM at the former site and 124.5 uM at the latter. Several
computer models were generated to direct further synthesis. Based on the structure-activity relationship of the aryldenedamino-guanidino compounds, a pharmacophore model of the agmatine binding site of the NMDAR was proposed.

KEYWORDS: Agmatine, N-Methyl-D-Aspartate Receptors, NMDAR Inhibition, Polyamine Binding Sites, Aryldenedamino-Guanidine

Joshua Roderick Ring
March 30, 2006
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March 30, 2006
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CHAPTER 1: BACKGROUND TO RESEARCH

1.1 Introduction

The N-Methyl-D-Aspartate receptor complex has become one of the most important and studied targets for drug development due to its importance in the central nervous system, as well as its complexity and incredible number of modulatory molecules, hence individual targets. NMDA receptors are highly regulated ligand-gated ion channels, which are affected by many substrates, including the endogenous ligand, L-glutamate. The cation channel is nonselectively permeable to Na\(^+\), K\(^+\), and Ca\(^{2+}\), but is blocked by Mg\(^{2+}\) at physiological conditions. Overactivation of the NMDA receptor can lead to hyperexcitability and a number of neurotoxic effects and neurological diseases such as epilepsy, as well as Parkinson’s, Alzheimer’s, and Huntington diseases. Antagonism of the NMDA channel has also been shown to reduce toxicity due to alcohol withdrawal (Littleton et al., 2001). However, the physiological function of this channel is crucial in learning and memory, and thus a therapeutic agent cannot be one which irreversibly inhibits channel flux.

Overexcitation of this receptor and neurotoxicity can result from activation of an NMDA coreceptor, NR2B, by spermine and spermidine, polyamines which are biosynthesized from putrescine (the ureahydrolyzed form of agmatine). Agmatine, a polyamine found in mammalian tissue (Li et al., 1994), has been investigated recently for its role in pain regulation, and may also be a neurotransmitter with its own transporter across the blood-brain barrier (Reis and Regunathan, 1999). An endogenous ligand at imidazoline binding sites (Raasch et al., 2001), this compound has also been shown to have effects on learning and memory in rodents (McKay et al., 2002). In addition, agmatine and its analogue arcaine are antagonists of the NMDA receptor in the brain, facilitating neuronal recovery. This background focuses on the roles of polyamines, specifically agmatine, as allosteric modulators of the NMDA receptor complex.
1.2 NMDA Receptors

The majority of mammalian fast excitatory synaptic transmission is regulated in the brain by the excitatory amino acid (EAA) L-glutamate. Therefore, its receptor channels have been identified as some of the most crucial in terms of physiological response as well as associated pathology.

Glutamate is synthesized in multiple metabolic compartments (Aureli et al., 1997), and is present at resting conditions in the synaptic cleft at concentrations around 0.6uM (Bouvier et al., 1992). During excitatory synaptic transmission, this extracellular concentration will very briefly increase twenty-fold (Clements et al., 1992), after which the L-glutamate undergoes reuptake by the pre-synaptic and post-synaptic cell membranes of the neurons (Danbolt, 2001).

There exist two major classifications of glutaminergic receptors in the brain, the ionotropic receptors (iGluR) and the metabotropic receptors (mGluR). The metabotropic glutamate receptors are G-protein coupled and eight genes for these have been identified. The ionotropic glutamate receptors are coded across at least fifteen genes and consist of three major and well-known families, named for their earliest discovered specific agonists: a-amino-3-hydroxy-5-methylisoxazole-4-propionic acid (AMPA), kainic acid (KA)(McKay et al.), and N-methyl-D-aspartate (NMDA).

Unlike the other subfamilies of iGluRs, NMDA receptors at resting potential are blocked in their channel by Mg$^{2+}$ in a voltage-dependent manner. Also, while every iGluR is permeable to Na$^+$ and K$^+$ ions, the NMDA channels are also highly permeable to Ca$^{2+}$ upon opening. Among all of the glutamate receptors, the NMDARs have many crucial roles in neural physiology, and their implication in many pathological conditions have opened this receptor to much intense scrutiny as a target for fine modulation.

1.2.1 NMDA Receptor Location and Structure

NMDA receptors exist as ligand-gated ion channels, formed as heteromultimeric protein complexes. The two major subunit families are termed NR1 and NR2; the latter consists of four separate proteins (termed NR2A-NR2D) coded on four separate genes,
while the former is a single protein with only one associated gene. In addition, an NR3A subunit has been shown to be expressed in the first post-natal week in mice; this subunit is only coimmunoprecipitated with NR1 and NR2B subunits, forming a receptor with lessened activity indicative of an unknown regulatory system (Das et al., 1998).

Figure 1.1 Representation of the NMDA receptor NR1 and NR2 subunits, showing NR1 exons involved in splice variants (adapted from Hynd et al., 2004)

Each NMDAR is assembled in vivo as either a tetrameric or pentameric channel complex, although it remains unknown as to which composition is exclusively formed, if not both. The majority of NMDA receptors are composed of at least one NR1 subunit and at least one NR2 subunit. It has been suggested that each NMDAR is likely to contain two NR1-subunits in each channel complex (Behe et al., 1995), and in vivo studies have shown that some NMDA receptor complexes are composed of more than one different NR2-subunit in addition to the NR1-subunit or subunits (Luo et al., 1997). Factors affected by the type of NR2 subunit, or subunits, incorporated into the channel complex include affinity for agonists, antagonists, and polyamines, sensitivity to blockage by Mg$^{2+}$ and Zn$^{2+}$, and channel conductance, as well as kinetics of deactivation. In *Xenopus* oocytes, a functional homomeric NR1 subunit complex is formed, which is indeed responsive to L-glutamate and glycine (Moriyoshi et al., 1991; Yamazaki et al., 1992). However, in mammalian expression systems, this NR1-only channel is able to bind the agonists as well as channel blockers, but does not display any functional
properties (Chazot et al., 1992; Grimwood et al., 1995). Many studies have been performed to determine specific stoichiometry of NMDA receptors, and while many different combinations of subtypes have been proposed, a definitive conclusion as to the exact number and composition of subunits remains hazy.

The gene which expresses the NR1 subunit is composed of 22 exons over 31 kb in rats (Zimmer et al., 1995). The human gene is very similar to the rat gene, save for the exclusion of rat exon 3 (Hollmann et al., 1993), leaving 21 exons. In both species, this gene undergoes alternative splicing at three different exons, and these coding regions give identical amino acid sequences in both rat and human (Foldes et al., 1994). These three exons code 21, 37, and 38 amino acids are titled N1, C1, and C2 respectively from the 5’ to 3’ end (Figure 1.1); they are exons 4, 20, and 21 in humans and 5,21, and 22 in rats (Durand et al., 1993). Alternative splicing of these three exons results in eight variants of the NR1 subunit, which are denoted in subscript to designate the inclusion (1), exclusion (0), or undetermined (X) state of N1, C1, and C2 (Durand et al., 1993). The relative abundance of these eight splice variants is 67% for the most commonly-found NR1_{011}, 15% total for the four NR1_{1XX} variants, and 18% total for the remaining three NR1_{0XX} variants (Sugihara et al., 1992). Alternative splicing has also been demonstrated in the NR2C subunit to some extent, however it has not been well characterized nor the functional differences well-researched (Ishii et al., 1993).

As the NR1 subunits are believed to be found in every functional NMDA receptor complex, they are well-distributed among most brain neurons, but the distribution of NMDA receptor complexes containing the specific NR2 subunits is more differentiated by location. NR2A mRNA is almost as well-distributed as that of NR1, while the mRNA of the NR2B and NR2C subunits seem to have a more complementary distribution (Monyer et al., 1992). Receptors which include the NR2A and NR2B subunits are found in most neurons throughout the cerebral cortex, hippocampus, and thalamus (Hollmann and Heinemann, 1994), and mRNA of both NR2A and NR2B have been found in the amygdaloid nuclei as well as the caudate-putamen (Monyer et al., 1992). The NR2B subunits are also highly expressed in the medium-sized spiny projection neurons (MSNs) of the striata, relative to other NR2 subtypes (Christie et al., 2000; Landwehrmeyer et al., 1995; Rigby et al., 1996). The cerebellum contains the majority of the NR2C subunits,
and NR2A subunits are also expressed there; NR2A-C subunits are all expressed in the thalamic nuclei, but the hypothalamus does not contain the mRNA of any of the three subunits. The cell type distribution is also different for these subtypes: while NR1 and NR2A mRNA are expressed in most cell types, the mRNA of NR2B is mainly found in granule cells, and NR2C mRNA is mainly expressed in tufted and mitral cells (Monyer et al., 1992). The NR2D subunits are exclusively expressed in the brain stem and spinal cord (Hollmann and Heinemann, 1994).

1.2.2 NMDAR Function

The NMDARs have been indicated in the governing of such important physiological functions as synapse formation, neuronal differentiation, and long-term potentiation; and are implicated in memory and special learning function, primarily during development. As these receptors require very high levels of synaptic membrane depolarization in order to activate by removing the voltage-dependent Mg\(^{2+}\) channel block and allow Ca\(^{2+}\) to flow, they are well-suited for the induction of synaptic plasticity (Bliss and Collingridge, 1993). It has also been found that NMDA receptor stimulation promotes the survival, maturation, and neurite outgrowth of neurons which have been isolated from cerebellum and hippocampus.

Receptor function is also closely related to the subunit composition of the NMDA receptor complex, as is seen in other types of EAA channels (Unwin, 1989). In studies of whole-cell currents, the subunit combination of NMDA receptors affected the strength of voltage blockage by magnesium. NR1/NR2C and NR1/NR2D complexes possess much weaker Mg\(^{2+}\) blocking action than the NR1/NR2A and NR1/NR2B NMDAR complexes, indicating that sensitivity to the endogenous voltage blocker differs directly according to the subunits involved (Liu et al., 1996; Monyer et al., 1992).

1.2.3 NMDAR Pathology

Excitatory transmission, while being an integral part of NMDA receptor function,
has also been implicated in neuronal destruction. L-Glutamate is the excitatory amino acid responsible for activation, and subsequently overactivation at excess levels, of NMDA receptors; the potential for overexcitation is normally overcome by uptake mechanisms removing extracellular L-Glu on both pre- and post-synaptic neuronal cell membranes as well as the membranes of adjacent glial cells. Involved in this toxicity are overactivation of NMDA receptors and increase in the intracellular concentration of Ca\(^{2+}\) ions. As AMPA and kainite receptors are not highly-permeable to calcium ions, NMDARs possess a much greater potential for introduction of excess intracellular Ca\(^{2+}\) concentrations, which initiate a neurodegenerative cascade leading to cell death. In vivo studies supporting the involvement of NMDARs with neurodegeneration have correlated the accumulation of radiolabeled calcium with toxicity (Koh and Choi, 1991), as well as demonstrating that an application of toxic L-Glu levels in hippocampal neurons elevates intracellular Ca\(^{2+}\) concentrations for a full hour and leads to the death of 85% of the neurons in a 24-hour period (Dubinsky, 1993). Excessive calcium ion influx may also be causative in neuronal death by triggering the activation of degradative enzymes such as calpains and capsases (Nicotera and Lipton, 1999). In addition, many studies have shown that this neuronal cell death can be prevented by blocking NMDARs, and thus excess calcium influx. Some of the pathology may be due in part to the rise in polyamine levels seen in various conditions, as some polyamines act as channel agonists (Paschen, 1992).

Seizures, and in particular epilepsy, have been long tied to glutamate receptors, specifically N-methyl-D-aspartate receptors. L-Glutamate, and its interaction with EAA channels, has been implicated in the initiation and proliferation of some types of seizure activity (Meldrum et al., 1999). NMDAR-mediated excitotoxicity may be the cause of the neuronal death following status epilepticus (Choi, 1988). In vivo epilepsy models have shown that NMDA receptor activation is necessary in the development of seizure susceptibility, while other studies have suggested a role for NMDA receptors in the appearance of epileptiform discharges. All classes of NMDAR antagonists have shown anticonvulsant properties including channel blockers, glycine site antagonists, and polyamine site antagonists (Chapman, 2000).

The actions of NMDA receptors, in particular with respect to polyamines, have been well investigated for their role in Alzheimer’s disease (AD). While the excitotoxic
necrosis of neurons in AD may be due to the pathological persistent depolarization of the cell membrane leading to excess calcium influx, the levels of brain polyamines which are seen in Alzheimer’s may be the more direct effect which leads to said NMDAR overactivation (Dodd, 2002; Morrison and Kish, 1995). In the temporal cortex of AD patients, the levels of spermidine (an NMDAR agonist) are greatly increased over control levels, while the levels of putrescine (a partial NMDAR antagonist) are decreased. These two effects are likely caused by the increase in brain S-adenosylmethionine decarboxylase activity, which should increase the rate of conversion of putrescine into spermidine (see Figure 1.2, page 18) (Morrison et al., 1996). In addition, the activity of ornithine decarboxylase (the main enzyme responsible for the biosynthesis of putrescine) is reduced in the occipital cortex, but increased in the temporal cortex in Alzheimer’s disease patients (Morrison et al., 1998). There is evidence that there is a loss of NMDA receptor complexes containing the specific splice-variant NR1_{1XX} subunit in the areas of the brain afflicted by AD and most subject to neuronal cell loss (Mattson, 1997), but whether this is a cause or effect of the disease is unknown. However, post-mortem brain tissue of Alzheimer’s disease patients is indeed observed to contain pathologically-modified excitatory glutaminergic pyramidal neurons (Braak et al., 1993; Francis et al., 1992).

Huntington disease, an inherited neurodegenerative disorder, has also been linked to NMDAR-mediated excitotoxicity (Li et al., 2003). Specifically, Huntington disease (HD) affects the striatal medium-sized spiny projection neurons (MSNs), which contain NMDAR complexes with a relatively larger amount of NR2B subunits expressed than other NR2 subunits. Indeed, brain tissue removed post-mortem from HD sufferers demonstrates the degenerative vulnerability of neurons with high levels of NMDA receptors (Albin et al., 1990; Young et al., 1988). A mutation in the protein huntingtin, htt, is causative in the development of Huntington disease in humans (Huntington's Disease Collaborative Research Group, 1993). It has been shown that the mutant htt potentiates both the currents and excitotoxicity mediated by N-methyl-D-aspartate receptor complexes which include at least one NR2B subunit (Li et al., 2003), as well as having a role in the induction of mitochondrial dysfunction (Panov et al., 2002). This increase in NMDA activity over long periods of time and with patient aging may be too
much for the natural regulatory mechanisms to overcome, with the resulting oxidative stress and excess $\text{Ca}^{2+}$ levels leading to increased calpain and capsase activity, htt proteolysis, and cell death (Li et al., 2003). In studies done on rodents and non-human primates, it was found that striatal NMDAR overactivation indeed resembles the neurological and behavioral changes seen in Huntington disease patients (Beal, 1992).

Brain damage associated with concussive head injury as well as ischemia, hypoxia, and hypoglycemia may also be a direct result of overactivation of NMDA receptors following these conditions. This excitotoxicity leads directly to necrosis or apoptosis (Johnston et al., 2000).

The NMDA receptors, specifically with regards to their regulation by polyamines, are crucial in the development and continuation of neuronal pathology demonstrated in drug abuse. It has been postulated that the NMDARs are involved in the mechanisms of alcohol as well as cocaine and opioid drugs. Chronic use of ethanol, an inhibitor of NMDARs (particularly those containing the NR2B subunit), can cause adaptations and desensitization which can lead to alcohol dependence (Lovinger et al., 1989). Furthermore, this NMDAR up-regulation which results from excess ethanol intake is known to directly result in the neurotoxicity associated with hyperexcitability demonstrated during the withdrawal period afterwards (Dodd et al., 2000). Fetal alcohol syndrome may also be due to the interactions between ethanol and polyamines at NMDAR polyamine sites. As polyamines positively modulate the NMDAR effects which facilitate neuronal growth and synapse formation during fetal development (Johnson, 1996), the inhibition of these potentiating effects by alcohol may indeed harm the fetal CNS.

1.3 Regulation of NMDA Receptors

1.3.1 Direct Channel Blockers

Regulatory sites on the NMDA receptor are abundant, and the list of modulators and binding sites is extensive. The endogenous agonist is the excitatory amino acid L-
glutamate, but as N-methyl-D-aspartate is specific for this receptor over the other EAA receptor types, the latter compound received naming privileges. Biochemical and electrophysiological studies have shown that glycine enhances the effects of glutamate or NMDA on channel activation, and is therefore considered a co-agonist. In fact, the gating occurs quite poorly in the absence of both of these neurotransmitters, a unique property of NMDARs among known neurotransmitter receptors. Site-directed mutagenesis has shown that the binding of glycine probably occurs on the outer membrane-spanning domains of the NR1 subunits of the receptor complex (Kuryatov et al., 1994), while L-glutamate binds in a similar location of the NR2A and NR2B subunits (Anson et al., 1998; Laube et al., 1997) (Figure 1.1).

Magnesium ions block the channel at resting membrane potential in a highly voltage-dependent manner, on a location deep within the pore, closer to the intracellular side of the receptor. The possibility of two separate Mg$^{2+}$ binding sites has also been suggested due to studies showing voltage-dependent NMDA ion-channel blockage by both intracellular and extracellular magnesium (Johnson, 1996). Curiously, in the absence of the endogenous agonist and co-agonist, low levels of Mg$^{2+}$ enhances the binding of the standard channel-pore blocker $[^3]$HMK-801, however, in the presence of the amino acids, Mg$^{2+}$ reduces $[^3]$HMK-801 binding. The type of NR2 subunit associated with the NMDA complex also has an effect on Mg$^{2+}$ activity, as those containing NR2C or NR2D subunits are less sensitive to blockage by Mg$^{2+}$ than are those containing NR2A or NR2B subunits (Liu et al., 1996).

Zinc ions also have a complicated array of effects on the NMDA receptors. Zn$^{2+}$ seems to act as both a voltage-dependent channel blocker and as a non-competitive, voltage-independent inhibitor (Peters et al., 1987). This channel blockage by zinc is much weaker than the voltage-dependent channel block by Mg$^{2+}$. In addition, Zn$^{2+}$ may also potentiate a current induced by bound agonists at the NMDAR (Hollmann et al., 1993). While it is important to note that Zn$^{2+}$ and Mg$^{2+}$ bind at separate sites, it has been suggested that Zn$^{2+}$ binds at two distinct sites on the NMDAR as well, one within the channel, and another outside the channel which is more sensitive to the membrane potential (Christine and Choi, 1990). The subunit composition of the NMDA receptor also impacts the sensitivity to Zn$^{2+}$: complexes containing the NR2A subunit have a
higher affinity for voltage-independent Zn$^{2+}$ inhibition (Mori et al., 1992) than complexes containing the other three NR2 subunits, and those NMDA complexes which contain the NR2A or NR2B subunits also display more of the low-affinity voltage-dependent Zn$^{2+}$ inhibition than the others (Paoletti et al., 1997).

Blockage of the NMDA receptor channel can also be achieved by a large number of synthesized molecules which are either drugs, or were designed as drug candidates. Phencyclidine (PCP) and the structurally similar drug ketamine are both NMDA channel blockers with slow kinetics, which have been used as dissociative anesthetics, but both are also commonly known to possess high abuse potential. (+)MK-801, also known as dizocilpine or (+)-[3H]-5-methyl-10,11-dihydro-5H-dibenzo[a,d]cyclohepten-5,10-imine maleate, is a very potent NMDAR channel blocker, which also has exhibited high abuse potential and is primarily used in displacement assays in order to judge the inhibitory properties of other molecules. Dextromethorphan, an antitusive, is another effective channel blocker, with a stronger selectivity for NMDA complexes containing the NR2A subunit over those lacking it (Avenet et al., 1997). A pair of similar amino-adamantane molecules, memantine and amantadine, are also potent channel blockers, which offer more potential as drugs than the previously mentioned channel blockers due to their faster kinetic profiles (Parsons et al., 1996). Memantine and amantadine exhibit an improved clinical tolerance compared to MK-801 and phencyclidine due to faster blocking/unblocking of the NMDA channel, and have been used clinically to treat dementia and Parkinson’s disease without the neurotoxic and abuse effects of the other channel blockers (Kornhuber et al., 1994).

1.3.2 Allosteric Modulators

Function of the NMDA receptor complex is also regulated in part by the pH levels of the extracellular solution (Traynelis and Cull-Candy, 1990). The concentration of H$^+$ ions at physiological pH are, in fact, very close to that of the IC$_{50}$ of H$^+$ ions for the NMDA receptor, thus any change in pH will alter channel activity to some degree (Tang et al., 1990). The location of pH effect on the channel complex, or the H$^+$ binding site, is located near the extracellular terminal of the NR1 protein subunit. As protons do not
directly interfere with the channel, they are said to act as allosteric inhibitors of NMDAR activity. NMDA receptor complexes containing the NR2C subunit have an IC$_{50}$ for H$^+$ inhibition around pH 6.2, while complexes containing the NR2A, B, or D subunits tend to have IC$_{50}$ levels around pH 7.2-7.3 (Traynelis et al., 1995).

Ethanol also is a concentration-dependent inhibitor of NMDAR function (Koltchine et al., 1993; Mirshahi and Woodward, 1995). Protein kinase inhibitors have been shown to counter the ethanol-induced inhibition in cerebellar granule cells (Snell et al., 1994), and it has been demonstrated that ethanol may enhance phosphorylation of tyrosine residues in the NR2B subunit, thereby increasing the receptor’s tolerance to ethanol inhibition (Miyakawa et al., 1997). Homomeric NR1$_{0XX}$ NMDAR complexes are less sensitive to ethanol-induced inhibition than the homomeric channels composed of NR1 subunits containing exon 4 (Koltchine et al., 1993), although the NR1 splice variant involved in NR1/NR2 heteromeric channels does not seem to have any bearing on ethanol sensitivity (Kuner et al., 1993). NMDAR complexes containing the NR2C subunit are less sensitive to the modulatory effects of ethanol than those containing NR2A or NR2B subunits (Kuner et al., 1993; Masood et al., 1994).

Endogenous oxidizing and reducing agents can also modulate NMDAR channel function from the extracellular milieu, with the former acting as an inhibitor and the latter in an activity-enhancing manner (Aizenman et al., 1989; McBain and Mayer, 1994). This modulatory function is governed by the equilibrium between reduced (thiol) and oxidized (disulphide, or possibly sulphenic acid) states of two cysteine residues located on an extracellular loop of the NR1 subunit, Cys-744 and 798 (Lipton et al., 1996; Sullivan et al., 1994). Not surprisingly, the subunit composition of the NMDA receptor complex governs the sensitivity to these redox substances; complexes containing the NR2A subunit undergo both reversible and persistent potentiation effects by reducing agents, while the NR2B-D subunits are only involved with complexes that undergo the persistent effects which can only be undone by the addition of an oxidizing agent (Kohr et al., 1994). This suggests separate, or additional, redox sites on the NR2A subunit, while the modulation demonstrated in other NMDAR complexes may be entirely governed by the previously mentioned cysteine residues of the NR1 subunit. However, homomeric NR1 channel complexes are completely insensitive to redox modulation (Sullivan et al., 1994).
Polyamines exhibit their modulatory activity based on several key structural features, including number of amines, distance between amines, charge, and lipophilic and hydrophilic domains. Both spermine and spermidine act as glycine-independent agonists, and potentiate binding of open-channel blockers. Antagonists at the polyamine sites include putrescine (1,4-diaminobutane) and diethylenetriamine (DET), which serve to block or displace spermidine and spermine, as well as arcaine (1,4-diguanidinobutane) and long-chain diamines with eight or more carbons between primary amines (Romano et al., 1992). Agmatine is believed to act as an inhibitory modulator at NMDARs at a separate site. Inhibitory and potentiative effects are seen with short-chain diamines, with the general observation that lipophilic 1,3-diamines act as NMDA channel antagonists whereas hydrophilic 1,3-diamines have agonist properties (Lewin et al., 1998). A wide range of activities was also found in several studies of synthetic spermine and spermine analogues, containing linear as well as cyclic linkers.

While many channel-blockers and EAA binding site inhibitors have been shown to possess strong binding affinity, these classes of antagonist have very high abuse potential and exhibit neurotoxicity. The polyamine sites are an interesting topic for research, as the antagonists at this site exhibit neither of these detrimental qualities, yet possess strong binding affinity. However, of the modulatory sites of the NMDAR complex, the polyamine site is least well-understood in terms of its location, binding domain, and even the number of polyamine recognition sites.

1.4 Polyamines

1.4.1 Polyamines Background

The ubiquitous endogenous polyamines in eukaryotic tissue include spermidine, spermine, putrescine, and agmatine. These compounds are all enzymatically synthesized products of the amino acid arginine, via the polyamine pathway (Figure 1.2). While agmatine is formed exclusively from the decarboxylation of arginine by arginine decarboxylase (ADC), the favored route for the synthesis of putrescine proceeds by the
ureahydrolysis of arginine to ornithine, followed by the subsequent decarboxylation to
putrescine by the enzyme ornithine decarboxylase, or ODC. ODC is believed to be one
of the major regulatory enzymes for maintaining proper cellular polyamine levels, along
with S-adenosylmethionine decarboxylase later in the polyamine pathway (Figure 1.2).
Ornithine decarboxylase itself is regulated by levels of cAMP and Ca$^{2+}$ (Veldhuis and
Hammond, 1981), and the degradation of the enzyme is accelerated by antizyme (Heller
and Canellakis, 1981). While the synthesis of putrescine from arginine through the
agmatine intermediate is believed to exist in mammals, neither the ADC nor agmatinase
enzyme is well-characterized (see Section 1.4.3). Spermidine is the resulting product of
the addition of aminopropyl moieties to one terminal putrescine amino moiety by
putrescine aminopropyltransferase (PAPT); this step is followed by the same addition
onto the other terminal “putrescine” amino moiety, catalyzed by spermidine
aminopropyltransferase (SAPT). The aminopropyl group that is transferred in both cases
originates from decarboxylated S-adenosylmethionine (dcAdoMet), which is made
available by the enzymatic decarboxylation of S-adenosylmethionine, a process that
partially serves to regulate the cellular levels of spermine and spermidine (Morrison et
al., 1993). Composed of a propyl and a butyl chain, spermidine is not a symmetrical
molecule, but it should be noted that SAPT specifically adds the aminopropyl group to
the butylamine.
The biosynthetic polyamine pathway

The classic three polyamines, putrescine, spermidine, and spermine, are present in relatively high concentrations in the CNS, putrescine at levels of roughly 4-70 nmol/g of tissue and the others around 50-1400 nmol/g (Seiler and Schmidt-Glenewinkel, 1975; Shimizu et al., 1964). These molecules have essential roles in the growth and differentiation of cells in many different systems and tissue types, with a wide range of
concentrations throughout mammalian systems (Pegg, 1986; Tabor and Tabor, 1984). Their levels are regulated by a complex system of enzymatic synthesis and degradation, as well as uptake and export (Pegg, 1988). In addition, polyamines have been implicated in protein synthesis (Lenzen et al., 1986), stabilization of nucleic acids (Igarashi and Kashiwagi, 2000), gene expression regulation (Celano et al., 1989), free radical scavenging (Ha et al., 1998), membrane permeability regulation (Lapidus and Sokolove, 1992), and programmed cell death (Ha et al., 1997). Polyamines are also present in the mammalian brain at micromolar concentrations (Sarhan and Seiler, 1989), where they possess regulatory roles at ion channels (Scott et al., 1993), including many effects on the NMDA receptor complexes. It has been suggested that these polyamines act as neurotransmitters, or possibly regulators of synaptic transmission (Harman and Shaw, 1981; Shaw, 1979).

1.4.2 Polyamine Activity at the NMDAR Complex

As with most NMDA inhibitors, the subunit composition of the receptor complex has a large bearing on the activity of the polyamines, individually and as a group (Hess et al., 1998; Zhang et al., 1994). While at least three separate NMDA receptor polyamine binding sites have been identified (Yoneda and Ogita, 1991), the polyamines also display effects at other modulatory sites on the NMDAR complex. These compounds, in addition to their inherent properties, also are able to enhance or reduce the modulatory properties of glycine and glutamate, as well as reducing the Ca$^{2+}$-influx inhibitory effects mediated by protons.

The final two polyamines in the metabolic pathway, spermine and spermidine, possess at least four modulatory effects on the complex (denoted in parentheses below as 1-4), and act as both allosteric agonists and antagonists. The two inhibitory effects of polyamines consist of (1) a voltage-independent inhibition, as they can serve to decrease the NMDAR binding affinity of the endogenous excitatory amino acid, L-glutamate (Hess et al., 1996); and (2) a voltage-dependent NMDAR inhibition, in which these compounds can act directly in the channel pore at the Mg$^{2+}$ binding site to interrupt the cation flux through the channel (Araneda et al., 1999; Rock and MacDonald, 1992).
voltage-dependent inhibition was reported as the low-affinity part of a biphasic nature exhibited by spermine and spermidine on [³H]MK-801 binding. While these compounds potentiate binding at lower concentrations, the effect reverses around 300μM, at which point the polyamine begins to displace the channel-bound ligand, presumably through the previously noted steric hindrance of the pore (Ransom and Stec, 1988). The lower affinity potentiation of [³H]MK-801 binding is an example of the positive modulatory effects of polyamines. These effects, while producing similar outcomes, are markedly different, depending on the levels of glycine present. At saturation concentrations of glycine, polyamines (3) increase the channel currents which are induced by L-glutamate, independent of glycine; and at lower glycine levels, polyamines (4) increase the receptor affinity for glycine, thereby potentiating the channel-opening effects of glycine, relative to its concentration.

Putrescine itself has no effect on the binding of [³H]MK-801 to NMDA receptors in the presence of physiological levels of glutamate and glycine (Reynolds and Miller, 1989). Putrescine does, however, inhibit the stimulatory effects of spermine and spermidine, regardless of the presence of the excitatory amino acids. This effect was shown to be non-competitive, suggesting that the effects of putrescine may be mediated at a separate polyamine recognition site (Sacaan and Johnson, 1990).

The polyamine arcaine acts as a displacer of MK-801 in the presence and absence of spermine and spermidine (Sacaan and Johnson, 1990). This compound is structurally similar to the endogenous polyamine agmatine, but has a guanidine moiety on both ends. As arcaine is able to inhibit the ligand binding to baseline levels, it is believed that this compound acts as an antagonist or inverse agonist at one of the polyamine binding sites (Reynolds, 1990).

A long-chain diamine, 1,10-diaminododecane (DA10 or N-10-N), does not replicate the effects of similarly-structured spermine and spermidine. In fact, in the absence of other polyamines, this compound inhibits the binding of [³H]MK-801 (Williams et al., 1989), due to a decrease in the rate of association of the channel-blocker. Experiments performed in a range of glutamate and glycine concentrations have shown that DA10 does not act at the amino acid sites; in fact, a high concentration of spermidine served to attenuate the effects of DA10. Another study performed with longer-chain diamines
possessing 12, 14, and 16 carbon-chains produced similar results, with higher potency (Berger et al., 1992). These data indicate that long diamines may act as inverse agonists, producing effects opposite to those of spermine and spermidine, but at the same site (Williams et al., 1990).

Diethylenetriamine (DET), a linear polyamine, has also been identified as an NMDA receptor antagonist. However, it does not directly reduce $[^3H]$MK-801 binding in the absence of spermine or spermidine, indicating that it acts as either a competitive antagonist at the binding site for longer-chain polyamines, or possibly as an allosteric displacer of them (Williams et al., 1989). Further studies showed that increasing DET concentrations resulted in a parallel shift of the concentration-effect curve of spermidine-potentiation, which confirms the former hypothesis of a direct effect of DET as a competitive antagonist at the long-chain polyamine binding site (Williams et al., 1990). Interestingly, high concentrations of DET (>100 μM) are able to slightly potentiate MK-801 binding, indicating that it acts as a weak partial agonist at a polyamine recognition site (Williams et al., 1991).

A large study undertaken by Yukio Yoneda (Yoneda et al., 1995) examined over 60 polyamine derivatives for their ability to affect the binding of $[^3H]$MK-801 from whole rat brain preparation in the presence and absence of spermidine (1 mM). These screenings of primarily synthetic polyamines demonstrated again that diamines with less than eight methylene groups have no effect on NMDARs, whereas those longer acted as channel antagonists. Triamines with between five and ten combined methylene groups between the two chains demonstrated the MK-801 binding potentiation of spermidine, but the eleven-methylene N-(3-aminopropyl)octanediamine (TA38) appeared to act as a competitive antagonist for spermidine by attenuating the spermidine-potententiating effects while having no statistical effect on binding in the absence of the endogenous polyamine. Tetraamines containing less than a combined fifteen methylene groups had similar activity to spermine, while those containing greater than fifteen displaced the MK-801 in the presence and absence of spermidine. The synthetic polyamine bis-(3-aminopropyl)nonane-diamine (TE393) was the only tetraamine which had no effect on the binding in the absence of added spermidine, but significantly inhibited binding in its presence (Yoneda et al., 1995). This structure-activity experiment demonstrated that the
chain length and number of amines in a polyamine can significantly impact its effects on the NMDAR complex, perhaps at different polyamine recognition sites.

Another structure-activity study studied the effects of various linear and cyclic synthetic tetraamines over a range of concentrations and compared the pKₐ values of the nitrogen atoms in the compounds. This study again detailed the complex nature of polyamine modulation of the NMDAR complex. While diethylspermine acts as a potent inhibitor of [³H]MK-801 binding with an IC₅₀ value of 10μM, a geometric homologue with fluorine atoms substituted for each of the terminal methyl hydrogen atoms possesses no activity. This latter compound, N¹,N¹₂-bis(2,2,2-trifluoroethyl)spermine, is only protonated at the two central nitrogen atoms at physiological pH, thus the compound has pKₐ values similar to putrescine, and maintains putrescine’s lack of NMDAR activity. Many other examples in this study belie the importance of charge as opposed to number of amines possessed by a given polyamine (Bergeron et al., 1996).

Insertion of an aromatic moiety into long-chain diamines has also been shown to increase the affinity of these compounds for the polyamine binding domains of the NMDAR complex (Berger et al., 1998). This study showed that introduction of a thiophene moiety between the fourth and fifth methylene groups of 1,12-dodecanediamine (DA12) results in a fifty-fold increase in [³H]MK-801 displacement in the presence and absence of 100μM spermine. Interestingly, shifting the thiophene group to a different position in the chain without varying the total number of methylene groups resulted in a decrease in potency. This increase in potency may be due to the conformational restraint of such a grouping, or possibly point to an affinity for aromatic groupings at one or more of the polyamine recognition sites.

Many additional series of inhibitors which act at these sites have been synthesized and reported, including several novel series in our laboratory (Bence et al., 2000; Worthen et al., 2001).

1.4.3 Agmatine:

Agmatine, 4-(aminobutyl)-guanidine, has been long known as a part of the polyamine pathway in plants, bacteria, and invertebrates, as it is converted into putrescine
by the enzyme agmatinase (Tabor and Tabor, 1984; see Figure 1.2). This biosynthetic route represents a second method of polyamine synthesis, independent of ornithine and ODC levels, which is believed to be favored in the lower life forms mentioned above.

A study in the mid-1990’s which was searching in mammalian brain tissue for an endogenous ligand at imidazoline binding sites led to the discovery of agmatine in mammalian tissue for the first time (Li et al., 1994). Shortly afterwards, subsequent studies discovered ADC and agmatinase in mammalian brain tissue, indicating that agmatine is locally synthesized (Li et al., 1995; Sastre et al., 1996). The heterogeneous distribution of agmatine throughout rat brain tissue was characterized immunocytochemically (Otake et al., 1998) using a polyclonal antibody developed to recognize agmatine but not the other polyamines (Wang et al., 1995). Based on this study, agmatine was proposed to be primarily localized in neurons of the hypothalamus, rostral midbrain, laterodorsal nucleus, locus coeruleus nucleus raphe dorsalis, and the periaqueductal gray. Agmatine is also found in neurons of the cerebral cortex, hippocampus, subcortical telencephalon, and thalamus. Interestingly, agmatine was not found in astrocytes, although ADC activity has been demonstrated there (Regunathan et al., 1995). The same immunocytochemical method was used to reveal that agmatine in the hippocampus is primarily located in axon terminals that form excitatory synapses on pyramidal cells (Reis et al., 1998).

The study by Regunathan et al. which first indicated the activity of ADC in the mammalian brain has been debated, and the group of Anthony Pegg published a study which concluded that there is not enough evidence to prove this alternative pathway of agamatine synthesis (Coleman et al., 2004). However, the group of Soudar Regunathan has since then published a study identifying a mammalian cDNA which codes for a distinct ADC enzyme (Zhu et al., 2004). More recent articles have reported the decarboxylation of arginine to agmatine in rat liver (Horyn et al., 2005), and analyzed the expression of ADC in the regions of rat brains (Iyo et al., 2006).

Agmatine was also found by HPLC methodology to be well-distributed throughout mammalian tissues (Raasch et al., 1995), largely in the lumen of the stomach. This high gastrointestinal content is due to several factors: the formation and release of agmatine from physiological gut microflora bacteria and pathogens (Molderings et al.,
the absorption from ingested food, and also to a lesser extent from shed GI epithelial cells (Benamouzig et al., 1997). Studies have shown that radio-labeled agmatine is transported into the stomach wall in a temperature-dependent manner by means of a specific agmatine transporter (Molderings et al., 2002), and it has been postulated that this absorbed agmatine is an important source for function in other organs of the body, especially the liver (Molderings et al., 2003). While agmatine is metabolized by two different enzymes, ADC and diamine oxidase (DAO, which converts agmatine into guanidinobutyraldehyde) (Lortie et al., 1996), the activity of ADC is relatively low compared to that of DAO in rat liver and kidney, while DAO metabolism of agmatine is nearly non-existent in brain tissue (Holt and Baker, 1995).

Agmatine is known to possess bioactivity, as it causes the release of a number of hormones and neurotransmitters including insulin from pancreatic islet cells (Sener et al., 1989), catecholamines from adrenomedullary chromafin cells (Li et al., 1994), and luteinizing hormone from pituitary cells (Kalra et al., 1995). Agmatine administered systematically also impairs specific types of learning and memory as well as behavior (McKay et al., 2002).

It has been proposed by the group of Donald Reis that agmatine may be an endogenous neurotransmitter in the mammalian brain (Reis and Regunathan, 2000) based on a number of factors: It is synthesized in the CNS (Li et al., 1994), stored in synaptic vesicles in axon terminals (Reis et al., 1998), released from synaptosomes by depolarization (Reis and Regunathan, 1998), reaccumulated and inactivated by reuptake (Sastre et al., 1997), degraded by the specific enzyme agmatinase (Sastre et al., 1996), and binds with high affinity to $\alpha_2$-adrenoceptors and imidazoline binding sites as well as to the NMDA receptor.

The activity of agmatine at NMDA receptors was first demonstrated in an MK-801 displacement study in rat cerebral cortex membrane (Anis et al., 1990). Enhancement of opioid analgesia, a characteristic of known NMDA receptor antagonists (Wong et al., 1996), is demonstrated by agmatine in vivo, as well as the prevention of opioid tolerance (Kolesnikov et al., 1996).

Yang and Reis also studied the direct effects of agmatine at varying concentrations on the voltage current through the NMDA receptor channel (Yang and
Reis, 1999). The data indicated that agmatine is a concentration- and voltage-dependent antagonist of the NMDA receptor channel. Using whole-cell currents recorded from hippocampal neurons, a reversible channel-block was established which was more potent at hyperpolarizing membrane potentials than at positive voltages. This blockage was obtained in the presence of currents elicited by N-methyl-D-aspartate, but in the absence of NMDA, no current effect was observed, indicating that agmatine has no agonist properties. A competitive antagonist at the NMDA binding site, (±)-2-amino-5-phosphonopentanoic acid (AP5), inhibited the NMDA current in both directions; however, simultaneous administration of agmatine and AP5 yielded an additional current blockage, indicating that agmatine is not a competitive antagonist at the same site, but acts to allosterically modulate the channel. This study also investigated the activity of agmatine on other glutamate receptor channels activated by AMPA or kainite, and found that the concentrations necessary to block the NMDA channel completely yielded current reduction in these channel of no more than 20%, indicating a strong selectivity for the NMDA receptor channels. Additionally, the action of arcaine and agmatine were observed in the presence of spermine, which is known to potentiate NMDA current. This spermine-induced potentiation still occurred in the presence of these channel inhibitors, although it was diminished; these data indicate that the polyamines mostly likely act at different binding sites.

Previous studies have shown that in the absence of the spermidine potentiation, agmatine is only a weak channel inhibitor with an IC$_{50}$ value of approximately 1mM, while ifenprodil and arcaine, also polyamine binding site inhibitors, showed significant binding reduction at concentrations of 500nM and 5μM, respectively (Gibson et al., 2002). However, in the presence of 100μM spermidine, agmatine had a K$_i$ of 14.8μM for MK-801 binding, indicating that agmatine is not a direct channel antagonist, nor does it bind to the polyamine binding site without a simultaneous binding of spermidine. Thus, agmatine is believed to be the only competitive inhibitor of the polyamine binding site amongst these three antagonists.

While works have been published which have focused on synthesis of polyamine analogues and their effects on the NMDA receptor channel, none have been reported on agmatine analogues as such. The intriguing properties of agmatine as a potent and
selective allosteric competitive inhibitor of the NMDA receptor complex have led to interest in optimizing this molecule for use in drug development. Thus, this study will undertake to synthesize and screen a variety of agmatine analogues, in order to find lead compounds for pharmaceutical development.
1.5 Hypothesis and Specific Aims

HYPOTHESIS

The hypothesis set forth in this work is that a more potent and therapeutically useful agmatine analog can be found which will allosterically modulate the NMDA receptor channel from a polyamine binding site, specifically at the site which binds agmatine. To this end, the work will set forth to study the structure-activity relationship of agmatine congeners with the NMDAR complex, and attempt to discover new lead compounds for pharmaceutical development. The agents so produced may be therapeutically useful in the treatment of such conditions as Epilepsy, Alzheimer’s disease, Huntington disease, and the neurodegeneration associated with the use and withdrawal of drugs of abuse, specifically ethanol.

SPECIFIC AIMS

Specific Aim 1. Synthesize a library of novel agmatine analogues having selectivity for the polyamine binding site of the N-methyl-D-aspartate (NMDA) receptor complex. This study will undertake to develop and synthesize a varied library of novel analogs of agmatine, with the goal of achieving selectivity and potent inhibition of the NMDA receptor. Earlier work has shown the effectiveness of agmatine to bind in a selective antagonistic manner at the polyamine recognition site, or sites, of the NMDA receptor complex. Design and testing of structural congeners of agmatine will effectuate a more defined set of substructural motifs, leading to more potent compounds. These optimization studies will produce molecules with varied chain length and flexibility, as well as aromatic and heteroatomic substitution in the chain region and modification of the guanidine moiety.

Specific Aim 2. Evaluate the synthesized library for their ability to displace [³H]MK-801 from the N-methyl-D-aspartate receptor. Agmatine has been shown to
inhibit the binding of $[^3]$HMK-801 to the ion channel of the NMDA receptor by allosteric modulation at one of the polyamine binding sites, thus indicating its potential usefulness in modulation of channel opening. In this study, the synthesized analogs of agmatine will be screened for their ability to similarly inhibit the binding of $[^3]$HMK-801 to the NMDA receptors of male rat P2 brain tissue preparation using the method of Ransom and Stec, which was modified to be amenable to high-throughput screening (Ransom and Stec, 1988). Utilizing a programmable liquid handler, compounds can be diluted and dispensed into 96-well plates with remarkable speed and precision. Each compound will be initially evaluated at two concentrations, with promising candidates being more thoroughly analyzed utilizing a range of concentrations to determine IC$_{50}$ values for each active compound in the presence and absence of pathological levels of the long-chain polyamine spermidine.

**Specific Aim 3. Construct a pharmacophoric model of the most potent antagonists at the binding site in question using a neural network approach to molecular modeling.** The polyamine binding site of the NMDA receptor is not very well characterized, much less the crystal structure known. Without knowledge of the structural biology, the approach to *in silico* modeling will be more challenging: after screening large libraries of compounds synthesized in this work and elsewhere, a program will be used to design a neural network model based on the data yielded. From this model, new compounds will be selectively synthesized based on *in silico* prediction of activity. These new compounds will then be used to verify and modify the developed model, in addition to providing new, more selective inhibitors.
CHAPTER 2: SYNTHESIS AND EVALUATION OF AGMATINE ANALOGS

2.1 Introduction

The primary goal of this study was to synthesize and test agmatine analogs in order to seek out a more potent inhibitor of the spermidine/spermine potentiation of NMDA channels demonstrated in channel overactivation, while attempting to maintain the lower inhibitory potency of agmatine at physiological levels of longer-chain polyamines. A number of spermine and spermidine analogues have been made and reported, but a focus on agmatine analogs has been largely overlooked.

Agmatine is composed of three specific moieties, labeled as A, B, and C in figure X. Moiety A consists of a terminal amine, which is protonated and carries a formal charge of +1 at physiological pH, and may be involved in hydrogen bonding with a residue at the site, or sites, at which agmatine acts in the NMDAR complex. Moiety B is a lipophilic n-butyl chain which connects the two terminal groups. Moiety C is the terminal guanidino group, which is also charged at physiological pH, and has a +1 charge shared between the two omega-nitrogen atoms through resonance.

![Figure 2.1 Modification sites on agmatine molecule](image)

Figure 2.1 Modification sites on agmatine molecule

Modifications of each of these moieties, and combinations of the separate modifications, are numerous. “Moiety A”, the terminal amine, can be replaced by heteroatoms including oxygen as well as halogens, or even left off completely to leave a terminal methyl group. “Moiety B”, the butyl bridge, can be lengthened or shortened to
n-pentyl and propyl bridges, respectively. It is also possible to add a heteroatom into this chain, as seen in the amino acid canavanine. As will become apparent, replacing this group with a benzyl, phenyl, xylyl, or other aromatic moiety is also quite viable. The modification of “Moiety C” presents a plethora of opportunities. While the interior secondary amine is seldom changed in groupings similar to a guanidine, the two omega nitrogen atoms proved to be quite flexible. Urea and thiourea groups can be obtained by the conversion of region C$_1$ to an oxygen or sulfur molecule, respectively. In addition, the C$_1$ and C$_2$ groups can be affixed with a short alkyl group, or they can be joined together by either an ethyl or propyl bridge.

The first synthetic goal was to find simple and reliable procedures which would allow modifications of both the aliphatic chain moiety and the guanidine of the agmatine molecule. The former involved the attachment of a guanidine group to a range of primary amines, and it became evident that this synthetic route would need to fulfill several requirements: an ability to synthesize several compounds in parallel, a decent yield without necessitating purification for preliminary product screening, and also the ability to produce a very pure product for further screening of lead compounds. Much time and effort was spent to these ends, but a synthetic route meeting these goals was discovered utilizing $N,N'$-bis(tert-butoxy)-S-methylisothiopseudourea as a guanylation reagent (see General Reaction 1 below). A small parallel synthesizer was purchased which can run this reaction on twelve starting materials simultaneously, with yields normally in the range of 60 to 80%. Unlike other guanylation reagents, this di-boc-protected product is uncharged, yet polar enough to purify by silica gel chromatography before deprotection. Modification of the guanidine moiety has also proved amenable to parallel synthesis, as addition of a substituted guanidine group to the primary amine starting materials are similarly one-step reactions without the need for lengthy purification (see General Reactions 2-3 below).

The formation of a Schiff base between arylaldehydes and aminoguanidine hydrochloride to form arylidenamino-guanidine compounds (see General Reaction 4) also opened up new avenues of facile agmatine analog synthesis. These reactions produced very clean products in high yield, and many of them crystallized from the reaction solution upon cooling. Four series of analogs were created from the same set of
aldehydes, which included addition of an aminoguanidine, dimethylhydrazine, semicarbazide, and thiosemicarbazide (see General Reactions 4-7, respectively). Further explored in chapter 3, the substituted aryaldehyde chosen dictated the composition of moieties A and B of figure 1. Each of these reactions also replaced the moiety B in figure 1 with an aromatic imine. Moiety C was left unchanged in the case of aminoguanidine; the amine of group C₁ was replaced with an oxygen molecule in semicarbazide, and a sulfur molecule in thiosemicarbazide. The addition of dimethylhydrazine further modified the entire structure of moiety C, switching the interior carbon atom for a nitrogen atom, and the terminal amines with methyl groups.

2.2 Synthetic Chemistry

Several major synthetic routes were followed in the course of this study in order to obtain the agmatine analogs. From two base starting materials, seven synthetic General Reactions were used in order to obtain seven separate products with varying moieties similar to a guanidine. The general procedures utilized in this study are denoted as General Reactions 1-7. General Reactions 1-3 utilized a primary amine as the site of addition for an S-methyl guanidine analog, while General Reactions 4-7 relied on the Schiff base formation between a starting material aldehyde and an aminoguanidine or analog thereof. These reactions are described below:

General Reaction 1:

Guanidino-compounds can be prepared by addition of an equimolar amount of the primary amine and \( N,N'-\text{bis(tert-butoxy)}\)-S-methylisothiopseudourea in tetrahydrofuran (Verdini et al., 1992). The boc-protecting groups are then cleaved with excess trifluoroacetic acid in dichloromethane followed by rotary evaporation and separation with diethyl ether and water (See Scheme 1). The aqueous layer is then reacted with hydrochloric acid and lyophilized to form the hydrochloride salt of the guanidine product. This method has the distinct advantage of yielding an extremely lipophilic intermediate which can easily be separated from unreacted starting materials by silica gel column.
Alternatively, a hydrosulfide salt of the guanidine may be prepared by the addition of the primary amine (3 equivalents) to S-methylisothiopseudourea hemisulfate (1 equivalent). The solution is refluxed in methanol for six hours, and yields the guanylated product as a hemisulfate salt as a crystal solid after cooling to room temperature.

Scheme 2.1 Synthesis of guanidino-compounds from primary amines

General Reaction 2:

Bridged S-methyl guanidines are prepared in nearly quantitative yield by refluxing the corresponding bridged thiourea with methyl iodide in methanol (Kennedy et al., 1998). The crystallized product is amply pure for reaction in the next step with a primary amine in methanol at reflux for 2-carbon bridged guanidines (imidazole derivatives), and in ethanol at reflux for 3-carbon bridged guanidines (1,4,5,6-tetrahydropyrimidine derivatives) to yield the corresponding N-substituted product as an iodine salt.

Scheme 2.2 Synthesis of $N^\omega$-$N^{\omega'}$ bridged guanidino-compounds
General Reaction 3:

$N^\omega$-substituted $S$-methyl guanidines are prepared in nearly quantitative yield by refluxing the corresponding substitute thiourea (in this work, ethyl) with methyl iodide in methanol. The crystallized product is amply pure for reaction in the next step with a primary amine at reflux in ethanol to yield the corresponding $N$-substituted product as an iodine salt.

Scheme 2.3 Synthesis of $N^\omega$-ethyl guanidino-compounds

General Reaction 4:

The general method for preparation of arylidenamines proceeds as follows: aminoguanidine hydrochloride (1 equivalent) and an aryl aldehyde (1.5-2 equivalents) are dissolved in a minimal amount of methanol. The solution is then heated to reflux and stirred for a period of 4-12 hours with TLC monitoring until the aminoguanidine hydrochloride is consumed. Upon cooling to room temperature, the corresponding arylidenamino-guanidine is separated from the remaining aryl aldehyde. In some cases, the product crystallizes from the methanol solution during cooling, in which case the product is filtered and rinsed with methanol and chloroform. In most cases, the product would remain in solution upon cooling, and the solution is then removed on a rotary evaporator. The resulting solid or semi-solid is then stirred at room temperature in chloroform or diethyl ether, into which the remaining aryl aldehyde dissolves, and the remaining solid arylidenamino-guanidine is filtered and washed with the corresponding rinsing solution. Products are allowed to dry on filter plates, and then placed on vacuum overnight to remove any remaining solvent.
General Reaction 5:

Compounds containing an arylaldehyde-dimethylhydrazone moiety can be prepared by the formation of a Schiff base between an aryl aldehyde (1 equivalent) and dimethylhydrazine (1.25 equivalents). These compounds are dissolved in a minimal amount of methanol, heated to reflux, and stirred for a period of 2-3 hours. Upon cooling to room temperature, the product crystallizes from the methanol solution in some cases; otherwise, the product solution is evaporated at a moderate temperature in order to remove excess unreacted dimethylhydrazine starting material. Products are then placed on vacuum overnight to remove any remaining solvent.

General Reaction 6:

Arylaldehyde semicarbazones can be easily prepared by a Schiff base formation between an aryl aldehyde (1.5-2 equivalents) and semicarbazide hydrochloride. These compounds are dissolved in a minimal amount of methanol, heated to reflux, and stirred for a period of 4-6 hours. Upon cooling to room temperature, the corresponding semicarbazone crystallized from the methanol solution. The solid precipitate was then filtered, rinsed with methanol, and placed on vacuum overnight to remove any remaining solvent.
Scheme 2.6 Synthesis of arylaldehyde semicarbazone compounds

General Reaction 7:

The general method for preparation of arylaldehyde thiosemicarbazone compounds proceeds as follows: thiosemicarbazide (1 equivalent) and an aryl aldehyde (1.5-2 equivalents) are dissolved in a minimal amount of methanol, and the solution is heated to reflux and stirred for a period of 4-6 hours. In most cases, the corresponding arylaldehyde thiosemicarbazone crystallizes from the methanol solution during cooling to room temperature, in which case the product is filtered and rinsed with methanol and chloroform. In addition, the product may remain in solution upon cooling, at which point the solution is removed on a rotary evaporator, and the resulting solid or semi-solid is stirred at room temperature in chloroform. The remaining aryl aldehyde starting material is dissolves into this solution, and the solid product is filtered and washed with the corresponding rinsing solution. Products are allowed to dry on filter plates, and then placed on vacuum overnight to remove any remaining solvent.

Scheme 2.7 Synthesis of arylaldehyde thiosemicarbazone compounds

The following specific compounds were synthesized in our laboratory. Reaction details are noted for deviations from the general procedures described above in General Reaction 1-7. No starting material screened possessed any affinity for polyamine sites, which is not surprising in that the diamines used as starting materials for General Reactions 1-3 bore the most structural similarity to putrescine, which is inherently
inactive in the [³H]MK-801 displacement assay. Therefore, in order to expedite screening of the large number of total compounds, the products were purified to >85%. Active compounds were then reevaluated by NMR and in most cases ES+ mass spectroscopy for greater purity, and if necessary were further purified by crystallization or column chromatography. Pure products were then used to generate full dose-response curves over seven concentrations spanning three orders of magnitude, with n=3.

Figure 2.2 Summary of modifications of moiety C (guanidino group)

2.3 Pharmacology

2.3.1 [³H]MK-801 Displacement Screening Assay

The pharmacological assay utilized P2 brain membrane homogenate from male Sprague-Dawley rats (225-250 grams) and radiolabeled MK-801 in order to measure
The reduction of this bound ligand due to addition of a modulating agent. The P2 membrane was prepared from a homogenized mixture of rat cortex, hippocampus, and cerebellum in 50 mM Trizma acetate buffer containing 0.32 M sucrose at pH 7.25. This crude homogenate was centrifuged for 10 minutes at 4°C and 2800 rpm in a Sorvall RC 26 Plus centrifuge, after which the supernatant was saved and the procedure was repeated with the pellet dissolved in cold buffer with sucrose. The combined supernatants were then centrifuged for 20 minutes at 4°C and 20,500 rpm on the same instrument. The supernatant was decanted, and the pellet retained and resuspended in 50 mM Trizma acetate buffer at pH 7.25, without sucrose. Another 20-minute centrifugation was performed on the resuspended pellet with the above parameters, and two additional 10-minute washings, with the pellet retained between each. After these washings, the membrane was again suspended in the above buffer without sucrose, and the protein concentration determined using Pierce reagent. Aliquots of 15 mg of the concentrated P2 membrane preparation were dispensed into cryo-tubes, which were then stored at -80°C. With this amount of protein per tube, the final protein concentration could easily be brought to 1.5 mg/mL buffer by the addition of sufficient 50 mM Trizma acetate buffer at pH 7.25 by bringing the total solution volume to 10 mL.

The working \[^{3}H\]MK-801 solution is prepared by the addition of 30 μM \[^{3}H\]MK-801 in ethanol (2 μL) to the Trizma buffer (10 mL). In order to determine the concentration of \[^{3}H\]MK-801 in this working solution, 100 μL was dispensed into 2.5 mL of Microscint 20 in duplicate, and the counts per minute (CPM) were measured on the scintillation counter. As the stock ethanolic MK-801 solution slowly evaporated and became more concentrated, this procedure was necessary in order to calculate further buffer dilutions of the working solution required for the desired 6.0 nM final concentration.

High-throughput screens were performed at ambient room temperature in 96-well micro-titer plates with a total capacity of 350 μL per well. To each well was added 100 μL P2 membrane (thawed on ice and brought to the final concentration of 1.5 mg/mL as described above), 100 μL sample and 100 μL \[^{3}H\]MK-801 ligand (6 nM) for a total of 300 μL, with a 15-minute incubation period between the additions of the sample and MK-801. Three replicates of each of the following were performed per plate: a control with
100 μL Tris acetate buffer as the sample, 100 μL spermidine (300 μM) as a spermidine-potentiated control, and 100 μL dextromethorphan (3 mM) in order to measure non-specific binding. The non-specific binding levels were 20-30% of the buffer control, indicating that 70-80% of the bound [³H]MK-801 is indeed in the channels of NMDA receptors, and is displaced by the rather large final concentration of 1mM dextromethorphan. The spermidine was responsible for a potentiation in [³H]MK-801 binding of 60-90%, which is consistent with literature values. This potentiation is measured as an increase above buffer control level with the non-specific binding control as 0%. Therefore, with a disintegration count for the buffer control of 750 DPM, non-specific control of 150 DPM, and spermidine-potentiation control of 1250 DPM, the potentiation would be observed as 500DPM. However, the percent potentiation would be measured as:

\[
\frac{(DPM_{pot} - DPM_{control})}{(DPM_{control} - DPM_{non-specific})} \times 100\%
\]

or 500 DPM / 600 DPM, resulting in a percent potentiation of 80% in this example (see Figure 2.3). It should also be noted that while the counts are expressed as raw DPM instead of DPM/mg protein, a constant level of protein was maintained in each well across all experiments (0.15 mg/well).
Following the addition of P2 membrane, buffer, dextromethorphan, and spermidine, three replicates of each sample at concentrations of 6 mM and 300 μM were added, to yield total well concentrations of 1.0 mM and 50 μM. The plates were then allowed to incubate for 15 minutes in order to establish the equilibrium between the spermidine and samples, after which the $[^3H]$MK-801 ligand was added to each well. All micro-titer plates were allowed to incubate for 40 minutes following $[^3H]$MK-801 addition, and were then harvested into filter plates, which were then dried overnight in the hood at ambient temperature. After 12-16 hours, 35 μL of Microscint 20 was added to each membrane on the dried filter plate; each plate was sealed and then incubated for two hours before reading on a plate counter.

The compounds described below were evaluated for their ability to reduce the binding of $[^3H]$MK-801 at concentrations of 1 mM and 50 μM, based on the DPM measured by the plate counter. Percent reduction of MK-801 binding is reported below as the reduction in counts from the spermidine-potentiated control (0% reduction) to the non-specific dextromethorphan control (100% reduction) (see Figure 2.4 for examples).
Figure 2.4 Calculation of % Reduction of $[^3H]MK-801$ binding
Table 2.1 Reduction of $[^3]$HMK-801 binding in P2 membrane by ligands

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Table 2.1 (cont.) Reduction of $[^3]$H|MK-801 binding in P2 membrane by ligands

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Table 2.1 (cont.) Reduction of $[^3]$H|MK-801 binding in P2 membrane by ligands

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Table 2.1 (cont.) Reduction of $[^3]$H|MK-801 binding in P2 membrane by ligands

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<td>4.8</td>
</tr>
<tr>
<td>AH-10</td>
<td><img src="image5.png" alt="Structure AH-10" /></td>
<td>39.9</td>
<td>3.0</td>
</tr>
<tr>
<td>AH-13</td>
<td><img src="image6.png" alt="Structure AH-13" /></td>
<td>38.6</td>
<td>2.4</td>
</tr>
<tr>
<td>AH-14</td>
<td><img src="image7.png" alt="Structure AH-14" /></td>
<td>14.2</td>
<td>7.7</td>
</tr>
<tr>
<td>AH-15</td>
<td><img src="image8.png" alt="Structure AH-15" /></td>
<td>27.4</td>
<td>9.4</td>
</tr>
<tr>
<td>AH-16</td>
<td><img src="image9.png" alt="Structure AH-16" /></td>
<td>52.4</td>
<td>11.3</td>
</tr>
</tbody>
</table>
Further testing to obtain dose-response curves was performed on the most promising compounds screened in the above assay. For each of these compounds, three replicates of seven concentrations were tested, spanning an ample region to generate full dose-response curves in the absence and presence of spermidine.

The assay protocol proceeded similarly to the one described above. A blank control was measured in triplicate using only freshly-prepared 50 mM Trizma acetate buffer at pH 7.25. The 100 μM spermidine-potentiated MK-801 binding control and 1 mM dextromethorphan control to measure non-specific binding were each performed in triplicate as well. Samples were dissolved in buffer to make 19 mM stock solutions (for a final well concentration of 3.16 mM, or $10^{-2.5}$ M), and were diluted serially with 2.16 equivalents of buffer six times, to give final well concentrations at every $10^{-0.5}$ M, down to 3.16 μM, or $10^{-5.5}$ M. These seven concentrations were each added to the microtiter plate twice, with 50 μL spermidine (600 μM) in one set for a potentiated curve and 50 μL buffer in another set to obtain a non-potentiated curve. In some cases, the compounds were not soluble at 19 mM, and stock solutions were prepared at lower concentrations. These compounds underwent serial dilutions as stated above.

For each compound, the two curves were then plotted, and IC$_{50}$-values with and without spermidine were determined using GraphPad software. A third value, the IC$_{50}^\text{pot}$, was derived as the point on the spermidine-potentiated curve which was halfway between the spermidine-potentiated control and the buffer control. This value corresponds to the concentration of compound needed to displace half of the $[^3]$H]MK-801 bound due to the channel-agonist effects of spermidine. Some of the tested compounds demonstrated IC$_{50}$-values which were too low to be accurately gleaned in the previously mentioned concentration range; these compounds were prepared in a lower-concentration stock solution and re-evaluated at a lower range. In addition, agmatine, as well as the NMDAR antagonists arcaine, memantine, and MK-801 were evaluated by the same methods.
Table 2.2 IC$_{50}$-values in the presence and absence of spermidine, and IC$_{50}$ of potentiation

<table>
<thead>
<tr>
<th>Compound Code</th>
<th>Structure</th>
<th>IC$_{50}$ w/ SPD</th>
<th>IC$_{50}$ w/o SPD</th>
<th>IC$_{50}^{\text{Pot}}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>JR-3</td>
<td><img src="image" alt="Structure JR-3" /></td>
<td>862.8</td>
<td>767.7</td>
<td>364.8</td>
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<td>JR-5</td>
<td><img src="image" alt="Structure JR-5" /></td>
<td>359.2</td>
<td>221.7</td>
<td>162.0</td>
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<tr>
<td>JR-83</td>
<td><img src="image" alt="Structure JR-83" /></td>
<td>84.7</td>
<td>49.7</td>
<td>27.5</td>
</tr>
<tr>
<td>JR-84</td>
<td><img src="image" alt="Structure JR-84" /></td>
<td>68.3</td>
<td>50.8</td>
<td>15.5</td>
</tr>
<tr>
<td>JR-89</td>
<td><img src="image" alt="Structure JR-89" /></td>
<td>169.5</td>
<td>105.9</td>
<td>12.4</td>
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<tr>
<td>JR-93</td>
<td><img src="image" alt="Structure JR-93" /></td>
<td>1069</td>
<td>930.3</td>
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<tr>
<td>JR-106</td>
<td><img src="image" alt="Structure JR-106" /></td>
<td>615.5</td>
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<td>JR-107</td>
<td><img src="image" alt="Structure JR-107" /></td>
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</tr>
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<td>JR-108</td>
<td><img src="image" alt="Structure JR-108" /></td>
<td>1145</td>
<td>1348</td>
<td>97.3</td>
</tr>
<tr>
<td>JR-113</td>
<td><img src="image" alt="Structure JR-113" /></td>
<td>234.1</td>
<td>158.5</td>
<td>20.4</td>
</tr>
<tr>
<td>JR-117</td>
<td><img src="image" alt="Structure JR-117" /></td>
<td>207.0</td>
<td>100.0</td>
<td>104.7</td>
</tr>
</tbody>
</table>

1 - IC$_{50}$ w/ SPD: the measurement of IC$_{50}$ derived from the data collected in the spermidine-potentiated [$^3$H] MK-801 displacement assay.

2 - IC$_{50}$ w/o SPD: the measurement of IC$_{50}$ derived from the data collected in the non-spermidine-potentiated [$^3$H] MK-801 displacement assay, in which the spermidine solution was replaced with buffer.

3 - IC$_{50}^{\text{Pot}}$: from the spermidine-potentiated curve, the concentration corresponding to one-half of the difference in DPM between the SPD-potentiated control and the blank control.
Table 2.2 (cont.) IC$_{50}$-values in the presence and absence of spermidine, and IC$_{50}$ of potentiation

<table>
<thead>
<tr>
<th>Compound Code</th>
<th>Structure</th>
<th>IC$_{50}$ w/ SPD$^1$ (µM)</th>
<th>IC$_{50}$ w/o SPD$^2$ (µM)</th>
<th>IC$_{50}$ Pot$^3$ (µM)</th>
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<tbody>
<tr>
<td>JR-120</td>
<td><img src="image1" alt="Structure" /></td>
<td>1304</td>
<td>1136</td>
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<td><img src="image2" alt="Structure" /></td>
<td>169.4</td>
<td>218.7</td>
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<tr>
<td>JR-122</td>
<td><img src="image3" alt="Structure" /></td>
<td>149.3</td>
<td>325.9</td>
<td>293.8</td>
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<td>JR-124</td>
<td><img src="image4" alt="Structure" /></td>
<td>131.3</td>
<td>81.4</td>
<td>43.4</td>
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<td>JR-125</td>
<td><img src="image5" alt="Structure" /></td>
<td>442.5</td>
<td>414.4</td>
<td>231.2</td>
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<tr>
<td>JR-126</td>
<td><img src="image6" alt="Structure" /></td>
<td>69.7</td>
<td>16.0</td>
<td>25.8</td>
</tr>
<tr>
<td>JR-127</td>
<td><img src="image7" alt="Structure" /></td>
<td>232.1</td>
<td>147.2</td>
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<tr>
<td>JR-132</td>
<td><img src="image8" alt="Structure" /></td>
<td>67.4</td>
<td>32.1</td>
<td>32.2</td>
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<tr>
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<td><img src="image9" alt="Structure" /></td>
<td>1355</td>
<td>1428</td>
<td>539.5</td>
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<tr>
<td>JR-147</td>
<td><img src="image10" alt="Structure" /></td>
<td>855.0</td>
<td>835.5</td>
<td>464.5</td>
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</table>
Table 2.2 (cont.) IC_{50}-values in the presence and absence of spermidine, and IC_{50} of potentiation

<table>
<thead>
<tr>
<th>Compound Code</th>
<th>Structure</th>
<th>IC_{50} w/ SPD^{1} (μM)</th>
<th>IC_{50} w/o SPD^{2} (μM)</th>
<th>IC_{50}^{Pot}^{3} (μM)</th>
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<tbody>
<tr>
<td>JR-160</td>
<td><img src="image" alt="Structure JR-160" /></td>
<td>269.4</td>
<td>120.2</td>
<td>104.2</td>
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<tr>
<td>JR-197</td>
<td><img src="image" alt="Structure JR-197" /></td>
<td>654.6</td>
<td>778.9</td>
<td>199.5</td>
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<td>AH-8</td>
<td><img src="image" alt="Structure AH-8" /></td>
<td>32.17</td>
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<td>15.67</td>
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<tr>
<td>Agmatine</td>
<td><img src="image" alt="Structure Agmatine" /></td>
<td>902.3</td>
<td>1296</td>
<td>274.2</td>
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<td>Arcaine</td>
<td><img src="image" alt="Structure Arcaine" /></td>
<td>101.1</td>
<td>104.4</td>
<td>33.1</td>
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<tr>
<td>Memantine</td>
<td><img src="image" alt="Structure Memantine" /></td>
<td>4.77</td>
<td>3.53</td>
<td>0.73</td>
</tr>
<tr>
<td>MK-801</td>
<td><img src="image" alt="Structure MK-801" /></td>
<td>5.58 nM</td>
<td>5.92 nM</td>
<td>2.19 nM</td>
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</tbody>
</table>
2.4 Results and Discussion

This structure-activity study was undertaken to examine agmatine mimetics in an effort to learn more about the contribution of the different moieties in the molecule (see introduction) to potency, as well as selectivity. This was done by synthesizing a large library of over 200 structurally different agmatine analogs and testing their ability to displace \[^{3}\text{H}]\text{MK-801}\) in the membrane-bound proteins of rat brains, and comparing the results of these screens as well as the full dose-response curves of the most interesting compounds in the presence and absence of spermidine.

2.4.1 Early Lead Development:

The first set of screenings was performed on JR-1 through JR-91 and AH-1 through AH-6 and AH-9 through AH-14, evaluating these compounds in the spermidine-potentiated \[^{3}\text{H}]\text{MK-801}\) binding screen in order to determine their ability to modulate the NMDA receptor channel. These early assays were performed in the 96-well microtiter plates with 8 replicates of each sample at concentrations of 500 μM and 250 μM. Modification of the \[^{3}\text{H}]\text{MK-801}\) displacement assay utilized a fully automated Packard MultiProbe II HT liquid handling system, which was specifically programmed to perform dilutions of samples as well as distribution of samples and reagents into the 96-well microtiter plates according to the assay specifications. Data showed that precision was increased by utilizing this method, and the assay time was dramatically reduced.

A primary set of eight compounds was synthesized by addition of the guanidine moiety at a terminal amine to form agmatine congeners as in General Reaction 1 above; the resulting analogs included replacement of the terminal agmatine amine (moiety A in Figure 1) with a hydroxyl group as well as a bromine, and addition of a phenyl group into the aliphatic chain region (moiety B).

Results obtained were very promising for such a small selection of compounds. Agmatine analogues bearing terminal hydroxyl groups at the end of an aliphatic chain (JR-1 and JR-2, or \(N\)-(5-hydroxy-pentyl)-guanidine and \(N\)-(4-hydroxy-butyl)-guanidine, respectively) showed no activity at the concentrations tested, but several of the
compounds with substituted phenyl rings possessed a small amount of activity at both concentrations (JR-8 seen above). \( N\)-(2-Aminobenzyl)guanidine (JR-05) reduced \([^3]H\)MK-801 binding by 59.6% and 36.8% at 500 \( \mu \)M and 250 \( \mu \)M, respectively; \( N\)-(4-Aminobenzyl)guanidine (JR-03) showed reductions of 45.5% and 31.2% at the same concentrations. These aromatic-ring containing compounds were thus viewed as promising lead compounds upon proceeding to the next series of compounds, and many of the later compounds contained either benzene and pyridine rings. Another active lead compound from this series was \( N\)-(4-bromophenyl)guanidine (JR-08), which afforded binding inhibition of 84.6% at 500\( \mu \)M and 63.6% at 250\( \mu \)M.

The observed inhibition by \( N\)-(4-bromophenyl)guanidine led to an attempt to optimize this compound by moving the bromo group to the 3-position as well as by substitution at the 4-position with chlorine, fluorine, and iodine, and addition of a methylene group between the phenyl ring and the guanidine moiety. Of these compounds, only 4-fluoro and 4-chloro analogues afforded even weak inhibition of the receptor, with the other compounds slightly potentiated MK-801 binding. While the addition of a halogen at the \textit{para}-position on a ring provided an interesting early lead, the failure of the subsequent compounds to have any significant NMDAR-modifying activity combined with the relative lack of possible further modifications of these molecules signaled the end of their interest for development. Interestingly, much later studies revisited the usefulness of the halogeno-phenyl group in substitution of moieties A and B and produced very active arylidenamines (see Chapter 3).

With the terminal amines of moiety A intact, synthesized compounds in subsequent series focused on modifications of the guanidine moiety (moiety C) by substitution of the \( \omega' \)-amine (moiety \( C_1 \)) with an oxygen to form urea derivatives,
substitution of the ω-amine (moiety C₂) with a methyl group to form N-1-iminoethyl structures and introduction of a 2-carbon $N^\omega-N^\omega'$ bridge to form 4,5-dihydroimidazole substituted agmatine and arcaine analogues. The first of these modifications proved unsuccessful, in that the products of the reactions of the amine starting materials with urea (JR-41 through JR-53) were very pure, but completely water-insoluble, making them impossible to screen in the MK-801 displacement assay. The second of these modifications provided a set of compounds (JR-11 through JR-19) which were mostly inactive and also very dark in color, despite column chromatography for further purification. While the color of the compounds tested was taken into consideration by the TopCount binding program in order to convert CPM to DPM, the compounds which left the final test solution, and thus the wells on the filter plate, mostly opaque would always result in a dramatic drop in counts. Such a strong and consistent false positive always rendered the reported activity of dark samples as suspect, especially when the demonstrated “activity” closely coincided with the visible color of the filtered sample. While this small set of compounds was thus discounted, the problem of activity due to dark sample solution color became an important factor in the search for further false positives, and ensuring that the active compounds reported indeed possessed inherent ability to modify NMDA receptors, and not just the ability to render a solution opaque at a low concentration.

Of the seventeen evaluated 4,5-dihydroimidazole compounds, only $N^\omega$-(4,5-dihydro-1H-imidazol-2-yl)propane-1,3-diamine (JR-89) displayed inhibitory properties (52.9% at 500 μM). Of note is the observation that $N^\omega$-(4,5-dihydro-1H-imidazol-2-yl)butane-1,4-diamine (JR-90), which is agmatine with a $N^\omega-N^\omega'$ bridge, had slight potentiating effects on binding; yet JR-89, with one less methylene group than agmatine, acted as a channel antagonist by reducing observed [$^3$H]MK-801 binding in this assay. This interesting set of results shows that a modification of one moiety (in this case, the guanidino moiety) can affect binding independent of differences at a separate moiety (such as the aliphatic chain).
In addition, more structurally diverse linkers at moiety B were used; this set of agmatine congeners was a follow-up series based on the lead compounds from the first screenings, N-(2-aminobenzyl)guanidine (JR-5) and N-(4-aminobenzyl)guanidine (JR-3). The only two active JR-compounds to come out of this series were N-(4-aminomethyl-benzyl)guanidine (JR-83), and N-(3-aminomethyl-benzyl)guanidine (JR-84). The para-compound JR-83 reduced MK-801 binding by 74.4% at 250 μM and 86.8% at 500 μM, while its meta-isomer JR-84 inhibited binding by 95.4% at 250 μM and an astounding 97.2% at 500 μM. These two compounds immediately became a clear focus of further optimization.

This series also included many interesting pyridine- and pyrrolidine-based guanidine compounds, synthesized by Aaron Haubner for use in nicotinic receptor studies (AH-1 through AH-6 and AH-9 through AH-14). The majority of these compounds showed no inhibitory activity at either 250 μM or 500 μM, with two exceptions: N-[2-(1-methyl-pyrrolidin-2-yl)ethyl]guanidine (AH-13) and the di-guanidine N-(3-guanidino-pyridin-4-yl)guanidine (AH-9).

The first of these compounds, AH-13, was observed to slightly inhibit [³H]MK-801 binding by 21.5% at 250 μM and 50.2% at 500 μM; while this data is not
outstanding, the compound is quite different at its terminal amine from the majority of the other screened compounds in that the terminal amine (moiety A) is tertiary and attached to three aliphatic groups. The only other such compound was AH-10 (N-(2-pyrrolidin-1-yl-ethyl)guanidine), which was not only unable to reduce [$^3$H]MK-801 binding, but potentiated it by over 40% at both tested concentrations.

AH-9 inhibited binding by 93.4% at 250 μM and 96.0% at 500 μM, making this compound a strong new lead for further development. However, the inactivity of its isomer AH-6 was almost as interesting as the activity of AH-9: both of these compounds possessed two ortho-guanidines on their pyridine rings, but differed only in the position of the pyridyl amine. AH-6, which potentiated MK-801 binding in the screening assay, had the guanidine groups in the 2,3-position relative to the ring amine, while AH-9, which inhibited MK-801 binding by over 95% at 500 μM, had the guanidine groups in the 3,4-positions. Explanations for this remarkable difference in activity could be due to two rationales. 1) It is possible that AH-6 formed a six-membered ring by hydrogen bonding between the lone pair on the pyridine nitrogen and a hydrogen attached to the ω-nitrogen of the guanidine in the 2-position. This would hinder the free rotation of this guanidine, and perhaps modify the selectivity of the molecule, and cause it to bind in a different way at the same site, or even at a different polyamine site on the NMDA receptor complex. 2) Perhaps the ring amine itself is vital to the activity of the compound with relation to the binding site, and is only able to interact with a particular local site reside when in a certain location (by forming a hydrogen bond with a serine residue, for example). This created a very interesting set of possibilities which was shortly thereafter rendered moot by further testing:

A persistent and nagging issue at this point was the relative lack of spermidine potentiation demonstrated by the controls in this assay. While literature values consistently reported potentiation of [$^3$H]MK-801 binding by 100 μM of spermidine to be in the range of 60-100%, the controls of these assays were only on the order of 10-20%, a significant difference. In addition, the potentiation of binding demonstrated by certain compounds were not necessarily conclusive; while it would be no surprise that some polyamine analogs acted as channel agonists due to numerous published instances of such events, the possibility existed that spermidine was only demonstrating its full potentiation
in certain rows of the microtiter plates, and the compounds therein were merely “innocent bystanders”.

It was by months of filling plates by hand that the problem was revealed: the membrane and especially the spermidine solution needed to be kept thoroughly mixed during the screening, and it was necessary to vortex these solutions immediately prior each of to their additions to the plate. While the liquid handling system was reducing the amount of time required the fill multiple 96-well plates, it lacked the ability to swiftly and completely mix a large enough amount of these “reagents” kept in reservoirs on the deck of the instrument for quick fills of multiple wells.

While the above tests provided a good set of potential lead compounds for further optimization, and some interesting structure-activity relations between the compounds and their activities, it was necessary to re-screen them, especially the active compounds, with proper conditions and without the use of the liquid handling system.

2.4.2 Data Verification and Structural Optimization:

Further screening assays were performed to verify the above data and test more compounds based on these collected structure-activity relationship results. The [3H]MK-801 binding was repeatedly tested and brought to levels of 60-100%, usually around 70%. It was also determined that the screening assay would provide more useful information if the concentrations tested were further differentiated to reflect two points which would be separated on a dose-response curve. Therefore, instead of using 250 μM and 500 μM, which are separated by a factor of 2, these assays used concentrations of 50 μM and 1.0 mM, which are separated by a factor of 20. The full spectra of satisfactory compounds which were tested in this assay are divulged with structures in Table 1 above.

Of the previously tested compounds from the first series, the activity of JR-3, JR-5, and JR-9 were confirmed in the re-screening, with [3H]MK-801 binding reductions of 67.5%, 87.8%, and 93.6%, respectively, at concentrations of 1.0 mM. None of these compounds had significant binding reduction at the lower concentration of 50 μM.

The meta- and para-xylene guanidine compounds, JR-83 and JR-84, were again the most promising of the JR-series. Earlier screening tested the chloride salts of each of
these compounds, but for this assay and the full dose-response curves that followed, the hemisulfate salts were prepared with S-methylisothiopseudoura hemisulfate instead of $N,N'$-bis(tert-butoxy)-S-methylisothiopseudoura, and were recrystallized from methanol to give very pure products. This should not have any bearing on activity, and only affected molecular weight. **JR-83** ($N$-(4-aminomethyl-benzyl)guanidine) reduced [$^3$H]MK-801 binding by 92.0% at 1 mM and by 20.9% at 50 μM. **JR-84** ($N$-(3-aminomethyl-benzyl)guanidine) was slightly more potent, reducing ligand binding by 94.5% at 1 mM and by 40.0% at 50 μM. These two compounds remained strong lead compounds for optimization as well as for examination as potential antagonists, and were commissioned for evaluation with full dose-response curves, to follow the screening assays.

The previously-examined imidazole derivatives were the first to show differences between the two screening assays. **JR-89**, the 4,5-dihydroimidazole compound with an aminopropyl chain, was still the most active of those examined, reducing [$^3$H]MK-801 binding by 67.8% at 1 mM, but by a mere 6.4% at 50 μM. However, the structurally similar compounds **JR-90** ($N'$-(4,5-dihydro-1H-imidazol-2-yl)butane-1,4-diamine) and **JR-91** ($N'$-(4,5-dihydro-1H-imidazol-2-yl)pentane-1,5-diamine) possessed markedly different activities in this screen. These two compounds were both observed to cause an increase in radioligand binding to the membrane in the previous assay, with **JR-90** causing nearly twice the potentiation at 250 μM than at 500 μM. In the present assay, neither possessed significant activity at 50 μM, but at 1 mM, JR-90 decreased the binding of [$^3$H]MK-801 by 31.5%, and **JR-91** by 50.0%. While neither of these compounds is noteworthy as an NMDAR inhibitor, they do reflect the necessity for the re-screening of the compounds under consistent spermidine potentiation. In the early screen, if **JR-90** was indeed acting as a channel agonist, it would not likely have shown twice the potentiating activity at 250 μM than at 500 μM. To the contrary, this compound was most likely partially attenuating the effects of spermidine, which was demonstrating more potentiating activity in these wells than in the controls; thus a higher concentration of **JR-90** was not potentiating less radioligand binding, but instead inhibiting binding by a larger amount. Still, the interesting observation remains that while **JR-90** is most structurally similar to agmatine itself with their common n-butyl chain, both the
imidazole derivative with an additional methylene group (JR-91) and one less methylene group (JR-89) at moiety B possess greater antagonist activity.

The pyridine and pyrrolidine-containing compounds of the AH-series were also re-screened for activity at 1 mM and 50 μM. All of the tested pyridine-containing compounds demonstrated a small amount of activity as NMDAR antagonists, none of these molecules reduced $[^3]$HMK-801 binding by more than 50% at 1 mM. This was especially disappointing for AH-9 (N-(3-guanidino-pyridin-4-yl)-guanidine), which appeared to be so active in the previous screen. In contrast, this screen yielded a paltry 12.6% reduction in ligand binding due to AH-9 at 1 mM, and 4.8% at 50 μM. These results were repeated and verified, and the previous screening data for AH-9 must be attributable to either an error in solution preparation or perhaps a dispensing error by the liquid handling system previously used.

There were four additional compounds of the AH-series examined in this new set of screens, which included the first indole compound tested in AH-8 (N-(1H-indol-5-yl)guanidine hydrochloride). This compound appeared to be very active in the reduction of $[^3]$HMK-801 binding, eliciting a response of 91.1% at 1 mM and 68.3% at 50 μM. While these results were potentially tremendous, this compound, while 95%+ pure by NMR, existed as a pitch black semisolid. As seen above, very dark compounds tend to give false positives, especially when the tested solution is also visibly opaque. However, the results were encouraging in that the solution was too dilute to show color at 300 μM (the stock solution used to fill the low-concentration well for a final compound concentration of 50 μM), and AH-8 still demonstrated significant activity. This compound was also interesting as it conformationally restricted the entire “moiety B” of agmatine in figure 1, and connected a secondary amine to a guanidine with a separation of four methylene groups while only allowing rotation of the guanidine. Additionally, AH-7 (N-(4-methoxy-benzyl)-guanidine hydrochloride) was demonstrated to have some activity in the assay, reducing the $[^3]$HMK-801 binding by 77.0% at 1 mM and 20.2% at 50 μM. This was the first screened compound with a terminal methoxy group at moiety A (Figure 2.1), and the previously tested compounds with a hydroxyl group in the same location were completely inactive in this screen. While AH-7 was not tremendously active, the terminal methoxy group, as well as the indole group which was involved in the
more active AH-8, were revisited in further development of active NMDAR antagonists (see Chapter 3). Additionally, AH-8 was potent enough to evaluate further as an antagonist, and was designated for a full dose-response curve.

While the dimethyl pyridine group was used in the synthesis of tested compounds in the AH-series, the dimethyl phenyl (or phenyldiamine) group had been altogether ignored up to this point. It was also of interest as 1,4-phenyldiamine could undergo addition of a guanidine to form the structural backbone of the active indolyl compound, AH-8. However, phenyldiamines are also very dark compounds which readily polymerize, and even filtering with active charcoal was unable to render these solutions light enough to yield a clear testing solution. N-(3-Amino-phenyl)-guanidine dihydrochloride (JR-102), which was the only synthetic phenyldiamine derivative to yield a non-opaque solution at 6 mM (although it was a very dark, albeit lovely, purple solution), was screened at 1 mM and 50 μM final well concentrations. It did render the filter plate entirely too dark to be trusted at 1 mM, and not surprisingly produced a 99.3% reduction of counted CPM, but at 50 μM, JR-102 was completely devoid of activity. Therefore, this compound could not be judged to have antagonist activity in this assay, and even if the 1 mM reading was correct, the lack of any radioligand displacement at 50 μM indicated that it was not as potent as the structurally similar JR-83 or JR-84.

The next series of compounds included another set of imidazole derivatives, which incorporated aromatic groups at moiety C (see Figure 2.1), as follow-up compounds to JR-3, JR-5, JR-83, and JR-84. Replacement of the guanidine with an imidazole did not reduce the radioligand binding for any of the new compounds (JR-92, JR-93, JR-119, and JR-120) over their guanidine counterparts. All of these compounds were somewhat active as NMDA inhibitors at 1 mM, but at the lower concentration of 50
μM they each failed to reduce binding more than 20%. The most promising of these compounds was **JR-120** \((\text{N}^-\text{(4,5-dihydro-1H-imidazol-2-yl)}\text{-benzylamino-3-methylamine hydriodide})\), which reduced \(^{3}\text{H}\text{MK-801}\) binding by 62.4% at 1 mM and 19.3% at 50 μM, and **JR-93** \((\text{N}^-\text{(4,5-dihydro-1H-imidazol-2-yl)}\text{-benzylamino-2-amine hydriodide})\) which reduced ligand binding by 61.9% at 1 mM. However, the bis-imidazole arcaine analog, **JR-117** \((\text{N,N-bis(3,4-dihydro-1H-imidazol-2-yl)}\text{-1,4-bis(aminomethyl)benzene dihydriodide})\), was a bit more potent than any of the mono-substituted imidazole compounds at 1 mM, reducing radioligand binding by 82.4%. These three described compounds were set aside for further examination of their full dose-response curves.

In addition, a set of 1,4,5,6-tetrahydropyrimidine compounds (**JR-106** through **JR-116**) were synthesized and screened according to General Reaction 2 above. These compounds were structurally similar to the previously described imidazole derivatives, except that instead of a 2-methylene bridge connecting the \(\omega\)-nitrogen atoms of the guanidine groups, there exists a 3-methylene bridge. Each of the previously examined combinations of moiety A and moiety B from figure 1 which had adjoined imidazole and guanidine groups were synthesized with this new moiety C. The compounds in this series with the aliphatic chains at moiety B possessed behavior similar to those with the imidazole substituents. The 1,3-diaminopropane derivative, **JR-113**, was the most potent of the three, reducing \(^{3}\text{H}\text{MK-801}\) binding by 71.8% at 1 mM and 12.7% at 50 μM. The 1,4-diaminobutane and 1,5-diaminopentane derivatives, **JR-114** and **JR-115** respectively, carry only slight activity at 1 mM with 26.8% and 28.5% radioligand reduction, and negligible reduction at 50 μM. While none of the ring-containing 1,4,5,6-tetrahydropyrimidine compounds demonstrated activity at 50 μM, both of the
xylenediamine derivatives showed significant inhibitory ability at 1 mM. **JR-107** (4-aminomethyl-benzyl-(1,4,5,6-tetrahydro-pyrimidin-2-yl)-amine hydriodide), the *para*-compound, was able to reduce the binding of \[^{3}H\]MK-801 by nearly 80% at 1 mM, while **JR-108** (3-aminomethyl-benzyl-(1,4,5,6-tetrahydro-pyrimidin-2-yl)-amine hydriodide), the *meta*-compound, reduced binding by nearly 50% at the same concentration. As with the previous series, the bis-compound of *p*-xylenediamine was synthesized, and demonstrated decent inhibitory activity at 1 mM. The compound, \(N,N\)-bis(1,4,5,6-tetrahydro-pyrimidin-2-yl)-1,4-bis(aminomethyl)benzene diiodide (JR-106), reduced radioligand binding at the high concentration by 60.5%, and along with JR-113, JR-106, and JR-108 was judged to be amply effective to be further examined in the next step.

The next series of compounds is described in synthetic General Reaction 3, the *N*-ethyl-guanidine compounds. Two clean compounds were produced with this general reaction, using *meta* - and *para*-xylenediamine as the groups for moiety A and B of figure 1. These two compounds, **JR-126** (\(N\)-[4-(aminomethyl)-benzyl]-*N*-’-ethyl-guanidine hydriodide) and **JR-127** (\(N\)-[3-(aminomethyl)-benzyl]-*N*-’-ethyl-guanidine hydriodide), were quite effective at both concentrations. The former reduced the amount of radioligand bound to the P2 membrane by a hearty 98.8% at 1 mM and 40.8% at 50 \(\mu\)M, while the latter was responsible for reducing \[^{3}H\]MK-801 binding by a 96.3% at 1 mM and 27.6% at 50 \(\mu\)M. These compounds each possess similar activity to their guanylated counterparts, **JR-83** and **JR-84**. Additionally, these two compounds are clear candidates for further examination.
The following several series of compounds deviate from the first three synthetic general reactions, and involve the formation of Schiff bases from substituted benzylamines and aminoguanidine congeners, as seen in General Reactions 4-7 of the chemical synthesis section above. A set of the arylidenamines demonstrated in General Reaction 4 were synthesized by Dr. Sundar Neelakantan (unpublished work in the laboratory of Dr. Peter Crooks), and were shown to be active in assays performed by Robert Holley in the laboratory of Dr. John Littleton. Such activity opened another door in the synthesis of aromatic agmatine analogs, with a facile reaction which was amenable to parallel synthesis, and yielded pure products, often crystalline. These arylidenaminoguanidino compounds are further examined in Chapter 3 of this work.

A primary series of nine compounds were prepared by reaction of various nitrogen-containing or nitrogen-substituted arylaldehydes with aminoguanidine hydrochloride to form arylidenamines. These included the para- and meta-nitro compounds (JR-121 and JR-122), the meta- and para-cyano compounds (JR-124 and JR-125), the 1,4-bis-compound (JR-132), the 4-pyridyl and 3-pyridyl compounds (JR-146 and JR-147), and the para-amino and meta-amino compounds (JR-160 and JR-197). Each of these nine compounds possessed significant antagonist activity at the NMDAR channels at 1 mM, substantiating the data previously obtained about the arylidenamines. Each of these compounds was thus appropriated for further examination. The most active of these compounds according to this assay were, with % reduction of \([^{3}H]MK-801\) binding at 1 mM and 50 μM: (3-nitrobenzylidenamino)-guanidine hydrochloride (JR-122) with 98.9% and 8.4%, (3-cyanobenzylidenamino)-guanidine hydrochloride (JR-124) with 98.4% and -5.9%, (4-nitrobenzylidenamino)-guanidine hydrochloride (JR-121) with 94.2% and 21.5%, (4-cyanobenzylidenamino)-guanidine hydrochloride (JR-123) with 97.3% and 9.8%.
hydrochloride (JR-125) with 93.1% and 11.6%, (4-aminobenzylidenamino)-guanidine hydrochloride (JR-160) with 92.3% and -3.6%, p-bis-benzylidenamino-guanidine hydrochloride (JR-132) with 90.6% and 46.7%, and (3-aminobenzylidenamino)-guanidine hydrochloride (JR-197) with 71.1% and -16.6%. While neither of the pyridyl compounds appeared to be extremely useful as an NMDA antagonist, they did possess some activity and were deemed to be worth further examination due to the promise of this group as a whole. To reiterate, these compounds are discussed further in Chapter 3.

The success of the arylidenamino compounds begat the notion that modification of the aminoguanidine molecule could bring about an increase in potency of these compounds. The first of these modifications was in fact the biggest deviation from aminoguanidine, by using 1,1-dimethylhydrazine in its place in order to produce aromatic agmatine analogs without terminal amines in moiety C (see Figure 2.1). This reaction is described in General Reaction 5 above. Unfortunately, the nature of these compounds is such that the guanidine congener group is not charged in water, making them rather water insoluble. Even at the maximal allowed amount of DMSO in the test solution, many of the synthesized products in this set were unable to be tested in the P2 membrane MK-801 displacement assay. Those compounds that were sufficiently soluble for testing primarily included those with hydroxy or methoxy substituents, or nitrogen-containing compounds such as amines and pyridines. However, none of these compounds demonstrated any significant activity in the screen, even at 1 mM. As the para-amino compound (JR-159, N’-(4-amino-benzylidene)-N,N-dimethyl-hydrazone) as well as the 4-pyridyl and 3-pyridyl compounds (JR-150, pyridine-4-carbaldeyde-dimethylhydrazone, and JR-151, pyridine-3-carbaldeyde-dimethylhydrazone, respectively) shared the same Moiety A and Moiety B as arylidenamines which had been previously prepared and found to be active (JR-160, JR-146, and JR-147), it is easily deductible that the dimethylhydrazone analogs are not active and there was thus no reason to further pursue them.
A second attempt to examine aminoguanidine derivative mimetics involved the replacement of Moiety C₁ in order to form semicarbazine compounds, with a terminal urea group. As the pKa of urea is 0.10, these compounds share the same solubility problems as the dimethylhydrazine compounds, in that their solubility relies on the groups found in Moieties A and B, and is not assisted by a charged group at Moiety C. The synthesis of these compounds is detailed in reaction General Reaction 6 above. Again, the para-amino compound (JR-195, 4-amino-benzaldehyde semicarbazone) and the 4-pyridyl and 3-pyridyl compounds (JR-148, pyridine-4-carbaldehyde semicarbazone, and JR-149, pyridine-3-carbaldehyde semicarbazone, respectively) were synthesized and thus were able to be directly compared to the corresponding arylidenamines; and again they were found wanting. None of these three compounds possessed any activity in the [³H]MK-801 displacement assay, at any concentration. However, the meta-amino compound, 3-amino-benzaldehyde semicarbazone (JR-199), was able to reduce radioligand binding to the NMDAR complex by 23.7% at 1 mM. While this result is hardly indicative of a possible lead compound, it is interesting to note that there is a difference between the 3-amino and 4-amino semicarbazine compounds (JR-195 and JR-199).
The third and final attempt to improve upon the arylidenamines was a similar substitution as the one above, but substituting a sulfur atom at Moiety C₁ instead of an oxygen atom, in order to form a terminal thiourea, as detailed in General Reaction 7. As could be expected, however, these thiosemicarbazine compounds shared the same solubility issues and were, in fact, less water-soluble than the semicabazine compounds. Only four of these compounds were able to be tested, and even then, their complete solubility was in doubt. These compounds consisted of hydroxyl-substituted derivatives (JR-185 and JR-187), the \textit{para} -aldehyde (JR-194) which precipitated from the methanolic reaction solution before it was able to form the bis-compound, and the \textit{meta} -amino compound (JR-200). None of these compounds demonstrated any significant activity, and every compound save JR-185 (2,4-dihydroxy-benzaldehyde thiosemicarbazone), which reduced $[^3\text{H}]\text{MK-801}$ binding by 14.9\% at 1 mM, potentiated radioligand binding by at least 10\%.

2.4.3 \textit{Full Dose-Response Curve Examination}

The twenty-four compounds which have been indicated so far through this discussion as worthy of further examination underwent further screening at seven concentrations ranging from 16 mM to 1.0 $\mu$M, as described in Section 2.3.2 above. These compounds were chosen based primarily on their activity in the screens, but also on their structure and structural similarity to other active compounds.

A solution of each compound was prepared freshly in order to ensure accurate readings. Each compound was then tested at seven concentrations which were chosen based on the screening assay, so that the IC$\text{_{50}}$ was estimated to be as close to the middle
of the concentration range as possible. The compounds were then each screened in the presence of 100 μM spermidine in order to closely replicate the commonly-seen pathological levels of the long-chain polyamine, and again in the absence of spermidine, in order to more accurately represent its physiological levels. While the actual physiological levels of spermidine are closer to 10 μM, the freshly-prepared and frozen P2 rat brain membrane should have normal levels of spermidine and spermine still bound to the NMDA receptor complex, as is the case for glycine and L-glutamate.

These two separate sets of screening data will be used to establish the difference in activity mediated by the compound under pathological and physiological conditions, at least in the case of conditions which are caused by excessive amounts of long-chain polyamines, or those which induce the release of neurotoxic levels of these polyamines.

Previous work performed in this laboratory (Gibson et al., 2002) demonstrated that agmatine demonstrates the ability to reduce spermidine-potentiated [³H]MK-801 binding in this assay down to a level which corresponded to the baseline levels of bound [³H]MK-801 in the absence of spermidine, and that the further reduction of bound radioligand below this non-potentiated level appears to proceed at a similar rate in both conditions. This data indicates that agmatine, while not a potent complete inhibitor of NMDAR channel opening (like MK-801 or memantine), acts as a selective inhibitor of over-NMDAR complex overactivation.

While a goal of this study is to seek out structures, or structural motifs, which modulate NMDAR complex activity at one of the polyamine sites instead of directly in the channel pore, it is certainly also a goal to attempt to optimize the intriguing effects of agmatine: attenuation of the potentiating effects of long-chain polyamines on the NMDAR without affinity effects on channel activity at physiological levels of these polyamines.

When performed in this assay with the same procedure as was followed for the tested compounds, agmatine had an IC₅₀-value of 902 μM from the spermidine-potentiated curve, and greater than 1 mM in the absence of added spermidine (see Figure 2.5). It was impossible to obtain an accurate value for the latter value, as the threshold of agmatine solubility in the buffer used was met at approximately 6 mM. These numbers are somewhat higher than previously reported values, but all assays differ slightly, and
the important factor to note is that the “Agmatine + SPD” curve is essentially on top of the “Agmatine” curve at and above 1 mM. While it is assumed that both curves would proceed together to the non-specific control levels, the solubility threshold of agmatine was met, as previously mentioned.

![Agmatine dose-response curves](image)

**Figure 2.5 Agmatine dose-response curves in the presence and absence of added spermidine (100 μM)**

The focus of this study was to find a more potent compound than agmatine, while maintaining the agmatine activity profile, and thus a comparison of the IC$_{50}$-values obtained from neither the spermidine-potentiated dose-response curve nor that without spermidine potentiation would accurately indicate the activity which leads to the desired response, which is that point at which the curves meet. Therefore, a value must be derived from the spermidine-potentiated curve to attest to the ability of a compound to reduce $[^3]$HMK-801 binding from the spermidine-potentiated control to the non-potentiated control.

Thus, in conjunction with the non-potentiated dose-response curve, an “IC$_{50}^{pot}$” value was taken from the spermidine-potentiated curve which corresponds to the concentration at which 50% of the $[^3]$HMK-801 bound solely due to spermidine-
potentiation is disallowed. In other words, the IC$_{50}^{\text{pot}}$ value is the point on the spermidine-potentiated curve which corresponds to the average of the spermidine-potentiated and non-potentiated controls. For example, in the case of agmatine in Figure 2.4, this would be 274 μM, which is the concentration corresponding to 940 DPM, the average of the spermidine-potentiated control (1074 DPM) and non-potentiated control (805 DPM). This value is directly taken from the curve using GraphPad Prism, which offers the ability to view the points on the curves in a database format, thereby granting the ability to garner an X-value (concentration) from a Y-value (DPM) for any curve.

Each of the IC$_{50}$-values from the two dose-response curves as well as the derived IC$_{50}^{\text{pot}}$ are divulged in Table 2.2 of Section 2.3.2 above, and the set of curves for each of the tested compounds are displayed with the individual compound synthetic details in Section 2.6 below.

In addition to the potency gleaned from the IC$_{50}$-values, the derived curves can also be utilized together to learn more about the possible binding sites of the compounds tested, or at least which sites are not occupied by these molecules.

Primarily, a direct channel inhibitor, one binding in the pore itself, should have the same IC$_{50}$-value in the presence and the absence of spermidine. As the long-chain polyamines are known to bind at a site separate from the channel domain, they only serve to increase channel opening frequency. This effect will result in an increase in the amount of channel inhibitor which binds, but should not modify either the rate of binding or the concentration necessary to inhibit the channel; these parameters are reflected in the dose-response curves as the slope and IC$_{50}$ values. Therefore, these two values will be the same regardless of spermidine concentrating in dose-response curves generated by compounds such as non-radiolabeled MK-801 (see Figure 2.6) which bind directly to the channel pore. While it is indeed possible that a compound could have the same slope and IC$_{50}$ values in both situations by binding at a separate allosteric site, a significant difference in these values between curves is indicative of binding away from the channel.
Figure 2.6 Dose-response curves for cold MK-801 in the presence and absence of 100 μM spermidine (IC$_{50}$ = 5.58 ± 0.07 nM with SPD, 5.92 ± 0.11 nM without SPD; hill slope = -1.09 ± 0.07 with SPD, -0.96 ± 0.11 without SPD)

In addition, the ratio of the two IC$_{50}$-values discloses information about the possibility of the tested compounds binding at the same polyamine recognition site at which spermidine acts. If the compound being tested is acting as an antagonist, or inverse agonist, at the same position where spermidine is acting as an agonist, these two molecules will be competitive. According to the Cheng-Prusoff equation, the IC$_{50}$ of a competitive inhibitor should increase linearly with the concentration of the competitive agonist (Berger et al., 1998; Cheng and Prusoff, 1973):

$$IC_{50,[agonist]} = IC_{50,[agonist]} = 0^* (1 + X/EC_{50})$$

The EC$_{50}$ of spermidine in this assay has been examined at different time points, as the equilibrium is slowly achieved between spermidine and its polyamine recognition site. At 45 minutes, this value was roughly 6.3 μM. For the purposes of each assay performed in this study, this corresponds to the 40-45 minute incubation time between the addition
of the membrane, polyamine, and tested compound, and the addition of [\(^3\)H]MK-801. As 100 \(\mu\)M of spermidine was used in the generation of the SPD-potentiated curve, the two IC\(_{50}\)-values should follow this equation, if they are indeed competitive at the same site:

\[
\text{IC}_{50}, [\text{spermidine}] = 100 \, \mu\text{M} = \text{IC}_{50}, [\text{spermidine}] = 0 \times (1 + 100 \, \mu\text{M}/6.3 \, \mu\text{M})
\]

\[
\text{IC}_{50}, [\text{spermidine}] = 100 \, \mu\text{M} = \text{IC}_{50}, [\text{spermidine}] = 0 \times (16.9)
\]

Therefore, competition of these compounds at the same polyamine recognition site would be indicated by a 17-fold increase in the IC\(_{50}\) from the non-potentiated to the potentiated curve, which would be accompanied by no change in the slope of the curve, simply a shift of the curve to the right.

While a number of the tested agmatine analogs had a ratio of IC\(_{50}\) with spermidine to IC\(_{50}\) without spermidine of close to 1 (which would not eliminate the possibility of channel site binding), none of the ratios were nearly as high as 17, which indicates that none of the compounds act competitively at the same site as spermidine.

In comparing the values generated for each of these compounds, a number of factors are of importance towards dictating their potency as well as their selectivity. In addition to the ratios of the two curve-generated IC\(_{50}\)-values, it is also useful to compare the IC\(_{50}\)\(_{\text{pot}}\) to the IC\(_{50}\) without spermidine. This ratio assumes that the compounds bind in separate sites, activity that is indicated by the curves displayed by agmatine. Agmatine has a ratio for IC\(_{50}\) without spermidine to IC\(_{50}\) of potentiation (ratio of potentiation, or Ratio\(_{\text{pot}}\)) of nearly 6:1, whereas compounds such as MK-801 have ratios on the range of 2-3:1. Higher ratios are therefore indicative of greater separation between polyamine site binding and channel binding, or a shallow slope, which also indicates multi-site binding.

The five guanidine compounds which were evaluated at the full concentration range, as mentioned above, were JR-3, JR-5, JR-83, JR-84, and AH-8. All of these were aromatic compounds, and all contained another amine; a primary amine in the case of the first four, and a secondary amine of an indole ring in the latter. While the two earliest leads, JR-3 and JR-5, showed activity in the early screens, they did not prove to be extremely potent when tested across the entire range of concentrations. The former possessed an IC\(_{50}\)\(_{\text{pot}}\) of 364.8 \(\mu\)M, with the two other values being roughly equal. The
latter was roughly twice as potent, with a 162.0 μM IC₅₀, again with roughly equal curve-derived IC₅₀-values. The two xylenediamine compounds bettered these by a full order of magnitude. The para-compound, JR-83, was observed to have an IC₅₀ of 27.5 μM; however, the IC₅₀ value for this compound in the presence of spermidine was nearly twice that of the value in its absence, and the Ratioᵢₚ was less than 2:1. The meta-compound, JR-84, was nearly twice as potent with a 15.5 μM IC₅₀. In addition, the Ratioᵢₚ was greater than 3:1. This compound had a shorter inter-atomic distance between the amine group and the guanidine group, making it more structurally similar to agmatine, which may explain the increase in selectivity over JR-83. The final guanidine compound examined, AH-8, while quite potent with a 15.7 μM IC₅₀, did not display any of the signs of selectivity expected of an agmatine analog. The ratio of IC₅₀ values for this compound in the presence to absence of spermidine was greater than 2:1, and the Ratioᵢₚ was less than 1:1.

The imidazole derivates, or ethyl-bridged guanidine compounds, were generally less potent than their unsubstituted guanidine counterparts. The two examined compounds in this set with the same moiety A and B (see Figure 2.1) as guanylated compounds were JR-93 and JR-120, which corresponded to JR-5 and JR-84, respectively. Neither of these compounds had remarkable potency, with JR-93 being less than half as potent as JR-5 and JR-120 having an IC₅₀ nearly 30 times as high as that of JR-84. While the aromatic 1,4,5,6-tetrahydropyrimidine derivatives, or propyl-bridged guanidine compounds, appeared to be more potent than the similar imidazole derivatives, they also displayed less activity than their guanylated counterparts. JR-107 and JR-108, the para- and meta-xylenediamine compounds, with IC₅₀-values of 52.7 μM and 97.3 μM, respectively, were less potent than their guanylated isomers, JR-83 and JR-84. Interestingly, it was the para-xylene 1,4,5,6-tetrahydropyrimidine compound which was nearly twice as potent as the meta-compound, while the meta-xylene guanylated compound was twice as active as the para-compound. However, the meta-xylene compound demonstrated more selectivity in both cases; while JR-107 had a Ratioᵢₚ of nearly 4:1, JR-108 yielded the same ratio at over 13:1. This observation reinforces the hypothesis that the shorter direct chain more closely mimics the selectivity
of agmatine, but in this case, the aromatic compound displayed greater selectivity than that of agmatine.

\(N^\omega-(4,5\text{-Dihydro-1}\text{-H-imidazol-2-yl})\text{-propane-1,3-diamine hydriodide (JR-89),}\) the imidazole derivative of agmatine with a propyl instead of butyl chain, demonstrated very good potency and selectivity, with a 12.4 \(\mu M\) IC\(_{50\text{pot}}\) and a Ratio\(_{pot}\) greater than 8.5:1. The 1,4,5,6-tetrahydropyrimidyl compound with the same propane bridge, JR-113, was also very promising, with its IC\(_{50\text{pot}}\) of 20.4 \(\mu M\) and Ratio\(_{pot}\) of 7.8:1.

Two symmetrical di-substituted bridged-guanylated compounds were tested, both with a para-xylene linker, JR-106 and JR-117. The bis-imidazole compound, JR-117, and the bis-1,4,5,6-tetrahydropyrimidyl compound, JR-106, demonstrated 104.7 \(\mu M\) and 254.7 \(\mu M\) IC\(_{50\text{pot}}\)-values, respectively. Neither of these values is remarkable, but these bis-compounds yielded very similar sets of dose-response curves: the hill slopes of the spermidine-potentiated curves are roughly twice as steep as the hill slopes of the curves in the absence of spermidine. As a result, the Ratio\(_{pot}\) for each compound is close to 1:1. These data indicate that neither of these compounds binds to the same polyamine site as agmatine, although the significance difference in hill slope indicates that neither act as inhibitors of the channel site. It is thus postulated that these bis-compounds bind at another polyamine recognition site, and act allosterically as more potent inhibitors in the absence of spermidine than in its presence.

The final two compounds to be tested with these sets of dose-response curves were the \(N^\omega\)-ethyl guanylated para- and meta-xylene-diamines, JR-126 and JR-127. The latter compound was unremarkable, with an IC\(_{50\text{pot}}\) of 75.3 \(\mu M\) and a Ratio\(_{pot}\) of 2:1. The former compound, \(N\)-[4-(aminomethyl)-benzyl]-\(N'\)-ethyl-guanidine hydriodide, had an IC\(_{50\text{pot}}\) of 25.8 \(\mu M\), but acted much like the bis-compounds in terms of selectivity. The ratio of hill slopes for the spermidine-potentiated curve to the non-potentiated curve was 2.3:1, and the Ratio\(_{pot}\) was 1:1.6. It is interesting that these compounds act in such different manners, while being very similar structurally. However, each of the mono-substituted meta-xylene compounds demonstrated greater selectivity for the polyamine site recognizing agmatine than their corresponding mono-substituted para-xylene compounds, an observation which holds true in this case as well.
The remaining compounds, the arylidenamino-guanidines, will be discussed in chapter 3 of this work, as they are part of a larger set of very similar compounds, both in structure and activity.

2.5 Conclusions

This study was undertaken to develop and evaluate agmatine analogs as inhibitors of the NMDA receptor complex polyamine recognition sites in order to develop leads and better understand the structure-activity relationship associated with the selectivity demonstrated by agmatine.

A library of nearly 200 compounds were designed and synthesized, and those amenable to the P2 membrane protein $[^3]$H]MK-801 displacement assay were screened for their ability to allosterically or directly modulate the opening of the NMDA receptor channel. The most active compounds were then re-examined at a comprehensive range of concentrations in the presence and absence of a pathological level of the polyamine spermidine, in order to evaluate their potency as well as selectivity for the NMDAR site which recognizes agmatine.

A majority of the potent compounds contained aromatic rings between a primary amine and a terminal guanidine congener. It was observed that the most potent connector groups were the xylenediamine moieties, with the meta-diamine demonstrating more selectivity than the para-diamine. These included $N$-(3-amino-methyl-benzyl)-guanidine dihydrochloride (JR-84), which had a 12.4 μM IC$_{50}^{pot}$ and greater than 3:1 Ratio$_{pot}$. Other aromatic groups also showed promise as linkers, such as $N$-(1H-indol-5-yl)guanidine hydrochloride (AH-8), which possessed a 15.7 μM IC$_{50}^{pot}$, but apparently little selectivity for the agmatine site.

There was also a pair of agmatine analogs with shorter aliphatic chains connecting the terminal amine to an ethyl- or propyl-bridged guanidine group that demonstrated both potency and selectivity for the agmatine site. $N'$-(4,5-dihydro-1H-imidazol-2-yl)-propane-1,3-diamine hydriodide (JR-89) and $N'$-(1,4,5,6-tetrahydro-pyrimidin-2-yl)-aminopropyl-3-amine hydriodide (JR-113) had IC$_{50}^{pot}$ values of 12.4 μM and 20.4 μM,
respectively, with ratios of potentiation of roughly 8:1 for each. These compounds demonstrate potency of a full order of magnitude better than agmatine, and demonstrate slightly greater selectivity.

A set of arylidenamino-guanidine compounds were all active in the \[^{3}\text{H}]\text{MK-801}\) displacement screenings, and these inhibitors are further examined for structure-activity relationship to potency and selectivity of NMDA receptor inhibition in the following chapter.

A number of compounds and structural motifs were examined in this study, and several leads were developed for further in vivo testing, as well as others which will be further optimized for therapeutic value as inhibitory modulators of the NMDA receptor complex.

### 2.6 Experimental Protocol

The following includes the reaction details of each of the screened compounds mentioned above in Table 1.1, including structures and analytical data. Each description refers to one of the seven general reactions from Section 2.2. In addition, the full dose-response curves are given for those compounds which were determined to be hits, and which were then subjected to this further pharmacological analysis. Again, it should also be noted that while the counts are reported as raw DPM instead of DPM/mg protein on the graphs below, a constant level of protein was maintained in each well across all experiments (0.15 mg/well).
N-(4-Amino-benzyl)-guanidine Dihydrochloride

p-Aminobenzylamine (567 μL, 611 mg, 5.0 mmol) and \(N,N'\)-bis(tert-butoxy)-S-methylisothiopseudourea (1.45 g, 5.0 mmol) were dissolved in tetrahydrofuran (10 mL) and reacted according to the above General Reaction 1. The final product was dissolved in water and lyophilized to yield a yellow solid product (998 mg, 4.21 mmol, 84% yield). Mp: 207-209°C; \(^1\)H NMR (300MHz, d-D_{2}O) \(\delta\) 7.44 (4H, m), 4.48 (2H, s); \(^{13}\)C NMR (300MHz, d-DMSO) \(\delta\) 157.0, 131.6, 130.2, 128.3, 122.9, 43.3; ES+ m/z 165.
p-Aminobenzylamine was reacted with di-tert-butyl dicarbonate in a 1:1 stoichiometric ratio in order to obtain (4-amino-benzyl)-carbamic acid tert-butyl ester in nearly quantitative yield. This ester product (1.11 g, 5.0 mmol) and \(N,N'\)-bis(tert-butoxy)-S-methylisothiopseudourea (1.45 g, 5.0 mmol) were dissolved in tetrahydrofuran (10 mL) and reacted according to the above General Reaction 1. The final product was dissolved in water and lyophilized to yield an off-white solid product (713 mg, 3.01 mmol, 60% yield). \(^1\)H NMR (300MHz, d-D\(_2\)O) \(\delta\) 7.5 (4H, m), 4.22 (2H, s).

N-(2-Amino-benzyl)-guanidine Dihydrochloride

o-Aminobenzylamine (611 mg, 5.0 mmol) and \(N,N'\)-bis(tert-butoxy)-S-methylisothiopseudourea (1.45 g, 5.0 mmol) were dissolved in tetrahydrofuran (10 mL) and reacted according to the above General Reaction 1. The final product was dissolved in water and lyophilized to yield a dark yellow solid product (566 mg, 2.39 mmol, 48% yield). Mp: 207-209°C; \(^1\)H NMR (300MHz, d-D\(_2\)O) \(\delta\) 7.51 (4H, m), 4.53 (2H, s); \(^{13}\)C NMR (300MHz, d-DMSO) \(\delta\) 156.9, 133.0, 129.3, 128.4, 127.4, 125.8, 122.1, 40.3; ES+ m/z 165.
4-Aminophenethyl alcohol (686 mg, 5.0 mmol) and \(N,N'\)-bis(tert-butoxy)-S-methylisothiopseudourea (1.45 g, 5.0 mmol) were dissolved in tetrahydrofuran (10 mL) and reacted according to the above General Reaction 1. The final product was dissolved in water and lyophilized to yield a tan-yellow solid powder product (388 mg, 1.80 mmol, 36% yield). \(^1\)H NMR (300MHz, d-D\(_2\)O) δ 7.36 (2H, d, J = 9Hz), 7.21 (2H, d, J = 9Hz), 3.83 (2H, t, J = 6.6Hz), 2.86 (2H, t, J = 6.6Hz)
N-(4-Bromo-phenyl)-guanidine Hydrochloride

4-Bromoaniline (860 mg, 5.0 mmol) and \(N,N'\)-bis(tert-butoxy)-S-methylisothiopseudourea (1.45 g, 5.0 mmol) were dissolved in tetrahydrofuran (10 mL) and reacted according to the above General Reaction 1. The final product was dissolved in water and lyophilized to yield a yellow solid product (210 mg, 0.84 mmol, 17% yield). \(^1\)H NMR (300MHz, d-D\(_2\)O) \(\delta\) 7.63 (2H, d, \(J = 9\)Hz), 7.21 (2H, d, \(J = 9\)Hz)

N-(5-Amino-pentyl)-guanidine Dihydrochloride

1,5-Diaminopentane was reacted with di-tert-butyl dicarbonate in a 1:1 stoichiometric ratio in order to obtain (5-amino-pentyl)-carbamic acid tert-butyl ester in nearly quantitative yield. This ester product (303 mg, 1.5 mmol) and \(N,N'\)-bis(tert-butoxy)-S-methylisothiopseudourea (480 mg, 1.75 mmol) were dissolved in tetrahydrofuran (5 mL) and reacted according to the above General Reaction 1. The final product was dissolved in water and lyophilized to yield a yellow semi-solid product (241 mg, 1.11 mmol, 74% yield). \(^1\)H NMR (300MHz, d-D\(_2\)O) \(\delta\) 3.21 (2H, t), 3.02 (2H, t), 1.71 (4H, m), 1.46 (2H, m)
p-Xylenediamine (163 mg, 1.2 mmol) and 2-methyl-2-thiopseudourea sulfate (111 mg, 0.4 mmol) were dissolved in methanol (5 mL) and reacted according to the alternate General Reaction 1 described above. Upon cooling to room temperature, the final product slowly crashed from solution as a white crystal product (82 mg, 0.36 mmol, 90% yield). Mp: > 300°C; $^1$H NMR (300MHz, d-D$_2$O and d-DMSO) $\delta$ 7.50 (2H, d, J = 8.4 Hz), 7.44 (2H, d, J = 8.4 Hz), 4.47 (2H, s), 4.17 (2H, s); ES$^+$ m/z 179.
m-Xylenediamine (158 μL, 163 mg, 1.2 mmol) and 2-methyl-2-thiopseudourea sulfate (111 mg, 0.4 mmol) were dissolved in methanol (5 mL) and reacted according to the alternate General Reaction 1 described above. Upon cooling to room temperature, the final product slowly crashed from solution as an off-white crystal product (59 mg, 0.26 mmol, 65% yield). Mp: > 300°C; \(^1\)H NMR (300MHz, d-D\(_2\)O) δ 7.44 (4H, m), 4.45 (2H, s), 4.19 (2H, s); \(^1\)\(^3\)C NMR δ 158.2, 138.6, 125.0, 131.1, 129.7, 129.1, 128.6, 127.9, 45.7, 44.4; ES+ m/z 179.
1,3-Diaminopropane (250 μL, 3.0 mmol) and 2-methylsulfanyl-4,5-dihydro-1H-imidazole hydriodide (610 mg, 2.5 mmol) were dissolved in methanol (15 mL) and reacted according to the above General Reaction 2. The product was evaporated to dryness on a rotary evaporator and then recrystallized from chloroform to yield white plate crystals (188 mg, 0.70 mmol, 29% yield). Mp: 136-138°C; $^1$H NMR (300MHz, d-DMSO) δ 3.23 (4H, m), 3.03 (2H, t, J = 6Hz), 2.64 (2H, t, J = 6Hz), 1.79 (2H, p, J = 6Hz); $^{13}$C NMR δ 153.0, 43.7, 40.8, 38.0, 19.8; ES+ m/z 143.
1,4-Diaminobutane (302 μL, 3.0 mmol) and 2-methylsulfanyl-4,5-dihydro-1H-imidazole hydriodide (610 mg, 2.5 mmol) were dissolved in methanol (15 mL) and reacted according to the above General Reaction 2. The product did not crystallize upon cooling, but was evaporated to dryness on a rotary evaporator and rinsed with chloroform to remove excess diamine starting material, yielding an off-white semi-solid (322 mg, 1.13 mmol, 45% yield). $^1$H NMR (300MHz, d-DMSO) δ 3.68 (4H, s), 3.22 (2H, m), 2.79 (2H, m), 1.60 (4H, m)

1,5-Diaminopentane (351 μL, 3.0 mmol) and 2-methylsulfanyl-4,5-dihydro-1H-imidazole hydriodide (610 mg, 2.5 mmol) were dissolved in methanol (15 mL) and reacted according to the above General Reaction 2. The product did not crystallize upon cooling, but was evaporated to dryness on a rotary evaporator and rinsed with chloroform to remove excess diamine starting material, yielding a dark orange liquid product (362 mg, 1.21 mmol, 49% yield). $^1$H NMR (300MHz, d-DMSO) δ 3.57 (4H, s), 3.10 (2H, m), 2.62 (2H, m), 1.4 (6H, m)
**JR-92**

\[ \text{N'}-(4,5\text{-Dihydro-1H-imidazol-2-yl})\text{-benzylamino-4-amine Hydriodide} \]

4-Aminobenzylamine (340 μL, 3.0 mmol) and 2-methylsulfanyl-4,5-dihydro-1H-imidazole hydriodide (610 mg, 2.5 mmol) were dissolved in methanol (15 mL) and reacted according to the above General Reaction 2. Upon cooling to room temperature, the product precipitated as orange crystals (335 mg, 1.05 mmol, 42% yield). Mp: 201-203°C; \(^1\)H NMR (300MHz, d-DMSO) δ 8.45 (1H, broad t), 6.97 (2H, d, J = 9Hz), 6.56 (2H, d, J = 9Hz), 5.12 (2H, broad s), 4.14 (2H, d, J = 6Hz), 3.59 (4H, s); \(^13\)C NMR δ 147.6, 127.9, 126.15, 113.6, 112.0, 46.4, 38.1.

**JR-93**

\[ \text{N'}-(4,5\text{-Dihydro-1H-imidazol-2-yl})\text{-benzylamino-2-amine Hydriodide} \]

4-Aminobenzylamine (366 mg, 3.0 mmol) and 2-methylsulfanyl-4,5-dihydro-1H-imidazole hydriodide (610 mg, 2.5 mmol) were dissolved in methanol (15 mL) and reacted according to the above General Reaction 2. Upon cooling to room temperature, the product precipitated as dull orange crystals (196 mg, 0.616 mmol, 25% yield). Mp: 195-197°C; \(^1\)H NMR (300MHz, d-DMSO) δ 8.37 (1H, broad t), 7.03 (1H, t, J = 7.2Hz), 7.01 (1H, d, J = 7.2Hz), 6.68 (1H, dt, J = 7.2, 1.5Hz), 6.56 (1H, td, J = 7.2Hz, 1.5Hz), 5.11 (2H, broad s), 4.18 (2H, d, J = 6Hz), 3.60 (4H, s); \(^13\)C NMR δ 159.2, 146.0, 128.4, 128.2, 119.2, 115.9, 115.0, 42.62, 42.56; ES+ m/z 191.
1,3-Phenyldiamine (324 mg, 3.0 mmol) and \(N,N'\)-bis(tert-butoxy)-S-methylisothiopseudourea (1.45 g, 5.0 mmol) were dissolved in tetrahydrofuran (10 mL) and reacted according to the above General Reaction 1. The final product was dried on a rotary evaporator to yield a clear orange liquid product (88 mg, 0.39 mmol, 13% yield). 

\(^1\)H NMR (300MHz, d-DMSO) \(\delta\) 9.11 (2H, broad s), 7.2-7.7 (4H, m).
\[ N,N\text{-bis}(1,4,5,6\text{-tetrahydro-pyrimidin-2-yl})\text{-}1,4\text{-bis(aminomethyl)}\text{benzene Diiodide} \]

\[ p\text{-Xylenediamine (272 mg, 2.0 mmol) and 2-methylsulfanyl-1,4,5,6-tetrahydro-pyrimidine hydriodide (1.29 g, 5.0 mmol) were dissolved in ethanol and reacted according to the above General Reaction 2. The product was evaporated to dryness on a rotary evaporator and then recrystallized from chloroform to yield white needle crystals (112 mg, 0.20 mmol, 10\% yield). Mp: 266-267^\circ\text{C}; ^1\text{H NMR (300MHz, d-DMSO)} \delta 7.79 (4H, broad s), 7.66 (2H, broad s), 7.41 (1H, t, J = 1.5Hz), 7.25 (2H, d, J = 1.5Hz), 7.23 (1H, s), 4.34 (4H, d, J = 2.4Hz), 3.26 (8H, t, J = 3Hz), 1.82 (4H, p, J = 3Hz); ^13\text{C NMR} \delta 152.3, 137.5, 128.8, 126.2, 125.8, 43.5, 40.3, 19.7; \text{ES}^+ \text{ m/z 151 (MW 302, 2 charges).} \]
4-Aminomethyl-benzyl-(1,4,5,6-tetrahydro-pyrimidin-2-yl)-amine Hydriodide

*p*-Xylenediamine (681 mg, 5.0 mmol) and 2-methylsulfanyl-1,4,5,6-tetrahydropyrimidine hydriodide (1.29 g, 5.0 mmol) were dissolved in ethanol and reacted according to the above General Reaction 2. The resulting solution was evaporated to dryness on a rotary evaporator followed by a vacuum pump to yield a light yellow solid product (208 mg, 0.60 mmol, 12% yield). ¹H NMR (300MHz, d-D₂O) δ 7.42 (4H, m), 4.41 (2H, d), 4.04 (2H, d), 3.33 (4H, t), 1.92 (2H, p)
3-Aminomethyl-benzyl-(1,4,5,6-tetrahydro-pyrimidin-2-yl)-amine Hydriodide

$m$-Xylenediamine (660 μL, 5.0 mmol) and 2-methylsulfanyl-1,4,5,6-tetrahydro-pyrimidine hydriodide (1.29 g, 5.0 mmol) were dissolved in ethanol and reacted according to the above General Reaction 2. The resulting solution was evaporated to dryness on a rotary evaporator followed by a vacuum pump to yield a dark orange semi-solid (314 mg, 0.91 mmol, 18% yield). $^1$H NMR (300MHz, d-DMSO) δ 7.24 (4H, m), 4.32 (2H, m), 3.74 (2H, d), 3.24 (4H, t), 1.83 (2H, p); $^{13}$C NMR δ 155.1, 148.2, 128.5, 123.5, 113.8, 44.3, 39.9, 36.1, 14.4.
**JR-109**

![Chemical Structure](image1.png)

*N'-(1,4,5,6-Tetrahydro-pyrimidin-2-yl)-benzylamino-4-amine Hydriodide*

4-Aminobenzylamine (567 μL, 5.0 mmol) and 2-methylsulfanyl-1,4,5,6-tetrahydro-pyrimidine hydriodide (1.29 g, 5.0 mmol) were dissolved in ethanol and reacted according to the above General Reaction 2. The resulting solution was evaporated to dryness on a rotary evaporator followed by a vacuum pump to yield a bright orange solid powder product (528 mg, 1.59 mmol, 32% yield). $^1$H NMR (300MHz, d-DMSO) $\delta$ 7.63 (2H, broad s), 7.40 (1H, broad t), 6.96 (2H, d, J = 9 Hz), 6.47 (2H, d, J = 9 Hz), 5.09 (2H, s), 4.07 (2H, d), 3.22 (4H, t), 1.82 (2H, p).

**JR-113**

![Chemical Structure](image2.png)

*N'-(1,4,5,6-Tetrahydro-pyrimidin-2-yl)-aminopropyl-3-amine Hydriodide*

1,3-Diaminopropane (416 μL, 5.0 mmol) and 2-methylsulfanyl-1,4,5,6-tetrahydro-pyrimidine hydriodide (1.29 g, 5.0 mmol) were dissolved in ethanol and reacted according to the above General Reaction 2. The resulting solution was evaporated to dryness on a rotary evaporator followed by a vacuum pump to yield a clear, colorless liquid product (597 mg, 2.1 mmol, 42% yield). $^1$H NMR (300MHz, d-DMSO) $\delta$ 3.22 (4H, t, J = 5.7 Hz), 3.11 (2H, t, J = 6.3 Hz), 2.67 (2H, t, J = 6.9 Hz), 1.79 (2H, p, J = 5.7 Hz), 1.60 (2H, t, J = 6.7 Hz).
JR-114

\[ \text{N'}-(1,4,5,6-Tetrahydro-pyrimidin-2-yl)-aminobutyl-4-amine Hydriodide} \]

1,4-Diaminobutane (502 μL, 5.0 mmol) and 2-methylsulfanyl-1,4,5,6-tetrahydro-pyrimidine hydriodide (1.29 g, 5.0 mmol) were dissolved in ethanol and reacted according to the above General Reaction 2. The resulting solution was evaporated to dryness on a rotary evaporator followed by a vacuum pump to yield an orange liquid, from which crashed light orange needle crystals (270 mg, 0.91 mmol, 18% yield). \(^1\)H NMR (300MHz, d-DMSO) δ 3.23 (4H, t, J = 5.7 Hz), 3.11 (2H, t), 2.69 (2H, t), 1.80 (2H, p, J = 5.7 Hz), 1.47 (4H, broad m).
1,5-Diaminopentane (587 μL, 5.0 mmol) and 2-methylsulfanyl-1,4,5,6-tetrahydro-pyrimidine hydriodide (1.29 g, 5.0 mmol) were dissolved in ethanol and reacted according to the above General Reaction 2. The resulting solution was evaporated to dryness on a rotary evaporator followed by a vacuum pump to yield a dark orange viscous liquid, from which crashed orange needle crystals (620 mg, 1.99 mmol, 40% yield). $^1$H NMR (300MHz, d-DMSO) δ 3.23 (2H, t), 3.07 (4H, t, J = 5.7 Hz), 2.70 (2H, t), 1.67 (2H, p, J = 5.7 Hz), 1.46 (4H, broad m) 1.32 (2H, broad m).

4-Amino-1-butanol (461 μL, 5.0 mmol) and 2-methylsulfanyl-1,4,5,6-tetrahydro-pyrimidine hydriodide (1.29 g, 5.0 mmol) were dissolved in ethanol and reacted according to the above General Reaction 2. The resulting solution was evaporated to dryness on a rotary evaporator followed by a vacuum pump to yield a light orange semi-solid (320 mg, 1.07 mmol, 21% yield). $^1$H NMR (300MHz, d-DMSO) δ 3.40 (2H, t, J = 6.3 Hz), 3.11 (4H, t, J = 5.7 Hz), 2.77 (2H, t, J = 7.5 Hz), 1.67 (2H, p, J = 5.7 Hz), 1.45 (2H, t).
N,N-bis(3,4-Dihydro-1H-imidazol-2-yl)-1,4-bis(aminomethyl)benzene Dihydriodide

*p*-Xylenediamine (341 mg, 2.5 mmol) and 2-methylsulfanyl-4,5-dihydro-1H-imidazole hydriodide (2.0 g, 8.2 mmol) were dissolved in methanol (15 mL) and reacted according to the above General Reaction 2. The product was evaporated to dryness on a rotary evaporator to yield a tan solid product (296 mg, 0.89 mmol, 36% yield). $^1$H NMR (300MHz, d-DMSO) $\delta$ 7.31 (4H, s), 4.36 (4H, s), 3.60 (8H, s); $^{13}$C NMR $\delta$ 43.3, 45.8, 128.2, 137.1, 159.9; ES+ m/z 137 (MW 274, 2 charges).
$N'-(4,5\text{-Dihydro}-1H\text{-imidazol-2-yl})\text{-benzylamino-4-methylamine Hydriodide}$

$p\text{-Xylenediamine (341 mg, 2.5 mmol) and 2-methylsulfanyl-4,5\text{-dihydro-1H-imidazole hydriodide (610 mg, 2.5 mmol) were dissolved in methanol (15 mL) and reacted according to the above General Reaction 2. The product was evaporated to dryness on a rotary evaporator to yield a yellow semi-solid (236 mg, 0.71 mmol, 28% yield).}$
$N'$-(4,5-Dihydro-1H-imidazol-2-yl)-benzylamino-3-methylamine Hydriodide

$m$-Xylenediamine (330 $\mu$L, 2.5 mmol) and 2-methylsulfanyl-4,5-dihydro-1H-imidazole hydriodide (610 mg, 2.5 mmol) were dissolved in methanol (15 mL) and reacted according to the above General Reaction 2. The product was evaporated to dryness on a rotary evaporator to yield a tan semi-solid (292 mg, 0.88 mmol, 35% yield). $^1$H NMR (300MHz, d-DMSO) $\delta$ 9.98 (2H, broad s), 8.66 (1H, s), 7.2-7.5 (4H, m), 4.40 (2H, d), 3.86 (2H, s), 3.61 (4H, s).
(4-Nitrobenzylidenamino)-guanidine Hydrochloride

4-Nitrobenzaldehyde (1.13 g, 7.5 mmol) and aminoguanidine hydrochloride (553 mg, 5.0 mmol) were dissolved in methanol (50 mL) and reacted according to the above General Reaction 4. The product solution was dissolved on the rotary evaporator, and the residue was stirred in chloroform and filtered to remove the remaining starting material. The filtered product was dried on vacuum overnight to afford a light-yellow powdery solid (768 mg, 3.15 mmol, 63% yield). Mp: 252-254°C; $^1$H NMR (300MHz, d- D$_2$O) $\delta$ 8.23 (2H, m), 8.0 (1H, m), 7.81 (2H, m); $^{13}$C NMR $\delta$ 155.4, 147.9, 144.2, 139.6, 128.4, 123.7; ES+ m/z 208.
3-Nitrobenzaldehyde (1.13 g, 7.5 mmol) and aminoguanidine hydrochloride (553 mg, 5.0 mmol) were dissolved in methanol (50 mL) and reacted according to General Reaction 4 above. The product solution was dissolved on the rotary evaporator, and the residue was stirred in chloroform and filtered to remove the remaining starting material. The filtered product was dried on vacuum overnight to afford a pale yellow powdery solid (1109 mg, 4.55 mmol, 91% yield). Mp: 267-268°C; $^1$H NMR (300MHz, d-D$_2$O) δ 8.53 (1H, t, J = 2.0Hz), 8.28 (1H, ddd, J = 9.0, 2.0, 1.3Hz), 8.09 (1H, s), 8.03 (1H, td, J = 9.0, 1.3Hz); 7.66 (1H, t, J = 9.0Hz); $^{13}$C NMR δ 155.5, 148.2, 146.1, 134.7, 133.7, 130.1, 125.1, 121.8; ES+ m/z 208.
3-Cyanobenzaldehyde (491 mg, 3.75 mmol) and aminoguanidine hydrochloride (276.4 mg, 2.5 mmol) were dissolved in methanol (50 mL) and reacted according to the above General Reaction 4. The product solution was dissolved on the rotary evaporator, and the residue was stirred in chloroform and filtered to remove the remaining starting material. The filtered product was dried on vacuum overnight to afford an off-white powdery solid (257 mg, 1.15 mmol, 46% yield). Mp: 235-239ºC; \(^1\)H NMR (300MHz, d- D\(_2\)O) \(\delta\) 12.18 (1H, s), 8.48 (1H, t, J = 1.5Hz), 8.19 (1H, s), 8.12 (1H, dt, J = 8.3, 1.5Hz), 7.87 (1H, dt, J = 8.3, 1.5Hz), 7.64 (1H, t, J = 8.3Hz); \(^{13}\)C NMR \(\delta\) 155.4, 144.2, 134.7, 133.2, 132.3, 130.3, 129.8, 118.4, 111.8; ES+ m/z 188.
4-Cyanobenzaldehyde (983 mg, 7.5 mmol) and aminoguanidine hydrochloride (553 mg, 5.0 mmol) were dissolved in methanol (50 mL) and reacted according to the above General Reaction 4. The product solution was dissolved on the rotary evaporator, and the residue was stirred in chloroform and filtered to remove the remaining starting material. The filtered product was dried on vacuum overnight to afford a white powdery solid (944 mg, 4.22 mmol, 84% yield). Mp: 264-267ºC; $^1$H NMR (300MHz, d- DMSO) δ 12.22 (1H, s), 8.22 (1H, s), 8.07 (2H, d, J = 9.0Hz), 7.91 (2H, d, J = 9.0Hz); $^{13}$C NMR δ 155.4, 144.6, 137.7, 132.4, 128.0, 118.5, 112.1; ES+ m/z 188.

![Graph](attachment:image.png)
$p$-Xylenediamine (340 mg, 2.5 mmol) and $N$-ethyl-$S$-methyl-isothiourea hydriodide (615 mg, 2.5 mmol) were dissolved in ethanol (10 mL) and reacted according to the above General Reaction 3. The product was evaporated to dryness on a rotary evaporator to a colorless liquid product (324 mg, 0.97 mmol, 39% yield). $^1$H NMR (300MHz, d-DMSO) δ 7.32 (2H, m), 7.24 (2H, m), 4.40 (2H, d, J = 12Hz), 3.69 (2H, d), 3.18 (2H, q, J = 8Hz), 1.08 (3H, t, J = 8Hz); ES+ m/z 206.
m-Xylenediamine (330 μL, 2.5 mmol) and N-ethyl-S-methyl-isothiourea hydriodide (615 mg, 2.5 mmol) were dissolved in ethanol (10 mL) and reacted according to the above General Reaction 3. The product was evaporated to dryness on a rotary evaporator to yield a colorless liquid product (478 mg, 1.43 mmol, 57% yield). $^1$H NMR (300MHz, d-D$_2$O) $\delta$ 7.31 (4H, m), 4.43 (2H, d, J = 5Hz), 3.81 (2H, d, J = 5Hz), 3.22 (2H, q, J = 7.5Hz), 1.18 (3H, t, J = 7.5Hz); $^{13}$C NMR $\delta$ 155.3, 143.7, 136.8, 128.2, 126.1, 125.9, 124.9, 45.3, 44.1, 36.0, 14.3; ES$^+$ m/z 207.
p-Terephthalaldehyde (402 mg, 3.0 mmol) and aminoguanidine hydrochloride (553 mg, 5.0 mmol) were dissolved in methanol (50 mL) and reacted according to the above General Reaction 4. The product solution was dissolved on the rotary evaporator, and the residue was stirred in chloroform and filtered to remove the remaining starting material. Two separate solid samples were collected as part of the solid product floated above the chloroform, and the remainder sunk to the bottom. The two solid fractions were then finely powdered and stirred again with chloroform to ensure separation. The light-yellow powder top product was characterized as a mixture of benzylidino-guanidine-4-carboxaldehyde and the bis product. The bottom product was isolated and characterized as p-terephthalaldehyde-*bis*-guanylhydrazone, a fine off-white powder (365 mg, 1.14 mmol, 38% yield). Mp: >300°C; $^1$H NMR (300MHz, d-DMSO) $\delta$ 12.24 (2H, s), 8.21 (2H, s), 7.94 (4H, s); $^{13}$C NMR $\delta$ 155.3, 145.8, 135.0, 127.7; ES+ m/z 124.
4-Fluorobenzaldehyde (408 µL, 3.8 mmol) and 1,1-dimethylhydrazine (360 µL, 4.74 mmol, 1.25 equiv) were dissolved in methanol (10mL) and reacted according to the above General Reaction 5. The product solution was dissolved on the rotary evaporator, and the residue was stirred in chloroform and filtered to remove the remaining starting material. The filtered product was dried on vacuum overnight to afford a light orange plate-like crystal solid (528 mg, 3.18 mmol, 84% yield). $^1$H NMR (300MHz, d-DMSO) $\delta$ 7.57 (2H, m), 7.30 (1H, s), 7.16 (2H, m), 2.88 (6H, s); GC/MS: 5.140 min, m/z 166.

2,4-Dimethoxybenzaldehyde (632 mg, 3.8 mmol) and 1,1-dimethylhydrazine (360 µL, 4.74 mmol, 1.25 equiv) were dissolved in methanol (10mL) and reacted according to the above General Reaction 5. The off-white product solution was dissolved on the rotary evaporator to remove the solvent and remaining 1,1-dimethylhydrazine (bp 62-64ºC), and the residue dried on vacuum overnight to afford a light yellow liquid product (594 mg, 2.85 mmol, 75% yield). $^1$H NMR (300MHz, d-DMSO) $\delta$ 7.59 (1H, d, $J = 8.7$ Hz), 7.42 (1H, s), 6.55 (1H, d, $J = 2.4$Hz), 6.50 (1H, dd, $J = 8.7, 2.4$Hz), 3.80 (3H, s), 3.77 (3H, s), 2.83 (6H, s).
2,4-Dihydroxybenzaldehyde (525 mg, 3.8 mmol) and 1,1-dimethylhydrazine (360 μL, 4.74 mmol, 1.25 equiv) were dissolved in methanol (10mL) and reacted according to the above General Reaction 5. The orange product solution was dissolved on the rotary evaporator to remove the solvent and remaining 1,1-dimethylhydrazine (bp 62-64°C), and the residue dried on vacuum overnight to afford a dark orange solid product (363 mg, 2.00 mmol, 53% yield). $^1$H NMR (300MHz, d-DMSO) δ 7.57 (1H, s), 7.11 (1H, d, J = 8.1 Hz), 6.29 (1H, dd, J = 8.1, 2.7Hz), 6.21 (1H, d, J = 2.7Hz), 2.79 (6H, s).

p-Dimethylamino-benzaldehyde (567 mg, 3.8 mmol) and 1,1-dimethylhydrazine (360 μL, 4.74 mmol, 1.25 equiv) were dissolved in methanol (10mL) and reacted according to the above General Reaction 5. The yellow product solution was dissolved on the rotary evaporator to remove the solvent and remaining 1,1-dimethylhydrazine (bp 62-64°C), and the residue dried on vacuum overnight to afford a yellow solid product (662 mg, 3.46 mmol, 91% yield). $^1$H NMR (300MHz, d-DMSO) δ 7.36 (2H, d, J = 8.7Hz), 7.27 (1H, s), 6.68 (2H, d, J = 8.7Hz), 2.90 (6H, s), 2.79 (6H, s)
4-Pyridinecarboxaldehyde (160 mg, 1.5 mmol) and aminoguanidine hydrochloride (110 mg, 1.0 mmol) were dissolved in methanol (15 mL) and reacted according to the above General Reaction 4. The product solution was dissolved on the rotary evaporator, and the residue was stirred in chloroform and filtered to remove the remaining starting material. The filtered product was dried on vacuum overnight to afford an off-white powdery solid (171 mg, 0.86 mmol, 86 % yield). Mp: 227-228°C; $^1$H NMR (300MHz, DMSO-$d_6$) $\delta$ 12.4 (1H, broad singlet), 8.65 (2H, m), 8.20 (1H, s), 8.0 (NH, broad s), 7.85 (2H, m); $^{13}$CNMR (DMSO-$d_6$) $\delta$ 155.4, 149.8, 144.1, 140.6, 121.2.
3-Pyridinecarboxaldehyde (160 mg, 1.5 mmol) and aminoguanidine hydrochloride (110 mg, 1.0 mmol) were dissolved in methanol (15 mL) and reacted according to General Reaction 4. The product solution was dissolved on the rotary evaporator, and the residue was stirred in chloroform and filtered to remove the remaining starting material. The filtered product was dried on vacuum overnight to afford an off white powdery solid (175 mg, 0.88 mmol, 88% yield). Mp: 228-229°C; $^1$H NMR (300MHz, D$_2$O) $\delta$ 8.70 (1H, s), 8.54 (1H, d), 8.15 (1H, d), 8.0 (1H, s), 7.45 (1H, m); $^{13}$C NMR $\delta$ 157.4, 152.6, 150.2, 147.6, 137.7, 132.0, 127.
4-Pyridinecarboxaldehyde (282.6 μL, 3.0 mmol) and semicabazide hydrochloride (223 mg, 2.0 mmol) were dissolved in methanol (10 mL) and reacted according to the above General Reaction 6. Upon cooling, the product crashed out as a white precipitate (297 mg, 1.81 mmol, 91% yield). $^1$H NMR (300MHz, d-DMSO) δ 11.15 (1H, s), 8.80 (2H, d, J = 8Hz), 8.32 (2H, d, J = 8Hz), 7.93 (1H, s), 6.94 (2H, broad s)

3-Pyridinecarboxaldehyde (282 μL, 3.0 mmol) and semicabazide hydrochloride (223 mg, 2.0 mmol) were dissolved in methanol (10 mL) and reacted according to the above General Reaction 6. Upon cooling, the product crashed out as a white precipitate (274 mg, 1.67 mmol, 84% yield). $^1$H NMR (300MHz, d-DMSO) δ 10.73 (1H, s), 9.19 (1H, s), 8.70 (2H, m), 7.92 (1H, s), 7.83 (1H, dd), 6.8 (2H, broad s)
4-Pyridinecarboxaldehyde (283 μL, 3.0 mmol) and 1,1-dimethylhydrazine (274 μL, 3.6 mmol) were dissolved in methanol (10 mL) and reacted according to the above General Reaction 5. The yellow product solution was dissolved on the rotary evaporator to remove the solvent and remaining 1,1-dimethylhydrazine (bp 62-64°C), and the residue dried on vacuum overnight to afford a light-orange solid product (371 mg, 2.49 mmol, 83% yield). ¹H NMR (300MHz, d-DMSO) δ 8.41 (2H, d, J = 8Hz), 7.40 (2H, d, J = 8Hz), 7.13 (1H, s), 3.01 (6H, s)

3-Pyridinecarboxaldehyde (282 μL, 3.0 mmol) and 1,1-dimethylhydrazine (274 μL, 3.6 mmol) were dissolved in methanol (10 mL) and reacted according to the above General Reaction 5. The light yellow product solution was dissolved on the rotary evaporator to remove the solvent and remaining 1,1-dimethylhydrazine (bp 62-64°C), and the residue dried on vacuum overnight to afford a yellow liquid product (413 mg, 2.77 mmol, 92% yield). ¹H NMR (300MHz, d-DMSO) δ 8.64 (1H, s), 8.39 (1H, d, J = 5Hz), 7.86 (1H, d, J = 6Hz), 7.35 (1H, dd, J = 5Hz, 6Hz), 7.27 (1H, s), 2.96 (6H, s)
2-Pyridinecarboxaldehyde (287 \mu L, 3.0 mmol) and semicabazide hydrochloride (223 mg, 2.0 mmol) were dissolved in methanol (10 mL) and reacted according to the above General Reaction 6. Upon cooling the lime green solution, the product crashed out as a tan precipitate (284 mg, 1.73 mmol, 87% yield). \textsuperscript{1}H NMR (300MHz, d-DMSO) \delta 11.18 (1H, s), 8.86 (1H, d, J = 6Hz), 8.41 (1H, t, J = 7Hz), 8.29 (1H, d, J = 7Hz), 7.98 (1H, s), 7.80 (1H, t, J = 6Hz)

2-Pyridinecarboxaldehyde-dimethylhydrazone (287 \mu L, 3.0 mmol) and 1,1-dimethylhydrazine (274 \mu L, 3.6 mmol) were dissolved in methanol (10 mL) and reacted according to the above General Reaction 5. The off-white product solution was dissolved on the rotary evaporator to remove the solvent and remaining 1,1-dimethylhydrazine (bp 62-64\degree C), and the residue dried on vacuum overnight to afford a yellow liquid product (397 mg, 2.66 mmol, 89% yield). \textsuperscript{1}H NMR (300MHz, d-DMSO) \delta 8.43 (1H, d, J = 6Hz), 7.67 (2H, m), 7.19 (1H, s), 7.16 (1H, m), 3.00 (6H, s)
4-Aminobenzaldehyde was prepared by the method of Bellamy and Ou (Tetrahedron Letters, Vol. 25 No. 8, 1984, pp. 839-842). p-Nitrobenzaldehyde (755 mg, 5 mmol) and tin(II) chloride dihydrate (5.64 g, 25 mmol) were refluxed at 70-75°C in ethanol (15 mL). The solution turned dark red and opaque, and a TLC taken at 20 minutes revealed that all aldehyde starting material had been consumed, and the solution was allowed to cool to ambient temperature. The reaction mixture was then poured over 30 mL of ice in a 50 mL beaker. An aqueous solution of 5% sodium bicarbonate (30 mL) was added, which brought the effervescing reaction mixture to pH 3-5. Extraction with ethyl acetate was impossible due to a lack of clear H2O/EtAc separation; an additional 200 mL of the sodium bicarbonate solution was added until the effervescence deceased and the solution became yellow. The ethyl acetate was removed, and the aqueous layer was again extracted with additional ethyl acetate. The yellow organic layers were combined and rinsed twice with brine. The ethyl acetate was boiled with charcoal, and filtered to yield a clear pale yellow liquid, which was then dried with sodium sulfate. The ethyl acetate was removed on a rotary evaporator, then overnight on a vacuum pump to yield an orange solid which was identified as 4-aminobenzaldehyde. This product (60 mg, 0.5 mmol) and 1,1-dimethylhydrazine (48 μL, 0.63 mmol, 1.25 equiv) were dissolved in methanol (5 mL) and reacted according to the above General Reaction 5. The off-yellow product solution was dissolved on the rotary evaporator to remove the solvent and remaining 1,1-dimethylhydrazine (bp 62-64°C), and the residue dried on vacuum overnight to afford a solid orange product (69 mg, 0.42 mmol, 85% yield). ¹H NMR (300MHz, d-DMSO) δ 7.23 (1H, s), 7.21 (2H, d, J = 9Hz), 6.51 (2H, d, J = 9Hz), 5.21 (2H, s), 2.77 (6H, s). GC/MS: 14.997 min, 100.0%, m/z 163.
4-Aminobenzaldehyde (60 mg, 0.5 mmol, prepared by the method of Bellamy and Ou, Tetrahedron Letters, Vol. 25 No. 8, 1984, pp. 839-842) and aminoguanidine hydrochloride (44 mg, 0.4 mmol) were dissolved in methanol (10 mL) and reacted according to General Reaction 4. The product solution was dissolved on the rotary evaporator, and the residue was stirred in chloroform and filtered to remove the remaining starting material. The filtered product was dried on vacuum overnight to afford a light brown powdery solid (46 mg, 0.22 mmol, 55% yield). 

Mp: 169-173°C; \(^1\)H NMR (300MHz, d-DMSO) \(\delta\) 11.42 (1H, s), 7.93 (1H, s), 7.50 (2H, d, \(J = 9\)Hz), 6.58 (2H, d, \(J = 9\)Hz), 5.74 (2H, s); \(^13\)C NMR (300MHz, d-D\(_2\)O) \(\delta\) 187.7, 152.3, 152.2, 131.9, 126.0, 118.6; ES+ m/z 178.
2,4-Dihydroxybenzaldehyde Thiosemicarbazone

2,4-Dihydroxybenzaldehyde (414 mg, 3.0 mmol) and thiosemicabazide (182 mg, 2.0 mmol) were dissolved in methanol (10 mL) and reacted according to the above General Reaction 7. Upon cooling, the product crashed out as a fine light-orange crystal precipitate (309 mg, 1.46 mmol, 73% yield). $^1$H NMR (300 MHz, d-DMSO) $\delta$ 11.18 (1H, s), 8.22 (1H, s), 7.93 (1H, s), 7.75 (1H, s), 7.64 (1H, d, $J = 8$ Hz), 6.26 (2H, m)

4-Hydroxy-benzaldehyde Thiosemicarbazone

4-Hydroxybenzaldehyde (366 mg, 3.0 mmol) and thiosemicabazide (182 mg, 2.0 mmol) were dissolved in methanol (10 mL) and reacted according to the above General Reaction 7. The product did not precipitate upon cooling to ambient temperature, and thus the solution was evaporated on a rotary evaporator, the resulting semi-solid stirred with chloroform to dissolve unreacted starting material, and the product filtered to yield a light yellow solid (322 mg, 1.64 mmol, 82% yield). $^1$H NMR (300 MHz, d-DMSO) $\delta$ 11.23 (1H, s), 8.03 (1H, broad s), 7.94 (1H, s), 7.81 (1H, broad s), 7.60 (2H, d, $J = 8$ Hz), 6.78 (2H, d, $J = 8$ Hz)
4-Terephthalaldehyde Mono Thiosemicarbazone

p-Terephthalaldehyde (489 mg, 3.0 mmol) and thiosemicabazide (182 mg, 2.0 mmol) were dissolved in methanol (10 mL) and reacted according to the above General Reaction 7. Upon cooling, the product crashed out as a light yellow solid precipitate (390 mg, 1.83 mmol, 92% yield). $^1$H NMR (300MHz, d-DMSO) δ 11.60 (1H, s), 9.98 (1H, s), 8.32 (1H, broad s), 8.15 (1H, broad s), 8.08 (1H, s), 8.00 (2H, d, J = 9Hz), 7.89 (2H, d, J = 9Hz)

4-Amino-benzaldehyde Semicarbazone

4-Aminobenzaldehyde (60 mg, 0.5 mmol, prepared by the method of Bellamy and Ou, Tetrahedron Letters, Vol. 25 No. 8, 1984, pp. 839-842) and semicarbazide hydrochloride (45 mg, 0.4 mmol) were dissolved in methanol (10 mL) and reacted according to General Reaction 6. Upon cooling the solution, the product crashed out as an orange powder precipitate (57 mg, 0.32 mmol, 80% yield). $^1$H NMR (300MHz, d-DMSO) δ 7.86 (2H, d, J = 9Hz), 7.74 (1H, s), 7.58 (2H, d, J = 9Hz), 6.94 (2H, d), 6.79 (2H, d), 6.62 (1H, d).
3-Aminobenzaldehyde was prepared by the dropwise addition of 2.0 M HCl in ethanol (6.0 mL) to the corresponding 3-aminobenzaldehyde ethylene acetal (902 mg, 5.97) in methanol (34 mL). The reaction solution darkened with the acid addition, from very pale orange to dark yellow, and during stirring at room temperature for 30 minutes, the color returned to a light orange, and TLC revealed a completed reaction. Aminoguanidine hydrochloride (111 mg, 1.0 mmol) was added to a 15-mL sample of the methanolic reaction solution (0.176 M, 2.64 mmol aldehyde), and the mixture refluxed for 3 hours. The reaction was then cooled to room temperature, and fine tan crystals (35.5 mg, 0.16 mmol, 16% yield) crashed out of solution, which were collected and rinsed with cold methanol. Mp: 240-246ºC; $^1$H NMR (300MHz, d-DMSO) $\delta$ 12.14 (1H, s), 8.20 (1H, s), 7.84 (1H, d, J = 7.8Hz), 7.63 (1H, s), 7.49 (1H, t, J = 7.8Hz), 7.36 (1H, d, J = 7.8Hz); $^{13}$C NMR $\delta$ 155.2, 145.8, 134.6, 129.8, 124.7, 123.5, 120.9; ES+ m/z 178.
3-Aminobenzaldehyde was prepared by the dropwise addition of 2.0M HCl in ethanol (6.0 mL) to the corresponding 3-aminobenzaldehyde ethylene acetal (902 mg, 5.97 mmol) in methanol (34 mL). The reaction solution darkened with the acid addition, from very pale orange to dark yellow, and during stirring at room temperature for 30 minutes, the color returned to a light orange, and TLC revealed a completed reaction.

Semicarbazide hydrochloride (112 mg, 1.0 mmol) was added to a 9-mL sample of the methanolic reaction solution (0.176 M, 1.58 mmol aldehyde), and the mixture refluxed for 3 hours. Upon cooling the solution, the product crashed out as an off-white powder precipitate (65 mg, 0.37 mmol, 37% yield). $^1$H NMR (300MHz, d-DMSO) δ 10.38 (1H, s), 7.83 (1H, s), 7.65 (1H, d, J = 7.8 Hz), 7.52 (1H, s), 7.42 (1H, t, J = 7.8Hz), 7.23 (1H, d, J = 7.8Hz), 6.51 (2H, broad s).
3-Amino-benzaldehyde was prepared by the dropwise addition of 2.0M HCl in ethanol (6.0 mL) to the corresponding 3-aminobenzaldehyde ethylene acetal (902 mg, 5.97 mmol) in methanol (34 mL). The reaction solution darkened with the acid addition, from very pale orange to dark yellow, and during stirring at room temperature for 30 minutes, the color returned to a light orange, and TLC revealed a completed reaction. Thiosemicarbazide (112 mg, 1.0 mmol) was added to a 9-mL sample of the methanolic reaction solution (0.176 M, 1.58 mmol aldehyde), and the mixture refluxed for 3 hours. Upon cooling, the product crashed out as a light brown powder solid precipitate (61 mg, 0.31 mmol, 31% yield). $^1$H NMR (300MHz, d-DMSO) $\delta$ 9.96 (1H, s), 7.3-7.7 (5H, m), 6.18 (2H, s).

Pyridin-2-yl-guanidine Hydrochloride

Prepared using General Reaction 1 above by Aaron Haubner. $^1$H NMR (300MHz, d-DMSO) $\delta$ 9.50 (1H, s, broad), 8.05 (1H, s), 7.85 (1H, m), 7.75 (1H, m), 7.62 (1H, d, J = 0.03Hz).
AH-2

Pyridin-3-yl-guanidine Hydrochloride

Prepared using General Reaction 1 above by Aaron Haubner. $^1$H NMR (300MHz, d-DMSO) δ 9.036 (1H, d, J=0.008Hz), 7.961 (1H, d, J=0.002Hz), 7.759 (1H, m, J = 0.005Hz), 7.73 (1H, m, J=0.01Hz), 6.69 (2H, s, broad).

AH-3

N-Pyridin-2-ylmethyl-guanidine Hydrochloride

Prepared using General Reaction 1 above by Aaron Haubner. $^1$H NMR (300MHz, d-DMSO) δ 11.50 (2H, s, broad), 8.80 (1H, d, J=0.018Hz), 8.425 (1H, t, J = 0.021Hz), 8.356 (1H, t, J=0.025), 7.781 (1H, t, J = 0.021Hz), 7.70 (2H, s, broad), 4.811 (2H, d, J=0.02Hz).
AH-4

N-Pyridin-3-ylmethyl-guanidine Hydrochloride

Prepared using General Reaction 1 above by Aaron Haubner. $^1$H NMR (300MHz, d-DMSO) $\delta$ 8.91 (1H, s), 8.88 (1H, d, J=0.018Hz), 8.688 (1H, t, J = 0.02Hz), 8.516 (1H, d, J=0.015Hz), 8.084 (1H, t, J = 0.021Hz), 7.68 (2H, s, broad), 4.711 (2H, d, J=0.021Hz).

AH-5

N-Pyridin-4-ylmethyl-guanidine Hydrochloride

Prepared using General Reaction 1 above by Aaron Haubner. $^1$H NMR (300MHz, d-DMSO) $\delta$ 8.92 (1H, d, J=0.021Hz), 8.758 (1H, t, J=0.02Hz), 7.91 (1H, d, J =0.021Hz), 7.727 (2H, s, broad), 4.842 (2H, d, J = 0.021Hz).
AH-6

\[ \text{2,3-Di-N-guanidyl-pyridine Dihydrochloride} \]

Prepared using General Reaction 1 above by Aaron Haubner. \(^1\)H NMR (300MHz, d-DMSO) \( \delta \) 9.62 (1H, s), 8.262 (1H, s, broad), 8.031 (1H, d, \( J = 0.02 \)Hz), 7.92 (1H, d, \( J=0.014 \)Hz), 7.685 (3H, s), 6.894 (1H, t, \( J = 0.022 \)Hz), 3.84 (2H, s, broad).

AH-7

\[ \text{N-(4-Methoxy-benzyl)-guanidine Hydrochloride} \]

Prepared using General Reaction 1 above by Aaron Haubner. \(^1\)H NMR (300MHz, d-DMSO) \( \delta \) 9.07(1H, s, broad), 8.20 (1H, s, broad), 7.077 (2H, s, broad), 6.918 (2H, m), 6.808 (2H, m), 3.691 (3H, s).
**AH-8**

\[
\begin{align*}
\text{H-Cl} \\
\text{H}_2\text{N} & \quad \text{NH} \\
\text{H}_2\text{N} & \quad \text{NH} \\
\end{align*}
\]

\textit{N-}(1\textit{H}-\textit{Indol-5-yl})\textit{guanidine Hydrochloride}

Prepared using General Reaction 1 above by Aaron Haubner. $^1$H NMR (300MHz, d-DMSO) $\delta$ 9.90 (1H, s, broad), 8.10 (2H, s, broad), 8.026 (1H, s) 8.00 (1H, d, J=0.044Hz), 7.50 (1H, m, J=0.04Hz), 7.256 (1H, s), 6.928 (1H, m)

---

![Graph showing DPM vs. log [M] Concentration for AH-8 and AH-8+SPD](image-url)
AH-9

3,4-Di-N-guanidyl-pyridine Dihydrochloride

Prepared using General Reaction 1 above by Aaron Haubner. $^1$H NMR (300MHz, d-DMSO) $\delta$ 11.07 (1H, s), 8.398 (1H, s), 8.138 (1H, s), 6.964 (1H, d, J=0.02), 6.10 (3H, s, broad).

AH-10

N-(2-pyrrolidin-1-yl-ethyl)-guanidine Hydrochloride

Prepared using General Reaction 1 above by Aaron Haubner. 1H NMR (300MHz, d-DMSO) $\delta$ 10.25 (1H, s, broad), 7.12 (2H, s, broad), 4.13 (1H, s, broad), 3.475 (2H, m, J=0.02Hz), 3.23 (2H, d, J = 0.02Hz), 2.750 (2H, d, J = 0.014Hz) 2.21 (2H, m), 1.908 (2H, m).
AH-13

\[
\begin{align*}
\text{N-NH} & \quad \text{H-Cl} \\
\text{CH}_3 & \quad \text{NH} \\
\end{align*}
\]

*N-[2-(1-methyl-pyrrolidin-2-yl)-ethyl]-guanidine Hydrochloride*

Prepared using General Reaction 1 above by Aaron Haubner. 1H NMR (300MHz, d-DMSO) δ 11.02 (1H, s), 7.12 (2H, s, broad), 4.13 (1H, s, broad), 3.475 (2H, m, J=0.02Hz), 3.23 (2H, d, J = 0.02Hz), 2.75 (2H, d, J = 0.014Hz), 2.21 (2H, m), 1.908 (2H, m).

AH-14

\[
\begin{align*}
\text{Cl} & \quad \text{H-Cl} \\
\text{NH} & \quad \text{NH} \\
\text{Cl} & \\
\end{align*}
\]

*5-Chloro-pyridin-2-yl-guanidine Hydrochloride*

Prepared using General Reaction 1 above by Aaron Haubner. 1H NMR (300MHz, d-DMSO) δ 9.20 (1H, d, J=0.08), 8.331 (1H, s), 7.99 (1H, d, J = 0.02Hz), 7.045 (1H, d, J=0.03), 6.45 (2H, s, broad).
AH-15

\[
\text{HN-Cl} \\
\text{Cl} \\
\text{HN} \\
\text{NH}_2 \\
\text{Cl}
\]

\textit{N-(7-Bromo-fluoren-2-yl)guanidine Hydrochloride}

Prepared using General Reaction 1 above by Aaron Haubner. \(^1\text{H}\) NMR (300MHz, d-DMSO) \(\delta\) 11.43 (1H, s), 10.14 (1H, s), 7.907 (2H, s), 7.88 (2H, s), 7.84 (2H, s) 7.818 (2H,m), 7.772 (2H,m), 7.579 (2H,m).

AH-16

\[
\text{H-I}
\]

\textit{N-(Pyridin-3-yl)-methyl-(1,4,5,6-tetrahydro-pyrimidin-2-yl)-amine Hydriodide}

Prepared using General Reaction 2 above by Aaron Haubner. \(^1\text{H}\) NMR (300MHz, d-DMSO) \(\delta\) 8.55 (1H, s), 8.521 (1H, d, J=0.02Hz), 7.71 (1H, d, J = 0.035Hz), 7.43 (1H, m), 6.02 (2H,s,broad), 4.370 (2H,s), 3.255 (4H, t, J=0.025 Hz), 3.074 (2H, m, J=0.02).
Agmatine sulfate

\[
\text{H}_3\text{N} - \text{SO}_4^\ominus - \text{NH}_2
\]

Purchased from commercial sources

### Agmatine +/- SPD

<table>
<thead>
<tr>
<th>log [M] Concentration</th>
<th>DPM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>118</td>
</tr>
<tr>
<td>Agmatine +/- SPD</td>
<td></td>
</tr>
<tr>
<td>Agmatine</td>
<td></td>
</tr>
</tbody>
</table>
Arcaine sulfate

Purchased from commercial sources

Arcaine

![Graph showing DPM values vs. log [M] Concentration for Arcaine and Arcaine+SPD with error bars.](image-url)
Memantine hydrochloride

Purchased from commerial sources

Memantine

![Graph showing the concentration of Memantine and Memantine+SPD](image-url)

- **X**: Memantine+SPD
- **•**: Memantine

Control

Log [M] Concentration

DPM

100 200 300 400 500 600 700 800 900 1000 1100 1200 1300

10 20 30 40 50 60 70 80 90 100
MK-801 (Dizocilpine)

Purchased from commercial sources

Cold MK-801
3.1 Introduction

After the synthesis of a library of several hundred agmatine analogs, and their subsequent screening and analysis, it became clear that the most consistently active series of compounds was the arylidenamino-guanidines. The nine compounds in the JR-series (JR-121, JR-122, JR-124, JR-125, JR-132, JR-146, JR-147, JR-160, and JR-197) were examined in the assays reported in chapter 2, and in addition, twenty arylidenamino-guanidines were synthesized by Dr. Sundar Neelakantan and screened using the same procedure as the others (the two pyridyl compounds were synthesized in each series, but each were screened to give significantly similar data). All 27 of the unique compounds were active in this assay, an impressive 100% hit rate. These data suggested the importance of further examination of this class of compounds, which began with a close look at the structural motif from which each of these molecules were derived, i.e. aminoguanidine.

Aminoguanidine hydrochloride, an agmatine analog which is devoid of the n-butyl chain, has been shown to be quite active in several aspects of neurobiology, which are closely related to polyamines. The activity of this compound was first shown in the inhibition of diamine oxidase, or histaminase, which is responsible for the deamination of histamine as well as the polyamine putrescine (Sculer, 1952). More recently, aminoguanidine has been demonstrated in the alleviation of several of the effects of diabetes, due to inhibition of advanced glycosylation end products (Brownlee et al., 1986). It has also been shown to inhibit nitric oxide synthase in vivo and in vitro (Corbett et al., 1992), the enzyme which catalyzes the biosynthesis of the neurotransmitter nitric oxide (NO) from L-arginine (also the amino acid at the top of the polyamine pathway).

Aminoguanidine also directly impacts the levels of the polyamines spermidine and spermine by affecting the activity of S-adenosylmethionine decarboxylase (SAMDC). SAMDC is the enzyme responsible for the conversion of S-
adenosylmethionine to decarboxylated S-adenosylmethionine, which acts as the donor of the aminopropyl group that is joined to putrescine to form spermidine, and thence to spermidine to form putrescine. Aminoguanidine at concentrations of ~1mM has been shown to stabilize SAMDC, and increase its levels and activity in mouse cell lines (Stjernborg and Persson, 1993).

Aminoguanidine has also been examined as a possible antagonist of the NMDA receptor complex, and has been shown to have no activity in the $[^3]H$MK-801 displacement assay in P2 membrane (see Figure 3.1). However, conjugates of aminoguanidine and an array of arylaldehydes were synthesized and screened in the above assay, and of the 45 compounds tested, all have demonstrated to be NMDA receptor inhibitory modulators. The lack of aminoguanidine activity in this assay is important for two reasons:

![Figure 3.1 $[^3]H$MK-801-displacement activity of aminoguanidine hydrochloride](image)

Firstly, the activity seen by the arylidenamino-guanidines is indeed due to the products, and not the aminoguanidine hydrochloride starting material. The proportions of starting materials used in the synthesis are chosen in such a way that aminoguanidine is the limiting reagent, as it is difficult to separate from the final product by
chromatography. While the products are mostly crystalline and appear to be very pure under scrutiny of $^1$H and $^{13}$C NMR, these analytical methodologies do little to display such a small compound without C-H bonds. In D$_2$O, deuterium exchange will render this molecule invisible to proton NMR, and even d-DMSO, the N-H peaks are hardly discernable as usually broader peaks which overlap the corresponding product peaks. In carbon NMR spectra, the peak for the lone quaternary carbon in this molecule is quite small even at a high concentration; even in the product which constitutes the vast majority of the compound in solution, the corresponding peak is very small, thus at a 5% starting material impurity, aminoguanidine would also be virtually invisible.

Secondly, the lack of activity of aminoguanidine is interesting in that it demonstrates that while it has been demonstrated to be a required moiety for the activity of the arylidenamino-guanidines (while semicarbazine, thiosemicarbazine, and dimethylhydrazine failed in the same position), this molecule itself does not possess any inhibitory activity. Indeed, this structure is the only entirely conserved moiety in a set of 45 active compounds, thus this observation may reveal much about the structure-activity of at least one of the polyamine binding sites of the NMDA receptor complex.

In order to further evaluate the usefulness of these compounds towards the specific goals of creating and optimizing agmatine analogs for use in NMDAR modulation, it was determined that a larger amount of similar compounds should be synthesized and tested. For the existing set of 27 arylidenamino-guanidines, full dose-response curves were obtained from the P2 membrane [$^3$H]MK-801 displacement assay, as described below. Using these data and 12 molecular descriptors, a partial least squares (PLS) model was developed, with an R-value for correlation of predicted vs. observed pIC$_{50}$-values of 0.902. This PLS model along with the subsequent full neural network model are described in detail in Chapter 5 of this work.

A set of an additional 22 arylidenamino-guanidine compounds were designed, each with predicted IC$_{50}$ values of less than 100 μM according to the above model. These compounds were then synthesized using the same synthetic schemes laid forth in Chapter 2, and yielded a set of 18 new molecules. These molecules were then tested as possible NMDAR inhibitory modulators, as well as being utilized in an effort to verify the developed PLS model, and provide a sufficient amount of data to produce a neural
network model. The synthesis of these molecules and the evaluation of the entire final set of 45 arylidenamino-guanidines are described below.

3.2 Synthetic Chemistry

The general method for preparation of arylidenamino-guanidines proceeds as follows: Aminoguanidine hydrochloride (1 equivalent) and an aryl aldehyde (1.5-2 equivalents) are dissolved in a minimal amount of methanol. The solution is then heated to reflux and stirred for a period of 4-12 hours with TLC monitoring until the aminoguanidine hydrochloride is consumed. Upon cooling to room temperature, the corresponding arylidenamino-guanidine is separated from the remaining aryl aldehyde. In some cases, the product crystallizes from the methanol solution during cooling, in which case the product is filtered and rinsed with methanol and chloroform. In most cases, the product would remain in solution upon cooling, and the solution is then removed on a rotary evaporator. The resulting solid or semi-solid is then stirred at room temperature in chloroform or diethyl ether, into which the remaining aryl aldehyde dissolves, and the remaining solid arylidenamino-guanidine is filtered and washed with the corresponding rinsing solution. Products are allowed to dry on filter plates, and then placed until vacuum overnight to remove any remaining solvent.

Scheme 3.1 One-step synthesis of arylidenamino-guanidines from aldehydes

The structures of all of the following compounds were verified using $^1$H NMR and $^{13}$C NMR, and in some cases mass spectroscopy; melting points were also obtained for each product.
3.3 Pharmacology

Each of these compounds were screened for their ability to reduce the binding of \( ^3\text{H} \)MK-801 in a P2 membrane preparation, comprised of membrane-bound proteins including the NMDA receptor complex. The range of concentrations tested spanned an ample region to generate full dose-response curves in the absence and presence of spermidine.

The pharmacological assay utilized P2 brain membrane homogenate from male Sprague-Dawley rats (225-250 grams) and radiolabeled MK-801 in order to measure reduction of this bound ligand due to addition of a modulating agent. The P2 membrane was prepared from a homogenized mixture of rat cortex, hippocampus, and cerebellum in 50 mM Trizma acetate buffer containing 0.32 M sucrose at pH 7.25. This crude homogenate was centrifuged for 10 minutes at 4°C and 2800 rpm in a Sorvall RC 26 Plus centrifuge, after which the supernatant was saved and the procedure was repeated with the pellet dissolved into cold buffer with sucrose. The combined supernatants were then centrifuged for 20 minutes at 4°C and 20,500 rpm on the same instrument. The supernatant was decanted, and the pellet retained and resuspended in 50 mM Trizma acetate buffer at pH 7.25, without sucrose. Another 20-minute centrifugation was performed on the resuspended pellet with the above parameters, and two additional 10-minute washings, with the pellet retained between each. After these washings, the membrane was again suspended in the above buffer without sucrose, and the protein concentration determined using Pierce reagent. Aliquots of 15 mg of the concentrated P2 membrane preparation were dispensed into cryo-tubes, which were then stored at -80°C. With this amount of protein per tube, the final protein concentration could easily be brought to 1.5 mg/mL buffer by the addition of sufficient 50 mM Trizma acetate buffer at pH 7.25 to bring the total solution volume to 10 mL.

The working \( ^3\text{H} \)MK-801 solution is prepared by the addition of 30 \( \mu \)M \( ^3\text{H} \)MK-801 in ethanol (2 \( \mu \)L) to the Trizma buffer (10 mL). In order to determine the concentration of \( ^3\text{H} \)MK-801 in this working solution, 100\( \mu \)L was dispensed into 2.5 mL
of Microscint 20 in duplicate, and the counts per minute (CPM) were measured on the scintillation counter. As the stock ethanolic MK-801 solution slowly evaporated and became more concentrated, this procedure was necessary in order to calculate further buffer dilutions of the working solution necessary to obtain the desired 6.0nM concentration.

Samples were dissolved in buffer to make 19 mM stock solutions (for a final well concentration of 3.16 mM, or $10^{-2.5}$ M), and were diluted serially with 2.16 equivalents of buffer six times, to give final well concentrations at every $10^{-0.5}$ M decrement, down to 3.16 µM, or $10^{-5.5}$ M. In some cases, the compounds were not soluble at 19 mM, and stock solutions were prepared at lower concentrations.

The assay was performed at ambient room temperature in 96-well micro-titer plates with a total capacity of 350 µL per well. To each well was added 100µL P2 membrane, thawed on ice and brought to the final concentration of 1.5mg/mL as described above. A blank control was measured in triplicate using only freshly-prepared 50 mM Trizma acetate buffer at pH 7.25. A 100 µM spermidine-potentiated control and 1 mM dextromethorphan control to measure non-specific binding were each performed in triplicate as well. The seven prepared concentrations of the compound being tested (50µL, n = 3) were added to the plate, with each sample being added in two separate sets: spermidine (50µL, 600µM) was added to one set for a potentiated curve and buffer (50µL) to the other set to obtain a non-potentiated curve. The plates were then allowed to incubate for 15 minutes in order to establish the equilibrium between the spermidine and samples, after which 100µL [$^3$H]MK-801 ligand (6nM) was added to each well for a total of 300µL. All micro-titer plates were allowed to incubate for 40 minutes following [$^3$H]MK-801 addition, and were then harvested into filter plates, which were then dried overnight in the hood at ambient temperature. After 12-16 hours, 35µL of Microscint 20 was added to each membrane on the dried filter plate; each plate was sealed and then incubated for two hours before reading on a plate counter.

For each compound, the potentiated and non-potentiated curves were plotted, and IC$_{50}$-values with and without spermidine were determined using GraphPad software. A third value, the IC$_{50}^{pot}$, was derived as the point on the spermidine-potentiated curve which was halfway between the spermidine-potentiated control and the buffer control.

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This value corresponds to the concentration of compound needed to displace half of the [³H]MK-801 bound due to the channel-agonist effects of spermidine. Some of the tested compounds demonstrated IC₅₀-values which were too low to be accurately gleaned in the previously mentioned concentration range; these compounds were prepared in a lower-concentration stock solution and re-evaluated at a lower range. In addition, agmatine and NMDAR antagonists aracaine, memantine, and MK-801 were evaluated by the same methods.
Table 3.1 IC<sub>50</sub>-values in the presence and absence of spermidine, and IC<sub>50</sub> of potentiation

<table>
<thead>
<tr>
<th>Compound # (Code)</th>
<th>Structure</th>
<th>IC&lt;sub&gt;50&lt;/sub&gt; w/ SPD&lt;sup&gt;1&lt;/sup&gt; (μM)</th>
<th>IC&lt;sub&gt;50&lt;/sub&gt; w/o SPD&lt;sup&gt;2&lt;/sup&gt; (μM)</th>
<th>IC&lt;sub&gt;50&lt;/sub&gt; Pot&lt;sup&gt;3&lt;/sup&gt; (μM)</th>
<th>Ratio&lt;sub&gt;Pot&lt;/sub&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 (AG-17)</td>
<td>![Structure 1]</td>
<td>232.1</td>
<td>339.4</td>
<td>73.1</td>
<td>4.6 : 1</td>
</tr>
<tr>
<td>2 (AG-6)</td>
<td>![Structure 2]</td>
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<td>149.6</td>
<td>80.7</td>
<td>1.9 : 1</td>
</tr>
<tr>
<td>3 (JR-202)</td>
<td>![Structure 3]</td>
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<td>6.0</td>
<td>4.6 : 1</td>
</tr>
<tr>
<td>4 (JR-212)</td>
<td>![Structure 4]</td>
<td>97.5</td>
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<td>42.5</td>
<td>2.5 : 1</td>
</tr>
<tr>
<td>5 (JR-211)</td>
<td>![Structure 5]</td>
<td>65.0</td>
<td>71.9</td>
<td>22.8</td>
<td>3.2 : 1</td>
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<tr>
<td>6 (JR-124)</td>
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<td>81.4</td>
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<tr>
<td>7 (JR-125)</td>
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<td>8 (JR-226)</td>
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<td>4.6 : 1</td>
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<tr>
<td>9 (JR-227)</td>
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<tr>
<td>10 (AG-20)</td>
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<td>67.2</td>
<td>71.1</td>
<td>25.1</td>
<td>2.8 : 1</td>
</tr>
</tbody>
</table>

<sup>1</sup> - IC<sub>50</sub> w/ SPD: the measurement of IC<sub>50</sub> derived from the data collected in the spermidine-potentiated [<sup>3</sup>H] MK-801 displacement assay.

<sup>2</sup> - IC<sub>50</sub> w/o SPD: the measurement of IC<sub>50</sub> derived from the data collected in the non-spermidine-potentiated [<sup>3</sup>H] MK-801 displacement assay, in which the spermidine solution was replaced with buffer.

<sup>3</sup> - IC<sub>50</sub> of Pot: from the spermidine-potentiated curve, the concentration corresponding to one-half of the difference in DPM between the SPD-potentiated control and the blank control.

* - This measurement is the IC<sub>50</sub> of the lower phase, as these SPD-potentiated curves were clearly biphasic in nature.
Table 3.1 (cont.) IC$_{50}$-values in the presence and absence of spermidine, and IC$_{50}$ of potentiation

<table>
<thead>
<tr>
<th>Compound # (Code)</th>
<th>Structure</th>
<th>IC$_{50}$ w/ SPD$^1$ ($\mu$M)</th>
<th>IC$_{50}$ w/o SPD$^2$ ($\mu$M)</th>
<th>IC$_{50}^{Pot}$</th>
<th>Ratio$_{Pot}$</th>
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<tr>
<td>11 (AG-13)</td>
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<tr>
<td>12 (AG-12)</td>
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<td>61.9</td>
<td>62.3</td>
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<tr>
<td>13 (AG-8)</td>
<td><img src="image3.png" alt="Structure" /></td>
<td>150.9</td>
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<td>14 (AG-10)</td>
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<td>2.4 : 1</td>
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<tr>
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<td><img src="image5.png" alt="Structure" /></td>
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<td>199.5</td>
<td>3.9 : 1</td>
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<tr>
<td>16 (JR-160)</td>
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<td>120.2</td>
<td>104.2</td>
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</tr>
<tr>
<td>17 (JR-219)</td>
<td><img src="image7.png" alt="Structure" /></td>
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<td>94.4</td>
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<td>11.9 : 1</td>
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<td>18 (JR-122)</td>
<td><img src="image8.png" alt="Structure" /></td>
<td>325.9</td>
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<td>149.3</td>
<td>2.0 : 1</td>
</tr>
<tr>
<td>19 (JR-121)</td>
<td><img src="image9.png" alt="Structure" /></td>
<td>169.4</td>
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<td>78.0</td>
<td>2.8 : 1</td>
</tr>
<tr>
<td>20 (JR-228)</td>
<td><img src="image10.png" alt="Structure" /></td>
<td>157.9</td>
<td>161.7</td>
<td>63.1</td>
<td>2.6 : 1</td>
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</table>
Table 3.1 (cont.) IC<sub>50</sub>-values in the presence and absence of spermidine, and IC<sub>50</sub> of potentiation

<table>
<thead>
<tr>
<th>Compound # (Code)</th>
<th>Structure</th>
<th>IC&lt;sub&gt;50&lt;/sub&gt; w/ SPD&lt;sup&gt;1&lt;/sup&gt; (μM)</th>
<th>IC&lt;sub&gt;50&lt;/sub&gt; w/o SPD&lt;sup&gt;2&lt;/sup&gt; (μM)</th>
<th>IC&lt;sub&gt;50&lt;/sub&gt;&lt;sup&gt;Pot&lt;/sup&gt; 3 (μM)</th>
<th>Ratio&lt;sup&gt;Pot&lt;/sup&gt;</th>
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<td>21 (JR-223)</td>
<td><img src="image" alt="" /></td>
<td>259.4*</td>
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<td>22 (JR-218)</td>
<td><img src="image" alt="" /></td>
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<td>7.4 : 1</td>
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<tr>
<td>23 (JR-217)</td>
<td><img src="image" alt="" /></td>
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<td>117.6</td>
<td>18.4</td>
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<tr>
<td>24 (JR-220)</td>
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<td>124.5</td>
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<td>25 (AG-9)</td>
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<td>42.3</td>
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<td>26 (AG-23)</td>
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<td>225.5</td>
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<td>3.0 : 1</td>
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<tr>
<td>27 (AG-18)</td>
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<td>4.0 : 1</td>
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<tr>
<td>28 (AG-24)</td>
<td><img src="image" alt="" /></td>
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<td>29 (JR-209)</td>
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<td>13.6</td>
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<tr>
<td>30 (AG-4)</td>
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<td>46.6</td>
<td>21.4</td>
<td>2.2 : 1</td>
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</table>
Table 3.1 (cont.) IC\textsubscript{50}-values in the presence and absence of spermidine, and IC\textsubscript{50} of potentiation

<table>
<thead>
<tr>
<th>Compound # (Code)</th>
<th>Structure</th>
<th>IC\textsubscript{50} w/ SPD\textsuperscript{1} (μM)</th>
<th>IC\textsubscript{50} w/o SPD\textsuperscript{2} (μM)</th>
<th>IC\textsubscript{50}\textsuperscript{3} Pot</th>
<th>Ratio\textsubscript{Pot}</th>
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<td>31 (JR-213)</td>
<td><img src="image1" alt="Structure" /></td>
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<td>1.5 : 1</td>
</tr>
<tr>
<td>33 (JR-204)</td>
<td><img src="image3" alt="Structure" /></td>
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<td>7.0 : 1</td>
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<td>34 (AG-19)</td>
<td><img src="image4" alt="Structure" /></td>
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<td>39 (AG-3)</td>
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<tr>
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<td>102.6</td>
<td>64.6</td>
<td>50.6</td>
<td>1.3 : 1</td>
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</tbody>
</table>
Table 3.1 (cont.) IC<sub>50</sub>-values in the presence and absence of spermidine, and IC<sub>50</sub> of potentiation

<table>
<thead>
<tr>
<th>Compound # (Code)</th>
<th>Structure</th>
<th>IC&lt;sub&gt;50&lt;/sub&gt; w/ SPD&lt;sup&gt;1&lt;/sup&gt; (μM)</th>
<th>IC&lt;sub&gt;50&lt;/sub&gt; w/o SPD&lt;sup&gt;2&lt;/sup&gt; (μM)</th>
<th>IC&lt;sub&gt;50&lt;/sub&gt; Pot&lt;sup&gt;3&lt;/sup&gt; (μM)</th>
<th>Ratio&lt;sub&gt;Pot&lt;/sub&gt;</th>
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</thead>
<tbody>
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<td><img src="image1" alt="Structure" /></td>
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<tr>
<td>43 (AG-5)</td>
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<td>334.8</td>
<td>290.1</td>
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<tr>
<td>44 (AG-14)</td>
<td><img src="image4" alt="Structure" /></td>
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<td>MK-801</td>
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<td>5.58 nM</td>
<td>5.92 nM</td>
<td>2.19 nM</td>
<td>2.7 : 1</td>
</tr>
</tbody>
</table>
3.4 Results and Discussion

3.4.1 Synthesis of the Optimized Set of Arylidenamino-Guanidines

Following the success of the previously prepared and screened arylidenamino-guanidines, an additional 22 compounds were designed based on additional structural diversity and the IC\textsubscript{50}-values predicted for them from the PLS model. As with so many of the best-laid plans of Sprague-Dawley rats and men, the finalized set of 18 compounds which were tested in the radioligand displacement assay were not the same as these 22 originally designed, but they introduced many new structural entities to this set of arylidenamino-guanidines nonetheless.

The synthesis of a \textit{meta}-vinyl substituted benzylidamine (JR-201, (3-vinyl-benzylidenamino)-guanidine hydrochloride) was unsuccessful, possibly due to polymerization of the reactive vinyl substituent. In addition, the use of 2- and 4-imidazole groups as the aromatic moiety of the arylaldehyde did not proceed via the facile Schiff base formation. These starting materials, 2-imidazolecarboxaldehyde and 4(5)-imidazolecarboxaldehyde, may exist in resonance forms which leave the aldehyde less reactive to the Schiff base formation with aminoguanidine.

While (2-biphenyl-carboxylidenamino)-guanidine hydrochloride (JR-209) was prepared and evaluated in this assay without impediment, the 4-isomer (JR-208, (4-biphenyl-carboxylidenamino)-guanidine hydrochloride) was prepared and identified as the desired product, but was completely water-insoluble. This was an unforeseen conundrum which did not manifest itself in any of the other arylidenamino-guanidines. While biphenyl is understandably not highly hydrophilic, it was indeed curious that the addition of the idenamino-guanidine moiety at the 2-position would render the biphenyl analog water-soluble, but that addition at the 4-position would not.

The solubility problem was also seen with the three carboxy-substituted compounds in this series. (2-Carboxybenzylidenamino)-guanidine hydrochloride (JR-221), (3-carboxybenzylidenamino)-guanidine hydrochloride (JR-210), and (4-carboxybenzylidenamino)-guanidine hydrochloride (JR-222) were all prepared as clean products. Each of these compounds would be predicted to form water-soluble
zwitterions; however, none of these three products proved to be even sparingly soluble in the prepared buffer. Thus, while these three compounds were not testable, the conversion of the carboxyl groups of the meta- and para-compounds into the corresponding methyl esters via treatment with thionyl chloride in methanol resulted in benzylidenaminoguanidine-3-methyl ester hydrochloride (JR-226, compound 8) and benzylidenaminoguanidine-4-methyl ester hydrochloride (JR-227, compound 9), respectively, which were readily soluble in buffer. The conversion of JR-221 to the corresponding ortho-methyl ester compound did not proceed, possibly due to steric hindrance around the carboxyl group.

In addition to the two methyl ester compounds, a third unplanned addition to this series was (4-methoxy-3-hydroxybenzylidenamino)-guanidine hydrochloride (JR-228, compound 20). This compound was synthesized after the preparation and testing of JR-201 through JR-223, because of the structural similarity to (4-methoxy-2-nitrobenzylidenamino)-guanidine hydrochloride (JR-223, compound 21), which displayed interesting activity in the [3H][MK-801 displacement assay, as described in Section 3.4.2.

3.4.2 Evaluation of the Full Set of Aryldenamino-Guanidines as Modulatory Inhibitors

The set of forty-five arylidenamino-guanidino compounds were each evaluated at seven concentrations ranging from 16 mM to 1.0 μM, as described in Section 3.3 above. Further details of their analysis are prefaced in Section 2.4.3 of Chapter 2. Each of the arylidenamino-guanidines were renumbered in order to compare them in a more natural progression, roughly corresponding to electronegativity of substitutions. Therefore, similar groups of compounds are numbered together, in order to ease comparison of the effects of similar ring substituents on NMDAR inhibitory activity.

There were twenty-eight substituted benzylidenamine-guanidine compounds that incorporated only one aromatic moiety (compounds 1-28), with a wide range of attached groups. These compounds were synthesized and examined in the comparative dose-response curve assay. The compounds were either mono- or di-substituted at different positions around the benzene ring; structure-activity relationship information can be
gained by comparing and contrasting the IC\textsubscript{50}\textsubscript{pot} and Ratio\textsubscript{pot} values of these substituted benzyl compounds with those of the unsubstituted benzylidenamino-guanidine.

**Compound 1**, (benzylidenamino)-guanidine hydrochloride, incorporated an unsubstituted benzene ring. This compound had a 73.1 μM IC\textsubscript{50}\textsubscript{pot}, and a 4.6:1 Ratio\textsubscript{pot}. While the potency is not exceptional, the selectivity garnered from the Ratio\textsubscript{pot} is close to that of agmatine, which is surprising, considering that there are no attached groups which could interact in the same way as the terminal amino group. This compound serves as a good basis from which to compare the effects of additional groups on their potency and selectivity.

```
\begin{center}
\includegraphics[width=0.5\textwidth]{compound_1.png}
\end{center}

**Compound 1**

IC\textsubscript{50}\textsubscript{pot} = 73.1 μM
Ratio\textsubscript{pot} = 4.6 : 1

**Figure 3.2 Dose-response curves generated by compound 21, in the presence and absence of 100 μM spermidine**
Four aliphatic-substituted benzylidenamino-guanidines were prepared, all with substitutions at the para-position. These compounds should not contribute in any way to direct interaction with binding site residues, but these groups should contribute to the hydrophobicity of the Moiety A (see Figure 2.1), and to some degree, to the molecule as a whole. In addition, steric effects may alter the position of the molecules in the recognition domain, or the bulkier groups may even keep them from binding in certain sites. The curve-derived IC\textsubscript{50}\textsuperscript{pot}-values indicated that the 4-methyl compound was more potent than the unsubstituted compound, but the IC\textsubscript{50}\textsuperscript{pot} was slightly higher, and it was significantly less selective based on the Ratio\textsubscript{pot}. The most potent and selective of these compounds was the 4-isopropyl benzylidenamine guanidine (\textbf{Compound 3}), which had the same 4.6:1 Ratio\textsubscript{pot}, but was a full order of magnitude more potent than the unsubstituted compound, with an IC\textsubscript{50}\textsuperscript{pot} of 6.0 \textmu M. The 4-n-butyl and 4-\textit{tert}-butyl compounds laid between the previous two in terms of potency and selectivity (see Table 3.1). It would thus appear that the addition of a certain amount of lipophilicity contributes to the potency of these compounds (or perhaps this is due to simply steric effects), but these small aliphatic substituents only serve to decrease their selectivity.

\begin{table}[h]
\centering
\begin{tabular}{|c|c|c|c|}
\hline
\textbf{Compound} & \textbf{IC\textsubscript{50}\textsuperscript{pot}} & \textbf{Ratio\textsubscript{pot}} \\
\hline
2 & 80.7 \textmu M & 1.9:1 \\
3 & 6.0 \textmu M & 4.6:1 \\
4 & 42.5 \textmu M & 2.5:1 \\
5 & 22.8 \textmu M & 3.2:1 \\
\hline
\end{tabular}
\end{table}

A set of five more mono-substituted benzylidenamine-guanidines contained substituents which included heteroatoms directly connected to the phenyl ring. \textbf{Compounds 6 and 7} were defined by 3- and 4-cyano groups; while these compounds did possess terminal nitrogen-containing functionalities, the tertiary nitrile nitrogen atom contributes to any hydrogen bonding as an acceptor of hydrogen bonds, while most primary amines act as hydrogen bond donors at physiological pH. These compounds
seemed to suffer from the decrease in selectivity seen with small aliphatic groups, both having $\text{Ratio}_{\text{pot}}$ values lower than 2:1, with the *meta*-substituted nitrile compound showing five times the potency of the *para*-compound. **Compound 10** possessed an *ortho*-trifluoromethyl group. This molecule demonstrated good potency with a 25.1 $\mu$M $\text{IC}_{50}^{\text{pot}}$, but only a 2.8:1 $\text{Ratio}_{\text{pot}}$. The two methyl-ester compounds described in Section 3.4.1 above (the *meta*-substituted **compound 8** and *para*-substituted **compound 9**) have more intrinsic ability to interact with protein residues, with the $\text{C}=\text{O}$ electron pairs being very likely to form hydrogen bonds with protons from any number of amino acid groups. However, neither of these molecules displayed greater potency than the unsubstituted **compound 1**, and they appeared to be significantly less selective for the agmatine recognition site.

Four benzylidenamine-guanidines with exclusively mono- or di-substituted hydroxy or methoxy groups were originally prepared and evaluated. Each of these molecules should possess greater hydrophilicity than the unsubstituted **compound 1**. **Compound 11**, with 2,4-dihydroxy substitution, was significantly less potent and selective than the unsubstituted compound, but each of the methoxy compounds had more promising activity. Of these three, the 2-methoxy substituted **compound 12**, (2-
methoxybenzylidenamino)-guanidine hydrochloride, was most potent and selective with a 12.8 μM IC$_{50}^{\text{pot}}$ and a 4.9:1 Ratio$_{\text{pot}}$. **Compound 13**, a mono-methoxy compound substituted at the *para*-position, displayed slightly decreased selectivity and potency. **Compound 14**, 2,4-methoxybenzylidenamine-guanidine, was even less selective than either of the mono-substituted compounds. Nevertheless, each of these compounds was at least twice as potent as **compound 1**, indicating that the methoxy group is beneficial in an inhibitory molecule of this type, perhaps by simple steric effects, as is seen with **compound 3**.

A set of five compounds with mono-substituted nitrogen-containing groups were also prepared, including primary amine, dimethylamine, and nitro groups. The primary amines, being structurally more similar to agmatine, as well as **JR-83** and **JR-84**, were expected to afford inhibition similar to these polyamines, but the results were disappointing. **Compound 15**, containing a *meta*-amino substituent, had a 200 μM IC$_{50}^{\text{pot}}$ accompanied by a solid 3.9:1 Ratio$_{\text{pot}}$; **compound 16**, with a *para*-amino substituent, was twice as potent as **compound 15**, but had a lower Ratio$_{\text{pot}}$ of 1.2:1. None of these data were as promising as the previously examined xylenediamines from Chapter 2, nor the unsubstituted benzylidenamine-guanidine (**compound 1**). It is apparent that the terminal amino group needs to be more sterically flexible, since it is attached to a methylene group in the cases of **JR-83** and **JR-84**, and in agmatine itself. This would also explain the reduced activity seen in **JR-3** and **JR-5**, both of which have amino groups directly attached to the benzene ring. Another difference in the comparison of a methyl-amino group and a phenyl-amino group is that of pKa: the former would be protonated at physiological pH, while the latter is mostly unprotonated. The *para-*
dimethylaminobenzyl **compound 17** was observed to have the most potential as a therapeutic entity thus far: with a 7.9 μM IC<sub>50</sub><sup>pot</sup> and an excellent 12:1 Ratio<sub>pot</sub>, this compound was the most selective of any drug tested, due to a very shallow spermidine-potentiated curve slope. The lack of activity in **compound 16** would indicate that the presence of a para-amino group itself is not enough to contribute to agmatine site binding; however, this compound sterically resembles the isopropyl **compound 3**, which had nearly the same IC<sub>50</sub><sup>pot</sup>. The pKa of N,N-dimethylaniline is 5.15, thus in pH 7.25 buffer, the amine will not be significantly protonated; therefore the nitrogen in the dimethylamino substituent will have a lone pair which can act as a hydrogen bond acceptor, unlike the isopropyl group of **compound 3**. It is indeed possible that the steric effects of these two similar compounds contribute to their potency, while the hydrogen bond accepting ability of **compound 17** increases its selectivity for the agmatine binding site over the low-affinity NMDAR site, at which both of these compounds act to completely displace [³H]MK-801 at high concentrations. As can be seen in Section 3.6, the two curves generated by **compound 17** are similar to those of agmatine, but shifted to lower concentrations in the case of the former. The spermidine-potentiated and non-potentiated dose-response curves generated by **compound 17** nearly overlap at concentrations greater than 10 μM. However, at concentrations lower than 10 μM, **compound 17** inhibits [³H]MK-801 binding in the presence of spermidine, but has no effect in its absence. These activity data are consistent with the hypothesis that two binding sites for agmatine and its structural analogs exist. The activity of the two compounds with nitro-substituents, **compounds 18 and 19**, closely resemble those with the methyl ester substituents: these nitro-compounds are less potent and selective than the unsubstituted benzylidenamino-guanidine.
A single compound with a nitro- and methoxy-substituent, (4-methoxy-3-nitrobenzylidenamino)-guanidine hydrochloride, compound 21, was serendipitously chosen and synthesized based on availability of starting material in the lab. This compound was the first to show a clear separation between effects at the two postulated binding sites, and produced a biphasic curve in the presence of spermidine (Figure 3.3).

![Figure 3.3 Biphasic dose-response curve generated by compound 21](image)

Therefore, measurement of an $IC_{50}^{pot}$ was unnecessary, as this theoretical value was replaced by the $IC_{50}$ of the top phase of the curve, which was 7.8 $\mu$M. The midpoints of the two phases on the spermidine-potentiated curve were separated by 1.5 orders of magnitude; the bottom phase of the spermidine potentiated curve had an $IC_{50}$ of 259.4
µM, which was very close to the 219.8 µM IC₅₀ value of the non-spermdine potentiated curve. Structurally, this compound does little to explain its activity based on the previously examined benzylidenamine-guanidines. Neither the 3-nitro nor the 4-methoxy compounds (compounds 18 and 13, respectively) were as selective as the unsubstituted compound 1, much less this present compound 21. When attached to the same ring, however, these groups act harmoniously to produce a very selective NMDAR antagonist for the high affinity “agmatine” binding site. It is thus proposed that these substituents are each separately necessary for recognition at the agmatine binding site, and exclusion at the low-affinity site. An effort to distinguish the need for the 3-nitro group was undertaken in the synthesis of compound 20, (4-methoxy-3-hydroxybenzylidenamino)guanidine hydrochloride, in which the nitro group was replaced with a hydroxyl moiety. This compound was nearly an order of magnitude less potent than compound 21, and more than an order of magnitude less selective for the high-affinity agmatine binding site. The 4-methoxy-3-hydroxybenzyl moiety of compound 20 is structurally very similar to the second ring attached to compound 34, the only difference being that the terminal methyl group in compound 20 may rotate around the adjoining oxygen group, whereas in compound 34, it is replaced by a methylene group bridging both oxygen atoms. Compound 34, benzo[1,3]dioxol-5-ylmethyleneamino-guanidine hydrochloride, is a slightly better selective NMDA inhibitor than compound 20, but is again much less potent and selective than compound 21, again indicating the importance of the specific groups attached to the phenyl ring of compound 21, as opposed to simply steric effects.

<table>
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<tr>
<th>Compound</th>
<th>IC₅₀pot</th>
<th>Ratiopot</th>
</tr>
</thead>
<tbody>
<tr>
<td>Compound 20</td>
<td>199.5 µM</td>
<td>3.9 : 1</td>
</tr>
<tr>
<td>Compound 21</td>
<td>7.8 µM</td>
<td>28.2 : 1</td>
</tr>
<tr>
<td>Compound 34</td>
<td>7.9 µM</td>
<td>11.9 : 1</td>
</tr>
</tbody>
</table>

Seven mono- and di-halogeno benzylidenamino-guanidines were examined in this
work, with mixed but interesting results. Of the three chloro-substituted compounds, the least potent and selective was the meta-substituted compound, at 18.4 μM IC$_{50}^{\text{pot}}$ and 6.4:1 Ratio$_{\text{pot}}$. While these values were certainly an improvement over than the unsubstituted compound 1, the ortho-chloro analog possessed an IC$_{50}^{\text{pot}}$ of 7.1 μM and a 7.4:1 Ratio$_{\text{pot}}$, and the para-chloro analog, compound 24, afforded the second example of a biphasic spermidine-potentiation curve. The latter compound, (4-chlorobenzylidenameino)-guanidine hydrochloride, exhibited a 3.6 μM IC$_{50}$ for the top spermidine-potentiated phase of the curve. The IC$_{50}$ of the bottom phase of the potentiated curve and the IC$_{50}$ of the non-potentiated phase curve were identical at 124.5 μM. As with compound 21, there was a 1.5 order of magnitude difference between the two phases of the spermidine-potentiated curve, which corresponds in this case to a Ratio$_{\text{pot}}$ of nearly 35:1. The difference in activity between these three chloro analogs may be attributable to cavity accommodation of the large chloride molecule at the 4-position; however, the relative volumes of chloride and methyl groups are similar, and there was a vast difference in potency and selectivity between the para-chloro analog, compound 24, and the para-methyl analog, compound 2. Still, the fact that the ortho- and para-compounds were better on both counts than the meta-compound may be in part due to steric hindrance, but may also have to do with the electromeric and inductive effects of the highly electronegative chloro substituent. Electron-withdrawing groups at the ortho- and para-positions of a phenyl ring will result in a polarized bond with their adjoining ring carbon, rendering the 2- and 4-positions of the ring with partial positive charges, and a relatively negatively charged carbon at the 1- and 3-positions; the 1-position is the imine and the only other position of importance to activity. Conversely, electron-withdrawing groups at the meta-position of a phenyl ring will likewise create a partial-positive charge, but at the 3- and 1-positions of the phenyl ring. These electronic effects may partially explain the similarities between the ortho- and para-chlorobenzylidenameino-guanidine, and their difference to the meta-compound. However, if this were the only effect contributing to the activity, then compound 25, with chlorine substituents at both the 2- and 4-positions, would possess similar potency and selectivity. While its potency is still excellent with a 10.2 μM IC$_{50}^{\text{pot}}$, the 4.1:1 Ratio$_{\text{pot}}$ is far less remarkable than the ortho- or para-chloro mono-substituted compounds. In addition, 2-
fluoro-, 4-fluoro-, and 2,4-difluorobenzylidnamino-guanidine (compounds 26-28, respectively) are nearly an order of magnitude less potent than their chloro counterparts, with each possessing ratios of potentiation lower than compound 1. Fluorine is more electronegative than chlorine, although the size of carbon is more similar to that of fluorine than of chlorine, whose nonbonding electron pairs do not overlap very well with carbon’s 2p orbital. It is therefore deduced that while the electronegativity and position of the chlorine group may contribute to the inhibitory activity of compound 24, the size and molecular orbitals also appear to play important roles.

A single biphenyl compound was tested, with the guanidino-imine moiety in the 2-position. As discussed in section 4.1, the isomer with the 4-position substitution was water-insoluble and unable to be screened in this assay. Compound 29, the first of several screened with multiple aromatic rings, was among the most potent in this assay,
with an 8.5 μM IC$_{50}^{pot}$, but yielded a Ratio$_{pot}$ of a mere 1.6:1. These data indicate that this compound is not a good “agmatine binding site” antagonist, but is instead a very potent “low-affinity site” antagonist. It is apparent that this larger compound suffers from the same drawback as a number of the more bulky molecules screened in the study of chapter 2, in that these compounds appeared to be quite potent at the low-affinity binding site for agmatine and its analogs, which rendered them less therapeutically useful than compounds with a lower affinity for this site.

A series of four naphthylidenamino-guanidines were prepared, two of which were methylated, and all four possessed IC$_{50}^{pot}$ values lower than 30 μM. **Compounds 30**, (1-naphthylidenamino)-guanidine hydrochloride, and **32**, (2-methyl-1-naphthylidenamino)-guanidine hydrochloride, possessed very low selectivity for the agmatine binding site, with ratios of potentiation of 2.2:1 and 1.5:1, respectively. However, this series also yielded the third, albeit least definitive, biphasic curve in the study, from **compound 31**. This 2-naphthyl derivative possessed an 8.7 μM IC$_{50}$ for the top phase of the spermidine-potentiated curve (corresponding to the agmatine binding site), and a 117.8 μM IC$_{50}$ for the steeper bottom phase (corresponding to the low-affinity site). However, the non-potentiated curve and the lower phase of the spermidine-potentiated curve were not as closely overlapped as they were in the previous two biphasic compounds (see section 6), and the top-phase and bottom-phase IC$_{50}$ values were separated by barely one order of magnitude. **Compound 33** was also quite potent with an 11.5 IC$_{50}^{pot}$, and selective with a 7.0:1 Ratio$_{pot}$. This compound, (4-methyl-1-naphthylidenamino)-guanidine hydrochloride, is unexpectedly selective in that compounds 30 and 32 were the only other 1-naphthyl derivatives, and both demonstrated negligible selectivity. The only
explanation structurally is the movement of the methyl group from the 2-position to the 4-position on the naphthalene ring, which corresponds to a *para*-position on a phenyl group. The sterics in the case of compound 33 are slightly more aligned than those of compounds 30 or 32, as this molecule can be viewed as a benzylidenamino-guanidine with substituents at the *meta*- and *para*-positions. These two positions have adjoining groups in several of the most selective compounds, and the inclusion of the *para*-substituent (which is absent in compounds 30 and 32) appears to be vital in each of them (see Figure 3.4).
Nine compounds with heteroatom-containing aromatic rings were prepared and examined in this study, and while they were, as a whole, unable to improve on the previously-described arylidenamino-guanidines, they did contribute to the structural diversity of this library as well as that of the modeling study described in Chapter 5.

**Compound 35**, 5-(p-nitrophenyl)-2-furan carboxylidenamino)-guanidine hydrochloride, was synthesized due to the availability of the starting material, and its structural similarity to the biphenyl **compound 29**. While the compound did possess some activity with a 109.4 μM IC$_{50}^{pot}$, as with so many previously examined bulky compounds, the selectivity was ineffectual with a mere 2.1:1 Ratio$_{pot}$.

**Compound 36**, pyrrole with the imine substituent at the 2-position, proved to be the most selective of the compounds in this set. It yielded a meager 59.3 μM IC$_{50}^{pot}$, but the selectivity was on par with agmatine.

However, the 3- and 4-pyridino analogs (**compounds 37 and 38**, respectively), were the least potent of the entire arylidenamino-guanidine series, each with IC$_{50}$’s of potentiation near 0.5 mM. Including a 1.8:1 and 2.6:1 Ratio$_{pot}$ for the two compounds, these data were interesting, considering the structural similarity to **compound 1**. It is understandable that the pyridine nitrogen may interact with some resides in the low-affinity site to lower the selectivity of these compounds, but it is difficult to explain their
low potency at the high-affinity polyamine recognition site (the agmatine binding site) solely based on the substitution of a phenyl ring with a pyridyl ring. Even the 3-thiophenyl derivative (compound 43) was five times more potent than the pyridine derivatives, although it did not demonstrate any selectivity for the polyamine site with a 2.5:1 $\text{Ratio}_{\text{pot}}$. The remaining four heteroaromatic molecules screened in this study were substituted indole derivatives. The previous data obtained from compound AH-8, $N$-(1H-indol-5-yl)guanidine hydrochloride, showed this compound to be possess a high degree of potency, but a very low $\text{Ratio}_{\text{pot}}$; these observations held true in the case of the indolecarboxylidenaminoguanidines. The 5-methyl analog, compound 40, was 3 times less potent than its guanylated counterpart, but possessed a very similar 1.3:1 $\text{Ratio}_{\text{pot}}$. None of the remaining 3-indolyl analogs synthesized were as potent as AH-8 in the MK-801 displacement assay. The unsubstituted 3-indolyl analog, compound 39, demonstrated similar selectivity to compound 1, with a 4.7:1 $\text{Ratio}_{\text{pot}}$, but was less potent than agmatine; the 1- and 6-methyl compounds (41 and 42, respectively), lacked both the potency and selectivity of agmatine for the agmatine binding site.
The final two compounds examined in this study were trans-cinnamylidenamino-guanidine hydrochloride, an analog of compound 1 with an additional trans-double bond between the imine and the phenyl ring, and para-bis-benzylidenamino-guanidine hydrochloride, the only arylidenamino-guanidine screened containing two identical imine-guanidine groups, attached to the phenyl ring at the 1,4-positions. Neither of these compounds generated particularly interesting data. The former, compound 44, was slightly more potent than compound 1, with a decreased degree of selectivity for the agmatine binding site, and it is thus concluded that the addition of an extra conjugated double bond is not helpful towards developing a therapeutically useful molecule. The latter, compound 45, displayed the same lack of agmatine-site selectivity as the two di-guanidine compounds examined in chapter 2, JR-106 and JR-117, with all three possessing roughly 2:1:1 relative values for the IC$_{50}$ in the presence of spermidine, IC$_{50}$
in the absence of added spermidine, and $IC_{50}^{\text{pot}}$. These data reinforce the hypothesis that a single guanidine moiety is optimal for selectivity for the polyamine binding domain which recognizes agmatine.

![Chemical Structures](image)

<table>
<thead>
<tr>
<th>Compound 44</th>
<th>Compound 45</th>
</tr>
</thead>
<tbody>
<tr>
<td>$IC_{50}^{\text{pot}} = 58.3 \mu M$</td>
<td>$IC_{50}^{\text{pot}} = 32.2 \mu M$</td>
</tr>
<tr>
<td>Ratio$_{\text{pot}} = 2.8 : 1$</td>
<td>Ratio$_{\text{pot}} = 1.0 : 1$</td>
</tr>
</tbody>
</table>

3.5 Conclusions

A small library of forty-five arylidenamino-guanidine compounds were designed and synthesized in the pursuit of a more potent and selective NMDAR receptor inhibitor which would mimic the selective antagonist activity of agmatine. Previous studies performed in our laboratory generated a 100% hit rate for this class of compounds as NMDAR antagonists, which rendered this class worthy of further investigation. The earlier compounds with this structural motif, as well as new compounds which were based structurally on the success of the earlier compounds, were each evaluated in a P2 membrane assay for their ability to displace $[^3H]MK-801$ in the absence and presence of pathological levels of spermidine, and across a sufficient range of concentrations to obtain two full dose-response curves.

Each of these compounds were evaluated for NMDAR polyamine recognition site potency by examining their ability to reduce $[^3H]MK-801$ binding from spermidine-potentiated control levels to those in the absence of added spermidine. The term to indicate the MK-801 displacement potency of these compounds is “$IC_{50}$ of potentiation”, or $IC_{50}^{\text{pot}}$, which is measured by the concentration of substrate needed to displace MK-801 halfway from the spermidine-potentiated control level to the control level in the absence of added spermidine. A measure of selectivity of the “high-affinity” agmatine
site was obtained by comparing this potency to that derived from the non-spermidine potentiated curve, which corresponds to the “low-affinity” site, presumably the ion channel. This measure of selectivity is termed the “ratio of potentiation”, or $\text{Ratio}_{\text{pot}}$.

Three of these candidate molecules, (4-methoxy-3-nitrobenzylidenamino)-guanidine hydrochloride (compound 21), (4-chlorobenzylidenamino)-guanidine hydrochloride (compound 24), and (2-naphthylidenamino)-guanidine hydrochloride (compound 31), generated biphasic spermidine-potentiated curves, which clearly separated the inhibitory activity at the two binding sites, in the former two cases, by over 1.5 orders of magnitude. Each of these compounds was also quite potent at the agmatine binding site, with $\text{IC}_{50}$ values for the corresponding high-affinity phase of the curve being measured at less than 10 $\mu$M.

Important structure-activity data was interpreted by scrutiny of each compound in this set, and several observations have been made which postulate the importance of steric and electronic effects on both the agmatine-site affinity and selectivity of these molecules.

While several promising lead compounds have resulted from this study, the activity of this group of arylidenamino-guanidines as a whole attests to the importance of further examination of the structural descriptors of each molecule as they relate to inhibitory activity. Molecular modeling with a neural network approach will thus be undertaken with this set of structurally similar molecules in an effort to facilitate further development of this intriguing set of compounds (Chapter 5). Thereafter, an attempt will be made to postulate a pharmacophore which will be developed based on the results generated in the whole of this work (Chapter 6).
3.6 Experimental Protocol

(see notes in section 2.6 for further details)

**COMPOUND 1 (AG17)**

![Chemical structure](image)

*(Benzylidenamino)-guanidine Hydrochloride*

Benzaldehyde (159 mg, 1.5mmol) and aminoguanidine hydrochloride (110 mg, 1 mmol) were dissolved in methanol (15 mL) and reacted according to the above general method. The product solution was dissolved on the rotary evaporator, and the residue was stirred in chloroform and filtered to remove the remaining starting material. The filtered product was dried on vacuum overnight to afford an off white powdery solid (158 mg, 0.8 mmol, 80 % yield, 140-141 °C melting point). $^1$H NMR (300MHz,D$_2$O) δ 8.0 (1H s), 7.76-7.63 (2H,m), 7.50-7.48 (3H,m); $^{13}$C NMR (DMSO-d$_6$) δ 155.2, 146.5, 133.2, 130.3, 128.5, 127.4.

**SUN-AG17**

![Graph](image)
**COMPOUND 2 (AG6)**

![Chemical Structure](attachment:image.png)

*(4-Methylbenzylidenamino)-guanidine Hydrochloride*

\[\begin{array}{c}
\text{H}_3\text{C} & \text{N} \\
\text{H} & \text{N} \\
\text{N} & \text{NH}_2 \\
\text{NH}_2 & \text{Cl}
\end{array}\]

\[\text{p-Tolualdehyde (180mg, 1.5mmol) and aminoguanidine hydrochloride (110 mg, 1mmol) were dissolved in methanol (15mL) and reacted according to the above general method. The product solution was dissolved on the rotary evaporator, and the residue was stirred in chloroform and filtered to remove the remaining starting material. The filtered product was dried on vacuum overnight to afford a colorless crystalline solid (174 mg, 0.82 mmol, 82 % yield, 170-171°C melting point).}^{1}\text{H NMR (300MHz, D}_2\text{O)} \delta 7.88 (1H,s), 7.56 (2H,d, J=8.2 Hz), 7.30 (2H,d, J=8.2 Hz), 2.38 (3H,s); \]^{13}\text{C NMR (DMSO-d}_6\text{)} \delta 155.3, 146.5, 140.1, 130.5, 129.1, 127.4, 21.1.

**SUN-AG6**

![Graph](attachment:graph.png)

**SUN-AG6**

<table>
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<th>DPM</th>
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<tbody>
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</table>

153
4-Isopropylbenzaldehyde (222mg, 1.5mmol) and aminoguanidine hydrochloride (110mg, 1.0 mmol) were dissolved in methanol (15mL) and reacted according to the above general method. The product solution was dissolved on the rotary evaporator, and the yellow residue was stirred in diethyl ether and filtered to remove the remaining starting material. The filtered product was recrystallized from ethyl acetate to yield hygroscopic yellow needle crystals, which immediately became amorphous upon exposure to atmospheric water (65mg, 0.27mmol, 27% yield). $^1$H NMR (400MHz, d-DMSO) δ 12.02 (1H, s), 8.14 (1H, s), 7.78 (2H, d, J = 8.0Hz), 7.31 (2H, d, J = 8.4Hz), 2.92 (1H, m), 1.21 (6H, d, J = 6.8Hz); $^{13}$C NMR δ 155.4, 151.2, 146.8, 131.2, 127.7, 126.7, 33.4, 23.7; ES+ m/z 205.
COMPOUND 4 (JR-212)

(4-n-Butylbenzylidenamino)-guanidine Hydrochloride

4-Butylbenzaldehyde (243mg, 1.5 mmol) and aminoguanidine hydrochloride (110mg, 1.0 mmol) were dissolved in methanol (15mL) and reacted according to the above general method. The product solution was dissolved on the rotary evaporator, and the light yellow liquid residue was stirred in ethyl ether and filtered to remove the remaining starting material. The filtered product was dried on vacuum overnight to afford a coarse off-white powdery solid (231mg, 0.91 mmol, 91% yield, 134-135°C melting point). $^1$H NMR (400MHz, d-DMSO) $\delta$ 12.02 (1H, s), 8.14 (1H, s), 7.76 (2H, d, $J = 8$ Hz), 7.26 (2H, d, $J = 8$Hz), 2.60 (2H, t, $J = 7.6$ Hz), 1.55 (2H, p, $J = 7.6$ Hz), 1.29, (2H, sextet, $J = 7.7$Hz), 0.89 (3H, t, $J = 7.8$Hz); $^{13}$C NMR $\delta$ 155.4, 146.8, 145.2, 131.0, 128.6, 127.6, 34.7, 32.9, 21.8, 13.8; ES+ m/z 219.
**COMPOUND 5 (JR-211)**

(4-tert-Butylbenzylidnamino)-guanidine Hydrochloride

4-tert-Butylbenzaldehyde (243mg, 1.5 mmol) and aminoguanidine hydrochloride (110mg, 1.0 mmol) were dissolved in methanol (15mL) and reacted according to the above general method. The product solution was dissolved on the rotary evaporator, and the residue was stirred in ethyl ether and filtered to remove the remaining starting material. The filtered product was dried on vacuum overnight to afford an off-white powdery solid (203mg, 0.80mmol, 80% yield, 214-215°C melting point). $^1$H NMR (400MHz, d-DMSO) δ 12.12 (1H, s), 8.15, (1H, s), 7.77 (2H, dd, J = 8.0Hz), 7.45 (2H, m), 1.28 (9H, t, J = 2.8Hz); $^{13}$C NMR δ 155.4, 153.3, 146.6, 130.8, 127.4, 125.5, 34.6, 30.9; ES+ m/z 219.

**JR-211**

![Graph showing DPM vs. log [M] Concentration for JR-211 and JR-211+SPD](image)
COMPOUND 6 (JR-124)

(3-Cyanobenzylidenamino)-guanidine Hydrochloride

3-Cyanobenzaldehyde (491mg, 3.75 mmol) and aminoguanidine hydrochloride (276.4mg, 2.5 mmol) were dissolved in methanol (50mL) and reacted according to the above general method. The product solution was dissolved on the rotary evaporator, and the residue was stirred in chloroform and filtered to remove the remaining starting material. The filtered product was dried on vacuum overnight to afford an off-white powdery solid (257 mg, 1.15 mmol, 46% yield, 235-239ºC melting point). $^1$H NMR (300MHz, d- D$_2$O) $\delta$ 12.18 (1H, s), 8.48 (1H, t, $J = 1.5$Hz), 8.19 (1H, s), 8.12 (1H, dt, $J = 8.3, 1.5$Hz), 7.87 (1H, dt, $J = 8.3, 1.5$Hz), 7.64 (1H, t, $J = 8.3$Hz); $^{13}$C NMR $\delta$ 155.4, 144.2, 134.7, 133.2, 132.3, 130.3, 129.8, 118.4, 111.8; ES+ m/z 188.
4-Cyanobenzaldehyde (983mg, 7.5 mmol) and aminoguanidine hydrochloride (553mg, 5.0 mmol) were dissolved in methanol (50mL) and reacted according to the above general method. The product solution was dissolved on the rotary evaporator, and the residue was stirred in chloroform and filtered to remove the remaining starting material. The filtered product was dried on vacuum overnight to afford a white powdery solid (944 mg, 4.22 mmol, 84% yield, 264-267°C melting point). $^1$H NMR (300MHz, d-DMSO) $\delta$ 12.22 (1H, s), 8.22 (1H, s), 8.07 (2H, d, $J = 9.0$Hz), 7.91 (2H, d, $J = 9.0$Hz); $^{13}$C NMR $\delta$ 155.4, 144.6, 137.7, 132.4, 128.0, 118.5, 112.1; ES+ m/z 188.
COMPOUND 8 (JR-226)

Benzylidenaminoguanidine-3-methyl ester Hydrochloride

3-Carboxybenzaldehyde (223mg, 1.5 mmol) and aminoguanidine hydrochloride (110mg, 1.0 mmol) were dissolved in methanol (15mL) and reacted according to the above general method. The product solution was dissolved on the rotary evaporator, and the white solid residue was stirred in chloroform and filtered to remove the remaining starting material. The filtered product was dried on vacuum overnight to afford a (3-carboxybenzylidenamino)-guanidine hydrochloride as a white powdery solid (226mg, 0.93mmol, 93% yield, 276-278°C melting point). This intermediate (100 mg, 0.4 mmol) was dissolved in methanol (5mL), and thionyl chloride (2.0 mmol, 238 mg, 146μL) was added dropwise. The solution was stirred at room temperature for 18 hours, then the solvent removed by rotary evaporation and the resulting solid left on the vacuum pump overnight to yield the light yellow solid product (72mg, 0.28 mmol, 70% yield, 213-215°C melting point). $^1$H NMR (300MHz, d-DMSO) $\delta$ 12.18 (1H, s), 8.36 (1H, slight triplet), 8.28 (1H, s), 8.19 (1H, d, J = 7.9Hz), 8.00 (1H, d, J = 7.9Hz), 7.6 (1H, t, J = 8Hz), 3.88 (3H, s); $^{13}$C NMR $\delta$ 165.6, 155.3, 145.7, 133.9, 131.7, 130.6, 130.1, 129.0, 128.2, 52.3; ES+ m/z 221.

JR-226
4-Carboxybenzaldehyde (300mg, 2.0 mmol) and aminoguanidine hydrochloride (143mg, 1.3 mmol) were dissolved in methanol (15mL) and reacted according to the above general method. The product solution was dissolved on the rotary evaporator, and the white solid residue was stirred in chloroform and filtered to remove the remaining starting material. The filtered product was dried on vacuum overnight to afford a (3-carboxylbenzylidenamino)-guanidine hydrochloride as a cream-colored powdery solid (223mg, 0.92 mmol, 71% yield, 276-281ºC melting point). This intermediate (50 mg, 0.2 mmol) was dissolved in methanol (5mL), and thionyl chloride (1.0 mmol, 119 mg, 73µL) was added dropwise. The solution was stirred at room temperature for 18 hours, then the solvent removed by rotary evaporation and the resulting solid left on the vacuum pump overnight to yield the off-white solid product (36mg, 0.14 mmol, 70% yield, 251-253ºC melting point). $^1$H NMR (300MHz, d-DMSO) δ 8.26 (1H, s), 8.005 (2H, m), 7.999 (2H, m), 3.86 (3H, s); $^{13}$C NMR δ 165.6, 155.4, 145.2, 137.7, 130.6, 129.2, 127.6, 52.3; ES+ m/z 221.

**JR-227**

![Graph showing the concentration (log [M]) vs. DPM for JR-227 and JR-227+SPD](image)
2-(Trifluoromethyl)benzaldehyde (260 mg, 1.5 mmol) and aminoguanidine hydrochloride (110 mg, 1 mmol) were dissolved in methanol (15 mL) and reacted according to the above general method. The product solution was dissolved on the rotary evaporator, and the residue was stirred in chloroform and filtered to remove the remaining starting material. The filtered product was dried on vacuum overnight to afford an off white powdery solid (226 mg, 0.89 mmol, 89% yield, 172-173°C melting point). $^1$H NMR (300MHz,D$_2$O) δ 8.2 (1H,s), 8.1 (1H,d, J=8.1Hz), 7.6 (1H,d, J=7.5 Hz), 7.63 (2H, m); $^{13}$C NMR (DMSO- d$_6$) δ 155.2, 141.5, 132.5, 130.9, 130.4, 127.5, 126.4, 125.7, 109.1.
2,4-Dihydroxybenzaldehyde (207 mg, 1.5 mmol) and aminoguanidine hydrochloride (110 mg, 1 mmol) were dissolved in methanol (15 mL) and reacted according to the above general method. The product solution was dissolved on the rotary evaporator, and the residue was stirred in chloroform and filtered to remove the remaining starting material. The filtered product was dried on vacuum overnight to afford a light brown powdery solid (182 mg, 0.79 mmol, 79 % yield, 231-232 °C melting point). $^1$H NMR (300MHz, D$_2$O) δ 7.86(1H,s), 7.23 (1H,d, J=8.5 Hz, 6.4 (1H,dd, J= 2.7 Hz, J= 8.5Hz), 6.3 (1H,d,J=2.7Hz) ; $^{13}$C NMR (DMSO-d$_6$) δ 160.9, 158.0, 154.8, 143.9, 128.0, 110.9, 107.7, 102.2.

SUN-AG13

![Graph](image-url)
**COMPOUND 12 (AG-12)**

(2-Methoxybenzylidenamino)-guanidine Hydrochloride

_o-Anisaldehyde (204mg, 1.5mmol) and aminoguanidine hydrochloride (110 mg, 1 mmol) were dissolved in methanol (15 mL) and reacted according to the above general method. The product solution was dissolved on the rotary evaporator, and the residue was stirred in chloroform and filtered to remove the remaining starting material. The filtered product was dried on vacuum overnight to afford an off white powdery solid (169 mg, 0.74 mmol, 74 % yield, 232-233°C melting point)._\(^1\)H NMR (300MHz, DMSO-d\(_6\)) \(\delta\) 12.3 (1H, broad), 8.45 (1H, s), 8.1 (1H,m) 7.8 (NH, broad) 7.45 (1H,m), 7.1 (1H,d), 7.0 (1H,m) ; \(^13\)C NMR \(\delta\) 157.5, 155.1, 141.8, 132.0, 126.1, 121.1, 120.4, 111.7, 55.7

**SUN-AG12**

![Graph showing DPM vs log [M] Concentration](image-url)
p-Anisaldehyde (204 mg, 1.5 mmol) and aminoguanidine hydrochloride (110 mg, 1 mmol) were dissolved in methanol (15 mL) and reacted according to the above general method. The product solution was dissolved on the rotary evaporator, and the residue was stirred in chloroform and filtered to remove the remaining starting material. The filtered product was dried on vacuum overnight to afford a light brown powdery solid (157 mg, 0.69 mmol, 69% yield, 110-111°C melting point). $^1$H NMR (300MHz, D$_2$O) $\delta$ 7.70 (1H, s), 7.55 (2H, d, J=8.0 Hz), 6.95 (2H, d, J=8.0Hz), 3.85 (3H, s); $^{13}$C NMR (DMSO-d$_6$) $\delta$ 160.9, 155.2, 146.3, 129.1, 125.9, 114.0, 55.3.
2,4-Dimethoxybenzaldehyde (174 mg, 1.5 mmol) and aminoguanidine hydrochloride (110 mg, 1 mmol) were dissolved in methanol (15mL) and reacted according to the above general method. The product solution was dissolved on the rotary evaporator, and the residue was stirred in chloroform and filtered to remove the remaining starting material. The filtered product was dried on vacuum overnight to afford an off white powdery solid (157 mg, 0.61 mmol, 61% yield, 203-204 °C melting point). $^1$H NMR (300MHz, DMSO- $d_6$) δ 12.0 (1H,broad), 8.35 (1H,s), 8.0 (1H,d), 7.7 (NH, broad) 6.64 (1H,s), 6.60 (2H,m); $^{13}$C NMR δ 162.7, 159.1, 154.9, 127.4, 113.9, 106.5, 97.9, 55.9, 55.5.
3-Aminobenzaldehyde was prepared by the dropwise addition of 2.0M HCl in ethanol (6.0 mL) to the corresponding 3-aminobenzaldehyde ethylene acetal (902 mg, 5.97) in methanol (34 mL). The reaction solution darkened with the acid addition, from very pale orange to dark yellow, and during stirring at room temperature for 30 minutes, the color returned to a light orange, and TLC revealed a completed reaction. Aminoguanidine hydrochloride (111mg, 1.0 mmol) was added to a 15-mL sample of the methanolic reaction solution (0.176 M, 2.64 mmol aldehyde), and the mixture refluxed for 3 hours. The reaction was then cooled to room temperature, and fine tan crystals (35.5 mg, 0.16 mmol, 16% yield, 240-246ºC melting point) crashed out of solution, which were collected and rinsed with cold methanol. $^1$H NMR (300MHz, d-DMSO) δ 12.14 (1H, s), 8.20 (1H, s), 7.84 (1H, d, J = 7.8Hz), 7.63 (1H, s), 7.49 (1H, t, J = 7.8Hz), 7.36 (1H, d, J = 7.8Hz); $^{13}$C NMR δ 155.2, 145.8, 134.6, 129.8, 124.7, 123.5, 120.9; ES+ m/z 178.
(4-Aminobenzylidenamino)-guanidine Hydrochloride

4-Aminobenzaldehyde (60mg, 0.5 mmol, prepared by the method of Tetrahedron Letters, Vol. 25 No. 8, 1984, pp. 839-842) and aminoguanidine hydrochloride (44mg, 0.4 mmol) were dissolved in methanol (10mL) and reacted according to the above general method. The product solution was dissolved on the rotary evaporator, and the residue was stirred in chloroform and filtered to remove the remaining starting material. The filtered product was dried on vacuum overnight to afford a light brown powdery solid (46 mg, 0.22 mmol, 55% yield, 169-173°C melting point). \(^1\)H NMR (300MHz, d-DMSO) \(\delta\) 11.42 (1H, s), 7.93 (1H, s), 7.50 (2H, d, \(J = 9\)Hz), 6.58 (2H, d, \(J = 9\)Hz), 5.74 (2H, s); \(^13\)C NMR (300MHz, d-D\(_2\)O) \(\delta\) 187.7, 152.3, 152.2, 131.9, 126.0, 118.6; ES+ m/z 178.
(4-Dimethylamino-benzylidenamino)-guanidine Hydrochloride

*p*-Dimethylaminobenzaldehyde (298.3mg, 2.0 mmol) and aminoguanidine hydrochloride (144mg, 1.3 mmol) were dissolved in methanol (15mL) and reacted according to the above general method. The product solution was dissolved on the rotary evaporator, and the solid residue was stirred in chloroform and filtered to remove the remaining starting material. The filtered product was dried on vacuum overnight to afford a tan-yellow powdery solid (112mg, 0.46 mmol, 36% yield, 179-182°C melting point). $^1$H NMR (400MHz, d-DMSO) δ 8.02 (1H, s), 7.63 (2H, d, J = 8.8Hz), 6.71 (2H, d, J = 8.8Hz), 2.95 (6H, s); $^{13}$C NMR δ 155.0, 151.7, 147.5, 129.0, 120.6, 111.6, 39.9; ES+ m/z 206.

![Graph](https://example.com/graph.png)
COMPOUND 18 (JR-122)

(3-Nitrobenzyldenamino)-guanidine Hydrochloride

3-Nitrobenzaldehyde (1.13g, 7.5 mmol) and aminoguanidine hydrochloride (553mg, 5.0 mmol) were dissolved in methanol (50mL) and reacted according to the above general method. The product solution was dissolved on the rotary evaporator, and the residue was stirred in chloroform and filtered to remove the remaining starting material. The filtered product was dried on vacuum overnight to afford a pale yellow powdery solid (1109 mg, 4.55 mmol, 91% yield, 267-268°C melting point). $^1$H NMR (300MHz, d-D$_2$O) δ 8.53 (1H, t, J = 2.0Hz), 8.28 (1H, ddd, J = 9.0, 2.0, 1.3Hz), 8.09 (1H, s), 8.03 (1H, td, J = 9.0, 1.3Hz), 7.66 (1H, t, J = 9.0Hz); $^{13}$C NMR δ 155.5, 148.2, 146.1, 134.7, 133.7, 130.1, 125.1, 121.8; ES+ m/z 208.
4-Nitrobenzaldehyde (1.13g, 7.5 mmol) and aminoguanidine hydrochloride (553mg, 5.0 mmol) were dissolved in methanol (50mL) and reacted according to the above general method. The product solution was dissolved on the rotary evaporator, and the residue was stirred in chloroform and filtered to remove the remaining starting material. The filtered product was dried on vacuum overnight to afford a light-yellow powdery solid (768 mg, 3.15 mmol, 63% yield, 252-254°C melting point). $^1$H NMR (300MHz, d-D$_2$O) δ 8.23 (2H, m), 8.0 (1H, m), 7.81 (2H, m); $^{13}$C NMR δ 155.4, 147.9, 144.2, 139.6, 128.4, 123.7; ES+ m/z 208.
3-Hydroxy-p-anisaldehyde (456mg, 3.0 mmol) and aminoguanidine hydrochloride (110mg, 1.0 mmol) were dissolved in methanol (5mL) and reacted according to the above general method. The product solution was dissolved on the rotary evaporator, and the white solid residue was stirred in chloroform and filtered to remove the remaining starting material. The filtered product was dried on vacuum overnight to afford a white powdery solid (163mg, 0.79 mmol, 79% yield, 209-210°C melting point). $^1$H NMR (300MHz, d-DMSO) δ 11.84 (1H, s), 9.146 (1H, s), 8.02 (1H, s), 7.34 (1H, d, J = 2.1Hz), 7.20 (1H, dd, J = 8.4, 2.1Hz), 6.97 (1H, d, J = 8.4Hz); $^{13}$C NMR δ 155.1, 149.8, 146.9, 146.4, 126.1, 120.4, 113.4, 111.5, 55.65; ES+ m/z 209.
COMPOUND 21 (JR-223)

(4-Methoxy-3-Nitrobenzylidenamino)-guanidine Hydrochloride

4-Methoxy-3-nitrobenzaldehyde (287mg, 1.6 mmol) and aminoguanidine hydrochloride (110mg, 1.0 mmol) were dissolved in methanol (15mL) and reacted according to the above general method. The product solution was dissolved on the rotary evaporator, and the yellow residue was stirred in chloroform and filtered to remove the remaining starting material. The filtered product was dried on vacuum overnight to afford a yellow powdery solid (191mg, 0.70mmol, 70% yield, 244-247°C melting point). $^1$H NMR (400MHz, d-DMSO) $\delta$ 12.23 (1H, s), 8.47 (1H, d, $J = 2.0$Hz), 8.18 (1H, s), 8.11 (1H, dd, $J = 4.8$Hz, 2.0Hz), 7.42 (1H, d, $J = 4.8$Hz), 3.97 (3H, s); $^{13}$C NMR $\delta$ 155.5, 152.8, 144.3, 139.8, 133.5, 126.3, 123.0, 114.4, 57.0; ES+ m/z 238.

JR-223

![Graph showing DPM vs log [M] Concentration with JR-223 and JR-223+SPD]
2-Chlorobenzaldehyde (280mg, 2.0 mmol) and aminoguanidine hydrochloride (144 mg, 1.3 mmol) were dissolved in methanol (15mL) and reacted according to the above general method. The product solution was dissolved on the rotary evaporator, and the yellow liquid residue was left on a vacuum pump overnight, producing a white solid. This solid was then stirred in chloroform and filtered to remove the remaining starting material. The filtered product was dried on vacuum overnight to afford a white powdery solid (248mg, 1.06 mmol, 82% yield, 178-181°C melting point). "H NMR (400MHz, d-DMSO) δ 12.36 (1H, s), 8.55 (1H, s), 8.30 (1H, dd, J = 7.6Hz, 2.0Hz), 7.53 (1H, dd, J = 7.8Hz, 1.6Hz), 7.47 (1H, dt, J = 7.6Hz, 2.0Hz), 7.42 (1H, dt, J = 7.4Hz, 1.4Hz); C NMR δ 155.4, 142.6, 133.2, 131.9, 130.6, 129.9, 127.7, 127.5; ES+ m/z 197, 199.
COMPOUND 23 (JR-217)

(3-Chlorobenzylidenamino)-guanidine Hydrochloride

3-Chlorobenzaldehyde (280mg, 2.0 mmol) and aminoguanidine hydrochloride (144mg, 1.3 mmol) were dissolved in methanol (15mL) and reacted according to the above general method. The product solution was dissolved on the rotary evaporator, and the white solid residue was stirred in chloroform and filtered to remove the remaining starting material. The filtered product was dried on vacuum overnight to afford a white powdery solid (223mg, 1.13 mmol, 87% yield, 226-229ºC melting point). $^1$H NMR (300MHz, d-DMSO) δ 12.13 (1H, s), 8.16 (1H, s), 8.09 (1H, s), 7.75 (1H, m), 7.48 (1H, m), 7.46 (1H, m); $^{13}$C NMR δ 155.3, 145.0, 135.5, 133.5, 130.4, 129.9, 126.7, 126.2; ES+ m/z 197, 199.
4-Chlorobenzaldehyde (281mg, 2.0 mmol) and aminoguanidine hydrochloride (144mg, 1.3 mmol) were dissolved in methanol (15mL) and reacted according to the above general method. The product solution was dissolved on the rotary evaporator, and the white solid residue was stirred in chloroform and filtered to remove the remaining starting material. The filtered product was dried on vacuum overnight to afford a white powdery solid (173mg, 0.74 mmol, 57% yield, 171-173°C melting point). $^1$H NMR (400MHz, d-DMSO) δ 12.22 (1H, s), 8.18 (1H, s), 7.91 (2H, d, J = 8.8Hz), 7.51 (2H, d, J = 8.4Hz); $^{13}$C NMR δ 155.5, 145.4, 134.9, 132.5, 129.3, 128.8; ES+ m/z 197, 199.
2,4-Dichlorobenzaldehyde (262 mg, 1.5 mmol) and aminoguanidine hydrochloride (110 mg, 1 mmol) were dissolved in methanol (15 mL) and reacted according to the above general method. The product solution was dissolved on the rotary evaporator, and the residue was stirred in chloroform and filtered to remove the remaining starting material. The filtered product was dried on vacuum overnight to afford an off white powdery solid (150 mg, 0.56 mmol, 56 % yield, 222-223°C melting point). $^1$H NMR (300MHz, DMSO-d$_6$) δ 8.4 (1H, s), 8.3 (1H, d, J=8.4Hz), 7.89 (NH, broad hump), 7.6 (1H,m), 7.4 (1H,m). $^{13}$C NMR (DMSO-d$_6$) δ 155.3, 141.1, 135.3, 133.7, 129.7, 129.1, 128.7, 127.6.
COMPOUND 26 (AG-23)

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(2-Fluorobenzylidnamino)-guanidine Hydrochloride

2-Fluorobenzaldehyde (186 mg, 1.5 mmol) and aminoguanidine hydrochloride (110 mg, 1 mmol) were dissolved in methanol (15 mL) and reacted according to the above general method. The product solution was dissolved on the rotary evaporator, and the residue was stirred in chloroform and filtered to remove the remaining starting material. The filtered product was dried on vacuum overnight to afford an off white powdery solid (158 mg, 0.73 mmol, 73% yield, 170-171°C melting point).\(^1\)\(^1\)H NMR (300MHz, D\(_2\)O) \(\delta\) 8.0 (1H,s), 7.80 (1H,m), 7.70 (1H,m), 7.18 (1H,m); \(^1\)\(^3\)C NMR (DMSO-d\(_6\)) \(\delta\) 162.2, 155.2, 139.1, 132.4, 126.9, 124.6, 120.9, 115.9.

SUN-AG23
4-Fluorobenzaldehyde (186 mg, 1.5 mmol) and aminoguanidine hydrochloride (110 mg, 1 mmol) were dissolved in methanol (15 mL) and reacted according to the above general method. The product solution was dissolved on the rotary evaporator, and the residue was stirred in chloroform and filtered to remove the remaining starting material. The filtered product was dried on vacuum overnight to afford a white powdery solid (151 mg, 0.70 mmol, 70 % yield, 189-190ºC melting point). $^1$H NMR (300MHz,D$_2$O) δ 7.87 (1H,s), 7.6 (2H,m), 7.15 (2H,m); $^{13}$C NMR (DMSO- d$_6$) δ 161.0, 155.3, 145.3, 129.7, 115.7
2,4-Difluorobenzaldehyde (213 mg, 1.5 mmol) and aminoguanidine hydrochloride (110 mg, 1 mmol) were dissolved in methanol (15 mL) and reacted according to the above general method. The product solution was dissolved on the rotary evaporator, and the residue was stirred in chloroform and filtered to remove the remaining starting material. The filtered product was dried on vacuum overnight to afford a white powdery solid (187 mg, 0.80 mmol, 80 % yield, 217-218°C melting point). $^1$H NMR (300MHz, D$_2$O) δ 8.0 (1H,s), 7.84 (1H,m), 7.0 (2H,m); $^{13}$C NMR(DMSO-d$_6$) δ 161.6, 159.1, 155.2, 138.2, 128.5, 117.9, 112.4, 104.2.

SUN-AG24
2-Biphenylcarboxaldehyde (273mg, 1.5mmol) and aminoguanidine hydrochloride (110mg, 1.0 mmol) were dissolved in methanol (15mL) and reacted according to the above general method. The product solution was dissolved on the rotary evaporator, and the white solid residue was recrystallized in chloroform to afford white short crystals (131mg, 0.48 mmol, 48% yield, 205-208ºC melting point). $^1$H NMR (400MHz, d-DMSO) δ 12.02 (1H, s), 8.33 (1H, d, J = 7.6Hz), 8.06 (1H, s), 7.48 (5H, m), 7.34 (3H, m); $^{13}$C NMR δ 155.4, 145.0, 142.1, 138.8, 130.5, 130.3, 129.6, 128.9, 128.6, 128.5, 128.2, 127.7, 127.6, 126.4; ES+ m/z 239.
1-Naphthaldehyde (234 mg, 1.5 mmol) and aminoguanidine hydrochloride (110 mg, 1 mmol) were dissolved in methanol (15 mL) and reacted according to the above general method. The product solution was dissolved on the rotary evaporator, and the residue was stirred in chloroform and filtered to remove the remaining starting material. The filtered product was dried on vacuum overnight to afford a cream powdery solid (186 mg, 0.75 mmol, 75 % yield, 143-144°C melting point). $^1$H NMR (300MHz, D$_2$O) δ 8.24 (1H,m), 8.17 (1H,s), 7.90(2H,m), 7.66 (1H,d), 7.61 (2H,m), 7.46 (1H,m) ; $^{13}$C NMR (DMSO- d$_6$) δ 155.2, 145.4, 133.2, 130.7, 130.2, 128.6, 128.4, 127.2, 126.4, 126.1, 125.3, 123.1.
2-Naphthaldehyde (234mg, 1.5 mmol) and aminoguanidine hydrochloride (110mg, 1.0 mmol) were dissolved in methanol (15mL) and reacted according to the above general method. Upon cooling, clear white crystals fell out of the orange solution, and were filtered and collected. The product crystals were stirred in chloroform and filtered to remove the remaining starting material, leaving the fine white crystal product (164mg, 0.66 mmol, 66% yield, 213-215°C melting point). $^1$H NMR (400MHz, d-DMSO) δ 12.17 (1H, s), 8.35 (1H, s), 8.20 (2H, m), 7.96 (3H, m), 7.57 (2H, m); $^{13}$C NMR δ 155.4, 146.8, 133.9, 132.7, 131.2, 129.5, 128.4, 128.3, 127.8, 127.4, 126.8, 123.0; ES+ m/z 213.
2-Methyl-1-naphthaldehyde (510mg, 3.0 mmol) and aminoguanidine hydrochloride (110mg, 1.0 mmol) were dissolved in methanol (15mL) and reacted according to the above general method (a first synthetic attempt produced a very low yield, thus the procedure was repeated with a 5:1 excess of aldehyde starting material). The product solution was dissolved on the rotary evaporator, and the residue was stirred in chloroform and filtered to remove the remaining starting material. The filtered product was dried on vacuum overnight to afford a cream-colored powdery solid (190mg, 0.72 mmol, 72% yield, 130-133°C melting point). $^1$H NMR (300MHz, d-DMSO) δ 12.18 (1H, s), 8.91 (1H, s), 8.37 (1H, d, J = 7.5Hz), 7.94 (1H, d, J = 4.2Hz), 7.92 (1H, d, J = 5.4Hz), 7.55 (2H, m), 7.46 (1H, d, J = 8.4Hz), 2.59 (3H, s); $^{13}$C NMR δ 133.2, 146.4, 136.3, 131.7, 130.8, 129.7, 129.2, 128.2, 127.2, 126.6, 125.3, 124.3, 20.7; ES$^+$ m/z 227.
4-Methyl-1-naphthaldehyde (225mg, 1.5 mmol) and aminoguanidine hydrochloride (110mg, 1.0 mmol) were dissolved in methanol (15mL) and reacted according to the above general method. The product solution was dissolved on the rotary evaporator, and the yellow solid residue was stirred in chloroform and filtered to remove the remaining starting material. The filtered product was dried on vacuum overnight to afford an off-white powdery solid (209mg, 0.80mmol, 80% yield, 187-189°C melting point). $^1$H NMR (300MHz, d-DMSO) $\delta$ 12.01 (1H, s), 8.99 (1H, s), 8.48 (1H, dd, $J = 7.5$Hz), 8.12 (2H, m), 7.66 (2H, m), 7.47 (1H, d, $J = 7.8$Hz), 2.70 (3H, s); $^{13}$C NMR $\delta$ 155.1, 145.7, 137.4, 132.1, 130.3, 127.0, 126.8, 126.3, 126.2, 126.1, 124.8, 123.6, 19.5; ES+ m/z 227.
Piperonal (225 mg, 1.5 mmol) and aminoguanidine hydrochloride (110 mg, 1 mmol) were dissolved in methanol (15 mL) and reacted according to the above general method. The product solution was dissolved on the rotary evaporator, and the residue was stirred in chloroform and filtered to remove the remaining starting material. The filtered product was dried on vacuum overnight to afford an off white powdery solid (208 mg, 0.86 mmol, 86 % yield, 205-206°C melting point). $^1$H NMR (300MHz, D$_2$O) $\delta$ 7.8 (1H, s), 7.2 (1H, s), 7.0 (1H, d, J = 8.5 Hz), 6.8 (1H, d, J = 8.5 Hz); $^{13}$C NMR (DMSO-d$_6$) $\delta$ 155.2, 149.1, 147.8, 146.0, 127.8, 124.1, 108.0, 105.5, 101.4.
5-(\(p\)-Nitrophenyl)-2-furan carboxylidenamino) -guanidine Hydrochloride

5-(\(p\)-Nitrophenyl)furfural (325 mg, 1.5 mmol) and aminoguanidine hydrochloride (110 mg, 1 mmol) were dissolved in methanol (15 mL) and reacted according to the above general method. The product solution was dissolved on the rotary evaporator, and the residue was stirred in chloroform and filtered to remove the remaining starting material. The filtered product was dried on vacuum overnight to afford a yellow powdery solid (284 mg, 0.92 mmol, 92 % yield, 165-166 °C melting point). \(^1\)H NMR (300MHz, D\(_2\)O) \(\delta\) 8.14 (2H, d, J = 8.2Hz), 7.86 (1H, s), 7.7 (2H, d, J =8 .2Hz); \(^{13}\)C NMR(DMSO-d\(_6\)) \(\delta\) 155.0, 152.4, 149.8, 146.2, 135.8, 134.9, 124.6, 124.2, 116.7, 112.2.

**SUN-AG15**

![DPM vs Log [M] Concentration Graph](image-url)
**COMPOUND 36 (AG-2)**

(Pyrrole-2-carboxyldenamino)-guanidine Hydrochloride

Pyrrole-2-carboxaldehyde (142 mg, 1.5 mmol) and aminoguanidine hydrochloride (110 mg, 1 mmol) were dissolved in methanol (15 mL) and reacted according to the above general method. The product solution was dissolved on the rotary evaporator, and the residue was stirred in chloroform and filtered to remove the remaining starting material. The filtered product was dried on vacuum overnight to afford a purple powdery solid (151 mg, 0.81 mmol, 81 % yield, 129-130°C melting point). $^1$H NMR (300MHz, D$_2$O) $\delta$ 7.96 (1H,s), 7.10 (1H, m), 6.62 (1H, m), 6.3 (1H,m); $^{13}$C NMR (DMSO-d$_6$) $\delta$ 155.1, 137.6, 126.9, 122.1, 113.2, 109.1.
COMPOUND 37 (JR-147)

(3-Pyridylmethylenamino)-guanidine Hydrochloride

3-Pyridinecarboxaldehyde (160 mg, 1.5 mmol) and aminoguanidine hydrochloride (110 mg, 1 mmol) were dissolved in methanol (15mL) and reacted according to the above general method. The product solution was dissolved on the rotary evaporator, and the residue was stirred in chloroform and filtered to remove the remaining starting material. The filtered product was dried on vacuum overnight to afford an off white powdery solid (175 mg, 0.88mmol, 88 % yield, 228-229ºC melting point). $^1$H NMR (300MHz, D$_2$O) $\delta$ 8.70 (1H,s), 8.54 (1H,d), 8.15 (1H,d), 8.0 (1H,s), 7.45 (1H,m); $^{13}$C NMR $\delta$ 157.4, 152.6, 150.2, 147.6, 137.7, 132.0, 127.}

JR-147

![Graph showing DPM vs. log [M] Concentration for JR-147 and JR-147+SPD]
4-Pyridinecarboxaldehyde (160 mg, 1.5 mmol) and aminoguanidine hydrochloride (110 mg, 1 mmol) were dissolved in methanol (15 mL) and reacted according to the above general method. The product solution was dissolved on the rotary evaporator, and the residue was stirred in chloroform and filtered to remove the remaining starting material. The filtered product was dried on vacuum overnight to afford an off white powdery solid (171 mg, 0.86 mmol, 86 % yield, 227-228°C melting point). $^1$H NMR (300MHz, DMSO-d$_6$) δ 12.4 (1H, broad singlet), 8.65 (2H,m), 8.20 (1H,s), 8.0 (NH, broad), 7.85 (2H,m); $^{13}$CNMR (DMSO-d$_6$) δ 155.4, 149.8, 144.1, 140.6, 121.2.
Indole-3-carboxaldehyde (217 mg, 1.5 mmol) and aminoguanidine hydrochloride (110 mg, 1 mmol) were dissolved in methanol (15 mL) and reacted according to the above general method. The product solution was dissolved on the rotary evaporator, and the residue was stirred in chloroform and filtered to remove the remaining starting material. The filtered product was dried on vacuum overnight to afford a brown powdery solid (182 mg, 0.77 mmol, 77 % yield, 230-231°C melting point). $^1$H NMR (300MHz, D$_2$O) δ 8.10 (1H,d), 7.85 (1H,s), 7.59 (1H,s), 7.57 (1H,d), 7.36 (1H,m), 7.28 (1H,m) ; $^{13}$C NMR(DMSO-d$_6$) δ 154.6, 144.5, 136.9, 131.7, 123.7, 122.6, 122.2, 120.5, 111.7, 110.4.
COMPOUND 40 (JR-207)

(Indole-5-carboxyldenaminio)-guanidine Hydrochloride

Indole-5-carboxaldehyde (217.5mg, 1.5 mmol) and aminoguanidine hydrochloride (110mg, 1.0 mmol) were dissolved in methanol (15mL) and reacted according to the above general method. The product solution was dissolved on the rotary evaporator, and the light-orange residue was allowed to sit for two days at which point the residue had become a peach-colored solid. This solid was stirred in chloroform and filtered to remove the remaining starting material. The filtered product was dried on vacuum overnight to afford a peach-pink powdery solid (148mg, 0.62 mmol, 62% yield, 177-180°C melting point). $^1$H NMR (400MHz, d-DMSO) δ 11.88 (1H, s), 11.45 (1H, s), 8.83 (1H, s), 8.21 (1H, s), 7.95 (1H, s), 7.71 (1H, dd, J = 8.4Hz, 1.6Hz), 7.43 (1H, d, J = 8.8Hz), 7.40 (1H, t, J = 2.6Hz), 6.49 (1H, m); $^{13}$C NMR δ 155.3, 148.7, 137.2, 127.5, 126.6, 124.5, 121.5, 119.9, 111.8, 101.9; ES+ m/z 202.

JR-207

![Graph](image-url)

Control

NonSpec

200 300 400 500 600 700 800 900 1000 1100 1200

log [M] Concentration

DPM

JR-207+SPD

JR-207

191
(1-Methylindole-3-carboxylidenamino)-guanidine Hydrochloride

1-Methylindole-3-carboxaldehyde (234mg, 1.5 mmol) and aminoguanidine hydrochloride (110mg, 1.0 mmol) were dissolved in methanol (15mL) and reacted according to the above general method. The product solution was dissolved on the rotary evaporator, and the residue was stirred in diethyl ether and filtered to remove the remaining starting material. The filtered product was dried on vacuum overnight to afford a cream-colored powdery solid (209mg, 0.83 mmol, 83% yield, 136-138°C melting point). $^1$H NMR (400MHz, d-DMSO) $\delta$ 8.33 (1H, s), 8.31 (1H, d, J = 8.0Hz), 7.87 (1H, s), 7.49 (1H, d, J = 8.0 Hz), 7.29 (1H, t, J = 8.0Hz), 7.18 (1H, t, J = 8.0 Hz), 3.81 (3H, s); $^{13}$C NMR (300MHz, d-DMSO) $\delta$ 154.5, 144.1, 137.4, 135.2, 124.1, 122.8, 122.4, 120.9, 110.1, 109.5, 32.9; ES+ m/z 216.
COMPOUND 42 (JR-215)

(6-Methylindole-3-carboxylidenameino)-guanidine Hydrochloride

6-Methylindole-3-carboxaldehyde (119mg, 0.75 mmol) and aminoguanidine hydrochloride (55mg, 0.5 mmol) were dissolved in methanol (15mL) and reacted according to the above general method. The product solution was dissolved on the rotary evaporator, and the yellow solid residue was stirred in chloroform and filtered to remove the remaining starting material. The filtered product was dried on vacuum overnight to afford a tan powdery solid (104mg, 0.83 mmol, 83% yield, 128-132°C melting point). $^1$H NMR (400MHz, d-DMSO) $\delta$ 11.65 (1H, s), 8.32 (1H, s), 8.15 (1H, d, J = 8.0 Hz), 7.79 (1H, s), 7.23 (1H, s), 6.95 (1H, d, J = 8.0Hz), 2.40 (3H, s); $^{13}$C NMR (300MHz, d-DMSO) $\delta$ 154.6, 144.9, 137.5, 132.0, 131.4, 122.4, 122.0, 121.7, 111.7, 110.5, 21.6; ES+ m/z 216.
3-Thiophenecarboxaldehyde (168 mg, 1.5 mmol) and aminoguanidine hydrochloride (110 mg, 1 mmol) were dissolved in methanol (15 mL) and reacted according to the above general method. The product solution was dissolved on the rotary evaporator, and the residue was stirred in chloroform and filtered to remove the remaining starting material. The filtered product was dried on vacuum overnight to afford a brown powdery solid (167 mg, 0.82 mmol, 82 % yield, 181-182°C melting point). $^1$H NMR (300MHz, D$_2$O) δ 8.11 (1H, s), 7.81 (1H, m), 7.56-7.51 (2H, m) ; $^{13}$C NMR (DMSO-d$_6$) δ 155.2, 141.9, 136.6, 128.9, 127.3, 125.2.
trans-Cinnamylidenamino-guanidine Hydrochloride

trans-Cinnamaldehyde (198 mg, 1.5 mmol) and aminoguanidine hydrochloride (110 mg, 1 mmol) were dissolved in methanol (15 mL) and reacted according to the above general method. The product solution was dissolved on the rotary evaporator, and the residue was stirred in chloroform and filtered to remove the remaining starting material. The filtered product was dried on vacuum overnight to afford a very light yellow crystalline solid (161 mg, 0.72 mmol, 72 % yield, 81-82ºC melting point). $^1$H NMR (300MHz, DMSO-d$_6$) δ 12.04 (1H, s), 8.0 (1H,d, J = 8.4 Hz), 7.7 (2H, m), 7.55(2H, d, J = 8.4Hz), 7.40 (1H, m), 7.15 (1H, m), 6.90 (1H, m); $^{13}$C NMR (DMSO-d$_6$) δ 155.0, 148.7, 140.1, 135.3, 129.0, 128.8, 126.9, 123.9.

SUN-AG14

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![Graph](image-url)
**COMPOUND 45 (JR-132)**

![Chemical Structure]

*p-Bis-benzylidenamino-guanidine Hydrochloride*

*p*-Terephthalaldehyde (402mg, 3.0 mmol) and aminoguanidine hydrochloride (535mg, 5.0 mmol) were dissolved in methanol (50mL) and reacted according to the above general method. The product solution was dissolved on the rotary evaporator, and the residue was stirred in chloroform and filtered to remove the remaining starting material. Two separate solid samples were collected as part of the solid product floated above the chloroform, and the remainder sank to the bottom. The two solid fractions were then finely powdered and stirred again with chloroform to ensure separation. The light-yellow powder top product was characterized as a mixture of benzylidino-guanidine-4-carboxaldehyde and the bis product. The bottom product was isolated and characterized as p-terephthalaldehyde-bis-guanylhydrazone, a fine off-white powder (365 mg, >300°C melting point, m/z 124) 1H NMR (300MHz, d-DMSO) δ 12.24 (2H, s), 8.21 (2H, s), 7.94 (4H, s); 13C NMR δ 155.3, 145.8, 135.0, 127.7.

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**JR-132**

![Graph](image-url)
CHAPTER 4: X-RAY CRYSTALLOGRAPHY

4.1 Introduction

The N-methyl-D-aspartate (NMDA) receptors are ligand-gated ionotropic glutamate receptor channels, which are regulated by a variety of endogenous molecules and multiple receptor complex binding sites. Overactivation of the NMDA receptor can lead to hyperexcitability and a number of neurotoxic effects and neurological diseases; however, the physiological function of this channel is crucial in many neural functions such as learning and memory, and thus a therapeutic agent cannot be one which irreversibly inhibits channel flux.

Channel blockers at the NMDA receptor complex can be very potent antagonists, but they possess very high abuse potential and exhibit neurotoxicity. The polyamine sites are an interesting focus of research, as the antagonists at this site exhibited neither of these detrimental qualities, while allosterically inhibiting channel activity. The polyamine agmatine has been demonstrated to exhibit antagonist activity at the polyamine binding sites of the NR2B subunits of the NMDA receptor complex, but only in pathologically high levels of the longer-chain endogenous polyamine-site agonists such as spermine and spermidine (Chapter 1). These intriguing properties begat this entire study, in an effort to (1) increase the potency of agmatine analogs for the “high affinity” site, which is expected to reduce the neuropathological effects regulated by NMDARs, and to (2) maintain or increase the selectivity of agmatine analogs for this site as opposed to the “low-affinity” site, which likely causes the unwanted side effects seen with the direct channel blockers (see Chapters 1-3).

Aminoguanidine hydrochloride, an agmatine analog missing the n-butyl chain, has been examined as a possible NMDAR antagonist, but was shown to have no activity in the P2 membrane [³H]MK-801 displacement assay (Section 3.1). However, a small library of conjugates of aminoguanidine with an array of arylaldehydes were synthesized and screened in the above assay; the 45 compounds tested thus far have demonstrated a 100% hit rate as NMDAR inhibitors.
The structure-activity relationships of these compounds appears to be quite complex, and any additional structural data of these products could vastly improve our ability to construct a potential pharmacophore. Therefore, several of the arylidenamino-guanidine compounds from Chapter 3 were crystallized and submitted for X-ray structure determination to obtain more in-depth structural information.

Crystal structure determination by X-ray crystallography is a technique which has been used for almost fifty years, and has become a vital tool in inorganic and organic structure analysis. Particularly, X-ray crystallography is important for determining the structures of small molecules and macromolecules, such as protein structures and their interactions with drug molecules. In order to detect and analyze the diffraction pattern generated by a molecular structure, a large amount of symmetrical molecules in a crystal structure must be used. The crystallization process often presents difficulty, especially with proteins, but is much more facile for small organic molecules such as those presented in this chapter. Using the crystals in a small amount of an appropriate solvent, the structure can be interpreted from an electron density map generated by the diffraction pattern.
4.2 Methods

The three arylidenamino-guanidine compounds were synthesized by the Schiff base formation of the substituted benzaldehydes with aminoguanidine hydrochloride (Scheme 4.1).

Scheme 4.1 Synthesis of the three arylidenamino-guanidine compounds which underwent X-ray structure determination
Compound 7 (JR-125, (4-cyanobenzylidenamino)-guanidine hydrochloride), compound 18 (JR-122, (3-nitrobenzylidenamino)-guanidine hydrochloride), and compound 21 (JR-223, (4-methoxy-3-nitrobenzylidenamino)-guanidine hydrochloride) were prepared according to Scheme 4.1. Further experimental and analytical details are described in Section 3.6. Suitable crystals for X-ray analysis were prepared with the following solvents: compound 7 was crystallized from a mixture of ethanol, isopropanol, and water; compound 18 was crystallized from ethanol and water; and compound 21 was crystallized from methanol.

The crystals were submitted to Dr. Sean Parkin at the UK Chemistry X-Ray Laboratory for X-ray analysis. Software programs used in the analysis were as follows. Data collection: COLLECT (Nonius, 1999); cell refinement: SCALEPACK (Otwinowski and Minor, 1997); data reduction: DENZO-SMN (Otwinowski and Minor, 1997); program used to solve structure: SHELXS97 (Sheldrick, 1997); program used to refine structure: SHELXL97 (Sheldrick, 1997); molecular graphics: XP in SHELXTL/PC (Sheldrick, 1995); software used to prepare materials for publication: SHELXL97 (Sheldrick, 1997) and local procedures.
4.3 Results and Discussion

4.3.1 X-ray Structure Results:

**Compound 21** was discovered to have a low IC$_{50}$ value in the displacement of MK-801, and in addition, yielded a biphasic curve for this displacement in the presence of spermidine, a neurotoxic agent at high concentrations. The biphasic nature of the curve is indicative of a high degree of selectivity for the “agmatine” site of the NMDAR complex.

![Figure 4.1 X-ray structure of compound 21, (4-methoxy-3-nitrobenzylidenamino)-guanidine hydrochloride. Displacement ellipsoids are drawn at the 50% probability level with hydrogen atoms included.](image)

**Compound 18** was neither as selective for, nor as potent as **compound 21** at the high-affinity agmatine site of the NMDA receptor complex. However, the structures of these two compounds are quite similar, differing only by the inclusion of a methoxy group at position 4 of the phenyl ring in **compound 21**. Therefore, this compound was subjected to X-ray structure analysis in order to determine if the addition of the methoxy
group, which resulted in such a large difference between the activities of the two compounds, might have caused a significant structural change elsewhere in the molecule, particularly at the aminoguanidine moiety.

Figure 4.2 X-ray structure of compound 18, (3-nitrobenzylidenamino)-guanidine hydrochloride. Displacement ellipsoids are drawn at the 50% probability level with hydrogen atoms included.

**Compound 7** was one of the least potent and selective NMDAR complex agmatine site inhibitors of the arylidenamino-guanidine compounds synthesized. The structure of this compound was elucidated by X-ray crystallography in an attempt to determine if the presence of the cyano group had any noticeable effect on the structure of the molecule, particularly in the guanidino moiety, which could explain its lack of activity.
Figure 4.3 X-ray structure of compound 7, (4-cyanobenzylidenamino)-guanidine hemisulfate. Displacement ellipsoids are drawn at the 50% probability level, without hydrogen atoms

The crystal structure of the starting material, aminoguanidine hydrochloride, was among the first published X-ray data (Bryden, 1957). As the Schiff base formation with the aminoguanidine moiety is believed to be the crucial (and entirely conserved) part of the structural motif in the aryldenamino-guanidine series, the detailed structure of the starting material was deemed to be quite relevant to the interpretation of structure-activity in these products.
Figure 4.4 Structure of aminoguanidine hydrochloride with bond lengths and resonance structures. Location of Schiff base formation is denoted (R). (Bryden, 1957)
4.3.2 Discussion:

In order to gain further information about the class of arylidenamino-guanidine compounds utilized in these studies, suitable crystals of three of these compounds were prepared for X-ray analysis. Structures were elucidated for each of them (Figures 4.1-4.3). The main points from structural information obtained were as follows:

1) Carbon-nitrogen bond lengths within the guanidine moiety; the lengths of these three bonds are an indication of the relative dispersal of $\pi$-orbital electrons of the C-N double bonds, and in turn, reflect the relative partial charge distribution among the three nitrogen atoms.

2) The planarity of the molecule as a whole is an indication of a high degree of double-bond conjugation, specifically between the phenyl ring and the guanidine moiety.

3) Bond lengths and dihedral angles along the bridge between the phenyl ring and guanidine moiety are also factors that may be an indication of a high degree of conjugation.

4) Orientation of the double bond formed between the aldehyde and aminoguanidine, i.e. E or Z geometry; in addition, if any double-bond character is shared with the N-N bond in the aminoguanidine moiety, this bond may exist in the E or Z orientation.

5) The possibility of intramolecular hydrogen bonding (i.e. between the protons of the terminal nitrogen molecules in the aminoguanidine moiety and the oxygen molecules of the ring-substituted nitro groups for compound 23); with the correct double-bond orientation along the chain between the ring and the guanidine moiety, several of these are possible.

While aminoguanidine hydrochloride (and most terminal guanidine-containing compound such as arginine) exists in resonance stabilized forms with the double bond characteristic primarily in the exo-position (Figure 4.4), some compounds such as canavanine have a different resonance stabilized structure which favors the endo-
position, due to electronegativity effects of the neighboring oxygen atom. In the case of these arylidenamino-guanidine compounds, a resonance structure favoring the endo-position of the guanidino moiety would also confer additional conjugation, which would reach into the ring, and possibly provide another measure of stability and conformational rigidity to the molecule.

The values for the bond lengths in the guanidine moiety in each of the arylidenamino-guanidino compounds were comparable to those found in the starting material, aminoguanidine hydrochloride. The molecular distances reported for aminoguanidine hydrochloride were 1.32 Å for the exo C-N bonds, and 1.35 Å for the one in the endo position, which were calculated to correspond to a weighting of roughly 40% double-bond contribution for each of the exo double-bond forms, and 20% for the endo double-bond (Bryden, 1957). The values obtained for the corresponding bond lengths in the three arylidenamino-guanidine compounds are shown in Table 4.1 below.

Table 4.1 Carbon-nitrogen bond lengths within the guanidino group of Aminoguanidine hydrochloride and the three arylidenamino-guanidine compounds from this chapter (numbering of nitrogen atoms are denoted as in Figure 4.4)

<table>
<thead>
<tr>
<th></th>
<th>C_1 – N_2 (endo)</th>
<th>C_1-N_3 (exo)</th>
<th>C_1-N_4 (exo)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aminoguanidine</td>
<td>1.35 Å</td>
<td>1.32 Å</td>
<td>1.32 Å</td>
</tr>
<tr>
<td>Hydrochloride</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Compound 7</strong></td>
<td>1.346 (8) Å</td>
<td>1.310 (8) Å</td>
<td>1.326 (8) Å</td>
</tr>
<tr>
<td><strong>Compound 18</strong></td>
<td>1.341 (3) Å</td>
<td>1.327 (3) Å</td>
<td>1.315 (3) Å</td>
</tr>
<tr>
<td><strong>Compound 21</strong></td>
<td>1.350 (2) Å</td>
<td>1.324 (2) Å</td>
<td>1.317 (2) Å</td>
</tr>
</tbody>
</table>

The C-N bond length values observed in the structurally similar **compounds 18 and 21** were remarkably similar for both the endo and exo C-N bonds, and they were also very close to the bond lengths found in aminoguanidine hydrochloride. In both of these arylidenamino-guanidine compounds, the C_1-N_3 bond lengths were significantly longer than the C_1-N_4 bond lengths; the longer C_1-N_3 bonds corresponded to the exo nitrogen
atom which is located farthest from the phenyl ring (see Figure 4.4). However, while the C-N bond lengths of the guanidine moiety in compound 7 were also not significantly different from those in aminoguanidine hydrochloride, the two exo-position C-N bond lengths were different than the exo C-N bond lengths in compounds 18 and 21: in compound 7, the exo C-N bond farthest from the phenyl ring (C1 – N3) was the shorter of the two (1.310 (±8) Å, compared to 1.326 (±8) Å for the other exo C-N bond, C1-N4). This difference in the exo C-N bond lengths does not seem to be large enough to contribute significantly to charge distribution within the guanidine moiety, but nevertheless should be examined. The bond length difference is most likely due to the difference in environment of the two terminal nitrogen atoms, N3 and N4. Compounds 18 and 21 have an aromatic meta-nitro group in their structure, which may affect the way the crystals, specifically the guanidine moieties of these molecules, interact with the solvent or counter-ion. Compound 7 has an aromatic cyano group in the para-position, and the distance of this substituent from the guanidine moiety may be large enough to remove any effects on the exo C-N bond lengths. The overall observation remains that the Schiff base formation that yields these arylidenamino-guanidino products does not significantly change the double-bond distribution within the resulting products’ guanidine moieties from that observed in the starting material, aminoguanidine hydrochloride. Therefore, the double bond character in the exo C-N bonds is similar to what it was in the starting material, with the positive charge spread mostly between the two terminal nitrogen atoms, N3 and N4 (Figure 4.4). Nevertheless, even 20% double bond character in the endo C-N bond of the guanidine moiety, as seen in the starting material, contributes significantly to conjugation of the molecule as a whole.

While the classical structure of the guanidino moiety does not indicate double bond character in the endo C-N bond, a extended distribution of π-orbital electron characteristic throughout the bonds of the molecule is suggested by the planarity seen in each of the three compounds. As seen in Figures 4.5 - 4.7, the guanidino moiety, the phenyl ring, and the imine which lies between them are all planar to each other. A general structure of this resonance stabilization is seen in figure 4.9.
Figure 4.5 Crystal packing of compound 7, viewed along the A-direction
Figure 4.6 Crystal packing of compound 18, viewed along the B-direction
Figure 4.7 Crystal packing of compound 21, viewed along the C-direction; nitro-groups appear as the only non-planar parts of each molecule
While the crystal packing diagrams of compounds 7, 18, and 21 illustrated in Figures 4.5 - 4.7 appear to show the planarity of the three molecules, the torsion angles have been measured and provide numerical evidence of the planarity. In the molecules illustrated in Figure 4.8, it can be seen that the torsion angles would be $+180^\circ$ (or $-180^\circ$) in a planar chain.

![Figure 4.8 Torsion and bond angle measurements, with Newman projections.](image)

A general representation of the resonance stabilization between the phenyl ring and the guanidine moiety is shown in Figure 4.9, which demonstrates the conjugation of the molecule when the guanidino moiety C-N double bond exists in the endo position, and the accompanying planarity. The torsion angles along the bonds between the phenyl ring and the guanidine moiety are given in Table 4.2.
Figure 4.9 General planar arylidenamino-guanidine compound structure, as conferred by resonance stabilization (relevant atoms are numbered for torsion and bond angle measurements in Tables 4.2 and 4.3, respectively)
Table 4.2 Torsion angles which link the phenyl and guanidine moieties in Figure 4.9 for compounds 7, 18, and 21

<table>
<thead>
<tr>
<th>Torsion Angle</th>
<th>Compound 7</th>
<th>Compound 18</th>
<th>Compound 21</th>
</tr>
</thead>
<tbody>
<tr>
<td>C₄-C₃-C₂-N₁</td>
<td>177.6° (6)</td>
<td>177.0° (2)</td>
<td>176.38° (16)</td>
</tr>
<tr>
<td>C₃-C₂-N₁-N₂</td>
<td>178.5° (6)</td>
<td>179.2° (2)</td>
<td>178.78° (14)</td>
</tr>
<tr>
<td>C₂-N₁-N₂-C₁</td>
<td>182.4° (6)</td>
<td>182.5° (2)</td>
<td>176.16° (15)</td>
</tr>
<tr>
<td>N₁-N₂-C₁-N₃</td>
<td>178.6° (6)</td>
<td>173.8° (2)</td>
<td>187.91° (14)</td>
</tr>
</tbody>
</table>

None of the torsion angles deviate more than 4 degrees from the plane of the molecule, excepting the torsion angle which ends at the exo guanidino N₃ amine (Figure 4.9). While this final torsion angle does not deviate more than 8 degrees from 180, it could be indicative of a slight rotational freedom along the C₁-N₂ bond, the endo guanidino C-N bond.

Planarity, however, is not necessarily an indication of $sp^2$ hybridization, which would be accompanied by double-bond conjugation or the delocalization of $\pi$-bond electrons throughout the molecule. As demonstrated in Figure 4.8B, single-bonds can also have 180° torsion angles, and thus four carbon atoms of an aliphatic chain (e.g. n-butyl) can all be coplanar; this planarity is likely a transient state in solution, as it is not due to the lack of rotational freedom associated with double bonds. However, in crystal structures, the packing will force such unsaturated chains into a single form, and it is important to attribute the planarity of the arylidenamino-guanidino compounds to its proper cause by defining the multiplicity of the bonds. Single and double bonds can easily be differentiated by their bond angles. A double bond compound will have $sp^2$ hybridization and roughly 120° bond angles will be formed between the two bonded atoms and their substituents (Figure 4.8A), while a single bond compound will have $sp^3$ hybridization and form roughly 109.5° bond angles between the two bonded atoms and their substituents (Figure 4.8B). The angles of the bonds which arise between the phenyl ring and the guanidine moiety due to the Schiff base formation of these compounds are shown in Table 4.3.
Table 4.3 Bond angles formed between the phenyl and guanidine moieties in Figure 4.9 for compounds 7, 18, and 21

<table>
<thead>
<tr>
<th>Bond Angle</th>
<th>Compound 7</th>
<th>Compound 18</th>
<th>Compound 21</th>
</tr>
</thead>
<tbody>
<tr>
<td>C3-C2-N1</td>
<td>120.5° (6)</td>
<td>119.9° (2)</td>
<td>120.63° (15)</td>
</tr>
<tr>
<td>C2-N1-N2</td>
<td>116.0° (6)</td>
<td>116.3° (2)</td>
<td>115.00° (14)</td>
</tr>
<tr>
<td>N1-N2-C1</td>
<td>118.8° (6)</td>
<td>117.4° (2)</td>
<td>118.48° (14)</td>
</tr>
<tr>
<td>N2-C1-N3</td>
<td>119.8° (6)</td>
<td>118.7° (2)</td>
<td>117.05° (15)</td>
</tr>
</tbody>
</table>

These data reveal bond angles within 3° of a perfect $sp^2$ 120° for each of the angles associated with the imino moiety and the aminoguanidine moiety, except for the bond angles of the C2-N1-N2 atoms (Figure 4.9), which were in the range of 115.0° to 116.3° for each compound. This bond angle lies in between the ideal $sp^2$ and $sp^3$ dihedral angles; this is unexpected, considering the fact that the C2-N1 imine bond is the only bond between the phenyl ring and the guanidino moiety which is expected to have double-bond characteristics. Resonance structures for these specific compounds cannot justify the N1 atom converting to an $sp^3$-hybridized state (such a resonance structure can be drawn in the presence of electron-donor substituents on the phenyl ring, but compounds 7 and 18 have no such substituent). In addition, there are no ortho-position phenyl substituents which could be sterically repulsing the exo N4 atom in the guanidine moiety, which could lower the C2-N1-N2 bond angle. Regardless, this bond angle is clearly larger than the 109.5° bond angle expected in a single bond. The observation remains that the C2, N1, and N2 atoms that serve to connect the phenyl ring to the guanidine moiety display primarily $sp^2$ hybridization, based on the bond angles and torsion angles they form. This double-bond characteristic causes an extended conjugation of the molecules (Figure 4.9), resulting in a hybridization of these Lewis structures and distribution of $\pi$-bond electrons from the phenyl ring through to the guanidine moiety.

In addition to the bond angles and torsion angles, it can be observed that formation of the Schiff base at the C2-N1 bond also impacted the length of the N1-N2 bond. This bond was reported as 1.42 Å in the aminoguanidine hydrochloride starting material, but in the products was observed as having a length of 1.372 (±7) Å in
compound 7, 1.374 (±3) Å in compound 18, and 1.381(±2) Å in compound 21. These data are further evidence of the significant π-bond characteristics of this bond.

Data obtained from the X-ray analysis clearly demonstrated an exclusive E-geometry in the newly formed imine double-bond, for each of the three products examined; in addition, the N1-N2 (Figure 4.9) bond has significant double-bond characteristics, and also has the “E-geometry” in each compound. In this way, the rigid C3-C2-N1-N2-C1 chain is elongated and extends the guanidine moiety to a maximum distance from the phenyl ring, eliminating the possibility of intramolecular hydrogen bonding between the guanidino group and the phenyl ring or its substituents (Figure 4.9, along with the X-ray structures of Figures 4.1-4.3). This information about the orientation of the double bond is crucial, as it is important to know that each of these compounds had the same orientation, and it is likely that the same geometry of the imino moiety is present in all the arylidenamino-guanidine compounds synthesized.

The structural conservation between the three compounds subjected to X-ray analysis is especially important in the development of a pharmacophore model. The lack of rotational freedom resulting from the double-bond conjugation through the molecules from the guanidine moiety to the phenyl ring provides an excellent basis for establishing the locations of agmatine binding site residues which interact with this novel class of ligands. While the data from Chapter 3 clearly indicates that structural modification of the aromatic ring moiety, as well as the ring substituents, can result in increased potency and selectivity for the agmatine binding site of the NMDAR complex, the definitive X-ray data of the conserved “backbone” moiety (Figure 4.10) allows the SAR study to focus on these substituents with knowledge of the structurally integrity of the rest of the molecule.
4.4 Conclusions

Three of the arylidenamino-guanidine compounds which were synthesized and screened (see Chapter 3) have been subjected to X-ray analysis in order to obtain more structural information that may be pertinent to their interaction with the agmatine binding site of the NMDA receptor complex. The three compounds were compound 21, one of the most potent and selective agmatine-site NMDAR inhibitors, compound 18, which was structurally similar to compound 21 but significantly less active, and compound 7, which was one of the least potent and selective agmatine-site NMDAR inhibitors in this set.

The data obtained yielded important structural information, which was very similar within the subset of these three compounds. The guanidine moiety in these compounds has double bond character primarily shared between the exo C-N bonds, as is seen in the starting material, aminoguanidine hydrochloride. However, the endo C-N bond is shorter than a standard C-N single bond, indicating that it has double-bond
character as well, and the formal positive charge is shared between the endo nitrogen (N_2 in Figure 4.9) and the two exo nitrogen atoms (N_3 and N_4 in Figure 4.9). The N_1-N_2 bond, which was observed as a typical single bond in the starting material, aminoguanidine hydrochloride, has shortened significantly in the process of the Schiff base formation. This is very significant as this bond is clearly taking part in resonance stabilization of the molecule, and joining the \( \pi \)-electron clouds of the guanidine moiety with that of the phenyl ring, which confers a rigid planarity to these molecules.

The X-ray structure (Figures 4.4 - 4.7) clearly shows that these compounds are almost completely planar. Each of the torsion angles which connect the imino moiety to the guanidine moiety and the phenyl ring are close to 180°, and the dihedral angles along this bridge were very close to 120°, data which reveal the \( sp^2 \) characteristic of the atoms between the guanidine moiety to the phenyl ring. The C_2-N_1 and N_1-N_2 bonds (Figure 4.9), both of which have double bond character, are observed to be strictly in the E geometrical form, for all three compounds. These \textit{trans} double bonds are very significant in that they form a rigid backbone which distances the guanidine moiety from the phenyl ring. The space between these two moieties will disallow any interactions (e.g. hydrogen bonding) between the guanidine and the ring substituents (at least at the \textit{meta-} and \textit{para-} positions).

While none of the data obtained from these X-ray structures was sufficient to substantially differentiate these compounds from each other based on structural characteristics, it is of obvious importance in the development of a pharmacophore model. The conformational rigidity of these structures dramatically simplifies SAR within this set of arylidenamino-guanidine compounds, and molecular modeling and pharmacophore model development will be easier to carry out as a result this information.
CHAPTER 5: MOLECULAR MODELING

5.1 Introduction

With many structure-activity studies, optimization of lead compounds is a continuous process which is never complete as long as there are untested molecules; this work is no exception. While a number of promising inhibitory modulators of the NMDA receptor complex have been developed and will be further examined in pharmacological assays, these leads may be rudimentary precursors of therapeutically useful drug molecules yet to be discovered. The search for a quantitative structure-activity relationship between these molecules and their activity is indeed a very important and relevant step towards understanding the agmatine binding site and the way drug molecules will interact with it.

Important structure-activity data was interpreted by scrutiny of each compound in the set of arylidenamino-guanidine compounds, and several observations have been made which postulate the importance of steric and electronic effects on both the agmatine-site affinity and selectivity of these molecules.

While several promising lead compounds have resulted from this study, the activity of this group of arylidenamino-guanidines as a whole attests to the importance of further examination of the structural descriptors of each molecule as they relate to inhibitory activity. Molecular modeling with partial least squares and neural network approaches will thus be undertaken with this set of structurally similar molecules in an effort to facilitate further development of this intriguing set of compounds. Indeed, the phenylidenamino-guanidine moiety (Figure 4.10) is predominantly conserved in this set of 45 active compounds, thus a structure-activity examination of the ring substituents may reveal much about the polyamine binding sites of the NMDA receptor complex.

As stated above, many of the structural features of the arylidenamino-guanidines were examined and discussed in Chapter 3 of this work. However, using the technology of the present time, computational methodologies can be used to discover and differentiate molecular descriptors that are less readily apparent to even the most well-trained chemist. Utilizing computer-generated descriptors, a number of models can be
generated, which highlight important features to include (and in some cases, exclude) in the design of potentially potent candidates. In addition, using these models to evaluate potential inhibitors *in silico* can save synthetic time and money, while dramatically increasing the hit rate of those molecules which are generated from such a process.

Two different computational models were developed in order to inspect the relationships between structural descriptors and the observed SAR in the NMDA receptor assay. These included utilization of partial least squares (PLS) regression, as well as a back-propagation artificial neural network (ANN) analysis method. The former is a linear model which generates an equation weighting the values of the descriptors to afford an equation which predicts IC\(_{50}\) values of compounds based on their molecular properties. The latter is a more complex non-linear modeling system, which imitates the function of the human brain by propagating signals of multivariable data through processing elements called neural nodes, which analyze and process the data. These neural nodes are arranged into a sequence of layers, and they are interconnected to form a feed-forward back-propagation network. In the input layer, each input node corresponds to one of the variables (or descriptors) used in the model; from the input layer, signals are propagated through a hidden layer and thence to the output layer, generating the predicted values.

### 5.2 Methods

The modeling process consisted of three steps: (i) construction and minimization of molecular structure, (ii) selection of descriptors according to the experimental data, and (iii) determination of the neural network configuration.

Molecular structure was modeled *in silico* with the use of the Sybyl discovery software 7.0. Each molecule used in the study was drawn using the program, and formal charges were assigned. The geometry optimization (energy minimization) was first performed by using the molecular mechanics (MM) method with the Tripos force field and the default convergence criterion. To consider various possible conformation of a molecule, a variety of conformations for a given molecule were obtained that were
associated with local minima on the potential energy surface from RANDOMSEARCH in Sybyl. Each of these conformations was geometrically optimized with semi-empirical molecular orbital (MO) energy calculations at the PM3 level to locate the most stable conformation, i.e. the ones with the lowest energy.

For each set of molecules examined, a set 313 molecular variables was generated by using the DRAGON program; these variables consisted of zero-dimensional (constitutional) descriptors, one-dimensional descriptors (empirical and physical descriptors as well as functional group indications), two-dimensional (topological) descriptors, and three-dimensional (WHIM) descriptors. These descriptors were then transferred to a Microsoft Excel database, along with the pIC$\text{50}$ values of the corresponding molecules; a value for the correlation of each descriptor to the pIC$\text{50}$ values of each compound was generated using the CORREL function, and the descriptors with the best correlation were selected to be further examined, while those with an extremely low correlation were discarded. For example, in the first set of compounds modeled, which included the preliminary 28 arylidenamino-guanidines, only the 134 descriptors with a CORREL function absolute value of greater than 0.3 were examined.

The partial least squares (PLS) model, used to examine the set of arylidenamino-guanidines mentioned above, was developed mainly by tedious selection and improvement upon inclusion of individual descriptors. Using the PLS modeling program in the Sybyl software package combined with the refined list of descriptors, R & $r_{cv}$-squared values could be generated for selected parameters. Thus, a model could be swiftly generated for any combination of descriptors, with corresponding cross-validated $r_{cv2}$ values and standard error. However, in order to generate a predictive model, selection of descriptors proceeded by using the PLS method to determine the highest $R^2$-value of a model including each descriptor one at a time, selecting the best model, then repeating the process of adding each remaining descriptor to form a new model. Each iteration therefore necessitated the generation of over 100 models and selection of the “best” individual model, while at the same time indicating the descriptors which were most related to the compound’s activity. Each added descriptor resulted in an increase in the $R^2$-value, but eventually this increase became negligible or 0, at which point the model was deemed complete, and addition of further descriptors would only serve to
complicate the linear equation without contributing significantly to the model itself. Addition of further descriptors would also dilute the importance of the descriptors which were most related to activity.

The descriptors which accounted for the best PLS model were then used in the artificial neural network (ANN) model, using methods refined by Dr. Fang Zheng in the laboratory of Dr. Crooks. This method of feed-forward back-propagation model development used the neural network C program. Each compound in the data set was singularly presented to the working ANN model for the learning process, with its descriptors being input to the input layer, and the final output from the output layer being compared to the experimental value for experimental pIC$_{50}$ which was generated in the laboratory. Leave-one-out cross validation was employed in the modeling, and the validity of each model was evaluated based on the discrepancy of the set of output values and the set of experimental values. The model assessment was based on the Pearson correlation coefficient ($R$), the root mean square deviation (RMSD), and the predictive coefficient ($r_{cv}$). The latter value was defined as:

\[
    r_{cv} = \sqrt{\frac{SD - PRESS}{SD}}
\]

Where $SD$ is the sum of squared deviations of each measured log(1/IC$_{50}$) value from its mean and $PRESS$ is the predictive sum of squared differences (the sum of squared differences between actual and predicted values).

### 5.3 Results and Discussion

The modeling work described in this dissertation was performed in order to lend insight into the structure-activity relationships between the molecules synthesized and their inhibitory activity demonstrated by the displacement of [$^3$H]MK-801 from the NMDA receptor complex, as described in Chapters 2 and 3. In addition, the models have contributed, and will continue to contribute towards the development of more active arylidenamino-guanidine compounds at this site, while reducing the cost and time
associated with the laboratory synthesis by indicating which structures should be synthesized based solely on predicted structures and activities.

Attempts were made to model the “agmatine binding site” based on the agmatine analogs prepared and evaluated in Chapter 2. These compounds possessed a wide spectrum of activity in the NMDAR assay utilized in this study, and represented a wide variety of structures as well. However, when these compounds were assigned descriptors, and each descriptor correlated with their pIC\textsubscript{50}-values, it became readily apparent by the utter lack of any correlation that such a set was too varied in structure to model as a single group of compounds. This may be due to the structural variation, but more likely that the wide range of functional groups allowed the molecules to act at different polyamine recognition sites as well as the channel site, and thus the activity was not uniformly attributable to their action at a single binding site.

It was therefore necessary to examine a smaller subset of compounds which were more structurally similar, and thus hypothesized to act at the same binding site. The group of arylidenamino-guanidino compounds provided a subset which met these qualifications. At that time, a preliminary set of 28 compounds had been tested, which included the AG-compounds synthesized by Dr. Sundar Neelakantan, and the JR-compounds in the range of JR-121 to JR-197. There compounds maintained the aromatic “arylidenamino-guanidine” moiety of Figure 4.10 (the only exception being AG-14, in which the aromatic ring is separated from the guanidino moiety of the molecule with an E-double bond linker), and possessed a variety of differing aromatic rings as well as aromatic ring-substituents. The pIC\textsubscript{50}-values of these compounds spanned the range of -3.0 to -5.0, ensuring that there would be a sufficient difference in activity that might be assignable to structural and electronic differences. As a minimum of roughly 40 compounds are normally required in order to obtain an artificial neural network model, the partial least squares method was chosen as the preliminary computer model for this subset of compounds.

The set of 313 descriptors was entered into a Microsoft Excel spreadsheet, and using the CORREL function, each descriptor was compared to the set of experimental pIC\textsubscript{50}-values for correlation. The 134 descriptors with a CORREL function absolute value of greater than 0.3 were then transferred to the Sybyl program in order to evaluate
them in the PLS model. To prevent confusion, it should be explained that the Sybyl software additionally assigned several title columns, as well as one to the observed IC$_{50}$ values, and thus the # values below, which go above 134, are not the descriptor numbers but the Sybyl-assigned column numbers. As stated above, each descriptor was added individually, and a model was “made” in order to discover the descriptor which produced the greatest cross-validated $r_{cv}^2$ value, either by itself in the case of the first addition, or in conjunction with the previously added descriptors. While the following description of the selection of descriptors for the PLS model is tedious and perhaps will not enthrall the reader, it is an important demonstration of the procedure utilized to generate such a model.

The first descriptor added in this procedure was #85 (see the descriptions below in Table 5.1), which, not coincidentally, produced the largest CORREL function value of 0.654. The $r_{cv}^2$ value with only this descriptor and the experimental pIC$_{50}$-values was 0.308, significantly higher than the values obtained by a model with any other single descriptor. Each of the remaining 133 descriptors was individually added to this model, and it was found that the addition of #135 (again, one of the most highly-correlated descriptors from the CORREL function) resulted in an $r_{cv}^2$ value of 0.650. The subsequent inclusion of descriptor #128 resulted in a PLS model with an $r_{cv}^2$ value of 0.715. Addition of a fourth descriptor afforded a meager improvement: #119 was the only descriptor which improved the model at all, to an $r_{cv}^2$ value of 0.717. The model was significantly improved upon addition of a fifth descriptor, #127, which increased the $r_{cv}^2$ value to 0.760. Unfortunately, supplementing the model with a sixth descriptor only served to worsen the PLS model, with the best $r_{cv}^2$ value being 0.758 in the presence of #7. It was determined that the best progress might be made by removing each of the six chosen descriptors one at a time, and examining the $r_{cv}^2$ values resulting from the combinations of the five remaining descriptors. Surprisingly, removing #128 while retaining #85, #135, #119, #127, and #7 resulted in an $r_{cv}^2$ value of 0.771, a slight improvement over the previous model, which included six descriptors. From thence forward, every descriptor addition was followed by individual removal of each descriptor that had been added to that point, in order to assess the potential for the model to improve by condensing the number of descriptors utilized. The iterative addition of descriptors
began anew, with the addition of #56 to yield an $r_{cv}^2$ value of 0.788. A series of small improvements were obtained with the further addition of descriptors #66, #125, and #31, resulting in a 9-descriptor model with a 0.805 $r_{cv}^2$-value. In the same manner, the subsequent addition of #86 brought the $r_{cv}^2$-value to 0.809, with addition of #61 affording an $r_{cv}^2$ of 0.812, and the addition of #35 affording an $r_{cv}^2$ of 0.814. While these small increases in $r_{cv}^2$ values were unremarkable, this process was continued until, with a model containing 12 descriptors, neither addition nor subtraction of descriptors improved at all upon the model. For the sake of comparison with the ANN models, which generate “training” $R^2$-values in addition to the $r_{cv}^2$-values, the $R^2$ value for this PLS model was 0.887 (or $R = 0.942$) with a standard error of estimate of 0.168. The computer-generated “probability of $R^2 = 0$” was 0.000.

The regression equation generated, which linearly correlates the predicted $pIC_{50}$ to the descriptors is:

$$pIC_{50} = 4.992 - (0.113 \times \text{Desc7}) + (0.158 \times \text{Desc31}) - (0.077 \times \text{Desc35})$$
$$+ (0.041 \times \text{Desc56}) + (2.229 \times \text{Desc61}) + (0.496 \times \text{Desc66}) + (8.235 \times \text{Desc85})$$
$$+ (12.258 \times \text{Desc86}) - (0.008 \times \text{Desc119}) - (11.71 \times \text{Desc125})$$
$$+ (2.347 \times \text{Desc127}) - (0.002 \times \text{Desc135})$$

$$R^2 = 0.887 (R = 0.942); \ r_{cv}^2 = 0.814 (r_{cv} = 0.902); \text{Standard Error} = 0.168; \ F = 8.495$$

The definitions of the descriptors are shown in Table 5.1 below, and their values are listed in Table 5.2, together with the predicted $IC_{50}$-values from this PLS model and the observed $IC_{50}$-values from the $[^3]H$MK-801 displacement assay. In addition, the weight was calculated for each descriptor in the determination of the predicted $pIC_{50}$-value. These weights were determined by standard deviation of the descriptors as a group, and then multiplying by the factor from the model-generated regression equation. Each of these products was then divided by the sum of the twelve products generated, and multiplied by 100 to generate a percentage weight. It is evident that the descriptors are of nearly equal importance, with the largest being worth 15.0% and the smallest 6.9%.
Table 5.1 Definitions of descriptors used in the 12-descriptor PLS model for the preliminary set of 28 arylidenamino-guanidine compounds

<table>
<thead>
<tr>
<th>Desc. #</th>
<th>Name</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>7</td>
<td>C-027</td>
<td>R--CH—X, instances of a heteroatom (N, S) being covalently bound to a mono-protonated carbon molecule</td>
</tr>
<tr>
<td>31</td>
<td>nNHR</td>
<td>Number of secondary amines (aliphatic)</td>
</tr>
<tr>
<td>35</td>
<td>GGI1</td>
<td>Topological charge index of order 1</td>
</tr>
<tr>
<td>56</td>
<td>HATS&lt;sub&gt;m&lt;/sub&gt;</td>
<td>Leverage-weighted total index / weighted by atomic masses</td>
</tr>
<tr>
<td>61</td>
<td>HATS&lt;sub&gt;4v&lt;/sub&gt;</td>
<td>Leverage-weighted autocorrelation of lag 4/weighted by atomic van der Waals volumes</td>
</tr>
<tr>
<td>66</td>
<td>R&lt;sub&gt;2u&lt;/sub&gt;</td>
<td>R autocorrelation of lag 2 / unweighted</td>
</tr>
<tr>
<td>85</td>
<td>R&lt;sub&gt;4v&lt;/sub&gt;</td>
<td>R maximal autocorrelation of lag 4 / weighted by atomic van der Waals volumes</td>
</tr>
<tr>
<td>86</td>
<td>R&lt;sub&gt;5v&lt;/sub&gt;</td>
<td>R maximal autocorrelation of lag 5 / weighted by atomic van der Waals volumes</td>
</tr>
<tr>
<td>119</td>
<td>T(N..N)</td>
<td>Sum of topological distances between N..N</td>
</tr>
<tr>
<td>125</td>
<td>G&lt;sub&gt;2m&lt;/sub&gt;</td>
<td>2nd component symmetry directional WHIM index / weighted by atomic masses</td>
</tr>
<tr>
<td>127</td>
<td>E&lt;sub&gt;2v&lt;/sub&gt;</td>
<td>2nd component accessibility directional WHIM index / weighted by atomic van der Waals volumes</td>
</tr>
<tr>
<td>135</td>
<td>PSA</td>
<td>Polar surface area (includes all O, N, S atoms and covalently bonded H’s) or fragment-based polar surface area</td>
</tr>
</tbody>
</table>
Table 5.2 Descriptors utilized in the preliminary 28-molecule PLS model

<table>
<thead>
<tr>
<th>Desc:</th>
<th>7</th>
<th>31</th>
<th>35</th>
<th>56</th>
<th>61</th>
<th>66</th>
<th>85</th>
</tr>
</thead>
<tbody>
<tr>
<td>Name</td>
<td>C-027</td>
<td>nNHR</td>
<td>GGI1</td>
<td>HATSm</td>
<td>HATS4v</td>
<td>R2u</td>
<td>R4v+</td>
</tr>
<tr>
<td>AG-2</td>
<td>0</td>
<td>1</td>
<td>2.0</td>
<td>1.138</td>
<td>0.096</td>
<td>1.428</td>
<td>0.012</td>
</tr>
<tr>
<td>AG-3</td>
<td>0</td>
<td>1</td>
<td>2.5</td>
<td>1.456</td>
<td>0.123</td>
<td>1.536</td>
<td>0.020</td>
</tr>
<tr>
<td>AG-4</td>
<td>0</td>
<td>0</td>
<td>2.5</td>
<td>1.411</td>
<td>0.131</td>
<td>1.510</td>
<td>0.021</td>
</tr>
<tr>
<td>AG-5</td>
<td>2</td>
<td>0</td>
<td>2.0</td>
<td>2.156</td>
<td>0.102</td>
<td>1.388</td>
<td>0.014</td>
</tr>
<tr>
<td>AG-6</td>
<td>0</td>
<td>0</td>
<td>3.0</td>
<td>0.693</td>
<td>0.097</td>
<td>1.666</td>
<td>0.015</td>
</tr>
<tr>
<td>AG-8</td>
<td>0</td>
<td>0</td>
<td>3.0</td>
<td>1.460</td>
<td>0.124</td>
<td>1.823</td>
<td>0.022</td>
</tr>
<tr>
<td>AG-9</td>
<td>0</td>
<td>0</td>
<td>3.5</td>
<td>6.107</td>
<td>0.211</td>
<td>1.363</td>
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<th>G2m</th>
<th>E2v</th>
<th>PSA</th>
<th>IC₅₀,OBS (Littleton et al.)</th>
<th>IC₅₀,Cal (Littleton et al.)</th>
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<tr>
<td>JR-121</td>
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<td>37</td>
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<td>0.268</td>
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<td>0.267</td>
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<td>0.265</td>
<td>223.62</td>
<td>224.0</td>
<td>139.0</td>
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</table>
A graphical representation of the observed versus PLS model-predicted IC\textsubscript{50}-values is shown in figure 1. The points are well-clustered along the ideal line, with the exception of the two pyridino compounds, JR-146 and JR-147 (compounds 37 and 38), which were predicted to possess approximately four times greater inhibitory activity than they demonstrated in the [\textsuperscript{3}H]MK-801 displacement assay.

![Graph showing comparison of observed pIC\textsubscript{50} values versus calculated pIC\textsubscript{50} values](image)

**Figure 5.1** Comparison of observed pIC\textsubscript{50} values versus calculated pIC\textsubscript{50} values using the 12-descriptor PLS model for the initial set of 28 compounds

Structures of the subsequent set of arylidenamino-guanidine compounds (JR-202 – JR-228) were drawn and input into the Sybyl software program, and the same twelve molecular descriptors were then generated with the DRAGON program. These compounds were predicted to have activity of the order of 4-5 pIC\textsubscript{50}, or IC\textsubscript{50}-values of 10-100 μM. When synthesized and screened in the same assay, the observed activities of the new set of compounds were compared to the predicted values from the linear PLS model.

While this new “training set” afforded mixed results in the comparison of predicted and observed inhibitory activity (see Figure 5.2), the observed IC\textsubscript{50} values that
differed the most from the predicted values were compounds which were more potent than predicted. In addition, while there were five points that differed in predicted and observed IC50-values by a full order of magnitude, three of these were the compounds which generated biphasic dose-response curves.

![Figure 5.2 Observed versus calculated IC50 correlation from the 12-component PLS model with original data points and testing set values](image)

With a larger set of compounds and a model which required refinement in order to better take into account the improved values generated by the compounds that exhibited a biphasic binding profile (and also to possibly explain the differences seen in comparing the predicted and observed inhibition of the pyridino compounds), it was decided to design a new partial-least squares model, and select descriptors for use in an artificial neural network model.

This second PLS model was generated by using the same techniques as described previously, with a new refined set of descriptors selected from the list of over 300 possible descriptors. The refined set was larger in this case than in the earlier model (comprising nearly 200 descriptors), and allowing the inclusion of more possible
descriptors rendered the analysis much more time-consuming; however, the added
degrees of freedom ensured that the best possible model was extruded from the data. It is
generally accepted that the number of descriptors chosen for an artificial neural network
model should be relatively small (roughly equal to or less than ten), and thus, while the
best PLS model for these data was obtained with fourteen descriptors, it was decided that
the best set of descriptors to use in the development of the ANN model was one which
contained eleven descriptors. The training $R^2$-value began to plateau around this number
of descriptors, and the value of 0.815 for the 11 descriptors was a significant
improvement over the 10-descriptor PLS model, which afforded an $R^2$-value of 0.762.
The standard error of estimate was also reduced from 0.267 in the 10 descriptor model to
0.229 in the 11 descriptor model, with a minimum value of 0.200 for the 14-descriptor
model.
This second PLS model, with eleven descriptors and based on data and structures from 45 molecules, had the following regression equation:

\[
pIC_{50} = 3.120 - (1.137 \times \text{Desc18}) - (0.186 \times \text{Desc21}) + (2.540 \times \text{Desc33})
+ (0.497 \times \text{Desc42}) - (0.395 \times \text{Desc78}) + (0.366 \times \text{Desc93})
- (0.717 \times \text{Desc109}) - (9.306 \times \text{Desc139}) + (18.626 \times \text{Desc167})
+ (2.419 \times \text{Desc172}) + (19.671 \times \text{Desc181})
\]

\[R^2 = 0.815 \ (R = 0.903); \ r_{cv}^2 = 0.718 \ (r_{cv} = 0.847); \ \text{Standard Error} = 0.229; \ F = 23.218\]

The definitions of the descriptors are shown in Table 5.3. While only one of these descriptors is also found in the earlier PLS model (C-027), many of the descriptors are very similar, and reflect molecular traits related to those which were judged to be important in the first set of descriptors. In addition, the values of each of the descriptors used in the final set of arylidenamino-guanidine compounds are shown in Table 5.4.
Table 5.3 Definitions of descriptors used in the 11-descriptor PLS model for the final set of 45 arylidenamino-guanidine compounds

<table>
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<th>Name</th>
<th>Definition</th>
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<td>3st component symmetry directional WHIM index/weighted by atomic masses</td>
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<tr>
<td>21</td>
<td>L2v</td>
<td>2nd component size directional WHIM index/weighted by atomic van der Waals volumes</td>
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<tr>
<td>33</td>
<td>Gu</td>
<td>G total symmetry index/unweighted</td>
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<td>42</td>
<td>nCIC</td>
<td>Total number of rings</td>
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<td>Moriguchi octanol-water partition coefficient (logP)</td>
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<td>leverage-weighted autocorrelation of lag 5/weighted by atomic van der Waals volumes</td>
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<td>167</td>
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<td>R maximal autocorrelation of lag 2/weighted by atomic van der Waals volumes</td>
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<tr>
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<td>R6e</td>
<td>R autocorrelation of lag 6/weighted by atomic Sanderson electronegativities</td>
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<td>181</td>
<td>R5p+</td>
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Table 5.4 Descriptors utilized in the final 45-molecule PLS and ANN models

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Table 5.4 (cont.) Descriptors utilized in the final 45-molecule PLS and ANN models

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Table 5.4 (cont.) Descriptors utilized in the final 45-molecule PLS and ANN models

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Table 5.4 (cont.) Descriptors utilized in the final 45-molecule PLS and ANN models

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For the second partial least squares model, the observed IC$_{50}$-values generated from the [$^3$H]MK-801 displacement assay were plotted versus the PLS model-predicted IC$_{50}$-values (see Figure 5.3). While the R$^2$ value of this model is lower than that obtained in the first PLS model (mainly due to an increase in the number of components), this new set of points is well-clustered around the ideal diagonal midline, with very few data points differing by more than half an order of magnitude. Specifically, the predicted values for the pyridino compounds are much closer to the observed values, and the three biphasic compounds are also much better predicted than in the previous model.

The data for this model are disclosed in Table 5.5 along with the artificial neural network-generated data.

Figure 5.3 Comparison of observed pIC$_{50}$ values versus calculated pIC$_{50}$ values using the 11-descriptor PLS model for the final set of 45 compounds
Table 5.5 Observed and predicted IC\textsubscript{50}-values of arylidenamino-guanidines

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<th>Comp. Code</th>
<th>Structure</th>
<th>Exper. IC\textsubscript{50} ((\mu)M)</th>
<th>PLS-11D IC\textsubscript{50} ((\mu)M)</th>
<th>NN-train. IC\textsubscript{50} ((\mu)M)</th>
<th>NN-LOO IC\textsubscript{50} ((\mu)M)</th>
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1 - PLS-11D: Predicted IC\textsubscript{50} value from the primary Partial Least Squared model with 11 descriptors
2 - NN-train: Predicted IC\textsubscript{50} value from the training set of the final Neural Network model
3 - NN-LOO: Predicted IC\textsubscript{50} value from the leave-one-out set of the final Neural Network model
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<th>Comp. No.</th>
<th>Comp. Code</th>
<th>Structure</th>
<th>Exper. IC&lt;sub&gt;50&lt;/sub&gt; (μM)</th>
<th>PLS-11D IC&lt;sub&gt;50&lt;/sub&gt; (μM)</th>
<th>NN-train. IC&lt;sub&gt;50&lt;/sub&gt; (μM)</th>
<th>NN-LOO IC&lt;sub&gt;50&lt;/sub&gt; (μM)</th>
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<td>143.4</td>
<td>169.4</td>
<td>168.1</td>
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<td>12</td>
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<td>13</td>
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<td>49.5</td>
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</tr>
<tr>
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<tr>
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<td>16</td>
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Table 5.5 (cont.) Observed and predicted IC$_{50}$-values of arylidenamino-guanidines

<table>
<thead>
<tr>
<th>Comp. No.</th>
<th>Comp. Code</th>
<th>Structure</th>
<th>Exper. IC$_{50}$ (μM)</th>
<th>PLS-11D IC$_{50}$ (μM)</th>
<th>NN-train. IC$_{50}$ (μM)</th>
<th>NN-LOO IC$_{50}$ (μM)</th>
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<tr>
<td>Comp. No.</td>
<td>Comp. Code</td>
<td>Structure</td>
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<td>PLS-11D IC$_{50}$ (µM)</td>
<td>NN-train. IC$_{50}$ (µM)</td>
<td>NN-LOO IC$_{50}$ (µM)</td>
</tr>
<tr>
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Table 5.5 (cont.) Observed and predicted IC<sub>50</sub>-values of arylidenamino-guanidines

<table>
<thead>
<tr>
<th>Comp. No.</th>
<th>Comp. Code</th>
<th>Structure</th>
<th>Exper. IC&lt;sub&gt;50&lt;/sub&gt; (μM)</th>
<th>PLS-11D IC&lt;sub&gt;50&lt;/sub&gt; (μM)</th>
<th>NN-train. IC&lt;sub&gt;50&lt;/sub&gt; (μM)</th>
<th>NN-LOO IC&lt;sub&gt;50&lt;/sub&gt; (μM)</th>
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Table 5.5 (cont.) Observed and predicted IC\textsubscript{50}-values of arylidenamino-guanidines

<table>
<thead>
<tr>
<th>Comp. No.</th>
<th>Comp. Code</th>
<th>Structure</th>
<th>Exper. IC\textsubscript{50} (µM)</th>
<th>PLS-11D IC\textsubscript{50} (µM)</th>
<th>NN-train. IC\textsubscript{50} (µM)</th>
<th>NN-LOO IC\textsubscript{50} (µM)</th>
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<tr>
<td>41</td>
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<td>62.5</td>
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<td>31.6</td>
<td>31.2</td>
<td>40.5</td>
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</tbody>
</table>
While this second PLS model was more accurate for the full set of forty-five arylidenamino-guanidine compounds than the PLS model for the preliminary set of twenty-eight compounds, it also yielded a set of eleven descriptors which were then used in the development of the artificial neural network model.

In order to determine the optimal configurations for the ANN model, parameters were modified while training, cross-validating, and testing the network in an effort to obtain the best predictive final model. These parameters, as well as the results obtained, are displayed in Table 5.6. It had been previously determined that the optimal learning rate (LR) was 0.01, and that the optimal momentum rate (MR) was 0.05; these parameters are consistent throughout the experiments. In addition, the number of nodes in the input layer were constant at 11 (the number of descriptors), as well as the number of output layers and nodes at 1. The parameters that were modified were the number of nodes in the hidden layer (from 1 to 4), and the number of total training cycles (from 10,000 to 100,000). Using each set of these two variables, models were generated and evaluated based on the correlation of predicted and observed data (R-value) and root-mean square deviation (RMSD) for the training results, as well as for the leave-one-out validation results, which generated separate correlation coefficients ($r_{cv}$) and error values (looRMSD) (see method section for definitions).
Table 5.6 Parameter modification of the artificial neural network model, using 45 molecules and 11 descriptors

<table>
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<tr>
<th>Config.</th>
<th>Cycles</th>
<th>LR</th>
<th>MR</th>
<th>R</th>
<th>RMSD</th>
<th>$r_{cv}^2$</th>
<th>$r_{cv}$</th>
<th>looRMSD</th>
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<td>PLS(11D)</td>
<td></td>
<td></td>
<td></td>
<td>0.903</td>
<td>0.718</td>
<td>0.847</td>
<td>S=0.229</td>
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<td>0.01</td>
<td>0.05</td>
<td>0.904</td>
<td>0.206</td>
<td>0.658</td>
<td>0.811</td>
<td>0.282</td>
</tr>
<tr>
<td>NN11-1-1</td>
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<td>0.05</td>
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<td>0.205</td>
<td>0.705</td>
<td>0.84</td>
<td>0.262</td>
</tr>
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<td>0.01</td>
<td>0.05</td>
<td>0.905</td>
<td>0.205</td>
<td>0.694</td>
<td>0.833</td>
<td>0.267</td>
</tr>
<tr>
<td>NN11-1-1</td>
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<td>0.01</td>
<td>0.05</td>
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<td>0.205</td>
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<td>0.828</td>
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<tr>
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<tr>
<td>NN11-3-1</td>
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<td>0.197</td>
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<td>0.05</td>
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</tr>
<tr>
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<td>0.05</td>
<td>0.903</td>
<td>0.209</td>
<td>0.632</td>
<td>0.795</td>
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<tr>
<td>NN11-4-1</td>
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<td>0.203</td>
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<td>0.909</td>
<td>0.201</td>
<td>0.695</td>
<td>0.834</td>
<td>0.266</td>
</tr>
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<td>NN11-4-1</td>
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<td>0.05</td>
<td>0.922</td>
<td>0.187</td>
<td></td>
<td></td>
<td></td>
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</tbody>
</table>
From the 3-dimensional display in Figure 5.4, it can be seen that the R-value from the training results increases with the number of training cycles and the RMSD decreases accordingly. These values are therefore not useable in the determination of the best model. However, it was also seen that the addition of a second and third node to the hidden layer yielded models with greater correlation than the models with 1 or 4 nodes in the hidden layer.

The leave-one-out validation paints a clearer picture, and a clear maximum of correlation is produced by the models with 1 hidden layer containing 3 hidden nodes, affording an \( r_{cv} \)-value of 0.849 at 30,000 training cycles (see Figure 5.5). The error is also minimized with this set of parameters to 0.250 looRMSD. While two hidden layers generated the best overall correlation, the 30,000 training cycles also generated the best \( r_{cv} \) values with each of the other hidden node values, resulting in the semispherical topology generated in Figure 5.5.

The correlation and error data from the second PLS model using the same eleven descriptors are also displayed in Table 5.6. While it is evident that the artificial neural network model did dramatically improve upon the training set R and its corresponding error, the correlation of the leave-one-out validation was only slightly better in the best ANN model, at 0.849 compared to 0.847 in the PLS model. However, the error was decreased significantly in the leave-one-out method, from 0.282 in the PLS model to 0.250 in the ANN model.
Figure 5.4 Optimization of R by variation of cycles and nodes using the training method of the artificial neural network model
Figure 5.5 Optimization of $r_{cv}$ by variation of cycles and hidden nodes using the leave-one-out method of the artificial neural network model.
The experimental IC$_{50}$-values for the arylidenamino-guanidino compounds which were generated from the screening data, as well as those predicted by both the training and leave-one-out values are displayed in Table 5.5. The observed values are plotted against the predicted values from the training method and the leave-one-out method in Figures 5.6 and 5.7, respectively.

Figure 5.6 Comparison of observed pIC$_{50}$ values versus pIC$_{50}$ training values calculated using the artificial neural network model
The plotted training values are, not surprisingly, very linear when compared to the observed values (see Figure 5.6). Each of the points lies within half an order of magnitude of the diagonal line of perfect correlation.

Figure 5.7 is very similar to 5.6, with most of the points lying within roughly one half of an order of magnitude of the ideal $r_{cv} = 1$ line. While this final predictive model had several points which were somewhat different from the values observed in the $[^{3}H]$MK-801 displacement assay, it should be remembered that this model is a tool to be used for pre-synthetic prediction of a molecule’s activity. This model should be able to make a good prediction for the IC$_{50}$ value of any compound with the general arylidenamino-guanidine structure, however there may be some structural features which impact site binding that have not yet been introduced into this ANN model, and would create anomalies with the current model. Therefore, the continual addition of more molecular structures would refine and re-train the model, making it more accurate and reducing the number of outlying points.

Regardless of future improvement, the ANN model in its current form is a successful predictive tool with excellent correlation values. The standard proof of a model’s high predictive ability is considered to be an $r_{cv}^2$ value of greater than 0.5. The best artificial neural network model obtained in this study possesses a cross-validation $r_{cv}$ of 0.849 ($r_{cv}^2$ of 0.720) and a correlation coefficient $r$ of 0.910.
3.0 3.5 4.0 4.5 5.0 5.5 6.0

pIC<sub>50</sub>, observed
pIC<sub>50</sub>, calculated

Figure 5.7 Comparison of observed pIC<sub>50</sub> values vs. pIC<sub>50</sub> leave-one-out values calculated using the artificial neural network model

While the predicted IC<sub>50</sub> values generated from the ANN method are generally nonlinear and thus quite complicated to compare directly to the descriptors, the two PLS models have given some new insight into structure-activity relationships of arylidenamino-guanidine compounds and their ability to reduce bound [³H]MK-801 in the NMDAR screening assay.

In both models, increasing the “C-027” descriptor would reduce the predicted inhibition, increasing the predicted IC<sub>50</sub>-value. This descriptor is a measure of the number of unsubstituted heteroatoms adjacent to a monoprotonated carbon, i.e. those contained within an aromatic ring (but without complete adjacent substitution as in the case of compound 35). Therefore, inclusion of pyridine, furan, or thiophene rings in the structure of analogs appears to be detrimental to the inhibitory activity of this class of compounds. Conversely, in the first model, the descriptor nNHR was shown to have a positive effect on inhibition; i.e. increasing the number of substituted protonated amino
groups increased receptor inhibition. While this latter descriptor was not used in the second PLS model nor the ANN model, these combined data indicate that indole and pyrrolidine rings, which contain non-basic protonated nitrogen atoms, are not detrimental to NMDA inhibition.

The first PLS model also demonstrated that decreasing the PSA descriptor increases the inhibitory activity of the molecule. This descriptor is a measure of polar surface area, and while it would be difficult to differentiate most of these compounds based on large differences in molecular surface area, this observation indicates that increasing molecular size (and thus PSA) could be detrimental to the inhibitory activity of the molecules.

Another structural detriment was the descriptor nCIC, defined as the total number of rings in the molecule. While the forty-five molecules used in the second computational model contained only two options for this descriptor, i.e. one or two rings, the model predicted that two rings were generally better than one for inhibitory activity.

Finally, an increase in the descriptor mlogP predicted an increase in the inhibitory activity of the arylidenamino-guanidino compounds in the second model. This Moriguchi octanol-water partition coefficient is a measure of the lipophilicity of the compound. Thus, increasing the mlogP value in these analog structures lowered the predicted IC$_{50}$-value. The compounds with the highest value for this descriptor included compound 9 [(2-biphenyl-carboxylidenamino)-guanidine hydrochloride] as well as compounds 32 and 33 (the 2- and 4-methylated 1-naphthylidenamino-guanidine hydrochloride compounds, respectively). This finding is interesting in that these more hydrophobic compounds were difficult to evaluate in the screens, due to their low solubility. This also raises issues with regard to such compounds as (4-biphenyl-carboxylidenamino)-guanidine hydrochloride, which was too insoluble in water to be screened. However, the general finding can be a valid consideration that testing further compounds which have increased lipophilicity may improve their inhibitory properties at the NMDA receptor.
5.4 Conclusions

Using a combination of both the partial least squares (PLS) and back-propagation artificial neural network (ANN) pattern recognition methods, several models have been developed to predict the activity of arylidenamino-guanidine compounds as inhibitory modulators of the N-methyl-D-aspartate receptor complex. This was done by correlating structural and physicochemical descriptors obtained from computation software to the observed $[^3]H$MK-801 displacement ability of 45 synthesized and screened arylidenamino-guanidine compounds.

A preliminary PLS model was developed using a smaller set of inhibitory compounds in this class, and this model was further refined with a set of additional arylidenamino-guanidines, while reducing the number of descriptors needed to yield a viable model. These models yielded correlation coefficients of $r = 0.941$ and $r = 0.903$, respectively; this decrease in correlation is due to the increase in sample size in addition to the inclusion of several biphasic compounds (see Chapter 3 for more details).

The ANN model was created with utilization of the eleven descriptors from the second PLS model. While this model would certainly benefit from the future addition of more structures to the dataset, it was still a viable and improved model, which resulted in a correlation coefficient of $R = 0.910$ and a cross-validated $r_{cv} = 0.849$. Each of the screened compounds tested had the same common structural feature, i.e. an aromatic ring attached to an imino-guanidine moiety. The majority of the molecules screened in the laboratory and predicted in these models have correlated well between observed and predicted inhibitory values (see Table 5.5 and Figures 5.3, 5.6, and 5.7).

These computational models are vital tools in the development of future generations of arylidenamino-guanidino compounds, as they are able to predict inhibition of the NMDAR by this series of compounds in silico. Arylidenamino-guanidino compounds with low predicted IC$_{50}^{Pot}$-values (i.e. high NMDAR inhibitory potency) will be synthesized and examined in the screening assays, while those compounds with high predicted IC$_{50}^{Pot}$-values (i.e. low NMDAR inhibitory potency) will be largely bypassed. In this way, a predictive structure-based in silico “pre-screening” of the compounds will save a substantial amount of otherwise wasted time and cost in synthesis.
Furthermore, the ANN model would benefit from the addition of further sub-libraries of compounds, which would add new structural “knowledge” as well as increase the accuracy of the model. The ability of these models to evolve in accuracy and complexity with the addition of further training sets of arylidenamino-guanidino compounds makes them invaluable in the further development of NMDA receptor inhibitory modulators as potential drug candidates.
CHAPTER 6: PHARMACOPHORE MODEL

6.1 Introduction

The information gathered in this study has been very informative, and the class of compounds which has resulted from the work is one which may be an excellent start towards identification of a potential candidate for pharmaceutical development. Each of the first two specific aims has been achieved. A library of several hundred agmatine analogs has been synthesized, screened, refined, and analyzed, and nine compounds in the arylidenamino-guanidine series have been identified as being more potent and selective for the agmatine binding site of the NMDA receptor complex than agmatine itself (Table 6.1). Furthermore, three of these compounds yielded clear biphasic dose-response curves for the displacement of $[^{3}H]$MK-801. These data reinforce the hypothesis that agmatine and its analogs can act at different sites, and specifically can interact with a high affinity site which can then be targeted therapeutically to inhibit the pathological overactivation of the NMDAR channel.

With the data that has already been obtained from the structure-activity studies undertaken in this work, along with the X-ray crystal structure data, a pharmacophore of this high-affinity agmatine binding site can be postulated. The introduction of this pharmacophore model will constitute a summary of the general structure-activity relationship conclusions drawn from this work, and will also serve as a useful tool to further direct the development of this novel class of compound as potential therapeutic agents.

The two factors which are to be considered in terms of identifying a useful NMDAR inhibitor are potency and selectivity for the agmatine binding site. As set forth in Chapter 1, the agmatine binding site is the site at which agmatine acts to reduce the channel-activating effects of the longer chain endogenous polyamines. It is apparent that agmatine also acts at a low-affinity site to displace $[^{3}H]$MK-801 down to the control levels of channel inhibitors such as dextromethorphan. However, the reason that agmatine is a valid therapeutic lead is that it acts at a higher affinity “agmatine binding site”, reducing pathological effects, at a lower concentration than it acts at the low-
affinity site, where it would reduce physiological effects mediated by the NMDA receptor. The measurement for potency at the agmatine binding site is approximated as the IC$_{50}^{\text{Pot}}$-value, which is the concentration of ligand necessary to reduce the amount of MK-801 bound to NMDA receptors by 50% of the difference between the spermidine-potentiated “pathological state” levels and the control “physiological state” levels. This measurement assumes that there are different mechanisms operating for a) a ligand to reduce binding from the SPD-potentiated levels to the non-potentiated control levels, and b) a ligand to reduce the binding from the non-potentiated control levels to the complete channel blockage control levels obtained with a high dose of dextromethorphan. With these assumptions, the IC$_{50}^{\text{Pot}}$ is an approximation of the IC$_{50}$ for mechanism “a”. The IC$_{50}$ value for mechanism “b” can easily be obtained from the dose-response curve obtained for MK-801 displacement in the absence of added spermidine. The selectivity for the agmatine binding site is given as a ratio of the IC$_{50}$-values for mechanisms “a” and “b”, and is expressed as a “ratio of potentiation”, or Ratio$_{\text{Pot}}$, which is simply the IC$_{50}^{\text{Pot}}$ divided by the IC$_{50}$ of the non-potentiated dose-response curve (i.e. the two values mentioned above). In the case of compounds 21, 24, and 31, which were sufficiently selective for the agmatine binding site to yield biphasic dose-response curves, the separation between these two mechanisms was visibly evident, and both the IC$_{50}^{\text{Pot}}$ and Ratio$_{\text{Pot}}$ values can be calculated from the graphical data without approximation.

Section 6.2 will examine closely the impact of the structural features of the compounds tested on the selectivity for, and potency at, the agmatine binding site of the NMDA receptor channel complex, detailing the observations drawn from each chapter.

6.2 Results and Discussion

6.2.1 Chapter 2 Insight

Chapter 2 focused largely on the early prepared compounds, which more closely resembled agmatine, while varying each part of the molecule in order to discriminate the structural features which might contribute to the potency and selectivity for the agmatine binding site of the NMDA receptor complex. While these series of compounds were
primarily a stepping stone towards the aryldenamino-guanidino compounds of Chapter 3, there were many important and interesting data which pertain to the interaction of these compounds with the agmatine binding sites.

![Figure 6.1 Modification sites on the agmatine molecule](image)

It was found that the best general substitution for Moiety B (Figure 6.1), the four carbon bridge between the terminal amine and the guanidino group, was an aromatic ring. In particular, a phenyl ring with an attached methylene bridge afforded more potent compounds than just a simple phenyl ring, and a xylene moiety with an attached methylene bridge on each side proved to be the best substitution of all the ones tested. While the larger intramolecular spacing may be important, the xylene linkers also provided more molecular flexibility than the simple aromatic linkers. In addition, agmatine analogs containing a meta-xylene linker proved to be more selective for the agmatine binding site (i.e. had higher $\text{Ratio}_{\text{pot}}$ values) than compounds with a para-xylene linker, which is likely due to the fact that the meta-xylene linker is closer in length to agmatine than the para-linker. While inclusion of an aromatic ring in the linker appears to increase the potency of these agmatine analogs at both the high-affinity agmatine site and the low-affinity agmatine site, these small changes in spacing accompanying the inclusion of the aromatic moiety may be crucial towards developing this new agmatine binding site pharmacophore.

As early attempts to substitute groups for the terminal amino functionality did not improve the inhibitory properties of the resulting agmatine analogs, Moiety A remained unchanged in the majority of the compounds described in Chapter 2. Variation of Moiety C (Figure 6.1 above) proved to be unproductive, and did not lead to any improvement in
NMDAR inhibition, as every structural modification of the guanidine group attempted resulted in a less potent compound than the parent molecule (i.e. the molecule with the intact, unsubstituted guanidine group.

The ratio of potentiation for each bis-guanidinium compound tested was close to 1:1. These data indicate that none of these compounds binds to the same polyamine site as agmatine, although the significance difference in hill slope indicates that none of these compounds act as inhibitors of the channel site. It is thus postulated that these bis-compounds, which are structurally related to arcaine, bind at another polyamine recognition site, and act allosterically as more potent inhibitors in the absence of spermidine than in its presence. Thus, while these arcaine analogs are NMDAR inhibitors, they do not act at the high affinity agmatine binding site.

6.2.2 Chapter 3 Insight

The research described in Chapter 3 was clearly focused around the lead sub-library of compounds, the arylidenamino-guanidino compounds. Each of the compounds within this series was shown to be an NMDAR inhibitor to some degree. The nine compounds which possessed both a low IC$_{50}^{\text{Pot}}$-value and a high ratio of potentiation (Ratio$_{\text{Pot}}$) are listed in Table 6.1.

A discussion of the effects of the different aromatic ring substituents on potency and selectivity within the arylidenamino-guanidino compounds will be summarized from the groups of compounds described previously in Chapter 3, with the focus on determining the best structural modifications which will increase both inhibitory potency and selectivity for the agmatine binding site. While these compounds can be broken down according to such structural factors as sterics and electronics, it is important to note that many of these compounds could possess multiple structural characteristics which could impact agmatine site binding. While a certain structural feature (for example, a hydrogen bond acceptor group at the 4-position of the aromatic ring) may seem to have a positive effect on selectivity for the agmatine binding site, such a compound might also be too sterically bulky to fit into the site, and thus the “negative” steric effect may override the “favorable” H-bond acceptor effect. Thus, the identification of an accurate
pharmacophore may be difficult, especially with a limited number of ligands and very little structural information about the actual binding site. Nevertheless, such data may be vital in the further refinement of this class of possible NMDAR antagonists.
Table 6.1 Selective agmatine-site NMDAR inhibitors; IC\textsubscript{50}-values in the presence and absence of spermidine, IC\textsubscript{50} of potentiation, and ratio of potentiation

<table>
<thead>
<tr>
<th>Compound</th>
<th>Structure</th>
<th>(\text{IC}_50) w/ SPD\textsuperscript{1}</th>
<th>(\text{IC}_50) w/o SPD\textsuperscript{2}</th>
<th>(\text{IC}_50) Pot \textsuperscript{3}</th>
<th>Ratio\textsubscript{Pot}</th>
</tr>
</thead>
<tbody>
<tr>
<td># (Code)</td>
<td></td>
<td>(µM)</td>
<td>(µM)</td>
<td>(µM)</td>
<td></td>
</tr>
<tr>
<td>12 (AG-12)</td>
<td></td>
<td>61.9</td>
<td>62.3</td>
<td>12.8</td>
<td>4.9 : 1</td>
</tr>
<tr>
<td>17 (JR-219)</td>
<td></td>
<td>71.7</td>
<td>94.4</td>
<td>7.9</td>
<td>11.9 : 1</td>
</tr>
<tr>
<td>21 (JR-223)</td>
<td></td>
<td>259.4*</td>
<td>219.8</td>
<td>7.8</td>
<td>28.2 : 1</td>
</tr>
<tr>
<td>22 (JR-218)</td>
<td></td>
<td>41.2</td>
<td>52.6</td>
<td>7.1</td>
<td>7.4 : 1</td>
</tr>
<tr>
<td>23 (JR-217)</td>
<td></td>
<td>78.7</td>
<td>117.6</td>
<td>18.4</td>
<td>6.4 : 1</td>
</tr>
<tr>
<td>24 (JR-220)</td>
<td></td>
<td>124.5*</td>
<td>124.5</td>
<td>3.6</td>
<td>34.6 : 1</td>
</tr>
<tr>
<td>31 (JR-213)</td>
<td></td>
<td>117.8*</td>
<td>77.2</td>
<td>8.7</td>
<td>8.9 : 1</td>
</tr>
<tr>
<td>33 (JR-204)</td>
<td></td>
<td>48.9</td>
<td>80.3</td>
<td>11.5</td>
<td>7.0 : 1</td>
</tr>
<tr>
<td>36 (AG-2)</td>
<td></td>
<td>279.6</td>
<td>443.7</td>
<td>59.3</td>
<td>7.5 : 1</td>
</tr>
<tr>
<td>Agmatine</td>
<td></td>
<td>902.3</td>
<td>1296</td>
<td>274.2</td>
<td>4.7 : 1</td>
</tr>
</tbody>
</table>

\textsuperscript{1} - \(\text{IC}_50\) w/ SPD: the measurement of \(\text{IC}_50\) derived from the data collected in the spermidine-potentiated [\(^3\text{H}\) MK-801 displacement assay.

\textsuperscript{2} - \(\text{IC}_50\) w/o SPD: the measurement of \(\text{IC}_50\) derived from the data collected in the non-spermidine-potentiated [\(^3\text{H}\) MK-801 displacement assay, in which the spermidine solution was replaced with buffer.

\textsuperscript{3} - \(\text{IC}_50\) of Pot: from the spermidine-potentiated curve, the concentration corresponding to one-half of the difference in DPM between the SPD-potentiated control and the blank control.

* - This measurement is the \(\text{IC}_50\) of the lower phase, as these SPD-potentiated curves were clearly biphasic in nature.
Aliphatic and Aromatic Substitution in Arylidenamino-Guanidino Compounds:

From the SAR data obtained on the arylidenamino-guanidino compounds with strictly aliphatic substituents on the aromatic ring, it appears that the addition of non-functional substituents, which strictly act as steric bulk, improves potency but not selectivity for the agmatine binding site. Compared to the unsubstituted compound 1 (which possesses a 4.6 $\text{Ratio}_{\text{pot}}$, very close to the 4.7 value of agmatine itself), none of the aliphatic-substituted compounds prepared had a high ratio of potentiation. These aliphatic substituents included methyl, isopropyl, $n$-butyl, and tert-butyl groups. Thus, \textit{addition of aliphatic substituents affords no improvement over either the parent unsubstituted molecule or agmatine}, and does not belong in the pharmacophore.

Aromatic substituents introduced into the main aromatic ring afford structure-activity relationships that are more difficult to interpret. As with a number of the more bulky molecules screened in the study described in Chapter 2, the biphenyl compound 29, as well as the indolyl compounds 39-42, appeared to be potent ligands at the low-affinity binding site, but not at the high-affinity agmatine binding site. However, the naphthyl analogs, compounds 30-33, did not fit this mold. This set of four bicyclic naphthyl compounds included two compounds (30 and 32) with low $\text{Ratio}_{\text{pot}}$ values (2.2 : 1 and 1.5 : 1, respectively), and two compounds (31 and 33) with high $\text{Ratio}_{\text{pot}}$ values (8.9 : 1 and 7.0 : 1, respectively). As was discussed in Chapter 3, the only discernable difference between these two sets of compounds is the location of their methyl and phenyl substituents. While compounds 30 and 32 have substituents at the \textit{ortho-} and \textit{meta-}positions, compounds 31 and 33 can be viewed as a benzylidenamino-guanidine with substituents at the \textit{meta-} and \textit{para-}positions. Interestingly, these two positions are
substituted in one of the most selective arylidenamino-guanidine compounds discovered in the entire work, compound 21 (Figure 6.2). It can thus be postulated that while a second ring is generally not an indicator of agmatine selectivity, an exception is made when there are two substituents at the meta- and para-positions.

Figure 6.2 Structurally-conserved substitutions in compounds 21, 31, and 33
Hydrogen Bond Donor Ligands:

A very important component in the binding of small molecule ligands to proteins is hydrogen bonding between the ligand and electronegative sites (normally from oxygen, sulfur, or nitrogen) on the surface of the protein molecule. These hydrogen bond interactions are roughly 10% as strong as covalent bonds. In an attempt to determine the residues which may be present in the binding site, the ligands which have the ability to form hydrogen bonds with receptor protein residues will be discussed (Figure 6.3).

The molecules most likely to exhibit hydrogen bond donation properties at the receptor binding site would be those containing protonic functionalities such as amino groups or hydroxyl groups. The former group includes compounds 15 and 16, which possess primary amino moieties at the meta- and para-positions of the phenyl ring, respectively, and compound 40, which is an indolyl compound with essentially a non-basic secondary amino group at the para-position. The latter group, with hydroxyl substituents, includes two arylidenamino-guanidino compounds, compound 11, disubstituted with hydroxyl groups at the ortho- and para-positions of the phenyl group, and compound 20, which has a hydroxyl group at the meta-position of the phenyl group. Not one of these compounds has a Ratio\textsubscript{pot}-value higher or equal to that of agmatine. While these compounds may be more potent NMDAR antagonists than agmatine, they are not as selective as agmatine for the agmatine binding site. Therefore, it is likely that the agmatine binding site does not contain any amino acid residues which could participate as hydrogen bond acceptors, in the binding of ligands with hydrogen-bond donating properties. Therefore, hydrogen bond donor groups which are directly attached to the phenyl ring of arylidenamino-guanidino compounds are not favored as selective agmatine binding site antagonists.
Figure 6.3 Interaction of direct ring-substituted hydrogen bond donor substituents (D) with an acceptor moiety (A) on the surface of the NMDAR protein.

Three compounds containing heteroatomic moieties in their structures that have potential to act as hydrogen bond donors were also synthesized. These compounds all have the imino-guanidine moiety attached to five-membered nitrogen-containing aromatic rings (Figure 6.4). **Compound 36** is a 2-pyrrole group which has a single proton attached to its ring nitrogen at physiological pH, and was shown to be more
selective and potent than agmatine in the [³H]MK-801 displacement assay. **Compounds 39** and 42 are 3-indolyl compounds, with the latter containing a 6-methyl group. **Compound 39** was shown to maintain the level of selectivity of agmatine, while **compound 42** was less selective than agmatine (perhaps due to the steric bulk of the added 6-methyl group). However, both compounds showed a 10-fold increase in NMDAR potency. These data appear to indicate that *protonated heteroatoms in the aromatic ring directly attached to the imino-guanidine moiety can act as hydrogen bond donors with residues on the surface of the agmatine binding site.*

![Chemical structures of compounds 36, 39, and 42]

<table>
<thead>
<tr>
<th>Compound 36</th>
<th>Compound 39</th>
<th>Compound 42</th>
</tr>
</thead>
<tbody>
<tr>
<td>IC₅₀^pot = 59.3 µM</td>
<td>IC₅₀^pot = 36.9 µM</td>
<td>IC₅₀^pot = 32.9 µM</td>
</tr>
<tr>
<td>Ratio^pot = 7.5 : 1</td>
<td>Ratio^pot = 4.7 : 1</td>
<td>Ratio^pot = 2.3 : 1</td>
</tr>
</tbody>
</table>

**Figure 6.4 Interaction of ligands containing ring heteroatoms that can act as a hydrogen bond donor (D) with an acceptor moiety (A) on the surface of the NMDA receptor protein**

Hydrogen Bond Acceptor Ligands:

Compounds that have aromatic ring substituents which may act as hydrogen bond acceptors were also synthesized. These structures were much more varied in structure,
selectivity, and potency. In addition, these molecules contained atoms which were capable of forming hydrogen bonds; these atoms were directly attached to the aromatic ring in these molecules, e.g. the oxygen atom of a methoxy group or a nitro group. It should also be noted that the oxygen atom of hydroxy group (as well as the nitrogen atom of an unprotonated amino group) can act as a hydrogen bond acceptor, as well as a hydrogen bond donor, as seen in the previous section.

Hydrogen bond acceptor moieties which are directly attached to the aromatic ring (Figure 6.5) in the ortho-position include the hydroxy group of compound 11, and the methoxy group substituents of compounds 12 and 14. Compound 12, with no other substituents, is more potent and slightly more selective than agmatine. Compound 11 is substituted with a second hydroxyl group at the para-position, and compound 14 possesses a second methoxy group substituent at the para-position; both are less selective than agmatine, and less potent than compound 12. As none of these compounds were subjected to X-ray structure determination, it cannot be definitively stated as to which phenyl ortho-position protamer is preferred; however, it is reasonable to assume that compounds 12 and 14 have their ortho-methoxy groups at the same position (due to favorable intramolecular hydrogen bonding, assumedly the 2-position of the phenyl ring, going clockwise from the imine). Thus, whether these compounds are all substituted at the 2- or 6-positions, as long as the substituent location is constant, it does not appear to have any particular effect on inhibitory activity. Therefore, while a hydrogen bond accepting moiety directly attached to the ring at the ortho-position may be beneficial to selectivity for the agmatine binding site, compounds with another such moiety at the para-position appear to afford lower potency and selectivity.

\[
\begin{align*}
\text{Compound 11} & \quad \text{IC}_{50}^{\text{pot}} = 167.5 \mu M \quad \text{Ratio}_{\text{pot}} = 1.7 : 1 \\
\text{Compound 12} & \quad \text{IC}_{50}^{\text{pot}} = 12.8 \mu M \quad \text{Ratio}_{\text{pot}} = 4.9 : 1 \\
\text{Compound 14} & \quad \text{IC}_{50}^{\text{pot}} = 28.8 \mu M \quad \text{Ratio}_{\text{pot}} = 2.4 : 1
\end{align*}
\]
Compounds with hydrogen bond acceptor moieties incorporated into the ring at the para-position were more numerous, and included compounds 11 and 14 mentioned above, as well as compounds 13, 17, 20, 21, and 34. Of these molecules, compounds 17 and 21 were the only ones that were more selective for the agmatine binding site than either compound 1 or agmatine. Compound 17, with an attached dimethylamino group, is the most sterically-hindered hydrogen bond acceptor group in this series of compounds. The other six compounds in this series all possess oxygen-containing moieties at the para-position of their phenyl rings, and may be more likely to act as hydrogen bond acceptors with agmatine binding site residues. Compound 13, contains a para-methoxy group, and has very similar activity to compound 14, which contains the same group (in addition to its ortho-methoxy substituent); both have low selectivity for the agmatine binding site. Likewise, compounds 20 and 34 both have hydrogen bond acceptor groups at the meta- and para-positions. Compound 21 has a para-methoxy group, just like compounds 13, 14, and 20. However, compound 21 has a nitro group at the meta-position of the aromatic ring, and is so potent and selective for the agmatine binding site that it yielded a biphasic dose-response curve with a 28.2 : 1 Ratio_Pot. Thus, while these results are not definitively conclusive, it appears that a hydrogen bond acceptor atom attached directly to the aromatic ring at the para-position can be present in a compound which is selective for the agmatine binding site, but it may also serve to reduce the selectivity of an otherwise promising compound (i.e. compound 11).
Figure 6.5 Interaction of ligands containing a direct ring-substituted hydrogen bond acceptor moiety (A) with a donor moiety (D) on the surface of the NMDA receptor protein

Compounds with hydrogen bond acceptor atoms separated from the aromatic ring by another atom (Figure 6.6) included three different types of moieties: a methyl-ester
group or a nitro group, both of which represent groups with two possible hydrogen bond acceptor interactions, or a cyano group, having a single site for hydrogen bonding. Compounds were prepared with all three of these aromatic substituents in either the meta- or para-positions: i.e. the meta-methyl-ester compound 8, the para-methyl-ester compound 9, the meta-nitro compound 18, the para-nitro compound 19, the meta-cyano compound 6, and the para-cyano compound 7. These six compounds were neither especially potent inhibitors at the NMDA receptor, nor more selective inhibitors of the agmatine binding site than compound 1. Interestingly, compound 21 did not fit the general SAR within their group; the inclusion of the meta-nitro group in compound 21 resulted in an increase in selectivity and potency over the para-methoxy compound 13, which was otherwise structurally the same. Thus, compounds with a hydrogen bond acceptor atom one atom separated from the aromatic ring were not notable inhibitors without additional functionalities, i.e. the para-methoxy group of compound 21.
Figure 6.6 Interaction of ligands containing a hydrogen bond acceptor moiety (A) one atom removed from the aromatic ring with a donor moiety (D) on the surface of the NMDA receptor protein.
In addition, there are four arylidenamino-guanidine compounds which contain heteroatoms in their aromatic moieties that have the potential to act as hydrogen bond acceptors (Figure 6.7). These are the 3-pyridyl and 4-pyridyl compounds (37 and 38, respectively), the 3-thiophene compound (43), and the 2-furano compound (which also has a 5-para-nitrophenyl substituent) (35). This small sub-set of compounds is entirely comprised of nonselective and relatively impotent compounds, and thus it can be concluded that aromatic rings containing hydrogen bond accepting heteroatoms do not interact in a positive way with the agmatine binding site of the NMDA receptor.

Electronic effects:

Aromatic substituents can have direct effects on the electronics of a phenyl or heteroaromatic ring, generally grouped into inductive and resonance effects. Inductive effects are exerted by electronegative atoms, which serve to pull σ electrons toward the more electronegative atom, and away from the phenyl ring or its other substituents. This has the effect of generating partial negative charges on electronegative atoms such as a halogen, while the carbons of the ring become slightly positively-charged, resulting in more polarized carbon-carbon bonds (Figure 6.8). The strongest electron-withdrawing
Inductive effects will be exerted by the most electronegative functional groups, which include halogeno, trifluoromethyl, nitro, and ester moieties.

![Figure 6.8 Inductive effects exerted by phenyl group substituents](image)

(Q = electron withdrawing group, e.g. Cl, CF₃, NO₂)

Resonance (or electromeric) effects serve to delocalize π electrons through conjugated double bonds, and are often observed in phenyl groups with electron-withdrawing functionalities such as nitro groups (Figure 6.9 A), and electron-donating functionalities with nonbonding electron pairs, such as amino, dimethylamino, hydroxy, and methoxy groups (Figure 6.9 B). Halogens, while possessing strong electron-withdrawing inductive effects, are also able to act as electron donors and resonance stabilize the aromatic ring. Fluorine is the most electronegative halogen, yet fluorobenzene is the most reactive halobenzene, due to the size of the fluorine atom, relative to carbon. Donation of an electron pair from fluorine arises from orbital overlap between the 2p orbital of fluorine and the 2p orbital of the adjoining carbon atom. The larger halogens, such as chlorine in this study, are relatively poor electron donors, as they are significantly larger than carbon and their nonbonding pairs are further from their nuclei and therefore do not overlap well with the 2p orbital of the carbon molecule to which they are bound.
Figure 6.9 Resonance effects observed with phenyl group substituents, whether electron-withdrawing (A.) or electron-donating (B.)

Phenyl ring substituents which have electron-withdrawing inductive and resonance effects include nitro, cyano, and ester groups. As previously mentioned, the two compounds with ester phenyl substituents, the meta-methyl-ester compound 8 and the para-methyl-ester compound 9, were neither potent nor selective for the agmatine binding site. Neither were the two compounds with a single nitro substituent, compounds 18 and 19. However, compound 21, with a meta-nitro and para-methoxy substituent, was extremely potent and selective. The meta-cyano and para-cyano compounds (6 and 7, respectively) had similar activity as their corresponding nitro-substituted compounds, i.e. low potency and selectivity for the agmatine binding site.

The ortho-trifluoromethyl aromatic substituent of compound 10 has a strong electron-withdrawing inductive effect on the ring, but is unable to form any resonance structures. This compound also possessed low potency and selectivity for the agmatine binding site.
Phenyl substituents which are the most electron-donating include amino groups, substituted amino groups, hydroxyl groups, and methoxy groups. While nitrogen and oxygen atoms are slightly more electrophilic than carbon, and can have a small electron-withdrawing inductive effect, the resonance effects of these compounds are much stronger, and in general, they freely donate their $2p$ electrons to the similar-sized carbon atoms of the phenyl ring to which they are attached. The substituents which have strong electron-donating substituents are those with nonbonded electron pairs, and thus this subset of arylidenamino-guanidino compounds mirrors the subset of compounds which have substituents that act as hydrogen bond acceptors (see the previous sections and Figure 6.5). The results remain true in this section as well, that electron-donating substituents attached directly to the aromatic ring at the ortho- or para-position generally do not improve the selectivity or potency of the parent compound for the agmatine binding site. However, in several cases such as compounds 21 and 34, a para-substituent with electron-donating character was present, but not as the sole substituent.

Perhaps the most interesting results in this sub-set of arylidenamino-guanidine compounds were produced by the halogeno-compounds. As previously described, the halogen atoms are electrophilic (having an electron-withdrawing inductive effect on the phenyl ring), but are also able to donate nonbonding electrons to the phenyl ring (having an electron-donating resonance effect on the phenyl ring). Four compounds in this group with aromatic fluoro substituents (the most electronegative halogen atom) were prepared and tested: i.e. compound 26, containing an aromatic ortho-fluoro substituent; compound 27, with a para-fluoro substituent; compound 28, containing both ortho- and para-fluoro substituents. None of these compounds exceeded a $4.0:1$ Ratio$_{Pot}$, nor were they remarkably potent NMDAR inhibitors. The four compounds that contained aromatic chloro substituents afforded better results. While the 2,4-difluoro compound 25 was potent, but not especially selective for the agmatine binding site, the three mono-chloro arylidenamino-guanidines were among the best lead compounds discovered. All of these compounds had IC$_{50}^{Pot}$ values below 20 μM. The Ratio$_{Pot}$ was 7.4 : 1 for the ortho-chloro compound 22, 6.4 : 1 for the meta-chloro compound 23, and a staggering 34.6 : 1 for the para-chloro compound 24. The differences between chlorine and fluorine atoms are mainly related to their size; a fluorine atom is a much smaller and
better electron-donating group to a phenyl ring due to its similar size to carbon. The nonbonding electrons in the $2p$ orbitals of fluorine overlap well with the $2p$ orbitals of carbon, but the $3p$ orbitals of chlorine atoms, which hold their nonbonding electron pairs, do not.

This group of data does not seem to follow an inflexible rule which pertains to the electronic effects of these substituents on activity, but several points can be made. 1) Electron-withdrawing substituents which exhibit inductive effects may contribute to selectivity, but 2) electron-withdrawing groups which induce resonance effects (i.e. electron sinks), pulling $\pi$-bond characteristic from the imine and guanidine moieties, do not contribute to agmatine-site selectivity, and may indeed reduce any activity conferred by beneficial inductive effects. In addition, the fact that the aromatic chloro analogs were more potent and selective agmatine binding site inhibitors than the aromatic fluoro analogs demonstrates that 3) electron-donating phenyl substituents may also reduce the inhibitory activity of arylidenamino-guanidino compounds.

6.2.3 Chapter 4 Insight

The information gleaned from the X-ray structure analysis was very important in that it provided a definitive look at several different arylidenamino-guanidine compounds, with bond geometries and angles. While there were not found to be any effects from the different ring substituents on bond conjugation, geometry, or length in the imino-guanidino part of the molecule, the similarities exhibited by the molecules provide a good reflection of the geometrical form in which this group of compounds binds to the high-affinity agmatine binding site.

Each of the salt forms of the tested molecules had a very similar structure, with regard to their phenylidena mino-guanidine moieties, and the boxed section of the structure of compound 21 in Figure 6.10 provides this general feature in these molecules. The guanidine moiety was shown to exist with the double bond characteristics primarily shared between the exo C-N bonds, and roughly 20% of the double bond characteristic was localized in the endo C-N bond. Thus, 80% of the unit positive charge of the protonated guanidine moiety is localized on the exo nitrogen atoms, which infers that these positively charged exo C-N moieties could interact with
similar negatively charged polar moieties, such as the ionized carboxylate of an aspartic or glutamic acid residue. Measurements of the torsion angles and bond angles between the phenyl ring and the guanidino group show that these compounds are almost completely planar: the torsion angles along the chain between the phenyl ring and the guanidine group are close to $180^\circ$, and the bond angles are very close to $120^\circ$. These data also show that the observed planarity is not just a result of the crystal packing, but a result of significant double-bond conjugation throughout the molecules. Each of the bonds between the phenyl ring and the guanidine moiety were observed to be strictly in the $E$ geometrical form, which extends the guanidine moiety away from the phenyl ring and its binding domain, and into a different region of the agmatine binding site.

Figure 6.10 X-ray structure of compound 21 with boxed “conserved moiety”
The information gleaned from the partial least squares and artificial neural network models (see Chapter 5) was very informative, and supported many of the observations thus far postulated in this chapter, with respect to the pharmacophore model. For instance, the ANN model predicted that the descriptor which measures unprotonated aromatic heteroatoms should be minimized; this is in agreement with the observations in Section 6.2.2 that hydrogen bond acceptors within the aromatic ring detract from the ability of arylidenamino-guanidino ligands to inhibit NMDAR activity.

However, there is a striking limitation in the therapeutic value of these models with regards to the pharmacophore of the agmatine binding site: in developing such models, the input variable to be optimized is the IC$_{50}^{\text{Pot}}$. For example, the computer models were instructed that \textbf{compound 3}, with an IC$_{50}^{\text{Pot}}$-value of 6.0 μM, is “better” than \textbf{compound 21}, which has an IC$_{50}^{\text{Pot}}$-value of 7.8 μM. However, the latter compound is over six times as selective for the agmatine binding site than the former compound, making it a much better therapeutic candidate.

The conclusions put forth in Chapter 5 are by no means without value or merit. Quite the contrary, several of them have been stated earlier in this chapter as relevant to our understanding of the agmatine binding site. More importantly, these models are a vital tool in the development of potent arylidenamino-guanidino NMDAR inhibitors, as they are able to predict potency \textit{in silico}. Therefore, structurally similar compounds with high predicted IC$_{50}^{\text{Pot}}$-values (i.e. low NMDAR inhibitory potency) can remain unsynthesized and untested in lieu of compounds with low predicted IC$_{50}^{\text{Pot}}$-values (i.e. high NMDAR inhibitory potency); while the agmatine site selectivity of the compounds will have to be determined experimentally, a substantial amount of cost and time can be saved by synthesis of only the arylidenamino-guanidino compounds with the highest predicted NMDAR potency. Regardless, the data from the models are not suitable for the current task of introducing a high-affinity agmatine binding site pharmacophore.
6.3 Conclusions

The data collected throughout this work has been examined with the intent of gathering structure-activity relationship information in order to propose a pharmacophore for the agmatine binding site on the NMDA receptor complex. The conclusions drawn from each section are compiled below.

I. Inclusion of an aromatic ring in the linker between the terminal amino group and the guanidino moiety of an agmatine analog seems to increase its potency for both the high-affinity agmatine site and the low-affinity site.

II. Every structural modification of the guanidine group attempted resulted in a less potent compound than the parent molecule (i.e. the molecule with the intact, unsubstituted guanidine group).

III. Bis-compounds, which are structurally related to arcaine, bind at another polyamine recognition site, and act allosterically as more potent inhibitors in the absence of spermidine than in its presence.

IV. Addition of aliphatic substituents to the phenyl ring affords no improvement over either the parent unsubstituted molecule or agmatine.

V. While a second aromatic ring adjoined to the first is generally not an indicator of agmatine selectivity, an exception is made when there are two substituents at the meta- and para-positions.

VI. Hydrogen bond donor groups which are directly attached to the phenyl ring of arylidenamino-guanidino compounds are not favored in selective agmatine binding site antagonists.

VII. Protonated heteroatoms in the aromatic ring directly attached to the imino-guanidine moiety can act as hydrogen bond donors with residues on the surface of the agmatine binding site.

VIII. Aromatic rings containing hydrogen bond accepting heteroatoms do not interact in a positive way with the agmatine binding site of the NMDA receptor.

IX. Direct ring-substituted hydrogen bond donors are not favored in selective ligands.
X. A hydrogen bond accepting moiety directly attached to the aromatic ring at the ortho-position may be beneficial to selectivity for the agmatine binding site.

XI. A hydrogen bond acceptor moiety attached directly to the aromatic ring at the para-position can be present in a compound which is selective for the agmatine binding site, but it may also serve to reduce the selectivity of an otherwise promising compound.

XII. Compounds with a hydrogen bond acceptor atom one atom separated from the aromatic ring were not notable inhibitors without additional functionalities.

XIII. Electron-donating substituents attached directly to the aromatic ring at the ortho- or para-position generally do not improve the selectivity or potency of the parent compound for the agmatine binding site. However, in several cases of selective and potent NMDAR agmatine binding site inhibitory molecules a para-substituent with electron-donating character was present, but not as the sole substituent.

XIV. Electron-withdrawing aromatic substituents which exhibit inductive effects may contribute to selectivity.

XV. Electron-withdrawing aromatic substituents which induce resonance effects (i.e. electron sinks), pulling π-bond characteristic from the imine and guanidine moieties, do not contribute to agmatine-site selectivity, and may indeed reduce any activity conferred by beneficial inductive effects.

XVI. Positively charged terminal amino groups of a guanidine moiety could interact with negatively charged polar residue, such as an aspartic or glutamic acid.

XVII. Each of the bonds between the phenyl ring and the guanidine moiety were observed to be strictly in the E geometrical form, which extends the guanidine moiety away from the phenyl ring and its binding domain, and into a different region of the agmatine binding site.

Based on these observations, along with the conserved moiety from the X-ray structure displayed in Figure 6.10, the following pharmacophore has been proposed (Figure 6.11). This important piece of information will be a helpful tool in the development of therapeutically useful drug molecules which target this agmatine binding site on the NMDA receptor complex.
Figure 6.11 Putative pharmacophore of the agmatine binding site of the NMDA receptor complex

D - H = hydrogen bond donor
A = hydrogen bond acceptor
(D)-H indicates that the aromatic ring can be a 5-membered ring with a non-basic protonated heteroatom which acts as a hydrogen bond donor.
Dashed oval represents the “planar surface” which interacts with the phenyl ring and possibly the conjugated imino and guanidino moieties.

“E” indicates the geometry of the imine double bond, or the N-N bond which possesses double-bond character
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