2005

Dual Mechanism Analgesia-Enhancing Agents

Shawquia Elithia Young

Virginia Commonwealth University

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DUAL MECHANISM ANALGESIA-ENHANCING AGENTS

A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science at Virginia Commonwealth University.

By

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August 2005
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<td>Analysis</td>
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<tr>
<td>ANOVA</td>
<td>Analysis of variance</td>
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<td>AR</td>
<td>Adrenoceptor</td>
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<td>ATP</td>
<td>Adenosine triphosphate</td>
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<td>BBB</td>
<td>Blood-brain barrier</td>
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<td>br.</td>
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<td>°C</td>
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<td>CL</td>
<td>Confidence limit</td>
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<td>CNS</td>
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<td>CNBr</td>
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<td>DRG</td>
<td>Dorsal root ganglion</td>
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<td>ED&lt;sub&gt;50&lt;/sub&gt;</td>
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<td>$K_i$</td>
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<td>Ligand-gated ion channel</td>
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<td>Log P</td>
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<td>Methanol</td>
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<td>TLC</td>
<td>Transmembrane</td>
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Abstract

DUAL MECHANISM ANALGESIA-ENHANCING AGENTS

By Shawquia Elithia Young, M.S.

A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science at Virginia Commonwealth University.

Virginia Commonwealth University, 2005

Major Director: Malgorzata Dukat
Associate Professor, Department of Medicinal Chemistry

Co-Director: Richard A. Glennon
Professor, Department of Medicinal Chemistry

Currently, there is an increasing need for novel analgesics that are potent but lack undesired side effects. Recent studies have shown that both 5-HT_3 receptors and α_{2B}-adrenoceptors play a role in antinociception. MD-354, N-(3-chlorophenyl)guanidine, has a high-affinity both for 5-HT_3 and α_{2B}-adrenoceptors and could be viewed as the first example of a rather selective 5-HT_3/α_{2B}-adrenoceptor ligand. MD-354, inactive by itself, potentiates the antinociceptive effects of an inactive dose of clonidine in the mouse tail-flick assay. An attempt to determine the underlying mechanism of this potentiating effect was the purpose of the present investigation. The studies focused on an examination of: i)
MD-354 in the mouse hot-plate assay, ii) a more lipophilic analog of MD-354 in the tail-flick assay, iii) various analogs of MD-354 with different binding profiles in both mouse tail-flick and hot-plate assays. The present investigation suggests that both 5-HT\textsubscript{3} and \( \alpha_{2B} \)-adrenoceptors are playing a role in the potentiation of clonidine analgesia by arylguanidines such as MD-354. Arylguanidines might represent a unique class of analgesia-enhancing agents with a dual (5-HT\textsubscript{3}/\( \alpha_{2} \)-adrenoceptor) mechanism of action.
I. Introduction

The development of an analgesic that is effective but free of serious side effects remains a major focus in the field of drug discovery. There are many drug classes that have analgesic properties and are being used to reduce all degrees of pain. Examples of these classes are nonsteroidal anti-inflammatory drugs (NSAIDs), local and general anesthetics, opiates, and $\alpha_2$-adrenoceptor agonists. These agents’ analgesic effects can often be enhanced when used in combination with other analgesics or with one another. Some of these agents have become very useful in the treatment of severe pain for patients in post-surgery. Morphine and clonidine are popular analgesics that are currently being used for this type of treatment.

Morphine was originally isolated in 1803 and was one of the first agents classified as an opiate. Morphine binds at opioid receptors and more specifically, mu opioid receptors. It is a potent agent and is often referred to as a narcotic analgesic. This means that morphine can induce sleep and/or a state of narcosis in addition to its analgesic effects. Although morphine has been very successful in the treatment of severe pain, there are some limitations with its use. Some of the serious side effects of morphine are tolerance, physical and psychological dependence, respiratory depression, and constipation.
Clonidine is an agent currently being used in regional anesthesia for the management of acute post-operative pain, labor pain, or chronic pain.\textsuperscript{7} Clonidine binds both at $\alpha_2$-adrenoceptors and imidazoline receptors.\textsuperscript{8} It binds nonselectively at $\alpha_2$-adrenoceptors, which means that it binds with similar affinity at all of the $\alpha_2$-adrenoceptor subtypes ($\alpha_{2A}$, $\alpha_{2B}$, $\alpha_{2C}$). Evidence shows that the analgesic effects of clonidine are due to its partial agonist activity at $\alpha_2$-adrenoceptors.\textsuperscript{3} Similar to morphine, clonidine is a potent analgesic but also has its limitations. Some of the side effects associated with this agent are sedation, hypotension, and bradycardia.\textsuperscript{3,7} Due to its ability to lower blood pressure, clonidine is also being used to treat hypertension.\textsuperscript{3} The sedative effects caused by clonidine also can be considered a desired effect, and its induced sedative effect is useful when clonidine is being used as an anesthetic. However, when it is being used as a post-operative analgesic, sedation can become a serious drawback.

Due to the undesired side effects associated with both morphine and clonidine, the development of novel agents without these effects has become a major focus in the improvement of analgesics. Several novel pathways have proven to play some role in nociception. One example is the dopaminergic pathway. Dopamine receptors have been related both to pronociception and antinociception.\textsuperscript{9} Specifically, $D_2$ receptors are involved with inducing antinociception.\textsuperscript{9} Therefore the use of $D_2$ agonists for pain relief can be a possible novel pathway. Many of the classes in the serotonin receptor family also play a role in nociception. It has been implicated that the 5-HT\textsubscript{3} receptor class plays a significant role in producing analgesia. However, there are currently available only a small number of selective 5-HT\textsubscript{3} receptor agonists. Since clonidine is already a well-used analgesic, another
therapeutic approach might be to reduce the side effects caused by this agent (i.e., sedation). Evidence shows that the sedative and hypotensive effects of clonidine are due to the activation of the $\alpha_{2A}$-adrenoceptor subtype.$^3$ Recent studies have shown that the $\alpha_{2B}$-adrenoceptor subtype contributes to antinociception but is not involved in sedation.$^3$ 5-HT$_3$ and $\alpha_{2B}$-adrenoceptors have become potential pathways that could contribute to the development of novel classes of analgesics without the drawbacks of morphine and clonidine. In our laboratory, a novel series of arylguanidines has been developed and has been shown to bind at 5-HT$_3$ and/or $\alpha_2$-adrenoceptors. This exciting development can possibly lead to a dual-mechanism analgesic agent. The purpose of the present study is to investigate the antinociceptive effects, and the mechanism of action, of the arylguanidines.
II. Background

A. Pain

Due to the inconvenient side effects associated with current analgesic therapies, pain is usually inadequately treated in most patients. Pain can be defined as "an unpleasant sensory and emotional experience associated with actual or potential damage". It consists of a mechanism that prevents any potential damage that can occur to the tissue. Therefore, any potential analgesic cannot compromise or prevent this protective mechanism from occurring which could lead to permanent damage. There are three types of pain: nociceptive, inflammatory, and neuropathic pain. Nociceptive pain is the most common type of pain experienced in everyday life. It is referred to as a usual response to a brief noxious stimulus that induces little tissue damage. Inflammatory pain is usually associated with trauma, infection, surgery, burns, or diseases that cause inflammation. Neuropathic pain is a painful response that is induced by damage involving the peripheral or central nervous system and is usually associated with infection, tissue trauma, and autoimmune diseases. In some cases, one mechanism can produce pain and in others, different mechanisms can coexist.

Pain can also be classified into different categories, e.g. acute, chronic, and cancer. Acute pain is defined as pain that is short with an identifiable cause and represents the largest class of pain being treated. It involves mild to moderately severe pain that is
usually managed by the NSAIDs.\textsuperscript{1,10,11} The side effects that accompany the use of NSAIDs are gastrointestinal, renal, and liver toxicity. Opioids are also used when the NSAIDs fail to manage moderate to severe acute pain. As mentioned earlier, the opioid class has its own side effects that limit its use. Chronic pain is typically defined as pain that extends for a duration of time in which the cause is unknown.\textsuperscript{10,12} Usually it is the common cause of major disability.\textsuperscript{12} Currently, there is no completely effective management or treatment for chronic pain. However, opioids have been used in an attempt to manage this type of pain as much as possible. Cancer pain is usually under-recognized or inadequately treated in many cancer patients.\textsuperscript{12} It can be acute or chronic depending on the stage of the disease. The pain can come from various sources associated with cancer such as the tumor itself, chemotherapy, radiotherapy, or surgery.\textsuperscript{10}

Pain can be associated with mechanical, chemical, and thermal stimuli. Mechanical stimulus can cause intense pain such as pinching of the skin, traumatic injury, or neuropathic conditions.\textsuperscript{10,13} Thermal stimuli involve the application of extreme temperatures to the peripheral tissues, e.g. heat or cold. Chemical stimuli involve application of acidic or basic chemicals to peripheral tissues.\textsuperscript{13} This latter type of stimulus is the slowest of the three in producing nociceptive responses, and the pain produced is inescapable.\textsuperscript{13} After the noxious stimulus interacts with the peripheral tissues, the primary afferent nociceptive pathways become activated. Nociceptor C and A\textdelta primary afferent fibers (PAFs) transduce the stimuli into electrical activity where the nociceptive information is then integrated in the dorsal horn located on the spinal cord.\textsuperscript{11,14} These two types of PAFs both are nocisponsive, which respond to noxious chemical, thermal, and
mechanical stimuli. The myelinated $A_\delta$ fibers elicit the first phase of pain that is rapid and sharp; whereas the unmyelinated $C$ fibers induce the second phase of pain which is slower and dull. PAFs can directly activate the projection neurons that can relay this information to the brain or indirectly activate them through the excitatory interneurons. This nociceptive information can also be transferred by the PAFs by stimulating the inhibitory interneurons. These interneurons can also interact with projection neurons, excitatory interneurons, and the PAFs’ terminals. This process plays an important role in the operation of the descending pathways of nociception. The descending pathways are comprised of two mechanisms: descending inhibition and descending facilitation. Descending inhibition is the primary mechanism that produces antinociception. This response is produced by reducing the release of neurotransmitters from the PAFs’ terminals that aid in transmitting the pronociceptive information. The projection neurons are inhibited by inhibiting the excitatory interneurons and simultaneously stimulating the inhibitory interneurons. This activity occurs postsynaptically to the terminals of the PAFs. Descending facilitation promotes pronociception. This pathway elicits stimulation of the excitatory interneurons and projection neurons which leads to an increase in nociceptive transmission.

The brainstem and other cerebral structures are the origin of the descending pathways. Many supraspinal regions play an essential role in the modulation of nociceptive information. After the message is integrated in the dorsal horn, it is conveyed to the brain through the spinothalamic and spinobrachial pathways. From the former pathway, the activity is transferred through the thalamus to the somatosensory cortex.
The parabranchial region is connected to other supraspinal sites such as the hippocampus, amygdala, and other regions which are involved in the nociceptive response. This message is then integrated in the periaqueductal gray which plays a major role in promoting and inhibiting pronociception.

Briefly mentioned earlier, the release of neurotransmitters is involved in pain transmission. The most investigated neurotransmitters involved in descending pathways are norepinephrine and serotonin (5-HT). These two neurotransmitters can modulate the flow of nociceptive information to the brain via its activity in the dorsal horn. Norepinephrine is a part of the descending noradrenergic pathway and it interacts with multiple classes of adrenoceptors (β, α1, α2). Serotonin is involved in the descending serotoninergic pathway and this neurotransmitter interacts with the 5-HT receptor superfamily (5-HT1-5-HT7).

B. 5-HT3 Receptors

1. Classification

Serotonin (5-hydroxytryptamine, 5-HT; 1) is a neurotransmitter that binds at several 5-HT receptor populations and is involved with a variety of physiological effects in the body. Some of these effects include vasoconstriction, vasodilation, body temperature regulation, appetite, mood, platelet aggregation, sleep, and hormonal regulation. These different physiological effects are due to the ability of 5-HT to bind at a variety of 5-HT receptors. 5-HT receptors are classified as belonging to seven major families (5-HT1-5-HT7).
All of the 5-HT receptors are G-protein coupled receptors, except 5-HT₃ receptors which are ligand-gated ion channel (LGIC) receptors. There are major differences in receptor function between G-protein coupled receptors and LGIC receptors. A G-protein coupled receptor's functions are mediated via second messenger systems. This mechanism is a multi-step receptor-effector cascade that elicits relatively slow cellular responses. These receptors transmit this signal by activating a guanine nucleotide binding protein (G-protein). G-Proteins consist of three subunits: α, β, γ, in which the α subunit modulates the activation of the G-protein. The ligand binds to the G-protein coupled receptor and causes the G-protein to bind to a target enzyme. This enzyme then triggers a second messenger that results in the cellular response. In the 5-HT family, the second messenger systems that are involved are stimulation or inhibition of adenylyl cyclase and stimulation of phospholipase C.¹⁶
Figure 1. G-Protein coupled receptor

5-HT₃ receptors are a member of the cys-loop superfamily which includes the nicotinic acetylcholine (nACh) receptors, GABAₐ, and glycine receptors. A LGIC receptor's major function is to modulate the rapid transmission of synaptic nerve impulses which are caused by the binding of neurotransmitters to the specific receptor. This process results in the opening of an ion-selective pore. In the case of 5-HT₃ receptors, 5-HT binds to the receptor and allows specific cations, such as Na⁺, K⁺, and Ca²⁺ to move through the lipid membrane. Activation of these receptors produces various effects including membrane depolarization, increase in intracellular Ca²⁺, and the modulation of neurotransmitter release.

2. Structure

The receptors in the cys-loop family typically are similar in structure. The most extensively studied receptor is the nACh receptor. These pentameric receptors have five homologous subunits (Figure 2) that consist of four transmembrane spanning domains.
(TM1-TM4) (Figure 3), an intracellular loop between TM3 and TM4, and a large extracellular N-terminal domain. It is suggested that the N-terminal domain is where the ligand-binding domain is located. In this domain, there are 2 cysteine residues that are linked by a disulfide bond. 5-HT₃ receptors share about 20-30% amino acid sequence identity with the nACh receptors. Since the crystal structure of the ACh binding protein (ACHBP) was recently determined, this facilitates the study of the 5-HT₃ ligand binding domain due to their structural similarities.

5-HT₃ receptors have been classified into three subunits: 5-HT₃A, 5-HT₃B, and 5-HT₃C. However, only the 5-HT₃A and 5-HT₃B subunits have been cloned. The 5-HT₃A subunit has been cloned from a mouse neuroblastoma/Chinese hamster embryonic brain cell hybrid, rat sympathetic neurons, human brain, guinea pig small intestine and ferret colon. The 5-HT₃B subunit has been cloned from human fetal kidney, human small intestine, and mouse and rat brain. The 5-HT₃A subunit is expressed in heterologous
expression systems and has structural similarities with the other receptors in the cys-loop superfamily. The protein sequence similarity between 5-HT$_3$A and the α-subunits of the other members is 22-30%. It has been suggested that the 5-HT$_3$ receptor is a common ancestor of all LGIC receptors because it is relatively similar to α7 nACh receptor subunits. The 5-HT$_3$A subunit consists of 487 amino acids and three potential N-glycosylation sequences that are located in the extracellular region. These N-glycosylation sites could potentially be involved in the assembly of the 5-HT$_3$ receptor. 5-HT$_3$A receptor is a protein that can form fully functional homomeric complexes. This subunit plays a pivotal role in the pharmacological effects elicited by 5-HT$_3$ receptors. Recently, three variant forms of the 5-HT$_3$A subunit have been identified in humans. These variants include normal, short, and long isoforms. The short variant is a polypeptide unable to form functional homomeric ion channels, and they are coexpressed with the normal 5-HT$_3$A receptor. It consists of 238 amino acids and forms only a single transmembrane domain. It has a very short C-terminus but encodes the ligand binding domain in the extracellular N-terminus. The long variant of 5-HT$_3$A consists of 510 amino acids which is 23 amino acids longer than the normal variant. It also does not form functional homomeric ion channels. These additional amino acids are inserted in the extracellular loop between the TM2 and TM3 domain.

The 5-HT$_3$B subunit cannot form homomeric complexes but can form heteromeric complexes with the 5-HT$_3$A subunit. This subunit has ~45% sequence identity with its 5-HT$_3$A homolog. The 5-HT$_3$B subunit has no function by itself, but elicits its function when it is coexpressed with the 5-HT$_3$A subunit. This receptor subunit has 441 amino acids and
also has extracellular N-glycosylation sites as seen in the 5-HT$_3^A$ subunit. The 5-HT$_3^B$ subunit does not possess any of the structural features needed to induce the conductance seen in the other LGIC receptors, and that causes its inability to form functional ion channels.\textsuperscript{22} When this subunit forms a heteromeric complex with the 5-HT$_3^A$ subunit, there are no relatively significant changes in the normal pharmacological function of the 5-HT$_3$ receptor. The only changes in function that the heteromeric complex shows are a larger single-channel conductance and a decrease in Ca$^{2+}$ permeability.\textsuperscript{20} The 5-HT$_3^C$ subunit was just recently patented and its function is still unclear.\textsuperscript{16,23} This receptor shows $\sim$39\% sequence identity to its 5-HT$_3^A$ homolog.\textsuperscript{23} It has been suggested that the 5-HT$_3^C$ subunit contributes to the regulation of the 5-HT$_3$ receptor’s responses.\textsuperscript{23}

3. Pharmacology

Evidence shows that 5-HT$_3$ receptors are distributed throughout the CNS among different species. In rats, these receptors are expressed in the forebrain, brainstem, and the spinal cord. Some of these regions include the solitary tract nucleus, nucleus of spinal tract of trigeminal nerve, dorsal horn of the spinal cord, and hippocampus.\textsuperscript{24,25} In mice, 5-HT$_3$ receptors are distributed in similar regions of the spinal cord and forebrain as in the rats. They are expressed in the hippocampus, dorsal tegmental nucleus, trochlear nerve nucleus, facial nerve nucleus, dorsal root ganglia, olfactory and somatosensory regions, and throughout the cortical regions and limbic system.\textsuperscript{26} In humans, the majority of 5-HT$_3$ receptors in the CNS are the 5-HT$_3^A$ subunit.\textsuperscript{20} These receptors are also distributed in the
forebrain, hindbrain, medulla oblongata, spinal cord and, to a lesser extent, in the nucleus accumbens, and striatum and substantia nigra.  

5-HT₃ ligands have become important tools in studying the 5-HT₃ receptor. For instance, 5-HT₃ antagonists are being used to determine the distribution of these receptors throughout the body. Various assays have been developed in order to differentiate between 5-HT₃ agonists and antagonists. One of the first in vivo assays that was used is the von Bezold-Jarisch reflex. In this assay, the cardiovascular system is the primary site of activity and the reflex causes a triad of responses that are apnea, bradycardia, and hypotension. When a 5-HT₃ agonist is administered it produces the von Bezold-Jarisch reflex and mainly causes bradycardia. If an agent blocks this response in the presence of the 5-HT₃ agonist, then it can be referred to as an antagonist in this functional assay. This in vivo assay is well known and is still currently being used to investigate 5-HT₃ agonists and antagonists. Another in vivo assay is the ferret and shrew emesis assay. In this assay, cisplatin, a vomiting-inducing agent, is administered to the animal to cause emesis. A 5-HT₃ antagonist will prevent this emetic response from occurring and a 5-HT₃ agonist induces emesis. Stimulation of 5-HT₃ receptors has been shown to also produce contractions in the gastrointestinal tract. An important in vitro functional assay that focuses on this response is the contraction of isolated guinea pig ileum. In this assay, administration of 5-HT₃ agonists produces contractions. If an agent can block these contractions it is referred to as an antagonist. In some cases, a 5-HT₃ ligand can show agonist activity in one functional assay and antagonist activity in another assay. The
determination of these ligands has played a pivotal role in fully understanding 5-HT3 receptors and their many functions.

4. Ligands and Structure-Activity Relationships (SAR)

a) Agonists

Over the years a variety of drug classes have been shown to bind selectively to 5-HT3 receptors. Both agonist and antagonists have been studied and some antagonists are currently being used clinically in the treatment of a variety of disorders. To date, there are few available 5-HT3 agonists to study their pharmacological effects. The first class of agonists found to bind to these receptors is the tryptamines. 5-HT (1), a member of this class, binds to 5-HT3 receptors nonselectively and with moderate affinity ($K_i = 1,000 \text{ nM}$). 2-Methyl 5-HT (2) was found to display more selectivity for 5-HT3 receptors than 5-HT but binds with slightly lower affinity ($K_i = 1,350 \text{ nM}$). 2-Methyl 5-HT is one of the few currently available 5-HT3 agonists being used. Recent investigations have shown, however, that 2 binds at 5-HT6 receptors ($K_i = 45 \text{ nM}$) with higher affinity than it displays for 5-HT3 receptors.

![Structure of 2-Methyl 5-HT (2)](image1)

![Structure of 5-HTQ (3)](image2)
Different analogs of 5-HT were studied and it was found that trimethylation of the terminal amine (5-HTQ, 3) resulted in a highly selective 5-HT$_3$ agonist ($K_i = 75$ nM). This quaternary amine binds with a ten times higher affinity than 5-HT (1), but has difficulty in penetrating the blood-brain barrier (BBB) and is of limited use for in vivo studies.

The arylbiguanides represent another class of 5-HT$_3$ agonists that are nontryptamines and selective for this receptor. Phenylbiguanide (PBG, 4) was the first agent of this class to be developed and it displayed moderate affinity ($K_i = 1,200$ nM) comparable to 5-HT. m-Chlorophenylbiguanide (m-CPBG, 5, $K_i = 17$ nM) was found to be a higher affinity agent than PBG. Structure-activity relationship (SAR) studies were conducted by Dukat et al. and the major focus was the modification of the aryl substituents and the biguanide chain. In aryl substitution, analogs with different functional groups at various positions on the phenyl ring were studied (Table 1). The primary goal of this study was to examine the electronic and lipophilic character of these substituents and their effect on affinity. Addition of a chloro group at the 2- or 4-position resulted in higher affinity than PBG but in lower affinity than m-CPBG (6, 7, $K_i = 62$ and 200 nM, respectively). Replacing the 3-chloro group with a nitro (8) or trifluoromethyl (17) group, stronger electron-withdrawing groups, resulted in a decrease in affinity ($K_i = 220$ nM and 700 nM, respectively). Also, replacing the chloro group with a methyl group, an equally lipophilic and more electron-donating group, resulted in decreased affinity (9, $K_i = 780$ nM). Replacement by a 4-methyl group abolishes affinity (10) but adding a chloro group at the 3-postion to this derivative enhances affinity (11, $K_i = 225$ nM). Addition of a methoxy group to the 2-position and a chloro group in the 5-position also enhances affinity.
when compared to PBG (12, $K_i=126$ nM). The 2-napthyl derivative 13 ($K_i=12$ nM) binds with high affinity that is similar to $m$-CPBG. From this study it was found that the 3-chloro substituent was optimal for binding. In separate studies, it was found that incorporating chloro substituents at the 4- and/or 5-positions resulted in even higher-affinity agents (14, 15, 16, $K_i=12, 1.8, and 2.7$ nM, respectively).30

Table 1. Observed Binding Affinities of Arylbiguanides for 5-HT3 Receptors.30

<table>
<thead>
<tr>
<th>R</th>
<th>$K_i$, nM</th>
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<tbody>
<tr>
<td>4</td>
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<tr>
<td>5</td>
<td>17</td>
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<tr>
<td>6</td>
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<td>&gt;10000</td>
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<td>15</td>
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<tr>
<td>16</td>
<td>2.7</td>
</tr>
<tr>
<td>17</td>
<td>700</td>
</tr>
</tbody>
</table>
Next, the biguanide chain was modified. Dimethylation of the terminal amine resulted in abolished affinity ($K_i > 10,000 \text{ nM}$). Various derivatives were studied and it was shown that any further modification (with one exception) of the biguanide chain also abolished affinity. However, the N-(2-phenylethyl)guanidine 19, ($K_i = 40 \text{ nM}$) displayed high affinity. More importantly, it was found that the entire biguanide chain was not necessary for binding and could be shortened; this resulted in the arylguanidines.

![Chemical structures](18.png) 18  

![Chemical structures](19.png) 19

This novel class of compounds, the arylguanidines, was found to bind with affinities comparable to the arylbiguanides. Between the two classes parallel structural changes led to parallel shifts in affinity. SAR studies were conducted and both aryl substitution and the guanidine moiety were examined (Table 2). As with the arylbiguanides, a chloro group at the 3-position is optimal. $m$-Chlorophenylguanidine (MD-354, 21) binds with high affinity and was the first agent developed in this class ($K_i = 35 \text{ nM}$). Also, as with the arylbiguanides, high affinity was not retained when the chloro group was replaced with a trifluoromethyl group (24, $K_i = 5700 \text{ nM}$). Addition of chloro groups at the 4- and/or 5-positions led to higher affinity agents as in the arylbiguanide series (27, 28, 29, $K_i = 3.1, 5.0, \text{ and } 0.7 \text{ nM}$, respectively). Conformationally constrained
analogs of MD-354 were also examined as possible ligands. Benzimidazole 30 and quinazoline 31 were developed and it was found that only the quinazoline displayed high affinity ($K_i = 725$ and $34 \text{ nM}$, respectively). Although some of the arylguanidines bind at 5-HT$_3$ receptors with high affinity, they might have difficulty penetrating the BBB. Theoretically, an agent should possess a Log P value that falls in the 1.5 to 2.5 range in order to penetrate the BBB. MD-354 (21) has a Log P value of -0.64 which suggests that this agent will have difficulty penetrating the BBB. This could limit the use of arylguanidines in behavioral studies, and their potential clinical use for treating central disorders.

Table 2. Observed Binding Affinities of Arylguanidines at 5-HT$_3$ Receptors.$^{30,33}$

<table>
<thead>
<tr>
<th>R</th>
<th>$K_i$, nM</th>
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<tbody>
<tr>
<td>20</td>
<td>H</td>
</tr>
<tr>
<td>21</td>
<td>3-Cl</td>
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<tr>
<td>26</td>
<td>3-OCH$_3$</td>
</tr>
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<td>28</td>
<td>3,5-di-Cl</td>
</tr>
<tr>
<td>29</td>
<td>3,4,5-tri-Cl</td>
</tr>
</tbody>
</table>
b) Antagonists

When compared with the 5-HT₃ agonists, there are definitely more available antagonists for research and on the market. ⁴⁴ 5-HT₃ antagonists are currently being used clinically in the treatment of irritable bowel syndrome and as antiemetics. These antagonists include zacopride (32), tropisetron (33), ondansetron (34), and granisetron (35), just to mention a few representative examples. ³⁴ Zacopride (32) is an extremely potent blocking agent that binds at 5-HT₃ receptors (Kᵢ= 0.1-1.9 nM), but it also acts as an agonist at 5-HT₄ receptors (Kᵢ= 1.1 n~).³⁴ Tropisetron (33) (formerly known as ICS 205-930) was one of the first clinically used antiemetics among the 5-HT₃ antagonists. It acts as an antagonist at both 5-HT₃ and 5-HT₄ receptors (Kᵢ= 0.4-3.2 and 930 nM, respectively).³⁵ Both ondansetron (34) and granisetron (35) bind selectively at 5-HT₃ receptors with high affinity (Kᵢ= 0.8-13 and 0.3-4.2 nM, respectively).³⁵ Radiolabeled versions of these agents have also been used as radioligands in binding studies, and were involved in determining the distribution of 5-HT₃ receptors in the CNS.³⁵ These agents also are useful in evaluating the functional roles of these receptors.
5-HT3 receptors have become major targets in the development of novel agents for the treatment of a variety of disorders due to their general lack of side effects.\textsuperscript{34} Ondansetron (34) is being used clinically to relieve nausea and vomiting associated with cancer chemotherapy, radiation, and anesthesia.\textsuperscript{34,36} 5-HT3 antagonists block emesis by producing antagonism of the receptors located at central sites.\textsuperscript{36} Ondansetron (34) is highly effective but does not completely eliminate nausea and vomiting.\textsuperscript{34} When compared with previous antiemetics (i.e., scopolamine), the 5-HT3 antagonists do not cause any sedation.\textsuperscript{34} 5-HT3 receptors also play a role in anxiety, and it is suggested that 5-HT3
antagonists could be used as potential anxiolytics.\textsuperscript{23,37} In clinical studies, however, it was shown that the 5-HT\textsubscript{3} antagonist granisetron (35) was not as effective as the benzodiazepines as an anxiolytic. However, these antagonists did not produce any of the side effects, such as sedation and rebound anxiety following chronic treatment, seen with the benzodiazepines.\textsuperscript{37} Another area in which 5-HT\textsubscript{3} receptors are involved is gut motility. The stimulation of these receptors could cause diarrhea, which is commonly referred to as intestinal hypermotility.\textsuperscript{36} This is usually associated with irritable bowel syndrome. 5-HT\textsubscript{3} antagonists could potentially be used in the treatment of diarrhea. On the other hand, 5-HT\textsubscript{3} agonists could also be used in the treatment of constipation.\textsuperscript{23} Since it is suggested that stimulation of 5-HT\textsubscript{3} receptors increases intestinal motility in this disorder, it could be beneficial to use a 5-HT\textsubscript{3} agonist which is effective and does not induce emesis and diarrhea.\textsuperscript{23}

As discussed earlier, 5-HT\textsubscript{3} receptors have been implicated as playing a role in nociception. This is summarized in Table 3; earlier radioligand binding studies showed that 5-HT\textsubscript{3} receptors are located on sensory nerve endings in the periphery, on the soma and axon membranes of sensory neurons, and on primary afferent terminals in the spinal cord.\textsuperscript{38,39} It was also shown that 5-HT\textsubscript{3} receptors located on peripheral sensory neurons are involved in inflammatory, but not mechanical or thermal, pain in animal models.\textsuperscript{36} For example, Giordano et al.\textsuperscript{40} showed that the subcutaneously (s.c.) administered 5-HT\textsubscript{3} receptor antagonist tropisetron (33) did not produce analgesia in thermal and mechanical nociceptive tests. However, tropisetron produced analgesia in the formalin test, a chemical nociceptive test.\textsuperscript{40} In this test, formalin induces inflammatory pain which is blocked by
tropisetron. Also in this study, both central and peripheral 5-HT₃ receptors were examined. When tropisetron was injected by intracerebroventricular (i.c.v.) administration, it was found to be ineffective in producing analgesia in acute thermal, mechanical, and chemical pain tests.⁴⁰ The lack of analgesic activity of 5-HT₃ receptor antagonists in thermal and mechanical pain tests supports the hypothesis that 5-HT₃ receptors do not mediate the transmission of nociceptive signals.⁴⁰ However, the antinociceptive effect produced by 5-HT₃ antagonists in the formalin-induced pain test further suggests that 5-HT₃ receptors induce a response to inflammatory pain.⁴⁰ These results also suggest that supraspinal 5-HT₃ receptors might not be involved in mediating this response since these antagonists were administered (i.c.v.) directly into the brain and did not produce antinociception.⁴⁰ In a separate study, the 5-HT₃ receptor agonist 2-methyl 5-HT (2) was also analyzed in the formalin-induced test.⁴¹ 2-Methyl 5-HT was administered i.t., and found to produce an antinociceptive response.⁴¹ This antinociceptive response was reduced by i.t. administered MDL-72222, a potent 5-HT₃ receptor antagonist.⁴¹ According to these results, it further suggests that spinal 5-HT₃ receptors induce antinociceptive responses to inflammatory pain. This analgesic activity could also be reduced by ketanserin (5-HT₂ antagonist), naloxone (opioid antagonist), and bicuculline (GABAₐ antagonist).⁴¹ Because this 5-HT₃ receptor-mediated antinociception could be attenuated by various other antagonists, this suggests that the 5-HT₃ descending inhibitory system is interacting with 5-HT₂, GABAₐ, and opioid receptors. When 2-methyl 5-HT (2) was administered i.c.v., it did not produce any antinociceptive activity.⁴¹ This also supports the idea that supraspinal 5-HT₃ receptors do not mediate nociceptive responses to inflammation. This receptor has been shown to
mediate a component of the nociceptive response to subacute and chronic inflammation caused by 5-HT and other noxious agents, however the initial phase of inflammation and acute pain does not seem to be reliant on the 5-HT3 receptor.\(^3\)\(^9\) 2-Methyl 5-HT and 5-HT were also analyzed in the writhing test.\(^3\)\(^9\) This test was used to study visceral 5-HT3 receptors’ role in modulating nociceptive responses. In this study intraperitoneally (i.p.) administered 5-HT produced a dose-dependent nociceptive writhing response that was blocked by tropisetron (33) (1 mg/kg, i.p.).\(^3\)\(^9\) However, 2-methyl 5-HT (2) did not produce any nociceptive responses or writhing when administered alone. But, when 2-methyl 5-HT was administered following a low dose of 5-HT, it showed enhancement in the antinociceptive activity of 5-HT.\(^3\)\(^9\) These results suggest that visceral 5-HT3 receptors could play a role in mediating inflammatory pain, but it seems to be dependent on other mechanisms to elicit its activity. This idea is demonstrated when 2-methyl 5-HT alone did not produce a nociceptive writhing response. Another possible explanation could be that since tropisetron is a 5-HT3 and 5-HT4 receptor antagonist, this specific dose could be interacting with 5-HT4 receptors which might be involved in this nociceptive activity.

5-HT3 receptors were also shown to have a possible role in spinal nociception. This hypothesis has been studied via thermal pain tests in rodents. In a study by Glaum et al.,\(^4\)\(^2\) it was shown that intrathecally (i.t.) administered 5-HT produced antinociceptive activity in the hot-plate test. This activity was attenuated by i.t. administered tropisetron (33) (0.01 mg/kg).\(^4\)\(^2\) This study further supports the concept that 5-HT3 receptors mediate spinal analgesia. As mentioned earlier, various 5-HT3 receptor antagonists were analyzed in the hot water bath test in rats for possible antinociceptive activity.\(^4\)\(^0\) These antagonists did not
<table>
<thead>
<tr>
<th>Model</th>
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<th>Agonist/Agonist</th>
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<th>Route of Admin.</th>
<th>Analgesic Activity</th>
<th>Ref.</th>
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<td>Antagonist</td>
<td>rats</td>
<td>i.c.v.</td>
<td>no</td>
<td>40</td>
</tr>
<tr>
<td></td>
<td>Ondansetron</td>
<td>Antagonist</td>
<td>rats</td>
<td>i.t.</td>
<td>no</td>
<td>40</td>
</tr>
<tr>
<td></td>
<td>2-Methyl 5-HT</td>
<td>Agonist</td>
<td>rats</td>
<td>i.t.</td>
<td>yes/blocked by 5-HT3, 5-HT2, opioid, GABA antagonists</td>
<td>41</td>
</tr>
<tr>
<td>Writhing test</td>
<td>rats</td>
<td>i.c.v.</td>
<td>no</td>
<td>41</td>
<td></td>
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</tr>
<tr>
<td></td>
<td>mice</td>
<td>i.p.</td>
<td>produces no nociceptive responses alone</td>
<td>39</td>
<td></td>
<td></td>
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<tr>
<td><strong>Hot plate/Hot water bath</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td></td>
<td>Tropisetron</td>
<td>Antagonist</td>
<td>rats</td>
<td>s.c.</td>
<td>no</td>
<td>40</td>
</tr>
<tr>
<td></td>
<td>MDL-72222</td>
<td>Antagonist</td>
<td>rats</td>
<td>i.c.v.</td>
<td>no</td>
<td>40</td>
</tr>
<tr>
<td></td>
<td>Ondansetron</td>
<td>Antagonist</td>
<td>rats</td>
<td>i.t.</td>
<td>no/blocks analgesic effect of 5-HT</td>
<td>40, 42</td>
</tr>
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<td>yes/reduced by 5-HT3, 5-HT2, opioid, and GABA antagonists</td>
<td>41</td>
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<td></td>
<td>PBG</td>
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<td>rats</td>
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<td>41</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>i.t.</td>
<td>yes</td>
<td>43</td>
</tr>
<tr>
<td><strong>Tail flick</strong></td>
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<td></td>
<td></td>
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<td>Antagonist</td>
<td>rats</td>
<td>s.c.</td>
<td>no</td>
<td>40</td>
</tr>
<tr>
<td></td>
<td></td>
<td>rats</td>
<td>i.c.v.</td>
<td>no</td>
<td>40</td>
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</tr>
<tr>
<td></td>
<td></td>
<td>rats</td>
<td>i.t.</td>
<td>no/blocks i.c.v. morphine-induced analgesia</td>
<td>40, 46</td>
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<td>MDL-72222</td>
<td>Antagonist</td>
<td>rats</td>
<td>i.t.</td>
<td>no/blocks i.t. 2-Methyl 5HT</td>
<td>40, 42</td>
</tr>
<tr>
<td></td>
<td>Zacopride</td>
<td>Antagonist</td>
<td>rats</td>
<td>i.t.</td>
<td>yes/blocked by 5-HT3, GABA, α2 and α-adrenoceptor antagonists</td>
<td>42-44</td>
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<td></td>
<td>2-Methyl 5-HT</td>
<td>Agonist</td>
<td>rats</td>
<td>i.t.</td>
<td>no</td>
<td>40</td>
</tr>
<tr>
<td></td>
<td>PBG</td>
<td>Agonist</td>
<td>rats</td>
<td>i.t.</td>
<td>yes</td>
<td>43</td>
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<td></td>
<td>Tropisetron</td>
<td>Antagonist</td>
<td>rats</td>
<td>s.c.</td>
<td>no</td>
<td>40</td>
</tr>
<tr>
<td></td>
<td>MDL-72222</td>
<td>Antagonist</td>
<td>rats</td>
<td>i.c.v.</td>
<td>no</td>
<td>40</td>
</tr>
<tr>
<td></td>
<td>Ondansetron</td>
<td>Antagonist</td>
<td>rats</td>
<td>i.t.</td>
<td>no</td>
<td>40</td>
</tr>
<tr>
<td></td>
<td>2-Methyl 5-HT</td>
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<td>rats</td>
<td>i.c.v.</td>
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<td>48</td>
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<tr>
<td></td>
<td>mCPBG</td>
<td>Agonist</td>
<td>rats</td>
<td>i.t.</td>
<td>yes/blocked by 5-HT3 antagonists</td>
<td>49</td>
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</table>
produce any antinociceptive activity when administered subcutaneously (s.c.) or via the i.c.v. route.\textsuperscript{40} 5-HT produces analgesic effects through influence on the CNS, and these effects can be mimicked by 2-methyl 5-HT (2).\textsuperscript{36,39} To further characterize the role of 5-HT$_3$ receptors in spinal analgesia, 2-methyl 5-HT was studied in the hot-plate test. Giordano \textit{et al.}\textsuperscript{41} showed that i.t. administered 2-methyl 5-HT produces antinociception in rats. This activity was reduced by 5-HT$_3$, 5-HT$_2$, opioid, and GABA$_A$ antagonists.\textsuperscript{41} This study also showed the involvement of other systems with 5-HT$_3$ receptors in mediating spinal analgesia. However, i.c.v. administered 2-methyl 5-HT did not produce antinociception.\textsuperscript{41} These results suggest that supraspinal 5-HT$_3$ receptors also might not mediate spinal analgesia. Another study showed that i.t. administered PBG (4), a 5-HT$_3$ agonist, produced analgesic activity.\textsuperscript{43} 5-HT$_3$ receptor ligands have also been analyzed in a second thermal test, the tail-flick test. This behavioral test focused on analgesic activity that was elicited by spinal 5-HT$_3$ receptors. In a study by Glaum \textit{et al.},\textsuperscript{42} both i.t. administered 5-HT and 2-methyl 5-HT produced antinociceptive activity that could be blocked by i.t. administered MDL-72222 and tropisetron (33). This study further supports the concept that 5-HT$_3$ receptors mediate spinal analgesia. In a separate study, i.t. administered 2-methyl 5-HT produced antinociception in mice that was also blocked by 5-HT$_3$ and GABA$_A$ antagonists.\textsuperscript{44} The antinociceptive activity was only partially blocked by phalcofen (GABA$_B$ antagonist).\textsuperscript{44} In earlier studies, it was suggested that 5-HT$_3$ receptors could mediate the release of GABA which elicits the antinociceptive activity.\textsuperscript{44} These results support this idea since both GABA$_A$ and GABA$_B$ antagonists can block the antinociceptive activity produced by 2-methyl 5-HT.\textsuperscript{44}
Noradrenergic receptors also play a role in mediating 5-HT-mediated antinociception.\textsuperscript{45} It was previously shown that i.t. administered norepinephrine and 5-HT produce a synergistic effect.\textsuperscript{45} Sawynok \textit{et al.}\textsuperscript{45} showed that both 5-HT and 2-methyl 5-HT's antinociceptive activity could be reduced by phentolamine (nonselective $\alpha$-AR antagonist) and yohimbine ($\alpha_2$-AR antagonist). PBG (4) was also analyzed in the tail-flick test for possible antinociceptive activity. When administered i.t., unlike 2-methyl 5-HT (2), PBG did not produce antinociception.\textsuperscript{43} The difference in activity with 2-methyl 5-HT and PBG suggests that 5-HT$_3$ receptors do not directly mediate spinal analgesia. 2-Methyl 5-HT could mediate the release of 5-HT, which then activates the 5-HT$_3$ receptors. Another possibility could be that 2-methyl 5-HT is inducing antinociception via another serotonin receptor subtype. The latter possibility is less likely because analgesic activity produced by 2-methyl 5-HT can be blocked by MDL-72222 which is selective for 5-HT$_3$ receptors. Since it has been implicated that 5-HT mediates morphine-induced antinociception, a specific role for 5-HT$_3$ receptors has been studied.\textsuperscript{46} It has been shown that i.t. administered morphine produces antinociception that can be blocked by tropisetron.\textsuperscript{46} In a later study, i.t. administered tropisetron (33) also blocked i.c.v. administered morphine-induced antinociceptive activity.\textsuperscript{47} It is apparent that 5-HT$_3$ receptors participate in a more complex system that mediates antinociception which involves a variety of receptor families. Figure 4 illustrates the overlapping of the different receptors and their possible roles in mediating analgesia.\textsuperscript{9}
Figure 4. Schematic diagram of 5-HT serotonergic system modulating nociception. This figure is used to show the involvement of the serotonergic system with other neurotransmitters.

Mechanical nociceptive tests have been used to study possible involvement of 5-HT₃ receptors in modulating an antinociceptive response to mechanical noxious stimuli in rodents. In a mechanical test, nociceptive responses are elicited by applying an increasing pressure to the paw or dorsal side of the distal tail of a rodent until a squeak or withdrawal response is obtained. As seen in certain other nociceptive tests, 5-HT₃ receptors do not seem to be conclusively involved in antinociception. 2-Methyl 5-HT (2), administered i.t. and i.c.v., was studied and did not produce an antinociceptive response in the paw pressure test. In another study, mCPBG (5) and 5-HT were also analyzed in the paw pressure test. Both i.t. administered 5-HT and mCPBG produced a dose-dependent antinociceptive
response which was blocked by tropisetron and granisetron. 5-HT produced higher antinociceptive effects than mCPBG, an agent which is more selective for 5-HT\(_3\) receptors than 5-HT. This suggests that 5-HT\(_3\) receptors might be partially involved in producing an antinociceptive response to a mechanical stimulus. In the paw pressure test, two 5-HT\(_3\) agonists, 2-methyl 5-HT (2) and mCPBG, showed different activities. Since mCPBG binds with higher affinity at 5-HT\(_3\) receptors, the findings suggest that the mechanism involved in the 5-HT-induced antinociception could be direct activation of 5-HT\(_3\) receptors. This activation could lead to the partial antinociceptive response seen by mCPBG. It has also been shown that mCPBG is involved with the dopaminergic pathway and causes the release of dopamine. This could also play a role in the partial antinociceptive actions of mCPBG since dopamine has been implicated in antinociception. Currently, results from animal studies that deal with the actual role of 5-HT\(_3\) receptors in modulating nociception are controversial. Although there is substantial evidence to suggest that 5-HT\(_3\) receptors could mediate spinal analgesia, the mechanism of this activity is still unclear. Thus, additional studies are required before it can be concluded that 5-HT\(_3\) receptor agonists may or may not be useful in the treatment of acute pain.

Many clinical studies have been conducted to determine the possible clinical utility of 5-HT\(_3\) receptor antagonists in the treatment of acute pain. Tropisetron (33) was one of the first selective 5-HT\(_3\) receptor antagonists to be used as a potential treatment of rheumatism, a condition characterized by inflammation or pain in muscles, joints, or fibrous tissue. Ferber et al. showed that tropisetron was effective compared with placebo in reducing pain in patients with fibromyalgia in a double-blind study. The most
frequent complaints reported were constipation and headache. Muller et al. reported that only up to 50% of patients with fibromyalgia actually responded to tropisetron with a reduction of pain. It was also hypothesized that 5-HT plays a key role in producing pain during an acute migraine attack. In an open pilot study, 5 out of 6 patients experienced a rapid relief of pain when treated with granisetron. In further studies, other 5-HT3 receptor antagonists (i.e., ondansetron and tropisetron) did not consistently relieve the pain caused by a migraine attack. Stratz and Muller showed that tropisetron was effective in the local treatment of rheumatoid arthritis, tendinopathies, peri-arthropathies, and myofasical pain syndrome. Overall these studies have shown that 5-HT3 receptor antagonists can be useful in situations linked to inflammatory stimuli.

It has been shown that 5-HT3 receptors might be involved in the modulation of nociception. The 5-HT3 receptor agonist 2-methyl 5-HT showed analgesic activity in the inflammatory and thermal but not mechanical tests, whereas another 5-HT3 receptor agonist, PBG, did not produce analgesia. However, the more selective 5-HT3 receptor agonist mCPBG produced analgesic activity in the mechanical test. The 5-HT3 receptor antagonists only produced analgesic activity in the formalin-induced inflammatory test when administered via the s.c. route. From these results, demonstrating a role for 5-HT3 receptors in mediating nociception has been quite difficult. Inconclusive findings with 5-HT3 receptor agonists could be related to their non-selectivity, low-affinity, and/or inability to penetrate the blood-brain barrier; thus, there is a definite need for the development of potent, centrally acting 5-HT3 agonists that have the ability to penetrate the blood-brain barrier. This controversy could also be due to factors dealing with the nociceptive tests
that were used. Other factors include route of administration, species, and 5-HT3 ligand used. Additional studies with more lipophilic 5-HT3 receptor agonists would certainly allow a clearer understanding of the involvement of 5-HT3 receptors in pain.

C. α2-Adrenoceptors

1. Classification

The adrenoceptors, a receptor family that has been extensively studied over the years, are membrane receptors that belong to the superfamily of G-protein coupled receptors (GPCRs). They bind the endogenous catecholamines, norepinephrine (36) and epinephrine (37), which elicit a variety of physiological effects in both the central and peripheral nervous systems. Some of these effects include control of blood pressure and blood flow, neuronal modulation, digestion, respiration, reproduction, pupil dilation and contraction, and energetic metabolism. Norepinephrine is released from the noradrenergic postganglionic nerve terminals, and both norepinephrine and epinephrine are secreted in the adrenal medulla.

\[ \text{Norepinephrine (36) } \]

\[ \text{Epinephrine (37) } \]
The adrenoceptors are divided into two broad categories, α- and β-adrenoceptors.\(^5^6\) α-Adrenoceptors can be further divided into \(\alpha_1\)- and \(\alpha_2\)-adrenoceptors.\(^5^6\) The α and β adrenoceptors were initially classified according to the rank order of potency of a series of adrenoceptor agonists.\(^5^7\) For the α-adrenoceptors, epinephrine (37) = norepinephrine (36) > isoproterenol (38) in terms of potency, and for the β-adrenoceptors, isoproterenol > epinephrine > norepinephrine.\(^5^7\) α-Adrenoceptors mediate mostly excitatory functions that include vasoconstriction, uterine musculature contraction, urethra contraction, and pupil dilation, and one inhibitory function, intestinal relaxation.\(^5^5\) β-Adrenoceptors mediate mostly inhibitory functions that include vasodilation, uterine musculature relaxation, bronchodilatation, and an excitatory function, cardiac function.\(^5^5\) α2-Adrenoceptors are classified into three subtypes: \(\alpha_{2A}\), \(\alpha_{2B}\), and \(\alpha_{2C}\)-adrenoceptor subtypes. As mentioned earlier, GPCRs mediate their physiological effects via second messenger systems (Figure 5). In the case of α2-adrenoceptors, the second messenger systems that are involved are inhibition or stimulation of adenylyl cyclase, activation of receptor-operated K\(^+\) channels, and inhibition of voltage-gated Ca\(^{2+}\) channels.\(^5^7\) It has been suggested that other G-protein dependent signaling pathways are mediated by activation of phospholipase D, stimulation
of phospholipase A₂ activity, and increased intracellular Ca²⁺ availability.⁵⁷,⁵⁸ The α₂-adrenoceptors are coupled to G_{i/0} types of G-proteins, which cause the inhibition of adenylyl cyclase and lead to a decrease in cAMP levels.⁸ These G-proteins are sensitive to inactivation by pertussis toxin, which prevents the dissociation of the α subunit from the βγ subunit.⁵⁸ However, it has been shown that α₂-adrenoceptors are also coupled to the stimulation of adenylyl cyclase via G_{s}-proteins in some cells transfected with α₂-adrenoceptors which leads to an elevation in cAMP levels.⁵⁹ All three subtypes are involved with decreasing cAMP levels, but it has been shown that only α₂A- and α₂C-adrenoceptors inhibit Ca²⁺ channels and activate K⁺ channels.⁵⁶

![Figure 5. Schematic diagram of the G-protein cycle.⁵⁹ PTX: Pertussis toxin and CTX: cholera toxin.](image)

2. Structure

The arrangement of the structure of α₂-adrenoceptors corresponds to a model of the protein rhodopsin, which has been extensively studied, and several subtypes have been
identified. In general, α2-adrenoceptors have seven transmembrane-spanning hydrophobic regions (TM1-TM7), with hydrophilic intracellular and extracellular loops. The transmembrane regions are made up of 20- to 25-amino acids forming alpha helices that are embedded in the membrane. The α2-adrenoceptor subtypes share about 75% amino acid identity in their transmembrane regions. The largest difference among the α2-adrenoceptor subtypes occurs in the N-terminus and in the third intracellular loop between TM5 and TM6. Among the subtypes, they share less than 25% sequence identity in the third intracellular loop. The third intracellular loops of the three subtypes vary in sequence and in the number of amino acids: α2A-adrenoceptors consist of 157 amino acids, α2B-adrenoceptors consist of 173 amino acids, and α2C-adrenoceptors consist of 148 amino acids (Table 4).

Table 4. Summary of α2-Adrenoceptor Subtype Characteristics.

<table>
<thead>
<tr>
<th></th>
<th>α2A</th>
<th>α2B</th>
<th>α2C</th>
</tr>
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<tbody>
<tr>
<td>Human chromosome</td>
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<td></td>
</tr>
<tr>
<td>Structure</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>number of amino acids</td>
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<td>2</td>
<td>4</td>
</tr>
<tr>
<td>transmembrane domains</td>
<td>7</td>
<td>7</td>
<td>7</td>
</tr>
<tr>
<td>number of amino acids in third intracellular loop</td>
<td>450</td>
<td>450</td>
<td>461</td>
</tr>
<tr>
<td>glycosylated</td>
<td>yes</td>
<td>no</td>
<td>yes</td>
</tr>
<tr>
<td>palmitoylated</td>
<td>yes</td>
<td>yes</td>
<td>no</td>
</tr>
<tr>
<td>Second messenger systems</td>
<td>$G_{i/o}$: ↓ cAMP, inhibit Ca$^{2+}$ channels, open K$^+$ channels</td>
<td>$G_{i/o}$: ↓ cAMP</td>
<td>$G_{i/o}$: ↓ cAMP, inhibit Ca$^{2+}$ channels, open K$^+$ channels</td>
</tr>
</tbody>
</table>
Interestingly, the third intracellular loop of the $\alpha_2B$-adrenoceptor subtype contains a higher number of glutamic acid residues (13%) when compared to $\alpha_2A$-adrenoceptors (6%) and $\alpha_2C$-adrenoceptors (3%). In this region, which is only seen in $\alpha_2B$-adrenoceptors, there is a stretch of 12 consecutive glutamic acid residues. The third intracellular loop is about two to three times longer in the $\alpha_2$-adrenoceptors than in the corresponding loops of the $\alpha_1$- and $\beta$-adrenoceptors. The second and third intracellular loops are involved with G-protein interactions. The $\alpha_2A$-adrenoceptor subtype consists of 450 amino acids and is glycosylated on the N-terminus and palmitoylated on the C-terminus. The $\alpha_2B$-adrenoceptor subtype also consists of 450 amino acids and is palmitoylated on the C-terminus but is not glycosylated, whereas the $\alpha_2C$-subtype consists of 461 amino acids and is only glycosylated on the N-terminus. The palmitoylated cysteine residue in the $\alpha_2A$- and $\alpha_2B$-adrenoceptors attaches the receptor to the lipid bilayer of the cell membrane. It is suggested that some of the TM regions form a ligand-binding pocket located in the upper part of the hydrophobic core of the receptor (Figure 6). There is a disulphide bridge between two cysteine residues in TM3 and the second extracellular loop that is important for maintaining the overall protein structure.
Figure 6. Model of a ligand binding to a GPCR.58

All of the α2-adrenoceptor subtypes have been cloned from human, rat, mouse, and guinea pig sources.55,58 The human α2-adrenoceptor subtypes have been designated α2-C10 (α2A), α2-C2 (α2B), and α2-C4 (α2C). These designations are based on their chromosomal location on the human gene that encodes these subtypes.57 These subtypes share approximately 50% amino acid identity. Also, the porcine and bovine α2A-, chicken α2A-, opossum α2C- and fish α2C-adrenoceptors are orthologs and have been cloned.58 The rodent α2A-adrenoceptor was initially classified as a new subtype, the α2D-adrenoceptor, but is now acknowledged as the rodent homolog of the human α2A-adrenoceptor subtype.58 The difference in these two subtypes is their affinity for an α2-adrenoceptor ligand. The rat α2D-adrenoceptor subtype has a lower affinity for the nonselective α2-adrenoceptor antagonist yohimbine (39) than does the human α2A-adrenoceptor.58
3. Pharmacology

α₂-Adrenoceptors play a major role in mediating several physiological effects in the CNS. The various α₂-adrenoceptor subtypes are distributed throughout the brainstem and spinal cord. In rats and mice, α₂-adrenoceptors are localized throughout the same regions in the CNS. The α₂A-adrenoceptor subtype is located in the brainstem, cerebral cortex, locus coeruleus, amygdala, pontine nuclei, nucleus tractus solitarii, dorsal horn, and the dorsal root ganglia. The α₂B-adrenoceptor subtype is found mainly in the thalamus, and the α₂C-adrenoceptor subtype is found in the olfactory bulb, cerebral cortex, hippocampus, striatum, and dorsal root ganglia. In humans, the α₂A-subtype is distributed in the cerebral cortex, brainstem, hippocampus, amygdala, locus coeruleus, and dorsal root ganglia. The α₂B-adrenoceptor subtype is distributed in the thalamus, dorsal root ganglia, and throughout the spinal cord, and the α₂C-adrenoceptor subtype is distributed in the cerebral cortex and lumbar spinal cord.
Both \( \alpha_2 \)-adrenoceptor agonists and antagonists have been analyzed to study the functions of \( \alpha_2 \)-adrenoceptors. There are various functional assays that have been used in order to characterize \( \alpha_2 \)-adrenoceptor ligands as agonists or antagonists. \( \alpha_2 \)-Adrenoceptors modulate blood pressure, therefore one \textit{in vivo} assay that has been used is a measurement of pressor responses in a pithed rat model.\(^6^6\) In this model, the spinal cord of the rat is severed by inserting a steel rod, which acts as a stimulating electrode, down the spinal cord.\(^6^6\) Then, arterial pressure is monitored by cannulating the right carotid artery. When an \( \alpha_2 \)-adrenoceptor agonist is administered, it causes a decrease in blood pressure (or a hypotensive effect).\(^6^6\) Thus an \( \alpha_2 \)-adrenoceptor antagonist blocks this decrease in blood pressure. An alternative method to studying pressor responses is in anaesthetized rats.\(^6^7\) The only difference in this model is that the rats are anaesthetized with pentobarbital instead of severing the spinal cord. As seen in the pithed rats, \( \alpha_2 \)-adrenoceptor agonists produce a hypotensive effect.\(^6^7\) An \textit{in vitro} assay used for \( \alpha_2 \)-adrenoceptors is contraction of the saphenous vein.\(^5^6\) In this assay, an \( \alpha_2 \)-adrenoceptor ligand is administered and if it causes the saphenous vein to contract, it is referred to as an agonist.\(^5^6\) \( \alpha_2 \)-Adrenoceptor ligands are characterized as antagonists if they can block this contraction. These functional assays have been helpful in further understanding the physiological effects produced by \( \alpha_2 \)-adrenoceptors.
4. Ligands and SAR

a) Agonists

Currently, there are several drug classes that bind at $\alpha_2$-adrenoceptor subtypes nonselectively. Both nonselective agonists and antagonists have a variety of therapeutic applications. The first drug class found to bind at $\alpha_2$-adrenoceptors was the phenylethylamines.\textsuperscript{68} This class consists of the two endogenous agonists norepinephrine (36, $K_i = 81$ nM) and epinephrine (37, $K_i = 15$ nM).\textsuperscript{69} However, these two agonists bind nonselectively at $\alpha_1$-, $\alpha_2$-, and $\beta$-adrenoceptors. Increasing the steric bulk of the N-substituent to ethyl (40), isopropyl (38), or tert-butyl (41) eliminates affinity for $\alpha_2$-adrenoceptors ($K_i > 10,000$).\textsuperscript{68} Maintaining the catechol moiety is also important for binding at these receptors.\textsuperscript{68} Removal of the $\beta$-hydroxyl group usually reduces the affinity at $\alpha_2$-adrenoceptors, but dopamine (42, $K_i = 250$ nM) retains high affinity.\textsuperscript{68} The phenylethylamines have been shown to possess higher affinity for $\beta$-adrenoceptors when compared to $\alpha_2$-adrenoceptors.
The next major class of compounds that binds at $\alpha_2$-adrenoceptors is the imidazolines. Most of the representative agents in this class are partial agonists, whereas others are either full agonists or antagonists. In the imidazoline class, the presence of a catechol moiety results in high affinity agents but is not required for binding at $\alpha_2$-adrenoceptors. An important nonselective $\alpha_2$-adrenoceptor agonist in this class is clonidine (43, $K_i = 1.8$ nM); 43 is an aminoimidazoline. In general, the aminoimidazolines are highly selective for $\alpha_2$-adrenoceptors and when they bear substituents at the 2- and/or 4-positions they can readily cross the blood-brain barrier. An analog of clonidine, ST-91 (44), was also found to bind at $\alpha_2$-adrenoceptors with high affinity as seen with clonidine and also behaves as an $\alpha_2$-adrenoceptor agonist. Since clonidine (43) possesses such high affinity at $\alpha_2$-adrenoceptors, its structure has been used as a model to develop novel ligands. For example, Hlasta et al. studied a series of indolin-2-yl (Table 5) and tetrahydroquinolin-2-yl imidazolines. It has been suggested that
the ortho di-chloro substitution pattern of clonidine (43) helps to restrict the imidazoline ring in a position that is perpendicular to the 2,6-dichlorophenyl ring. This conformation is supposed to be responsible for the α2-adrenoceptor agonist activity of clonidine. It was shown that the cis-indoline 45 (Ki = 6.9 nM) possesses high affinity that was comparable with clonidine. The trans-indoline 46 (Ki = 72.3 nM), which is less sterically hindered, showed a decrease in affinity at α2-adrenoceptors. Various heteroatoms were introduced into the side chain that was added to the indoline at the 2-position. These additions to the indoline resulted in high affinity for these agents at α2-adrenoceptors. Ring expansion of the indolines to, for example cis-tetrahydroquinoline 47 (Ki >10,000 nM) resulted in elimination of affinity.

Table 5. Observed Binding Affinities of Indolin-2-yl imidazolines at α2-Adrenoceptors.

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<td>H</td>
<td>CH3</td>
<td>H</td>
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</tr>
<tr>
<td>46</td>
<td>H</td>
<td>H</td>
<td>CH3</td>
<td>72.3</td>
</tr>
<tr>
<td>48</td>
<td>CH3</td>
<td>H</td>
<td>H</td>
<td>3.4</td>
</tr>
<tr>
<td>49</td>
<td>CH2CH=CH2</td>
<td>H</td>
<td>H</td>
<td>77</td>
</tr>
<tr>
<td>50</td>
<td>CH2CH2CH2</td>
<td>H</td>
<td>H</td>
<td>260</td>
</tr>
<tr>
<td>51</td>
<td>CH2OCH3</td>
<td>H</td>
<td>H</td>
<td>38</td>
</tr>
<tr>
<td>52</td>
<td>CH2SCH3</td>
<td>H</td>
<td>H</td>
<td>13.2</td>
</tr>
<tr>
<td>53</td>
<td>CH2N(i-Pr)2</td>
<td>H</td>
<td>H</td>
<td>25.1</td>
</tr>
<tr>
<td></td>
<td>clonidine</td>
<td></td>
<td></td>
<td>1.8</td>
</tr>
</tbody>
</table>
It has also been suggested that the structure of clonidine (43) has to be in a specific conformation to interact with the receptor. The basic nitrogen must lie at a short distance above the plane of the aromatic ring with the plane of the imidazoline ring perpendicular to the plane of the aromatic ring. This conformation prevents any resonance interaction between the aromatic portion and the imidazoline ring. So in order to study this conformation, a series of spiro-imidazolines was developed and analyzed (Table 6). The parent compound (i.e., 54) was found to bind at $\alpha_{2A}$-adrenoceptors with high affinity ($K_i = 34$ nM) and at $\alpha_{2B}$-adrenoceptors with decreased affinity ($K_i = 275$ nM). Adding a fluoro group at the $R_1$ position increased the binding affinity at both $\alpha_{2A}$- and $\alpha_{2B}$-adrenoceptors ($55$, $K_i = 12$ and 152 nM, respectively). Chloro groups in the $R_2$ and $R_4$ positions also increased the affinity at both $\alpha_{2A}$- and $\alpha_{2B}$-adrenoceptors ($56$, $K_i = 3$ and 95 nM, respectively). Adding an electron donating group in the $R_1$ position, a methoxy group (i.e., 57), decreased the affinity at the $\alpha_{2A}$-adrenoceptors ($K_i = 51$ nM) but had a similar affinity at $\alpha_{2B}$-adrenoceptors ($K_i = 99$ nM) when compared to 56. A methoxy group in the $R_2$ position (i.e., 58) increased the affinity at $\alpha_{2A}$-adrenoceptors but decreased the affinity at $\alpha_{2B}$-adrenoceptors ($K_i = 18$ and 193 nM, respectively). More analogs were studied and
it was shown that this conformation might be one of the ways that clonidine (43) binds at both $\alpha_{2A}$- and $\alpha_{2B}$-adrenoceptors.71

Table 6. Observed Binding Affinities of Imidazolines at $\alpha_{2A}$- and $\alpha_{2B}$-Adrenoceptors.71

<table>
<thead>
<tr>
<th>$K_i$, nM</th>
<th>$R_1$</th>
<th>$R_2$</th>
<th>$R_3$</th>
<th>$R_4$</th>
<th>$\alpha_{2A}$</th>
<th>$\alpha_{2B}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>54</td>
<td>H</td>
<td>H</td>
<td>H</td>
<td>H</td>
<td>34</td>
<td>275</td>
</tr>
<tr>
<td>55</td>
<td>F</td>
<td>H</td>
<td>H</td>
<td>H</td>
<td>12</td>
<td>152</td>
</tr>
<tr>
<td>56</td>
<td>H</td>
<td>Cl</td>
<td>H</td>
<td>Cl</td>
<td>3</td>
<td>95</td>
</tr>
<tr>
<td>57</td>
<td>CH$_3$O</td>
<td>H</td>
<td>H</td>
<td>H</td>
<td>51</td>
<td>99</td>
</tr>
<tr>
<td>58</td>
<td>H</td>
<td>CH$_3$O</td>
<td>H</td>
<td>H</td>
<td>18</td>
<td>193</td>
</tr>
<tr>
<td>59</td>
<td>H</td>
<td>H</td>
<td>CH$_3$O</td>
<td>H</td>
<td>87</td>
<td>1200</td>
</tr>
<tr>
<td>60</td>
<td>H</td>
<td>H</td>
<td>H</td>
<td>CH$_3$O</td>
<td>12</td>
<td>159</td>
</tr>
<tr>
<td>61</td>
<td>H</td>
<td>CH$_3$</td>
<td>H</td>
<td>H</td>
<td>7</td>
<td>50</td>
</tr>
</tbody>
</table>

Oxymetazoline (62) is another example of an imidazoline but it shows selectivity among the $\alpha_2$-adrenoceptor subtypes. Oxymetazoline (62) binds with higher affinity at $\alpha_{2A}$-adrenoceptors ($K_i = 5.6$ nM) than at $\alpha_{2B}$- and $\alpha_{3C}$-adrenoceptors ($K_i = 350$ and 72 nM,
respectively). This agent is one of the few selective $\alpha_{2A}$-adrenoceptor agonists that has been developed. Although oxymetazoline (62) is selective among the $\alpha_2$-adrenoceptor subtypes, it also binds at $\alpha_1$-adrenoceptors ($K_i = 29.8$ nM).

![Oxymetazoline (62)](image)

The substituted guanidines are a small class consisting of a couple of ligands that bind at $\alpha_2$-adrenoceptors nonselectively. Two representative agonists in this class are guanabenz (63, $K_i = 16.7$ nM) and guanfacine (64, $K_i = 24.9$ nM). It has been shown that the substituted guanidines also bind at another receptor population, the imidazoline receptors.

![Guanabenz (63)](image)  ![Guanfacine (64)](image)
An analog of the imidazolines, medetomidine (65, $K_i = 25$ nM), is a highly potent nonselective $\alpha_2$-adrenoceptor agonist. A series of conformationally restricted analogs of medetomidine was developed and analyzed as possible $\alpha_2$-adrenoceptor agonists. For example, tetralin 67 (Table 7) displayed about 4-fold lower affinity than 65. In this tetralin series, the 5-methoxy analog retained affinity at $\alpha_2$-adrenoceptors (66, $K_i = 98$ nM). Other analogs are shown in Table 7. In the 3,4-dihydronapthalene series (Table 7), a methoxy group in the 5-position decreased the affinity by more than 50-fold (72, $K_i = 1420$ nM). A methyl group (73, $K_i = 31$ nM) in the 5-position resulted in similar affinity as medetomidine (65). However adding methyl groups in the 5- and 7-positions resulted in a decrease in affinity (74, $K_i = 232$ nM). As seen in the 5-substituted tetralin series, a methoxy group in the 6- or 7-position decreased affinity (75, 76, $K_i = 580$ and 1570 nM, respectively). The 4-methylindan analog 77 ($K_i = 8.8$ nM) displayed enhanced affinity relative to 65.
Table 7. Observed Binding Affinities of Conformationally Restricted Analogs of Medetomidine at $\alpha_2$-Adrenoceptors.\textsuperscript{72}

<table>
<thead>
<tr>
<th>R</th>
<th>$K_i$, nM</th>
</tr>
</thead>
<tbody>
<tr>
<td>66 5-OCH$_3$</td>
<td>98</td>
</tr>
<tr>
<td>67 5-CH$_3$</td>
<td>95</td>
</tr>
<tr>
<td>68 5-OH</td>
<td>63</td>
</tr>
<tr>
<td>69 6-OCH$_3$</td>
<td>169</td>
</tr>
<tr>
<td>70 7-OCH$_3$</td>
<td>201</td>
</tr>
<tr>
<td>71 5,7-diCH$_3$</td>
<td>103</td>
</tr>
<tr>
<td>72 5-OCH$_3$</td>
<td>1420</td>
</tr>
<tr>
<td>73 5-CH$_3$</td>
<td>31</td>
</tr>
<tr>
<td>74 5,7-diCH$_3$</td>
<td>232</td>
</tr>
<tr>
<td>75 6-OCH$_3$</td>
<td>580</td>
</tr>
<tr>
<td>76 7-OCH$_3$</td>
<td>1570</td>
</tr>
<tr>
<td>77</td>
<td>8.8</td>
</tr>
</tbody>
</table>

In a previous study, Gentili et al.\textsuperscript{73} showed that modifying the structure of imidazoline compounds resulted in a significant difference in their affinity at $\alpha_2$-adrenoceptors. It was found that imidazolines 78 and 79 were antagonists at $\alpha_2$-adrenoceptors and weak agonists at $\alpha_1$-adrenoceptors.\textsuperscript{73} From this study, biphenyline (80) was also developed and found to be selective for $\alpha_2$-adrenoceptors and also behaved as an $\alpha_2$-adrenoceptor agonist in \textit{in vitro} assays.\textsuperscript{73} Biphenyline (80) was obtained by introducing a phenyl ring in the \textit{ortho} position of the 2-[1-(phenoxy)ethyl]-imidazoline basic structure.\textsuperscript{73} It was suggested that this phenyl group might be responsible for the agonist
activity seen with biphenyline (80). Various modifications were made to the phenyl ring in the ortho position and resulted in a series of biphenyline analogs (Table 8). The affinity and intrinsic activity were measured for these biphenyline analogs as well as biphenyline (80). Biphenyline (80) was found to bind with higher affinity at $\alpha_{2A}$-adrenoceptors when compared to $\alpha_{2B}$- and $\alpha_{2C}$-adrenoceptors ($K_i = 47, 501$, and $199$ nM, respectively). It also behaved as an agonist at both $\alpha_{2A}$- and $\alpha_{2C}$-adrenoceptors and a partial agonist at $\alpha_{2B}$-adrenoceptors. Therefore a major focus for this study was to modify the phenyl group in order to develop subtype selective $\alpha_2$-adrenoceptor agonists. The first approach was isosteric substitution with a pyridine or thiophene nucleus. The pyridine derivatives 81 and 83 resulted in lower affinity at $\alpha_2$-adrenoceptors when compared to
Table 8. Observed Binding Affinities of Biphenyl Analogs at $\alpha_2$-Adrenoceptor Subtypes.

<table>
<thead>
<tr>
<th>R</th>
<th>$K_i$, nM</th>
<th>$\alpha_2A$</th>
<th>$\alpha_2B$</th>
<th>$\alpha_2C$</th>
<th>$\alpha_2A$</th>
<th>$\alpha_2B$</th>
<th>$\alpha_2C$</th>
</tr>
</thead>
<tbody>
<tr>
<td>80</td>
<td>47</td>
<td>501</td>
<td>199</td>
<td>0.70</td>
<td>0.50</td>
<td>0.80</td>
<td></td>
</tr>
<tr>
<td>81</td>
<td>1202</td>
<td>3311</td>
<td>1259</td>
<td>NA</td>
<td>NA</td>
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<td></td>
</tr>
<tr>
<td>82</td>
<td>138</td>
<td>933</td>
<td>64</td>
<td>0.5</td>
<td>0.5</td>
<td>1.00</td>
<td></td>
</tr>
<tr>
<td>83</td>
<td>282</td>
<td>2692</td>
<td>1445</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td></td>
</tr>
<tr>
<td>84</td>
<td>17</td>
<td>214</td>
<td>72</td>
<td>0.8</td>
<td>0.6</td>
<td>0.8</td>
<td></td>
</tr>
<tr>
<td>85</td>
<td>46</td>
<td>417</td>
<td>132</td>
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<tr>
<td>86</td>
<td>47</td>
<td>832</td>
<td>234</td>
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<td>NA</td>
<td>0.45</td>
<td></td>
</tr>
<tr>
<td>87</td>
<td>51</td>
<td>537</td>
<td>245</td>
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<td>NA</td>
<td>0.6</td>
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</tr>
<tr>
<td>88</td>
<td>42</td>
<td>398</td>
<td>251</td>
<td>NA</td>
<td>NA</td>
<td>0.46</td>
<td></td>
</tr>
<tr>
<td>89</td>
<td>309</td>
<td>1000</td>
<td>1622</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td></td>
</tr>
<tr>
<td>90</td>
<td>28</td>
<td>355</td>
<td>18</td>
<td>NA</td>
<td>0.5</td>
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<td>91</td>
<td>34</td>
<td>813</td>
<td>331</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td></td>
</tr>
<tr>
<td>92</td>
<td>174</td>
<td>1549</td>
<td>1230</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td></td>
</tr>
</tbody>
</table>
biphenyline (80) and were inactive at all three subtypes. However, 82 possessed higher affinity at \( \alpha_2 \)-adrenoceptors than the other pyridine derivatives and showed selectivity at \( \alpha_{2C} \)-adrenoceptors. Compound 82 behaved as an agonist at \( \alpha_{2C} \)-adrenoceptors and a partial agonist at the other subtypes. The thiophene nucleus possesses aromaticity that is comparable to that of a phenyl ring. Both 84 and 85 behaved as agonists at \( \alpha_{2A} \)- and \( \alpha_{2C} \)-adrenoceptors and showed more selectivity in affinity for the \( \alpha_{2A} \)-adrenoceptor. The next approach was to incorporate various substitution at the *ortho*, *meta*, and *para* positions of the phenyl ring. A methyl group was added to the *ortho*, *meta*, or *para* position (86, 87, and 88, respectively) and none of these compounds were active at \( \alpha_{2A} \)- or \( \alpha_{2B} \)-adrenoceptors but behaved as partial agonists at \( \alpha_{2C} \)-adrenoceptors. When a hydroxy group was added at these three positions (89, 90, and 91), the only active compound was the *m*-hydroxy derivative 90 (\( K_i = 18 \text{ nM}, \text{ia} = 1.15 \)) that behaved as a full agonist with high affinity at \( \alpha_{2C} \)-adrenoceptors. To further test the possible role that an electronic effect has on binding and agonist activity, a nitro group was added at the *ortho*, *meta*, and

<table>
<thead>
<tr>
<th>Compound</th>
<th>( \text{K}_i ) (nM)</th>
<th>( \text{ia} )</th>
<th>Selectivity</th>
</tr>
</thead>
<tbody>
<tr>
<td>93</td>
<td>60 661 234</td>
<td>NA NA 0.6</td>
<td></td>
</tr>
<tr>
<td>94</td>
<td>123 501 302</td>
<td>NA NA NA</td>
<td></td>
</tr>
<tr>
<td>95</td>
<td>37 479 309</td>
<td>0.45 NA 0.5</td>
<td></td>
</tr>
<tr>
<td>96</td>
<td>15 269 17</td>
<td>0.58 NA 1.15</td>
<td></td>
</tr>
</tbody>
</table>

\( \text{ia} = \text{intrinsic activity, NA = not active (ia < 0.3)} \)
para positions (92, 93, and 94, respectively).\textsuperscript{73} All three nitro derivatives were inactive at \(\alpha_{2A}\)- and \(\alpha_{2B}\)-adrenoceptors.\textsuperscript{73} However, the \(m\)-nitro derivative 93 was the only nitro derivative that showed any activity at \(\alpha_{2C}\)-adrenoceptors.\textsuperscript{73} Since the meta position seemed to be optimal for agonist activity, several other substituents were introduced at this position. An amino derivative 95 possessed moderate affinity for all three \(\alpha_2\)-adrenoceptor subtypes but behaved as a partial agonist at \(\alpha_{2A}\)- and \(\alpha_{2C}\)-adrenoceptors.\textsuperscript{73} Addition of a fluoro group in the meta position, 96, showed an increase in affinity compared to biphenyline (80) and behaved as a full agonist at \(\alpha_{2C}\)-adrenoceptors and as a partial agonist at \(\alpha_{2A}\)-adrenoceptors.\textsuperscript{73} This was the first series of \(\alpha_2\)-adrenoceptor ligands that showed subtype selectivity for \(\alpha_{2C}\)-adrenoceptors.

Currently, there are available \(\alpha_2\)-adrenoceptor agonists that bind nonselectively at the \(\alpha_2\)-adrenoceptor subtypes. However, there are still only a limited number of agonists that show subtype selectivity for \(\alpha_{2A}\)-adrenoceptors versus \(\alpha_{2B}\)- and \(\alpha_{2C}\)-adrenoceptor subtypes. Although these agonists show subtype selectivity among the \(\alpha_2\)-adrenoceptor subtypes, they also bind at other receptor populations (i.e., 5-HT and \(\alpha_1\)-adrenoceptors). Still, there is no strictly subtype-selective \(\alpha_{2A}\)-adrenoceptor agonist that has been developed. There are also no subtype-selective \(\alpha_{2B}\)-adrenoceptor agonists available. Recently, subtype selective \(\alpha_{2C}\)-adrenoceptor agonists have been developed and might be used to determine the physiological effects of \(\alpha_{2C}\)-adrenoceptors.\textsuperscript{73} Selective \(\alpha_2\)-adrenoceptor subtype agonists can help to further understand the physiological effects of each subtype and also to manage any disorders that are mediated by the \(\alpha_2\)-adrenoceptor.
subtypes. There is an increasing clinical, and basic science, need for selective $\alpha_2$-adrenoceptor subtype-selective agonists.

b) Antagonists

Many $\alpha_2$-adrenoceptor antagonists have been developed over the years. These antagonists have been used to classify the $\alpha_2$-adrenoceptor subtypes via radioligand binding studies. $\alpha_2$-Adrenoceptor antagonists come from a variety of chemical classes. Some representative $\alpha_2$-adrenoceptor antagonists include yohimbine (39), rauwolscine (97), imiloxan (98), and SK&F 86466 (99). Yohimbine (39), an indole alkaloid that has been isolated from *Pausinystalia yohimbe* bark and *Rauwolfia* root, has been used in herbal medicines for centuries.\(^1\)

![Rauwolscine (97) and Imiloxan (98)](image)
Yohimbine (39) is an $\alpha_2$-adrenoceptor antagonist that binds at the $\alpha_{2A}$-adrenoceptor subtype ($K_i = 7.5$ nM), $\alpha_{2B}$-adrenoceptor subtype ($K_i = 4.6$ nM), and $\alpha_{2C}$-adrenoceptor subtype ($K_i = 2.3$ nM) with similar affinity. Since yohimbine (39) binds at $\alpha_2$-adrenoceptors with such high affinity, other yohimbine derivatives were examined and characterized as $\alpha_2$-adrenoceptor antagonists. Rauwolscine (97) is also an alkaloid that belongs to the same family as yohimbine, the yohimbanes. Rauwolscine (97) binds at the $\alpha_2$-adrenoceptor subtypes nonselectively: $\alpha_{2A}$-adrenoceptor subtype ($K_i = 4.6$ nM), $\alpha_{2B}$-adrenoceptor subtype ($K_i = 4.7$ nM), and $\alpha_{2C}$-adrenoceptor subtype ($K_i = 1.0$ nM), and with an affinity comparable to that of yohimbine (39). Several other analogs of yohimbine, raubasine (100) and akuammingine (101), are also selective $\alpha_2$-adrenoceptor antagonists. Both of these analogs have been isolated from the same sources as Rauwolscine (97).
yohimbine (39) and are known as heteroyohimbines.\textsuperscript{68} Raubasine (100) binds at all of the \(\alpha_2\)-adrenoceptor subtypes with high affinity: \(\alpha_{2A}\)-adrenoceptor subtype (\(K_i = 8.2\) nM), \(\alpha_{2B}\)-adrenoceptor subtype (\(K_i = 14.5\) nM), and \(\alpha_{2C}\)-adrenoceptor subtype (\(K_i = 5.0\) nM).\textsuperscript{68} Akuammingine (101) binds at each \(\alpha_2\)-adrenoceptors with lower affinity than yohimbine (39): \(\alpha_{2A}\)-adrenoceptor subtype (\(K_i = 106\) nM), \(\alpha_{2B}\)-adrenoceptor subtype (\(K_i = 116\) nM), and \(\alpha_{2C}\)-adrenoceptor subtype (\(K_i = 28\) nM).\textsuperscript{68}

Imiloxan (98) is an \(\alpha_2\)-adrenoceptor antagonist that belongs to another class, the benzodioxans.\textsuperscript{74} Imiloxan (98) is a benzodioxanyl N-ethylimidazole that binds selectively at the \(\alpha_{2B}\)-adrenoceptor subtype (\(K_i = 136\) nM) compared to \(\alpha_{2A}\)- and \(\alpha_{2C}\)-adrenoceptor (\(K_i = 1701\) and 1056 nM respectively).\textsuperscript{75} Imiloxan (98) has been used to characterize the physiological functions of \(\alpha_{2B}\)-adrenoceptors, since it is the only available \(\alpha_{2B}\)-adrenoceptor-selective antagonist. When imiloxan (98) originally was implicated as a selective \(\alpha_{2B}\)-adrenoceptor antagonist, its binding activity was studied only at \(\alpha_{2A}\)- and \(\alpha_{2B}\)-adrenoceptors.\textsuperscript{76,77} However, it has been shown that imiloxan (98) blocks the antinociceptive activity of the \(\alpha_2\)-adrenoceptor agonist, ST-91 (44), whose actions are thought to be mediated primarily through the \(\alpha_{2C}\)-adrenoceptor subtype.\textsuperscript{78} Currently, there is great controversy for which \(\alpha_2\)-adrenoceptor subtype imiloxan (98) is truly selective: \(\alpha_{2B}\)- or \(\alpha_{2C}\)-adrenoceptors. Idazoxan (102), a benzodioxanyl imidazoline, is also an \(\alpha_2\)-adrenoceptor antagonist. Unlike imiloxan (98), idazoxan (102) binds nonselectively at the \(\alpha_2\)-adrenoceptor subtypes: \(\alpha_{2A}\)-adrenoceptor subtype (\(K_i = 21\) nM), \(\alpha_{2B}\)-adrenoceptor subtype (\(K_i = 43\) nM), and \(\alpha_{2C}\)-adrenoceptor subtype (\(K_i = 35\) nM).\textsuperscript{59}
Another class of $\alpha_2$-adrenoceptor antagonists are the benzazepines (Table 9). SK&F 86466 (99) shows moderate selectivity for $\alpha_2$-adrenoceptors versus $\alpha_1$-adrenoceptors, but binds at the $\alpha_2$-adrenoceptor subtypes nonselectively: $\alpha_{2A}$-adrenoceptor subtype ($K_i = 9.4 \text{ nM}$), $\alpha_{2B}$-adrenoceptor subtype ($K_i = 16 \text{ nM}$), and $\alpha_{2C}$-adrenoceptor subtype ($K_i = 20 \text{ nM}$). SK&F 86466 (99) has been used to subclassify $\alpha_2$-adrenoceptor physiological responses in functional assays. A heterofused analog, SK&F 104856 (103) shows approximately 8-fold selectivity for the $\alpha_{2B}$-adrenoceptor subtype ($K_i = 3.4 \text{ nM}$), when compared to $\alpha_{2A}$-adrenoceptor subtype ($K_i = 24 \text{ nM}$) and $\alpha_{2C}$-adrenoceptor subtype ($K_i = 21 \text{ nM}$). Several related 3-benzazepine derivatives have been developed; some are shown in Table 9.
Table 9. Observed Binding Affinities of 3-Benzazepine Derivatives at $\alpha_2$-Adrenoceptor Subtypes.\textsuperscript{68}

<table>
<thead>
<tr>
<th></th>
<th>$\alpha_{2A}$</th>
<th>$\alpha_{2B}$</th>
<th>$\alpha_{2C}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>SK&amp;F 86466 (99)</td>
<td>9.4</td>
<td>16</td>
<td>20</td>
</tr>
<tr>
<td>SK&amp;F 104856 (103)</td>
<td>24</td>
<td>3.4</td>
<td>21</td>
</tr>
<tr>
<td>SK&amp;F 105854 (104)</td>
<td>54</td>
<td>14</td>
<td>28</td>
</tr>
<tr>
<td>SK&amp;F 104078 (105)</td>
<td>114</td>
<td>142</td>
<td>64</td>
</tr>
<tr>
<td>SK&amp;F 106686 (106)</td>
<td>31</td>
<td>15</td>
<td>35</td>
</tr>
</tbody>
</table>

A well-known $\alpha_2$-adrenoceptor antagonist belongs to a group, the quinazolines, that mostly behaves as $\alpha_1$-adrenoceptor antagonists. Quinazolines do not discriminate between the $\alpha_1$-adrenoceptors but several, including prazosin (107), have been shown to bind at $\alpha_2$-adrenoceptors. Evidence shows that prazosin (107) binds selectively at both $\alpha_{2B}$- and $\alpha_{2C}$-
adrenoceptors with high affinity \((K_i = 30.6 \text{ and } 10.7 \text{ nM, respectively})\) when compared to \(\alpha_{2A}\)-adrenoceptors \((K_i = 302 \text{ nM})\).^68

In general, a number of \(\alpha_2\)-selective antagonists have been identified, but with the exception of imiloxan (98), few show selectivity within the \(\alpha_2\)-adrenoceptor family. Furthermore, it is currently unknown whether imiloxan is an \(\alpha_{2B}, \alpha_{2C}\) or \(\alpha_{2B}/\alpha_{2C}\)-selective antagonist.

c) Therapeutic Application of \(\alpha_2\)-Adrenoceptor Agents

\(\alpha_2\)-Adrenoceptor agonists and antagonists have a wide range of therapeutic applications. \(\alpha_2\)-Adrenoceptor agonists have been used in the treatment of hypertension for many years.\(^1\) This is due to the fact that \(\alpha_2\)-adrenoceptors, which are present on blood vessels, can mediate vasoconstriction.\(^68\) Clonidine (43), guanabenz (63), and guanfacine (64) are still being used clinically for the treatment of hypertension.\(^1\) All of these agents also bind at imidazoline receptors and are thought to require both components, \(\alpha_2\)-
adrenoceptors and imidazoline receptors, to lower and maintain blood pressure.\textsuperscript{79} However, some of the drawbacks of these antihypertensives are that they cause sedation, bradycardia, and mental depression.\textsuperscript{79} $\alpha_2$-Adrenoceptors are also involved with non-insulin-dependent diabetes.\textsuperscript{68,79} Activation of $\alpha_2$-adrenoceptors has been shown to inhibit the secretion of insulin from pancreatic islet cells.\textsuperscript{68,79} Several $\alpha_2$-adrenoceptor antagonists, rauwolscine (97) and idazoxan (102), enhanced glucose-induced insulin secretion in the rat.\textsuperscript{68,79} Yohimbine (39) increases plasma insulin, and inhibits epinephrine-induced hyperglycemia in mice.\textsuperscript{68,79} Therefore, blocking $\alpha_2$-adrenoceptors seems to play a role in selectively enhancing glucose-stimulated insulin secretion in this type of diabetes.\textsuperscript{79}

Another possible target of $\alpha_2$-adrenoceptors' therapeutic applications is obesity. It has been shown that stimulating $\alpha_2$-adrenoceptors causes an inhibition of lipolysis in isolated human adipocytes. This stimulation of $\alpha_2$-adrenoceptors could possibly be used to promote weight loss.\textsuperscript{68,79} In rodents, SK&F 86466 (99) can block $\alpha_2$-adrenoceptors which then stimulates epinephrine-induced lipolysis.\textsuperscript{68,79} Studies have shown that long-term treatment with SK&F 86466 (99) results in a dose-related loss in body weight that was independent of the amount of food intake.\textsuperscript{68,79} Since activation of central $\alpha_2$-adrenoceptors results in sedative effects, $\alpha_2$-adrenoceptor agonists have been clinically used in anesthesia.\textsuperscript{68} Clonidine and other $\alpha_2$-adrenoceptor agonists are being used as adjuncts to general anesthesia.\textsuperscript{68,79} $\alpha_2$-Adrenoceptor agonists, detomidine and medetomidine (65), are being used as veterinary anesthetics.\textsuperscript{68,79} Evidence shows that pretreatment with
medetomidine (65) reduces the requirement for using gaseous anesthetics in both rats and dogs.68,79

The noradrenergic system inhibits nociception that is primarily mediated by \( \alpha_2 \)-adrenoceptors. Noradrenergic neurons descend from the brainstem to the spinal cord and terminate in the dorsal horn.80 This descending inhibitory pathway modulates pain by constituting a gating mechanism that controls impulse transmission in the dorsal horn.80 Norepinephrine (36) plays an important role in the modulation of pain transmission in the brain and spinal cord.80 I.t. administered norepinephrine (36) produces antinociceptive activity in rodents.8 \( \alpha_2 \)-Adrenoceptor agonists produce antinociception through the activation of the noradrenergic system.80 Clonidine (43), the most studied \( \alpha_2 \)-adrenoceptor agonist, produces antinociception both in rodents and humans.8 Evidence shows that clonidine produces its analgesic activity when administered systemically, epidurally, and intrathecally.8 The \( \alpha_2 \)-adrenoceptor’s role in nociception has been studied in nociceptive tests in rodents, especially thermal nociceptive tests. In both the hot-plate and tail-flick tests, clonidine produces dose-dependent antinociception when given via the intravenous (i.v.), i.t., or s.c. route.81 These studies showed that clonidine-induced antinociception is mediated by supraspinal and spinal \( \alpha_2 \)-adrenoceptor sites. Also, ST-91 (44), an analog of clonidine, produced antinociception in rats when administered via the i.t. route.82

Activation of \( \alpha_2 \)-adrenoceptors causes hyperpolarization of neurons by opening K\(^+\) channels, which seems to share a role in the antinociception produced by \( \alpha_2 \)-adrenoceptor agonists.83 Galeotti et al.83 showed that clonidine-induced antinociception was enhanced
by the $K_{ATP}$ channel openers minoxidil, pinacidil, and diazoxide in both the hot-plate and tail-flick tests, whereas the $K_{ATP}$ channel blocker gliquidone attenuated the antinociceptive activity of clonidine (43). $\alpha_2$-Adrenoceptors seem to be involved in the antinociception mediated by other descending inhibitory systems. 5-Methoxy-N,N-dimethyltryptamine (5-MeO-DMT), a 5-HT agonist, produced a dose-dependent antinociception that was blocked by yohimbine (39). This study suggested that 5-HT-induced antinociception might be dependent on the descending noradrenergic system. In another study, Svokos et al. showed that i.c.v. administered improgan, a histamine-2 receptor ligand, produced antinociceptive activity in rats. When i.p. or i.t. administered yohimbine (39) was given, yohimbine (39) attenuated the antinociceptive activity of improgan. However, i.c.v. administered yohimbine (39) did not attenuate the antinociceptive activity of improgan. These results showed that $\alpha_2$-adrenoceptors play a role in antinociception induced by histamine receptors but only the spinal $\alpha_2$-adrenoceptors were involved. $\alpha_2$-Adrenoceptors might also be involved in $GABA_B$ receptor-induced antinociception. Sabetkasai et al. showed that i.p. administered baclofen, a representative $GABA_B$ agonist, produced antinociception in mice in the tail-flick test. The antinociceptive activity of baclofen was blocked by i.p. administered yohimbine (39). This study further demonstrated the various systems that induce antinociception might be influenced by the noradrenergic system (Figure 7).
Figure 7. Schematic diagram of noradrenergic system modulating nociception.\textsuperscript{9} This figure is used to show the involvement of the noradrenergic system with other neurotransmitters.

Thermal nociceptive tests were also used to further understand which $\alpha_2$-adrenoceptor subtypes might be involved in antinociception mediated by $\alpha_2$-adrenoceptors. In a study by Millan,$\textsuperscript{87}$ s.c. administered UK 14,304 (108), an $\alpha_2$-adrenoceptor agonist, produced antinociceptive activity in mice in the hot-plate test. Several $\alpha_2$-adrenoceptor antagonists, both nonselective or subtype selective, were used to study $\alpha_2$-adrenoceptor subtype involvement.$\textsuperscript{87}$ The nonsubtype selective $\alpha_2$-adrenoceptor antagonist idazoxan (102) blocked the antinociceptive activity of UK 14,304 (108).$\textsuperscript{87}$ Both BRL 44408 (109) and RX 821002 (110), $\alpha_{2A}$-adrenoceptor antagonists, also blocked UK 14,304-induced antinociception.$\textsuperscript{87}$ However, BRL 41992 (111) and ARC-239 (112) and prazosin (107), selective $\alpha_{2B}$- and $\alpha_{2C}$-adrenoceptor antagonists, did not have any effect on the
antinociceptive activity of UK 14,304.\textsuperscript{87} This study suggested that among the supraspinal $\alpha_2$-adrenoceptor subtypes, only $\alpha_{2A}$-adrenoceptors are involved with antinociception induced by $\alpha_2$-adrenoceptor agonists.

\textbf{Figure 8.} Subtype selective $\alpha_2$-adrenoceptor ligands.\textsuperscript{87}
All of the previous studies in the hot-plate test showed that i.t. administered clonidine (43) and ST-91 (44) produced dose-dependent antinociception that activated supraspinal \( \alpha_2 \)-adrenoceptor sites.\(^8\)-\(^3\) However, it was also shown that the i.t.-administered \( \alpha_2 \)-adrenoceptor agonist dexmedetomidine was inactive in the hot-plate test but active in the tail-flick test.\(^8\) This could be due to the fact that clonidine (43) and ST-91 (44) are very lipid soluble compounds. This means that when these agents are administered directly into the spinal cord they are absorbed rapidly into the blood and diffused to other parts of the CNS.\(^8\) This allows these agents to activate \( \alpha_2 \)-adrenoceptors that are located in the brain. This idea was supported by the study by Ossipov et al.\(^8\) that showed that i.v. administered clonidine (43) produces antinociception in both the hot-plate and tail-flick tests. There has been controversy about the actual involvement of supraspinal \( \alpha_2 \)-adrenoceptors in the antinociception induced by spinally-administered \( \alpha_2 \)-adrenoceptor agonists. In another study, it was shown that i.t. administered clonidine (43) still produced potent antinociceptive activity after transection of the spinal cord.\(^8\) This suggests that although supraspinal \( \alpha_2 \)-adrenoceptors could possibly contribute to antinociception, activation of these sites might not be required to produce the maximal antinociceptive effect of clonidine (43).

Earlier studies showed that \( \alpha_2 \)-adrenoceptors have influence on the mediation of opioid-induced antinociception. Norepinephrine (36) is a modulator of the antinociceptive activity produced by opioid agonists in the spinal cord.\(^8\) I.t. administered yohimbine (39) blocks the antinociceptive activity of i.t.-administered morphine.\(^9\) This evidence further
suggests that $\alpha_2$-adrenoceptors are involved in morphine-induced antinociception. However, naloxone, an opioid antagonist, did not attenuate the antinociceptive activity of clonidine (43). Coadministration of $\alpha_2$-adrenoceptor agonists and opioid agonists resulted in a greater-than-additive antinociceptive effect, or synergy. These agents could be administered in lower doses than when administered separately to produce an equivalent antinociceptive effect. This is important because this combination could be used clinically in pain therapies. The morphine/clonidine synergism allows a more efficient treatment that involves lower doses which probably was associated with lower risk of undesired side effects. Separately, morphine and clonidine (43) are being used clinically in the treatment of pain, including postoperative and cancer pain. Therefore, the morphine /clonidine synergism has been extensively studied in rodents using both tail-flick and hot-plate tests. Systemically or spinally coadministered morphine and clonidine (43) produce a potentiation in antinociceptive activity in several animal studies. Since morphine produces the development of tolerance, the synergistic effect of morphine and clonidine (43) was studied in mice that were either acutely or chronically tolerant to morphine. Fairbanks and Wilcox showed that mice acutely tolerant to i.t.-administered morphine still produce a synergistic effect when morphine and clonidine (43) were coadministered via the i.t. route. Similar results were shown when the mice were chronically tolerant to i.t.-administered morphine. These results showed that even though tolerance develops from the use of morphine, coadministration of morphine and clonidine (43) could still be an efficient therapeutic treatment of pain.
Coadministration of clonidine (43) and morphine has been used clinically in patients for the treatment of postoperative pain. It was shown that epidural clonidine (3 μg/kg) plus morphine (0.05 mg/kg) provided better postoperative analgesia than epidural morphine alone after a gastrectomy. Evidence shows that the combination of these two analgesics can also be used in postcesarean analgesia. In a recent study, it was reported that the combination of subarachnoid morphine and clonidine (43) increased the duration of postcesarean analgesia when compared with morphine or clonidine (43) alone. This combination also reduced the opioid requirement and increased intraoperative sedation.

As mentioned earlier, studies have shown that the α2A-adrenoceptors are involved in antinociception. It was recently found that both α2B- and α2C-adrenoceptor subtypes could also play a role in mediating antinociception. Prazosin, which is selective for α2B- and α2C-adrenoceptors, inhibited neurotransmitter release from spinal cord preparations. This supports the idea of possible roles for both α2B- and α2C-adrenoceptors in antinociception. Sawamura et al. showed that activation of α2B-adrenoceptors could mediate the antinociceptive activity of nitrous oxide, an adjunctive general anesthetic. In this study, null mice for the α2B-adrenoceptor subtype produced a reduced or absent analgesic response to nitrous oxide.

α2-Adrenoceptor agonists have been classified as being potent analgesics. Although α2-adrenoceptor agonists are being used therapeutically as analgesics, they still produce the undesired side effects of sedation, hypotension, and bradycardia. The use of α2-adrenoceptor agonists in combination with other clinically known analgesics has been
studied over the years. This allows $\alpha_2$-adrenoceptor agonists to be used therapeutically but in lower doses than those that prevent the development of undesired side effects. It has been shown that the $\alpha_{2A}$-adrenoceptor subtype is responsible for the hypotensive, sedative, and bradycardic actions associated with $\alpha_2$-adrenoceptor agonists.\textsuperscript{78} The development of $\alpha_2$-adrenoceptor agonists that are selective for $\alpha_{2B}$- and/or $\alpha_{2C}$-adrenoceptors has become a major interest in the field of analgesia.

D. Behavioral Tests for Nociception

Measuring pain and analgesia in animals can be quite difficult. This is mainly because pain itself consists of a multiplicity of sensory entities.\textsuperscript{95} Also, the term “pain” is poorly defined when related to animals and their behavior.\textsuperscript{95} Pain is a subjective and personal psychological experience that cannot be measured directly in animals since an animal cannot describe how it feels.\textsuperscript{95} Therefore the term “pain” is usually avoided when describing the study of nociception in animal models,\textsuperscript{95} rather, the term “antinociception” is used. The antinociceptive effect of a test compound can be measured in animal models, and many factors can influence the outcome of the measurements in all animal models of nociception.\textsuperscript{95} Different species and strain of animals can have a major affect on the test results and is one of the more obvious factors.\textsuperscript{95} Another factor is that the time of day that the test is being conducted can produce a variation in results.\textsuperscript{95} Skin temperature of the stimulated area can influence the response to heat stimulus and is a factor that is important in thermal nociceptive tests.\textsuperscript{95,96} Nociceptive tests can be useful when they are carefully performed. In nociceptive tests, various types of stimuli can be used. Specifically, in
thermal nociceptive tests, heat stimulus is used to induce a response to nociception in rodents. In thermal nociceptive tests, the stimulatory response is fixed and a standard response is defined. The duration of the heat stimulus, when the nociceptive response occurs, is measured, and thermal nociceptive tests mainly focus on the threshold of the heat stimulus required to induce a specific response.

Two well-known thermal nociceptive tests, tail-flick and hot-plate tests, have been used extensively in studying nociception and antinociception in rodents. Both the tail-flick and hot-plate tests were first described more than 60 years ago. The tail-flick test was developed by D’Amour and Smith and is used both in rats and mice. In the tail-flick test, radiant heat is focused on the rodent’s tail and the time is measured for how long it takes for the animal to flick its tail away from the beam. This measurement is known as tail-flick latency, which is a measure of the sensitivity of the animal to heat stimulus. A maximal cut-off time is employed to prevent damage to the tail tissue. When an analgesic is administered to an animal, the agent can prolong the tail-flick latency. Spinal receptor sites are primarily involved in producing the nociceptive response seen in the tail-flick test. This idea was studied by transecting the spinal cord above the lumbar level. From such studies, it was shown that transection does not block the tail-flick response. The tail-flick test is very reliable for studying nociception in rodents. However, there is one factor that can possibly result in a variation in data. The skin temperature of the animal is negatively correlated with tail-flick latency. The tail-flick response is induced when the temperature at the nociceptors in the skin reach a critical level. The tail-flick latency depends strongly on the initial temperature of the skin which is influenced by local...
blood flow. So certain manipulations that affect thermoregulation could cause variation in the results; these include lesions of the descending noradrenergic or serotonergic systems, transection of the spinal cord, administration of serotonin or norepinephrine receptor agonists or antagonists, and stress and handling of the animals. Skin temperature is critical because reduction in the tail skin temperature can be interpreted as antinociception. This problem can be avoided by recording the tail skin temperature and making corrections to the tail-flick latency accordingly. In contrast, Lichtman et al. showed that tail-flick latency is not influenced by the skin temperature of the rodent. Thus there is some controversy whether skin temperature actually causes a variation in results from tail-flick tests.

The hot-plate test was developed by Woolfe and MacDonald and is also extensively used in studying nociception in rodents. When first described, the hot-plate test measured the nociceptive response of rodents placed on a hot-plate apparatus at temperatures varying from 55 to 70 °C. The hot-plate test was later modified and a constant temperature of 55 °C is the temperature that is consistently used today. The latency to a variety of behavioral responses, including jumping, kicking, shaking of the foot, holding a foot tightly against the body, and licking the forepaw and/or the hindpaw, is used to measure the sensitivity to pain in rodents. Usually, hindpaw licking latency is used as the end-point. A maximal cut-off time is also employed in the hot-plate test. The hot-plate test focuses on the supraspinal receptors that are involved in mediating nociception and antinociception. One problem seen in the hot-plate test is that mild analgesics possess no, or weak, antinociceptive activity at a constant temperature of 55
Therefore, the increasing temperature hot-plate test was developed to solve this problem. In this test, the temperature of the plate is set below the pain threshold and is gradually increased until the animal makes a response. The temperature at which the response occurs is then recorded as the nociceptive endpoint. The increasing temperature hot-plate test can be used with a conventional hot-plate which is initially set at 42-43 °C and the cut-off temperature is 50-52 °C. This test is more sensitive than the constant temperature hot-plate test. For example, earlier studies showed that the mild analgesics acetylsalicylic acid and paracetamol showed antinociceptive activity at 50 °C but not at 55 or 59 °C.

Thermal nociceptive tests are the most commonly used nociceptive tests in rodents. Since the development of both the tail-flick and hot-plate tests, there have been modifications made to each test to enhance the reliability of the results collected. The results from both of these tests might be affected by the skin temperature of the animal. However, the tail-flick test may be more susceptible to this problem than the hot-plate test. Use of the increasing temperature hot-plate test prevents the possible influence that skin temperature might have. In the case of the tail-flick test, this situation can be prevented by measuring the skin temperature of the animal before the test. However, this has been refuted. Both thermal nociceptive tests have been extremely reliable in studying nociception and antinociception in rodents.
III. Specific Aims and Rationale

It was previously shown by Dukat et al.\textsuperscript{30} that MD-354 (21) binds at 5-HT\textsubscript{3} receptors with high affinity (K\textsubscript{i} = 35 nM). Additional binding studies showed that this compound also binds at \(\alpha_2\)-adrenoceptors with high affinity.\textsuperscript{103} MD-354 (21) binds selectively at \(\alpha_2\textsubscript{B}\)-adrenoceptors (K\textsubscript{i} = 25 nM) when compared to \(\alpha_2\textsubscript{A}\) (K\textsubscript{i} = 825 nM) and \(\alpha_2\textsubscript{C}\)-adrenoceptors (K\textsubscript{i} = 140 nM).\textsuperscript{103} As such, it might be viewed as the first example of a rather selective 5-HT\textsubscript{3}/\(\alpha_2\textsubscript{B}\)-adrenoceptor ligand. Because 5-HT\textsubscript{3} receptors and \(\alpha_2\textsubscript{B}\)-adrenoceptors both have been implicated as playing a role in antinociception, MD-354 (21) might represent a novel, dual-mechanism type of analgesic agent. MD-354 (21) was first studied in the mouse tail-flick test. The antinociceptive activity of MD-354 (21) was compared with that of clonidine (43) as positive control.\textsuperscript{103} It was found that 1.0 mg/kg of clonidine (43) produced 76\% of the maximal possible effect (MPE) when administered via the s.c. route 20 min prior to the test.\textsuperscript{103} Other doses were evaluated and it was shown that clonidine (43) produced a dose-dependent antinociceptive effect (Figure 9).\textsuperscript{103}
Figure 9. Antinociceptive actions of clonidine following s.c. administration in the mouse tail-flick assay. Asterisks denote significant differences compared to control group (i.e., saline); *P < 0.01 and **P < 0.001.

Subcutaneous administration of MD-354 (21) at doses ranging from 1.0 to 30 mg/kg did not produce an antinociceptive effect when administered 45 min prior to testing (Figure 10). Results were not different using various other pretreatment times. MD-354 (21) was also examined as a possible antagonist of clonidine-induced antinociception. When administered 45 min prior to testing, doses of 1.0, 3.0, and 10 mg/kg of MD-354 did not antagonize the antinociceptive activity of the ED₅₀ dose of clonidine (43) administered 20 min prior to testing. Interestingly, a combination of the ED₅₀ dose of clonidine (43, MPE = 59%) and a dose of 10 mg/kg of MD-354 (21, MPE = 8%) was found to enhance antinociception in mice. It was also shown that an inactive dose of 0.25 mg/kg of clonidine (43, MPE = 13%) in combination with MD-354 doses ranging from 1.0 to 30 mg/kg dose-dependently enhanced the antinociceptive effect in what appears to be a bell-shaped manner. (Figure 11).
**Figure 10.** Effect of MD-354 doses administered 45 min prior to testing in the tail-flick assay as compared to saline control.\textsuperscript{103}

**MD-354 Dose (mg/kg)**

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**Figure 11.** Potentiation of the antinociceptive actions of clonidine (0.25 mg/kg) by MD-354 in the tail-flick assay. Asterisks denote significant differences compared to control group; *\(P< 0.05\) and **\(P< 0.01\).\textsuperscript{103}
Mechanistic studies were conducted to determine the mechanism behind the potentiating effect of clonidine antinociception by MD-354 in the mouse tail-flick test. Pretreatment with the putative $\alpha_{2B}$-adrenoceptor antagonist imiloxan (6.0 and 10 mg/kg i.p.) and the 5-HT$_3$ receptor antagonist zacopride (0.5 and 1.0 mg/kg i.p.) failed to attenuate the potentiation of clonidine antinociception (0.25 mg/kg s.c.) by MD-354 (21, 6.0 mg/kg s.c.). These results suggest that neither $\alpha_{2B}$-adrenoceptors nor 5-HT$_3$ receptors play a role in the potentiating effect of MD-354 (21). Since it is still controversial to which subtype of $\alpha_2$-adrenoceptors imiloxan binds, neither $\alpha_{2B}$- nor $\alpha_{2C}$-adrenoceptors can be ruled out as playing a role in the potentiating effect by MD-354 (21). As previously mentioned, clonidine potentiates the antinociceptive effects of morphine. Since MD-354 (21) enhances clonidine antinociception, the effect of MD-354 (21) on morphine antinociception was evaluated in the mouse tail-flick assay. In a combination study, MD-354 (21) did not enhance the antinociceptive action of morphine.

The purpose of the present studies was to further examine MD-354 (21) in a different pain model (i.e., the hot-plate assay), and to further investigate its potential mechanism(s) of action accounting for its potentiating effect on clonidine antinociception (in both the tail-flick and hot-plate assay). MD-354 (21) will be studied in the mouse hot-plate assay so that the activity in both thermal tests can be compared. Using the hot-plate assay will help further understand the mechanism of potentiation of clonidine (43) by MD-354 (21). If MD-354 (21) is active by itself or in combination with clonidine (43) in the hot-plate assay, it would suggest that MD-354 (21) could possibly cross the blood-brain barrier and bind at supraspinal 5-HT$_3$ and/or $\alpha_{2B}$-adrenoceptors. This will show that the
potentiating effect by MD-354 (21) works via both spinal and supraspinal receptor sites. Then, mechanistic studies will be conducted to determine which receptor(s) (i.e., $\alpha_{2B}$-adrenoceptors and/or 5-HT$_3$ receptors) are involved in this activity. If MD-354 (21) is inactive and does not potentiate clonidine-induced antinociception in the hot-plate assay, that could mean that MD-354 (21) is not lipophilic enough to cross the blood-brain barrier and may not reach the supraspinal receptors. MD-354 (21) is not a very lipophilic agent and has an experimentally determined Log P value of -0.64. This suggests that MD-354 (21) might have difficulty penetrating the blood-brain barrier. To study this, the carbamate derivative of MD-354 (21), 113, will be synthesized and evaluated in both thermal assays. Addition of the carbamate moiety might sufficiently potentiate the lipophilicity of 21 so that it can penetrate the blood-brain barrier. In theory, carbamate 113 should act as a prodrug and be hydrolyzed to MD-354 (21) in the brain. If 113 does reach the supraspinal receptors, then 113 should be hydrolyzed to MD-354 (21) and possess antinociceptive activity in the hot-plate assay. Compound 113 will also be evaluated in combination with clonidine in both thermal assays and these results will be used to compare with the actions of MD-354 (21). If 113 produces a similar dose-response curve as MD-354 (21) in the mouse tail-flick assay, this would suggest that 113 is hydrolyzed to MD-354 and is interacting in the same manner.
Next, mechanistic studies will be conducted to determine which pathway, 5-HT$_3$ and/or $\alpha_{2B}$-adrenoceptor, is responsible for potentiation of the clonidine effect. A related analog 3,4,5-trichlorophenylguanidine, 29, with a different binding profile will also be examined. Compound 29 binds with higher affinity than MD-354 (21) at 5-HT$_3$ receptors ($K_i = 0.7$ nM) but with an affinity similar to that of MD-354 at the $\alpha_{2B}$-adrenoceptors ($K_i = 30$ nM). However, unlike MD-354 (21), 29 does not show subtype selectivity ($\alpha_{2A}, \alpha_{2C}; K_i = 32$ and 30 nM, respectively). First, 29 will be evaluated in the mouse tail-flick assay. If the potentiating effect is due to 5-HT$_3$ receptors, then 29 should be more potent than MD-354 in potentiating clonidine (43). Because 29 possesses 50 times the affinity of MD-354 at 5-HT$_3$ receptors, it should have a comparatively greater potency relative to MD-354 (21) in its potentiating effect if the effect is primarily related to a 5-HT$_3$ receptor mechanism. If the potentiating effect is via the $\alpha_{2B}$-adrenoceptors, then 29 should have similar potency as MD-354. Antagonist studies also will be conducted to determine the involvement of 5-HT$_3$ and/or $\alpha_{2B}$-adrenoceptors. The 5-HT$_3$ antagonist tropisetron (33), the putative $\alpha_{2B}$-adrenoceptor antagonist imiloxan (98), and the $\alpha_2$-nonselective adrenoceptor antagonist yohimbine (39) will be used to attenuate the potentiating effect of 29. Compound 29 will
also be evaluated in the hot-plate assay to see if this compound reaches the supraspinal receptors and the results will be compared with those from MD-354 (21). Combination studies of 29 and morphine will be conducted to determine if 29 potentiates the antinociceptive effects of morphine. Since clonidine (43) has been shown to produce sedation via $\alpha_2$-adrenoceptors, 29 will be evaluated in the spontaneous activity assay in mice. If 29 produces sedative effects (as indicated by a decrease in locomotor activity), then its potentiating effect could possibly be due to induced sedation. If 29 does not produce sedation, this would further suggest that it possesses actions which selectively potentiate the antinociceptive effect of clonidine (43).

Another phenylguanidine, 3-methoxyphenylguanidine, 26, will be synthesized and examined for possible antinociceptive actions in the mouse tail-flick assay. The rationale for its evaluation is that it lacks significant affinity for 5-HT$_3$ receptors ($K_i = 1600$ nM), but binds nearly equally well at the three different subpopulations of $\alpha_2$-adrenoceptors ($\alpha_{2A}, \alpha_{2B}, \alpha_{2C}$; $K_i = 177, 152, \text{and} 135$ nM, respectively). Furthermore, it binds with only several-fold lower affinity than 29 at each of the $\alpha_2$-adrenoceptor subtypes. This analog will help determine whether 5-HT$_3$ receptors play a significant role in the potentiating effect seen with MD-354 (21). A constrained analog of MD-354 (i.e., 114) will also be synthesized and examined in antinociceptive assays. The constrained analog of MD-354, 114, can be used to further understand which conformation of MD-354 is more important for binding at 5-HT$_3$ and $\alpha_{2B}$-adrenoceptors. Compound 31 has been previously prepared as a conformationally-constrained analog of MD-354. Compound 114 represents a
conformationally-constrained rotamer of 31. If it possesses high affinity similar to MD-354, then this analog will also be evaluated in both thermal assays.

The overall focus of the studies that have been proposed is to determine the mechanism of the potentiating effect of clonidine-induced antinociception by MD-354 and MD-354-type compounds in the mouse tail-flick assay.
IV. Results and Discussion

A. Synthesis

a) N-(3-Chlorophenyl)guanidine Nitrate (MD-354; 21)

The method of Kreutzberger and Tantawy\(^\text{104}\) (Scheme 1) was used to prepare N-(3-chlorophenyl)guanidine nitrate (21). Cyanamide, 3-chloroaniline (115), and concentrated HCl in absolute EtOH were allowed to stir with heating at reflux for 48 h. Thin-layer chromatography (TLC) was used to monitor the status of the reaction, and after 48 h the reaction gave a single separate spot from the starting material. The EtOH was removed under reduced pressure and the resulting brown oil was kept at 0 °C overnight. Water and an excess of ammonium nitrate were added to give a white precipitate which was collected by filtration. The white precipitate was recrystallized from hot water and a drop of MeOH to give 21 as a white powder.

\[
\begin{align*}
\text{NH}_2\text{CN, EtOH, concentrated HCl, reflux; b. NH}_4\text{NO}_3, \text{H}_2\text{O.}
\end{align*}
\]

Scheme 1. a. NH$_2$CN, EtOH, concentrated HCl, reflux; b. NH$_4$NO$_3$, H$_2$O.
b) Carbamate analog of MD-354, 113

Several procedures were used in attempts to synthesize the carbamate analog of MD-354. The procedure of Goetz and Zeeh\textsuperscript{105} (Scheme 2) was first used. Cyanamide in H\textsubscript{2}O, methyl chloroformate, and 50\% NaOH were combined and the pH was maintained between 7 and 8. An exothermic reaction was supposed to increase the temperature to between 40 and 45 °C with regulation by cooling. However, the reaction temperature barely reached 40 °C and when it did, the temperature dropped quickly. The reaction was to be stirred for an additional hour at 50 °C, then freshly distilled aniline 115 was added. Concentrated HCl was added until a pH of about 3 to 3.5 and the reaction mixture was heated at reflux for 45 min. However, the reported precipitate did not form, but a sticky substance was obtained that was dried under vacuum. The melting point of the unidentified

\[
\begin{align*}
\text{NH}_2\text{CN} & \xrightarrow{\text{a}} \text{NC} \text{N} & \xrightarrow{\text{b}} \text{HN} \\
\text{Cl} \text{O} \text{O} \text{CH}_3 & \text{N} \text{H} & \text{Cl} \text{O} \text{O} \text{CH}_3 \\
\text{Cl} & \text{NH}_2 & \\
115 & 113
\end{align*}
\]

\textbf{Scheme 2.} a. H\textsubscript{2}O, 50\% NaOH in H\textsubscript{2}O; b. concentrated HCl, reflux
crude product (78-80 °C) did not correspond to the literature melting point of 128-129 °C. The product of this reaction was never identified.

The procedure of Khasanov (Scheme 3) was also applied in an attempt to synthesize the desired product using calcium cyanamide and methyl chloroformate. The reaction mixture was heated and the temperature was supposed to be maintained between 40 and 50 °C for 1 h. After 1 h, the reaction mixture was filtered and 3-chloroaniline (115) was added to the filtrate. Concentrated HCl was added to the filtrate until the pH was adjusted to 3 and the reaction was heated at reflux for 45 min. The desired product was reported as a white precipitate, but the product that was collected was an oily residue. Although the product was homogeneous to TLC, the 1H NMR spectrum showed that the oily residue was not the desired product. The identity of the product was not pursued (but see below discussion).

Scheme 3. a. H₂O, 40 to 50 °C; b. concentrated HCl, reflux
In yet another procedure (Scheme 4), a different approach was used to synthesize the carbamate. Methyl chloroformate in dry THF was added to a solution of the free base of 21 and triethylamine in dry THF at 0 °C under nitrogen. The reaction mixture was allowed to stir at 0 °C for 30 min and then at room temperature for 16 h. After 16 h, the precipitate was removed by filtration, and the THF was removed under reduced pressure to give a tan powder. The tan powder was recrystallized from hot EtOH, and white crystals were collected by filtration. $^1$H NMR spectral analysis indicated that the product was not the desired carbamate, but rather the bis-carbamate analog of MD-354, 116. This conclusion was supported by elemental microanalysis.

\[
\begin{align*}
\text{Cl} \quad \text{O} \quad \text{O} \quad \text{CH}_3 \\
\text{+} \\
\text{NH} \quad \text{NH} \\
\text{NH} \quad \text{NH}_2 \\
\text{21 (free base)} \\
\end{align*}
\]

\[
\begin{align*}
\text{N} \quad \text{N} \quad \text{O} \quad \text{CH}_3 \\
\text{H}_3 \text{C} \quad \text{O} \quad \text{NH} \\
\text{N} \quad \text{N} \quad \text{O} \quad \text{CH}_3 \\
\text{116} \\
\end{align*}
\]

\[
\begin{align*}
\text{Cl} \quad \text{N} \quad \text{NH} \quad \text{O} \\
\text{113} \\
\end{align*}
\]

Scheme 4. a. THF, triethylamine, 0 °C, N$_2$, 30 min; b. room temperature, 16 h.
Thin-layer chromatographic analysis of the above reaction mixture indicated the formation of a second, minor product that was suspected to be the desired product. The above procedure was attempted again but the starting material was dissolved in a larger amount of THF. It was hoped that this high-dilution technique would minimize formation of the bis-carbamate. Under these conditions, TLC analysis showed that there was still a small amount of starting material plus two products. The three were separated using column chromatography. Carbamate 113 was eventually obtained in low yield as a white powder.

Due to the low yield of the previous reaction, another attempt was made to use the procedure of Khasanov\textsuperscript{106} to synthesize the carbamate analog of MD-354 (Scheme 3). By using an addition funnel, calcium cyanamide in H\textsubscript{2}O was added slowly in a dropwise manner to methyl chloroformate. The reaction temperature was maintained at around 40 °C using a hot water bath and was cooled with an ice bath if the temperature exceeded 45 °C. The reaction mixture was allowed to stir for 20 min and the precipitate was removed by filtration; 3-chloroaniline hydrochloride was added to the filtrate. Several drops of concentrated HCl were added to achieve a pH of 3. The reaction mixture was heated at reflux for 30 min rather than 45 min. The reaction produced a brown oil which was kept at 0 °C overnight. The oil obtained was dried under vacuum to yield a solid that was recrystallized from hot H\textsubscript{2}O and a few drops of EtOH. The product was dried using a lyophilizer to give 113 as a white powder in low yield. The \textsuperscript{1}H NMR spectrum of the product was consistent for the carbamate hydrochloride. The product isolated from this reaction was also consistent with the product from the above reaction as determined by
comparison of $^1$H NMR spectra. The free base, but not the salt, of 113 has been previously reported.$^{105,106}$

c) N-(3-Methoxyphenyl)guanidine Nitrate (26).

The method of Kreutzberger and Tantawy$^{104}$ was used to prepare N-(3-methoxyphenyl)-
guanidine nitrate (26) (Scheme 5). Cyanamide, $m$-anisidine (117), and concentrated HCl in
absolute EtOH were allowed to stir while heating at reflux for 24 h. Thin-layer
chromatography was used to monitor the status of the reaction and after 24 h the reaction
gave a single spot distinct from the starting material. The solvent was removed under
reduced pressure and half of the resulting brown oil was used to make the salt form of the
desired product. Water and an excess of ammonium nitrate were added to give a white
precipitate which was collected by filtration. The white precipitate was recrystallized from
hot H$_2$O and a drop of MeOH to give 26 as a white powder.

\[
\begin{align*}
\text{NH}_2CN, \text{EtOH, concentrated HCl, reflux; } & \text{NH}_4\text{NO}_3, \text{H}_2\text{O}
\end{align*}
\]

Scheme 5. a. NH$_2$CN, EtOH, concentrated HCl, reflux; b. NH$_4$NO$_3$, H$_2$O
d) A conformationally-constrained analog of MD-354, 114

The method of Stadler\textsuperscript{107} (Scheme 6) was used to synthesize 114, a constrained analog of MD-354 (21). 2-Chloro-6-nitrotoluene (118), N-bromosuccinimide, and benzoyl

\[
\begin{align*}
\text{NO}_2 & \text{C}_6\text{H}_4\text{Cl} \\
\text{CH}_3 & + \\
\text{Br} & + \\
\text{CO} & \text{N} \equiv \text{N} \\
\text{O} & \text{C} \\
& \text{Cl} \\
\text{NO}_2 & \text{C}_6\text{H}_4\text{Br} \\
\text{Cl} & + \\
\text{C}_6\text{H}_4\text{Cl} & + \\
\text{O} & \text{N} \equiv \text{N} \\
\text{O} & \text{C} \\
& \text{Cl}
\end{align*}
\]

Scheme 6. a. C\textsubscript{4}H\textsubscript{10}, 250-Watt bulb, reflux; b. DMF, reflux; c. H\textsubscript{2}NNH\textsubscript{2}, MeOH, reflux; d. Raney Nickel, EtOH, H\textsubscript{2}; e. CNBr, EtOH, reflux; f. HBr, ether.
peroxide in carbon tetrachloride (CCl₄) under a 250-Watt light bulb were heated at reflux for 26 h. The reaction mixture was filtered and the solvent was removed under reduced pressure. TLC analysis showed that there was still a small amount of starting material plus one product. The two were separated using column chromatography. The yellow powder was collected and recrystallized from EtOH to give 119 as yellow crystals. Compound 119 and potassium phthalamide in DMF were allowed to stir at room temperature for 2 h. The reaction mixture was cooled to room temperature, CHCl₃ was added and the mixture was poured on to H₂O/ice. The CHCl₃ portion was washed with 0.2 N NaOH, brine and dried with Na₂SO₄. Solvent was removed under reduced pressure to give 120 as yellow crystals. Compound 120 and hydrazine hydrate in MeOH were heated at reflux for 3 h. Water was added to the reaction mixture and the MeOH then removed under reduced pressure. The solution was acidified with concentrated HCl and heated at reflux for 1 h. The precipitate was collected by filtration. A solution of 3 N NaOH was added to the filtrate to maintain a pH = 9 and extracted with Et₂O and brine. The Et₂O extract was dried with Na₂SO₄ and the solvent was removed under reduced pressure. TLC analysis showed that there was one product and one contaminant. The two were separated using Kugelrohr distillation to give 121 as a yellow oil. Compound 121 and Raney nickel in EtOH were placed on a Parr hydrogenator for 4 h. TLC analysis showed that two products were present. The Raney nickel was removed by filtration and washed with MeOH. The filtrates were combined and solvent was removed under reduced pressure. The crude product was dissolved in Et₂O, the solution was washed with H₂O and brine, and dried with Na₂SO₄. The product was converted to its HCl salt and recrystallized to give 122 HCl as a yellow powder. The
free base of 122 and cyanogen bromide in EtOH were heated at reflux for 1 h to provide the desired target 114.

In its protonated form, compound 114 could possibly exist as one of three tautomers (Figure 12). That is, any one of the three nitrogen atoms can be protonated. The desired product (structure A, Figure 12) is that with the double bond located at the nitrogen attached to the phenyl ring. The tautomeric identity of the product was verified by several different means. First, the $^1$H NMR spectrum of the free base of 114 was compared with the $^1$H NMR spectrum of the free base of the product reported in the literature. In the literature, it was reported that the NH and NH$_2$ of 114 (free base) gave a broad signal at 4.7 ppm. However, the $^1$H NMR spectrum of the free base of 114 prepared here did not show

![Figure 12. Compound 114 can exist as one of three tautomers.](image)

the signal for the two amines at 4.7 ppm. This difference in $^1$H NMR spectra could be because different $^1$H NMR spectrometers were used. This could also occur because the
spectrum was run in deuterated DMSO, and if H₂O is present in the DMSO the signal for the amines can exchange.

The predicted ¹H NMR spectra of the three protonated tautomers (using the program ChemDraw) were compared to that of 114. The major difference among the three tautomers was that the methylene signal appeared at different shifts. For tautomer A, the methylene proton signal is predicted to be at 4.5 ppm whereas for tautomer B it is at 3.91 ppm, and for tautomer C at 2.6 ppm. This difference helped to determine which of the three tautomers was actually obtained. That is, the actual signal for the methylene protons of 114 was a singlet at 4.5 ppm. Another source used was the ¹H NMR spectrum of a similar, previously reported compound, 123.¹⁰⁸ Compound 123 is similar to tautomer A except that it does not possess the chloro group on the phenyl ring. When comparing the two ¹H NMR spectra, the methylene signals are identical and show singlets at exactly 4.5 ppm. The evidence suggests that 114 is the desired tautomer (i.e., tautomer A as shown in Figure 12).
B. Behavioral Studies

1. Results

1.1. MD-354 (21)

1.1.1. Hot-plate assay

The antinociceptive activity of MD-354 (21) was compared with that of clonidine (43) in the hot-plate assay. When administered via the s.c. route 20 min prior to testing, 2 mg/kg of clonidine produced 80% of the maximal possible effect (MPE, Figure 13). Lower doses of clonidine produced less antinociception (Figure 13). In the hot-plate assay,

![Graph showing antinociceptive actions of clonidine in the hot-plate assay](image)

**Figure 13.** Antinociceptive actions (± S.E.M.) of subcutaneous clonidine in the hot-plate assay (*n* = 5-8 mice/treatment). Asterisks denote significant differences compared to control group; *P* < 0.001; one-way ANOVA followed by Newman-Keuls post hoc test.
the potency of clonidine was determined (ED$_{50}$ = 0.8 mg/kg; 95% CL = 0.6-1.1 mg/kg). For comparison, in the tail-flick assay, ED$_{50}$ = 0.51 mg/kg.$^{103}$ As seen in the tail-flick assay, MD-354 failed to produce statistically significant antinociceptive activity in the hot-plate assay (Figure 14). MD-354 produced 8-26% MPE at doses of 3, 10, and 30 mg/kg when administered from 10 to 90 min prior to the test. MD-354 at 30 mg/kg produced a slight, but not statistically significant time-dependent effect which diminished after the 45-min pretreatment time (Figure 14).

![Figure 14](#)

**Figure 14.** Effect (± S.E.M.) of subcutaneous MD-354 doses administered 20 to 90 min prior to examination in the hot-plate assay ($n = 6$-16 mice/treatment) as compared to saline control (8 ± 2% MPE).
1.1.2. Combination studies

In the hot-plate assay, MD-354 (21) was evaluated in combination with clonidine. Unlike in the tail-flick assay, MD-354 did not potentiate the antinociceptive activity of clonidine. Instead, a dose of 30 mg/kg of MD-354 (MPE = 11%) slightly antagonized the antinociceptive activity of clonidine (doses 0.8 and 2.0 mg/kg, 40% and 80% MPE; Figure 15). However, this slight antagonist effect was not statistically significant. In contrast, a dose of 0.25 mg/kg of clonidine (25% MPE) in combination with doses (10 and 30 mg/kg, 10% and 11% MPE, respectively) of MD-354 slightly elevated the antinociceptive activity (MPE = 33% and 32%, respectively; Figure 16). This slight elevation was not statistically significant.
Figure 16. Effect (± S.E.M.) of subcutaneous MD-354 doses administered in combination with subcutaneous clonidine (0.25 mg/kg) in the hot-plate assay (n = 5-26 mice/treatment).

1.1.3. Morphine/MD-354 combination studies

Because clonidine has been shown to potentiate the antinociceptive actions of morphine (see section II C), the effect of a morphine/MD-354 combination was examined. Morphine (10 mg/kg) produced 95% MPE (Figure 17) when administered via the s.c. route 30 min prior to the test. The potency of morphine was determined (ED$_{50}$ = 2 mg/kg; 95% CL = 1.2-3.2 mg/kg) and found to be consistent with that previously reported in the literature.$^{109}$ In a combination study, doses of 6, 10, and 30 mg/kg of MD-354 did not potentiate the antinociceptive activity of 1 mg/kg (29% MPE) of morphine (Figure 18). When a dose of 3 mg/kg of morphine (66% MPE) was administered in combination with MD-354, there was no significant increase in the antinociceptive activity (Figure 19).
Figure 17. Antinociceptive actions (± S.E.M.) of subcutaneous morphine in the tail-flick assay (n = 5-8 mice/treatment). Asterisks denote significant differences compared to control group; *P<0.05, **P<0.01, and ***P<0.001; one-way ANOVA followed by Newman-Keuls post hoc test.

Figure 18. Effect (± S.E.M) of subcutaneous MD-354 (21) doses administered alone and in combination with subcutaneous morphine (1 mg/kg) in the tail-flick assay (n = 5-11 mice/treatment).
Figure 19. Effect (± S.E.M) of subcutaneous MD-354 doses administered alone and in combination with subcutaneous morphine (3 mg/kg) in the tail-flick assay (n = 5-11 mice/treatment).

1.2. N-(3,4,5-Trichlorophenyl)guanidine (29)

1.2.1. Tail-flick and hot-plate assays

The antinociceptive activity of 29 was compared with that of MD-354 (21). Compound 29 generally produced <30% MPE at doses 0.003, 0.03, 0.3, 1.0, 3.0, 6.0, 10, and 30 mg/kg when administered 45 min prior to evaluation in the mouse tail-flick assay (Figure 20). In the mouse hot-plate assay, 29 produced <10 % MPE at doses of 0.3, 1.0, and 3.0 mg/kg when administered 45 min prior to testing (Figure 21).
Figure 20. Effect (± S.E.M.) of subcutaneous compound 29 doses administered 45 min prior to examination in the tail-flick assay (n = 6-16 mice/treatment) as compared to saline control (6 ± 4% MPE).

Figure 21. Effect (± S.E.M.) of subcutaneous compound 29 doses administered 45 min prior to examination in the hot-plate assay (n = 6-16 mice/treatment) as compared to saline control (8 ± 2% MPE).
1.2.2. Combination studies

Compound 29 was evaluated in both the tail-flick and hot-plate assays in combination with clonidine (43). In the tail-flick assay, a combination of the ED₅₀ dose of clonidine (0.5 mg/kg, MPE = 61%) and an inactive dose of 29 (0.3 mg/kg, MPE = 13%) potentiated the antinociceptive action of clonidine (MPE = 94%, Figure 22). However, 29 did not appear to potentiate the antinociceptive action of a very low (i.e., 0.1 mg/kg) dose of clonidine (Figure 22). But, an inactive dose of clonidine (0.25 mg/kg, MPE = 13%) in

![Graph showing the effect of compound 29 and clonidine in the tail-flick assay.](image)

Figure 22. Effect (± S.E.M.) of subcutaneous compound 29 (0.3 mg/kg) administered alone and in combination with subcutaneous clonidine (0.1 and 0.5 mg/kg) in the tail-flick assay (n = 8 mice/treatment). Asterisks denote significant differences compared to clonidine control; *P<0.05; one-way ANOVA followed by Newman-Keuls post hoc test.
combination with inactive doses (0.003, 0.03, 0.3, 1.0, 3.0, 6.0, 10, and 30 mg/kg) of 29 produced increases in antinociceptive activity (43-84% MPE; Figure 23). The combination of clonidine (0.25 mg/kg) and a dose of 0.03 mg/kg of 29 (MPE = 4%) gave the greatest potentiating effect of the antinociceptive activity of clonidine (MPE = 84%; Figure 23). The effect appeared to be biphasic. After an initial increase in antinociception, the effect was diminished at 3 mg/kg of 29, and was then increased at 10 mg/kg of 29 (Figure 23).

![Figure 23](image)

**Figure 23.** Potentiation of the antinociceptive actions (± S.E.M.) of subcutaneous clonidine (0.25 mg/kg) by subcutaneous compound 29 in the tail-flick assay (n = 8-9 mice/treatment). Asterisks denote significant differences compared to clonidine control; *P<0.05 and **P<0.01; one-way ANOVA followed by Newman-Keuls post hoc test.

In the hot-plate assay, a dose of 0.3 mg/kg of 29 (MPE = 9%) significantly potentiated the effect of the ED<sub>50</sub> dose (0.8 mg/kg, 40% MPE) of clonidine (combination:
69% MPE, Figure 24). In contrast, a 0.3 mg/kg dose of 29 in combination with lower doses of clonidine (0.5 and 0.25 mg/kg, 38% and 25% MPE; respectively) did not affect the antinociceptive effect (MPE = 34% and 15%; Figure 24).

![Graph showing the effect of 29 in combination with clonidine](image)

Figure 24. Effect (± S.E.M.) of 29 (0.3 mg/kg, s.c.) administered in combination with clonidine (0.25, 0.5, and 0.8 mg/kg, s.c.) in the hot-plate assay (n = 8-9 mice/treatment). Asterisks denote significant differences compared to clonidine control; *P<0.05; one-way ANOVA followed by Newman-Keuls post hoc test.

1.2.3. Mechanistic studies

An attempt was made to determine the mechanism behind the potentiation of clonidine antinociception by MD-354 (21) in the tail-flick assay. Neither imiloxan (98), an α2B-adrenoceptor antagonist, the 5-HT3 receptor antagonist tropisetron (33), nor yohimbine (39), an α2-adrenoceptor antagonist, produced a statistically significant antinociceptive
effect in the tail-flick assay when administered alone. Doses of 0.03, 0.1, 0.3, 1.0, and 3.0 mg/kg of imiloxan (98) produced <5% MPE when administered 55 min prior to testing (data not shown). Tropisetron (33) produced <10% MPE at doses 0.0000001, 0.000001, 0.00001, 0.0001, 0.001, and 0.0035 mg/kg when administered 50 min prior to testing (data not shown). Yohimbine (39) produced <1% MPE at doses of 0.1, 0.5, and 1.0 mg/kg when administered 60 min prior to testing (data not shown). Pretreatment with imiloxan (0.03-3.0 mg/kg) significantly attenuated the potentiation of clonidine (0.25 mg/kg) by 29 (0.03 mg/kg) (Figure 25). Interestingly, doses of 0.03, 0.1, 0.3, 1.0, and 3.0 mg/kg of imiloxan potentiated the antinociceptive actions of clonidine (0.25 mg/kg) (Figure 26).

**Figure 25.** Effect (± S.E.M.) of subcutaneous imiloxan on the antinociceptive actions of a combination (of subcutaneous clonidine (0.25 mg/kg) and compound 29 (0.03 mg/kg)) in the tail-flick assay (n = 8-10 mice/treatment). Asterisks denote significant differences compared to the combination control group; *P<0.05,**P<0.01, and ***P<0.001; one-way ANOVA followed by Newman-Keuls post hoc test.
Clonidine 0.03 0.1 0.3 1 3
0.25 mgkg

Figure 26. Potentiation of the antinociceptive actions (± S.E.M.) of subcutaneous clonidine (0.25 mg/kg) by subcutaneous imiloxan in the tail-flick assay (n = 6-15 mice/treatment). Asterisks denote significant differences compared to control group; *P<0.05; one-way ANOVA followed by Newman-Keuls post hoc test.

Pretreatment with the 5-HT3 antagonist tropisetron (33) (0.00001 and 0.0001 mg/kg) significantly attenuated the increase in the tail-flick latency produced by s.c. administration of 29 (0.03 mg/kg) in combination with clonidine (0.25 mg/kg; Figure 27). However, doses of 0.001 to 0.1 mg/kg of tropisetron (33) did not significantly attenuate the effect of the 29/clonidine combination (Figure 27). Interestingly, doses of 0.0000001, 0.000001, 0.00001, 0.0001, 0.001, 0.02, and 0.1 mg/kg potentiated the antinociceptive actions of clonidine (0.25 mg/kg) (Figure 28) although the effects were statistically significant only following doses of 0.02 and 0.1 mg/kg of tropisetron. Because both 29
and tropisetron (33) potentiate the antinociceptive actions of clonidine, a combination of a low, non-potentiating dose of 29 and a low, non-potentiating dose of tropisetron (33) should potentiate the action of clonidine. The ED50 dose for the potentiation of clonidine by tropisetron was calculated to be 0.0035 mg/kg (calculated using the results from the three highest doses shown in Figure 28). Administered with 0.25 mg/kg of clonidine, 0.0035 mg/kg of tropisetron (33) produced 51% MPE (Figure 29). In other words, the ED50 dose for tropisetron (33) did not potentiate the action of clonidine. The half-maximal dose for the potentiation of clonidine by 29 (i.e., 0.03 + 2 = 0.015 mg/kg) when
administered in combination with clonidine also failed to potentiate the antinociceptive effect (i.e., 42 ± 17 % MPE) (Figure 29). However, a combination of 0.0035 mg/kg of tropisetron (33) plus 0.015 mg/kg of 29 in combination with 0.25 mg/kg of clonidine produced an effect (79 ± 12 % MPE; Figure 29) that was statistically significant relative to the effect of clonidine alone.

Figure 28. Potentiation of the antinociceptive actions (± S.E.M.) of subcutaneous clonidine (0.25 mg/kg) by subcutaneous tropisetron in the tail-flick assay (n = 6-8 mice/treatment). Asterisks denote significant differences compared to control group; *P<0.05; one-way ANOVA followed by Newman-Keuls post hoc test.
**Figure 29.** Effect (± S.E.M.) of tropisetron (33)(s.c.) and 29 (s.c.) on the antinociceptive actions of clonidine (0.25 mg/kg; s.c) and 29 (s.c.) in the tail-flick assay (n = 5-7 mice/treatment). Asterisks denote significant differences compared to control group; *P<0.01, **P<0.001; one-way ANOVA followed by Newman-Keuls post hoc test. Saline produced 6 ± 4 % MPE.

Pretreatment with yohimbine (0.5 and 1 mg/kg) significantly attenuated the effect observed with the combination of 29 (0.03 mg/kg) and clonidine (0.25 mg/kg) (Figure 30). Doses of 0.1, 0.5, and 1.0 mg/kg of yohimbine did not significantly potentiate clonidine (Figure 31).
Figure 30. Effect (± S.E.M.) of subcutaneous yohimbine on the antinociceptive actions of a combination (of subcutaneous clonidine (0.25 mg/kg) and 29 (0.03 mg/kg)) in the tail-flick assay (n = 8 mice/treatment). Asterisks denote significant differences compared to control group; *P<0.05 and **P<0.01; one-way ANOVA followed by Newman-Keuls post hoc test.

Figure 31. Potentiation of the antinociceptive actions (± S.E.M.) of subcutaneous clonidine (0.25 mg/kg) by subcutaneous yohimbine in the tail-flick assay (n = 8-16 mice/treatment).
In the tail-flick assay, 2.0 mg/kg of morphine produced 39% MPE (Figure 32), which was similar with that previously reported in the literature. In a combination study, doses of 0.003, 0.03, and 1.0 mg/kg of 29 slightly potentiated the antinociceptive activity of 2.0 mg/kg of morphine (Figure 32). The observed effect was not statistically significant. However, a combination of 2 mg/kg of morphine plus 10 mg/kg of 29 significantly decreased the antinociceptive activity of morphine.

![Figure 32](image-url)  
**Figure 32.** Effect (± S.E.M.) of subcutaneous compound 29 doses administered in combination with morphine (2 mg/kg, s.c.) in the tail-flick assay (n = 6-8 mice/treatment). Asterisks denote significant differences compared to control group; *P<0.05; one-way ANOVA followed by Newman-Keuls post hoc test.

### 1.2.4. Spontaneous activity

Subcutaneous administration of 0.03 mg/kg of 29 after 15, 30, and 45 min produced saline-like effects in three main measures of locomotor activity. For example,
after 45 min the measure of (i) total movements was recorded as 350 ± 24 and 322 ± 28 for saline and 29, respectively; (ii) total movement time in seconds was 1472 ± 143 for saline and 1411 ± 165 for 29; (iii) total movement distance in centimeters was 5175 ± 919 and 4470 ± 625 for saline and 29, respectively. Subcutaneous administration of 29 (0.03 mg/kg) in combination with subcutaneous clonidine (0.25 mg/kg) after 15, 30, and 45 min produced clonidine-like effects in three main measures of activity. After 45 min the measure of (i) total movements were recorded as 123 ± 22 and 164 ± 154 for clonidine and 29 + clonidine, respectively; (ii) total movement time in seconds was 404 ± 76 for clonidine and 632 ± 154 for 29 + clonidine; (iii) total movement distance in centimeters was 1287 ± 272 and 2039 ± 480 for clonidine and 29 + clonidine, respectively.

1.3. The carbamate analog of MD-354, 113

1.3.1. Tail-flick assay

The antinociceptive properties of the carbamate analog of MD-354 (21), 113, were compared with those of MD-354. Compound 113 failed to produce a statistically significant antinociceptive effect when administered alone (Figure 33). Compound 113 produced <5% MPE at doses 0.3, 1.0, 6.0, and 10 mg/kg when administered 45 min prior to testing (Figure 33). To determine an optimal pretreatment time, selected 113 doses were examined using various pretreatment times (in addition to the 45-min pretreatment time) but did not produce >10% MPE (data shown in Figure 34 for 1 mg/kg of 113).
Figure 33. Effect (± S.E.M.) of 113 doses administered s.c. 45 min prior to examination in the tail-flick assay (n = 6-8 mice/treatment) as compared to saline control (6 ± 4% MPE). Note scale of y axis.

Figure 34. Effect (± S.E.M.) of a 113 dose (1 mg/kg, s.c.) administered 5 to 120 min prior to examination in the tail-flick assay (n = 6-8 mice/treatment) as compared to saline control (6 ± 4% MPE). Note scale of y axis.
1.3.2. Combination studies

In the tail-flick assay, doses of 0.3, 1.0, 3.0, 6.0, and 10 mg/kg of 113 potentiated the antinociceptive actions of clonidine, when tested 45 min after administration (Figure 35), but the effect was statistically significant only after the 1.0 and 10 mg/kg doses. To determine the optimal pretreatment time, a dose of 1.0 mg/kg of 113 with clonidine (0.25 mg/kg) was examined using various pretreatment times (in addition to the 45 min pretreatment time). The 113/clonidine combination was most effective following a 113 pretreatment time of 5 to 90 min (Figure 36).

![Figure 35](image)

**Figure 35.** Potentiation of the antinociceptive actions (± S.E.M.) of clonidine (0.25 mg/kg, s.c.) by subcutaneous 113 in the tail-flick assay (n = 7-9 mice/treatment) 45 min post administration of compound 113. Asterisks denote significant differences compared to control group; *P<0.05; one-way ANOVA followed by Newman-Keuls post hoc test.
Figure 36. Potentiation of the antinociceptive actions (± S.E.M.) of clonidine (0.25 mg/kg, s.c.) by 113 (1.0 mg/kg, s.c.) when administered 5 to 120 min prior to examination in the tail-flick assay (n = 8-9 mice/treatment). Asterisks denote significant differences compared to control group; *P<0.05; one-way ANOVA followed by Newman-Keuls post hoc test.

1.4. N-(3-Methoxyphenyl)guanidine (26)

1.4.1. Tail-flick assay

The antinociceptive actions of the methoxy counterpart of MD-354, 26, were compared with those of MD-354. N-(3-Methoxyphenyl)guanidine failed to produce a significant antinociceptive effect when administered alone (Figure 37). Compound 26 produced <10% MPE at doses 0.03, 0.3, 1.0, 3.0, 6.0, 10, and 30 mg/kg when administered 45 min prior to testing.
Figure 37. Effect (± S.E.M.) of subcutaneous 26 doses administered 45 min prior to examination in the tail-flick assay (n = 5-7 mice/treatment) as compared to saline control (6 ± 4% MPE). Note scale of y axis.

1.4.2. Combination studies

In the tail-flick assay, a combination of the ED$_{50}$ dose of clonidine (0.5 mg/kg) plus inactive doses of 26 (0.03, 0.3, 1.0, 3.0, 6.0, 10, and 30 mg/kg) potentiated the antinociceptive effect in mice (MPE = 50-100%; Figure 38) but this increase was not statistically significant except at doses of 0.3 and 6 mg/kg of 26. A lower dose of clonidine (0.25 mg/kg) in combination with doses (0.3, 1.0, and 10 mg/kg) of 26 did not result in statistically significant potentiation of the antinociceptive effect (31-47% MPE; Figure 39).
Figure 38. Potentiation (± S.E.M.) of the antinociceptive actions of clonidine (0.5 mg/kg, s.c.) by subcutaneous 26 in the tail-flick assay (n = 7 mice/treatment). Asterisks denote significant differences compared to control group; *P<0.05; one-way ANOVA followed by Newman-Keuls post hoc test.

Figure 39. Effect (± S.E.M.) of subcutaneous 26 doses administered in combination with clonidine (0.25 mg/kg, s.c.) in the tail-flick assay (n = 7 mice/treatment).
2. Discussion

MD-354 (21) binds selectively both to 5-HT\textsubscript{3} receptors and \(\alpha_{2B}\)-adrenoceptors, and since both receptor populations have been implicated as playing a role in nociception, the antinociceptive actions of MD-354 were evaluated. It was previously shown in our laboratory that MD-354 is inactive in the mouse tail-flick assay (Figure 10). Interestingly, MD-354 potentiated the antinociceptive actions of an inactive dose of clonidine (Figure 11). Based on these results, the purpose of the present study was to examine MD-354 in a different pain model, and to investigate its potential mechanism(s) of action accounting for its potentiating effect on clonidine analgesia. One of the first studies conducted was to determine whether MD-354 (21) is active in a different thermal pain test (i.e., hot-plate assay). Clonidine was used as a positive control and evaluated in the hot-plate assay (ED\textsubscript{50} = 0.8 mg/kg). The results obtained (Figure 13) were consistent with those reported in the literature.\textsuperscript{110} Subsequently, MD-354 was evaluated in the mouse hot-plate assay. As in the tail-flick assay, MD-354 did not produce any antinociceptive activity (Figure 14). However, unlike what was seen in the tail-flick assay, MD-354 did not potentiate the antinociceptive activity of clonidine in the mouse hot-plate assay (Figures 15 and 16). There are several possible explanations for these differences. The tail-flick assay might involve different receptor subpopulations or a different receptor mechanism than the hot-plate assay. For instance, the tail-flick assay involves spinal receptors,\textsuperscript{95} whereas the hot-plate assay involves supraspinal receptors.\textsuperscript{98} It has been suggested that supraspinal 5-HT\textsubscript{3} receptors might not be involved with spinal analgesia.\textsuperscript{41} This was supported by a study showing that the nonselective 5-HT\textsubscript{3} receptor agonist, 2-methyl-5-HT did not produce
antinociception when administered directly into the brain. Therefore, if supraspinal 5-HT3 receptors are not involved with nociception, then any possible antinociceptive activity observed with the arylguanidines in the hot-plate assay would primarily involve non-5-HT3 supraspinal receptors. Another possibility could be that there is a low density of α2B-adrenoceptors present in the brain, and/or that MD-354 may not reach these receptors. Since there is only a small number of supraspinal α2B-adrenoceptors and supraspinal 5-HT3 receptors, these might not be involved in nociception; this could possibly explain why MD-354 does not potentiate clonidine in the mouse hot-plate assay. Differences might also be related to the fact that MD-354 is not a very lipophilic agent and distributional factors could be involved. MD-354 has an experimentally determined Log P value of -0.64, indicating that it might have difficulty penetrating the blood-brain barrier.

The carbamate derivative of MD-354, 113, was synthesized and examined to study this factor. In theory, the more lipophilic 113 should more readily penetrate the blood-brain barrier than MD-354 (21), and be subsequently hydrolyzed by brain tissue esterases to MD-354. Compound 113 produced an effect similar to that of MD-354. As reported for MD-354, compound 113 (doses of 0.3 to 10 mg/kg) did not produce antinociceptive effects when administered 5 to 120 min prior to testing in the mouse tail-flick assay (Figures 33 and 34). This suggests that 113 might be rapidly hydrolyzed shortly after administration. When administered in combination with clonidine (0.25 mg/kg), however, 113 potentiated the antinociceptive actions of clonidine (Figure 35) much in the same manner as did MD-354. Previously we have shown that MD-354 potentiates the antinociceptive effect of clonidine in a bell-shaped manner (Figure 11). Re-examination
of 1 mg/kg of MD-354 (MPE = 1%) in combination with clonidine (0.25 mg/kg; MPE = 13%) resulted in a supraadditive (statistically significant) antinociceptive effect (MPE = 84%; data not shown). Furthermore, these new data clearly indicate that a potentiation of antinociceptive actions of clonidine by MD-354 occurs in a biphasic manner; this was also seen for its carbamate analog 113. Overall, these results suggest that 113 may be hydrolyzed to MD-354 in the periphery and behaves in the same manner as MD-354. The potentiating effect of clonidine with 113 was also examined at various pretreatment intervals (5 to 120 min) before the test (Figure 36). This might give an idea of how fast 113 is hydrolyzed in vivo and how long the observed potentiating effect lasts. When 113 was administered 15 min after clonidine and 5 min before the test, the effect was similar to when 113 was administered 45 min before the test (Figure 36). Although, the results suggest that 113 is probably hydrolyzed within at least 5 min of administration, the potentiating effect lasts up to 120 min, even though at 120 min the 113/clonidine combination produced a lower %MPE. Further studies will be required to examine the rate of hydrolysis of 113 in vivo. In any event, 113 seems to behave much like its parent: MD-354. It would seem that the carbamate is too rapidly hydrolyzed to show effects substantially different than those seen with MD-354. Therefore 113 was not examined in the mouse hot-plate assay. The concept, however, is still a valid one. Future studies might target carbamate analogs with bulkier substituents that are not as rapidly hydrolyzed as 113.

Next, mechanistic studies were conducted to help determine which pathway, 5-HT₃ and/or α₂B-adrenoceptors, is responsible for the potentiation of clonidine analgesia by
arylguanidines. Some of these studies utilized structurally modified arylguanidines whereas other studies utilized 5-HT$_3$ and adrenoceptor antagonists. In order to fully understand which pathway is involved, several analogs of MD-354 (21) with different binding profiles from MD-354 were studied. The first approach was to determine the importance of 5-HT$_3$ receptors in the potentiation of the antinociceptive actions of clonidine by arylguanidines. A MD-354-related analog, 29, with a different binding profile was examined. Compound 29 binds with about 50-fold higher affinity than MD-354 at 5-HT$_3$ receptors, and with similar affinity at $\alpha_2$B-adrenoceptors. However, unlike MD-354, 29 does not show subtype selectivity for $\alpha_2$-adrenoceptors (Table 10). Compound 29 alone did not produce an analgesic effect when administered 45 min before the test in the tail-flick assay (Figure 20). When administered in combination with clonidine, 29 (0.3 mg/kg) potentiated the antinociceptive actions of clonidine (Figure 23) and was about as effective as 1 mg/kg of MD-354. A much lower dose of 29 (0.03 mg/kg) still produced potentiation of the clonidine effect. That is, 29 is at least 33 times more potent than MD-354 in potentiating the antinociceptive actions of clonidine. Since 29 is 50 times more potent than MD-354 in binding at 5-HT$_3$ receptors, this might explain the comparatively greater potency of 29 relative to MD-354 in potentiating clonidine’s antinociceptive actions. In theory, if the potentiating effect of clonidine by MD-354 primarily involves 5-HT$_3$ receptors then 29 should be more potent than MD-354 in potentiating clonidine. If $\alpha_2$B-adrenoceptors primarily mediated the potentiating effect of
Table 10. Binding Data for MD-354 (21), 29, and 26 at 5-HT3 and \(\alpha_2\)-Adrenceptors.\(^{103,111}\)

<table>
<thead>
<tr>
<th></th>
<th>5-HT(_3)</th>
<th>(\alpha_{2A})</th>
<th>(\alpha_{2B})</th>
<th>(\alpha_{2C})</th>
</tr>
</thead>
<tbody>
<tr>
<td>MD-354</td>
<td>35 ± 5</td>
<td>825 ± 160</td>
<td>25 ± 5</td>
<td>140 ± 40</td>
</tr>
<tr>
<td>29</td>
<td>0.7 ± 0.1</td>
<td>32 ± 3</td>
<td>30 ± 5</td>
<td>30 ± 5</td>
</tr>
<tr>
<td>26</td>
<td>1600 ± 300</td>
<td>177 ± 6</td>
<td>152 ± 12</td>
<td>135 ± 6</td>
</tr>
</tbody>
</table>

clonidine, then 29 should have a similar potency relative to MD-354. Because 29 is more potent in potentiating clonidine than MD-354, this further suggests that 5-HT\(_3\) receptors may be involved in the potentiation of clonidine by arylguanidines. However, the involvement of \(\alpha_2\)-adrenoceptors cannot be ruled out. Compound 29 also has a higher affinity for \(\alpha_{2A}\)- and \(\alpha_{2C}\)-adrenoceptor subtypes than MD-354 (Table 10). Compound 29 is about 25 times more potent than MD-354 in binding at \(\alpha_{2A}\)-adrenoceptors and 5 times more potent in binding at \(\alpha_{2C}\)-adrenoceptors. Since it has been shown that the \(\alpha_{2A}\)-
adrenoceptor subtype plays a role in antinociception, the increase in potency might also involve the activation of $\alpha_{2A}$-adrenoceptors. It might also be noted that 29 is more lipophilic than MD-354 (21); hence, 29 might be more potent than MD-354 because more of it reaches supraspinal sites. Compound 29 was also studied in the mouse hot-plate assay. The results were similar to that of MD-354, when 29 (0.3 mg/kg) was administered in combination with doses of 0.25 and 0.5 mg/kg of clonidine, in that 29 did not potentiate the antinociceptive activity of clonidine. Interestingly, 29 significantly potentiated the antinociceptive activity of the ED$_{50}$ dose of clonidine (0.8 mg/kg) in the mouse hot-plate assay (Figure 24). These results suggest that 29 could be lipophilic enough to cross the blood-brain barrier and reach supraspinal sites. Compound 29 possesses higher affinity for 5-HT$_3$ receptors, $\alpha_{2A}$- and $\alpha_{2C}$-adrenoceptors than MD-354 and this could also explain the potentiation of clonidine in the mouse hot-plate assay.

Another phenylguanidine, 26, was examined for possible antinociceptive actions in the tail-flick assay. Compound 26 lacks significant affinity for 5-HT$_3$ receptors but binds nearly equally well at the three different subpopulations of $\alpha_2$-adrenoceptors (Table 10). Furthermore, it binds with only several-fold lower affinity than 29 at each of the adrenoceptor subtypes. Compound 26 did not produce antinociception when administered alone (Figure 37). Interestingly, 26 did not potentiate the antinociceptive actions of an inactive dose of clonidine (0.25 mg/kg) (Figure 39). The lack of potentiating effect of 26 on the antinociceptive action of clonidine can be directly attributed to its low affinity for 5-HT$_3$ receptors. Methoxy-compound 26 binds with 46-fold and $>2,000$ fold lower affinity than MD-354 (21) and 29, respectively. Its reduced $\alpha_{2B}$-adrenoceptor affinity might also
play a role here. Interestingly, 26 (at doses 0.3 and 6.0 mg/kg) potentiated the antinociceptive activity of the ED₉₀ dose of clonidine (0.5 mg/kg) (Figure 38).

In the antagonist studies conducted, the potentiating effect of 29 in combination with clonidine was significantly antagonized by the 5-HT₃ antagonist tropisetron (doses 0.00001 and 0.0001 mg/kg), the α₂B-adrenoceptor antagonist imiloxan (doses 0.03 to 3.0 mg/kg), and the nonselective α₂-adrenoceptor antagonist yohimbine (doses 0.5 and 1.0 mg/kg). The non-selective α₂-adrenoceptor antagonist yohimbine completely abolished the potentiating effect of 29 on clonidine (Figure 30). This observation is fairly simple to explain. Because clonidine is believed to produce its antinociceptive effects via an α₂-adrenoceptor mechanism,³ unavailability of α₂-adrenoceptors (by yohimbine blockade) would be expected to result in inactivity. Clonidine, alone or in combination with an inactive arylguanidine, would not be expected to show any antinociceptive effect in the presence of yohimbine.

The effect of clonidine (or clonidine/arylguanidine combinations) with tropisetron or imiloxan is more complex, and might be dose related. Results with tropisetron and imiloxan support the concept that the potentiation of clonidine by arylguanidines might involve both 5-HT₃ and α₂B-adrenoceptors. The α₂B/α₂C-adrenoceptor antagonist imiloxan failed to block the potentiating effect of MD-354 (21) on clonidine antinociception.¹⁰³ However, imiloxan was shown to potently antagonize the potentiating effect of 29 (Figure 25). The different results will require additional study, but they certainly suggest involvement of α₂B/α₂C-adrenoceptors in the action of 29. However at doses of 0.3 and 3.0
mg/kg, imiloxan (98), in the absence of 29, potentiated the antinociceptive activity of clonidine (0.25 mg/kg). The potentiating effect of clonidine by imiloxan might be explained by several possibilities. Clonidine binds nonselectively at the three $\alpha_2$-adrenoceptor subtypes. It has been shown that both $\alpha_{2A}$- and $\alpha_{2B}$-adrenoceptor subtypes are involved with antinociception.3,94 When given in combination with clonidine, imiloxan could bind to $\alpha_{2B/2c}$-adrenoceptors and prevent clonidine from binding at these receptors. This would allow clonidine to bind at more spinal and supraspinal $\alpha_{2A}$-adrenoceptors than before and could possibly potentiate its antinociceptive actions. Also, $\alpha_{2A}$-adrenoceptors mediate the sedative effects caused by clonidine.78 Therefore, if more $\alpha_{2A}$-adrenoceptors are being activated then this could possibly potentiate the sedative effect as well. An increase in sedation might be mistaken for an increase in antinociceptive properties. There are several approaches that could be used to determine if sedative effects are involved in the enhancing effect of clonidine antinociception by imiloxan. One approach is to examine imiloxan in combination with clonidine in the locomotor activity assay. If imiloxan does not potentiate the locomotor effects of clonidine, then this could suggest that sedative effects are not responsible for the enhancing effect of clonidine’s antinociceptive action by imiloxan. Another approach is to try to block the greater than additive effect of imiloxan and clonidine with an $\alpha_{2A}$-adrenoceptor antagonist (e.g. BRL 44408).

Evidence was obtained that 5-HT$_3$ receptors also play a role. That is, the 5-HT$_3$ antagonist tropisetron was able to block the potentiation of clonidine antinociception by 29 (Figure 27). Interestingly, however, administration of tropisetron potentiated the
antinociceptive effects of clonidine in the absence of 29 (Figure 28). It might be argued that 29, at least in part, potentiates the effect of clonidine by a 5-HT3 agonist mechanism; this is consistent with the observation that the effect of clonidine/29 is blocked by the 5-HT3 antagonist tropisetron. It is much more difficult to reconcile the observation that tropisetron by itself potentiates the action of clonidine. Overall, the results imply that 5-HT3 antagonists are capable of potentiating the antinociceptive actions of clonidine. Evidently, this is more complex than it would first seem to be. Taken together, the results of the present investigation suggest that arylguanidines potentiate the antinociceptive actions of clonidine via both an α2-adrenoceptor and 5-HT3 receptor mechanism. Although the arylguanidines might behave as α2-adrenoceptor agonists (or partial agonists), the specific α2-adrenoceptor subtype(s) involved remains to be elucidated. Several of the studies argue in favor of a role for 5-HT3 receptors; but here, it is unclear whether the agents act via a 5-HT3 agonist or antagonist (or both) mechanism.

Surprisingly, at doses 0.02 and 0.1 mg/kg, tropisetron potentiated the antinociceptive actions of clonidine. These results further suggest that the mechanism of antinociception via 5-HT3 and α2-adrenoceptors are interrelated. It has been shown that the combination of 5-HT (1) and norepinephrine (36) produces a synergistic effect on antinociception.9,45 Because tropisetron is both a 5-HT3 and 5-HT4 antagonist, another explanation is that tropisetron is blocking both 5-HT3 and 5-HT4 receptors and the antagonist activity at one or both of these serotonin receptors could influence levels of 5-HT. The available 5-HT could mediate the release (e.g. via heteroreceptors) of other neurotransmitters which activate their corresponding descending inhibitory systems (e.g.
NE, GABA). Serotonin, by interacting with 5-HT₃ receptors, can also modulate the release of other neurotransmitters and neuropeptides (i.e., dopamine, acetylcholine, GABA, substance P, cholecystokinin). Another possibility could be that α₂-adrenoceptor antagonists block the antinociception mediated by 5-HT₃ receptors because the two systems are functionally related.

The effect of the ED₅₀ dose of the potentiating dose of tropisetron (33) and half of the potentiating dose of 29 in combination with clonidine (43) was studied. In theory because both tropisetron and 29 potentiate clonidine when administered alone, the low doses of tropisetron and 29 together should potentiate clonidine in a similar manner. An ED₅₀ dose of 0.0035 mg/kg of tropisetron and half the active dose of 29 (0.015 mg/kg) potentiated the antinociceptive actions of clonidine in a similar manner as the 29/clonidine combination. These results suggest that 29 is potentiating clonidine in the same manner as tropisetron (i.e., via a 5-HT₃ receptor antagonist mechanism). These findings of tropisetron and 29 potentiating clonidine could be used clinically. Since tropisetron is already being used as an antiemetic, using tropisetron with clonidine, or using the three agents (i.e., tropisetron, clonidine, and an arylguanidine) in combination could be clinically useful in the treatment of pain associated with cancer.

The results with 5-HT₃ and/or α₂B-adrenoceptor antagonists show that the potentiating effect of 29 with clonidine might involve a biphasic mechanism. This means that at extremely low doses, 5-HT₃ receptors might be involved and that at higher doses, α₂B-adrenoceptors could be involved in the action of 29. It might be noted that 5-HT₃
receptor ligands commonly show potent actions (i.e., at sub-mg/kg doses) in a variety of pharmacological assays.\textsuperscript{42}

Clonidine \textsuperscript{(43)}, a nonselective $\alpha_2$-adrenoceptor agonist, potentiates the antinociceptive actions of morphine but the exact adrenoceptor population responsible is still unknown.\textsuperscript{90} Since the potentiation of clonidine by \textsuperscript{29} might involve $\alpha_2$-adrenoceptors via an agonist mechanism, \textsuperscript{29} was also examined in combination with morphine in the tail-flick assay. In theory, if the potentiating effect of \textsuperscript{29} involves $\alpha_2$-adrenoceptors as with clonidine, then \textsuperscript{29} should potentiate the antinociceptive actions of morphine. Compound \textsuperscript{29} potentiated the antinociceptive effect of morphine (2 mg/kg) (Figure 32) only at doses of 0.003, 0.03, and 1 mg/kg of \textsuperscript{29} but the effect was not statistically significant. In contrast, doses of 0.1 and 3 mg/kg of \textsuperscript{29} slightly attenuated the antinociceptive action of morphine (2 mg/kg); but here, too, the effect was not statistically significant. However a dose of 10 mg/kg of \textsuperscript{29} blocked the actions of morphine and these results were statistically significant (Figure 32). The studies of \textsuperscript{29} in combination with morphine can be explained in several ways. As mentioned earlier, co-administration of $\alpha_2$-adrenoceptor agonists and opioid agonists produce a synergistic effect.\textsuperscript{90} These results suggest that certain doses of \textsuperscript{29} possibly potentiate morphine's antinociception via an $\alpha_2$-adrenoceptor agonist mechanism. Earlier studies also showed that i.t. or i.c.v. administered morphine produces antinociception that can be blocked by the 5-HT\textsubscript{3} antagonist tropisetron.\textsuperscript{46} Consequently, any adrenergically-mediated potentiating effects of \textsuperscript{29} might be counteracted by the antagonist actions of \textsuperscript{29} at 5-HT\textsubscript{3} receptors if \textsuperscript{29} is a 5-HT\textsubscript{3} partial agonist. These results
from the 29/morphine studies suggest that 29 could be a 5-HT₃ antagonist and an α₂-
adrenoceptor agonist.

In the studies of MD-354 in combination with morphine, MD-354 (at doses 6 and 10 mg/kg) did not seem to affect the antinociceptive action of morphine (1 mg/kg) (Figure 18). However, a dose of 30 mg/kg of MD-354 (21) attenuated morphine antinociception but these results were not statistically significant. This difference in activity seen with MD-354 and 29 might be explained by the compounds' different binding profiles. Since it was proposed earlier that the attenuation seen with 29 might involve a 5-HT₃ receptor antagonist mechanism, the difference in activity could be due to the fact that 29 binds with a 50-fold higher affinity at 5-HT₃ receptors than MD-354 (Table 10). Also, different doses were used in the two studies of MD-354 or 29 in combination with morphine. These different doses could also explain the difference in activity with these two arylguanidines. MD-354 was studied in combination with a lower dose of morphine (1.0 mg/kg) than 29, whereas 29 was studied with the ED₅₀ dose of morphine (2.0 mg/kg). This difference suggests that the higher dose of morphine (2.0 mg/kg) could possibly require a lower dose of 29 (10 mg/kg) to block its antinociceptive actions. In the case of MD-354, using a dose of 1.0 mg/kg of morphine might require a higher dose of 30 mg/kg of MD-354 to block its antinociceptive actions. Further studies are needed to fully explain the difference in activity seen with MD-354 and 29 in combination with morphine.

Clonidine has been shown to produce sedation through activation of α₂-
adrenoceptors. In order to determine whether the potentiating effect by 29 is caused by
antinociceptive effects or sedation, 29 was studied in a locomotor activity assay. Compound 29 did not produce sedation in mice; this further suggests that 29 produces its potentiating effect via an antinociceptive action and not a sedative effect. The combination of 29 with clonidine was also studied and compared with the results of clonidine by itself. The purpose of studying the combination was to see if 29 only potentiates clonidine’s antinociceptive actions or whether it also potentiates clonidine’s sedative effects. The combination of 29 and clonidine produced clonidine-like effects in the locomotor activity assay and suggests that 29 selectively potentiates the antinociceptive, but not sedative effects of clonidine. This finding could be beneficial if 29 was clinically used in combination with clonidine because sedation caused by clonidine is an undesired side effect.
V. Conclusions

In summary, MD-354 is a rather selective 5-HT₃/α₂B-adrenoceptor ligand. However, MD-354 was found to lack antinociceptive action by itself in the mouse tail-flick assay,¹⁰³ and in the mouse hot-plate assay (present study). Although MD-354 potentiated the antinociceptive effect of clonidine in the tail-flick assay, it did not potentiate the action of clonidine in the hot-plate assay. These differences might reflect roles for spinal versus supraspinal loci of action. Compound 113, a carbamate derivative of MD-354, produced a similar biphasic dose-response curve as MD-354. This study suggests that 113 is rapidly hydrolyzed in vivo to MD-354. Compound 29, which binds at 5-HT₃ receptors with 50 times the affinity of MD-354, was extremely potent in potentiating the antinociceptive effects of clonidine and was about 30 times more potent than MD-354. The potentiation of clonidine by 29 can be blocked by the 5-HT₃ receptor antagonist tropisetron, the α₂B-adrenoceptor antagonist imiloxan, and the α₂-adrenoceptor antagonist yohimbine. This shows that both 5-HT₃ and α₂-adrenoceptors are involved in the potentiation of clonidine antinociception by 29. Compound 26, an analog of MD-354 that lacks affinity at 5-HT₃ receptors, did not potentiate the effect of an inactive dose of clonidine in the tail-flick assay. However, 26 potentiated the effect of the ED₅₀ dose of clonidine (0.5 mg/kg); however, this potentiation was statistically significant only at doses of 0.3 and 6.0 mg/kg. The mechanism underlying clonidine potentiation requires further investigation;
nevertheless a role for 5-HT₃ and α₂-adrenoceptors has been established. At least some aryguanidines might be referred to as “dual-mechanism” potentiating agents of the antinociceptive actions of clonidine. Another major finding of the present investigation is that the 5-HT₃ antagonist tropisetron, which is currently being used as an antiemetic agent in cancer chemotherapy, can potentiate the analgesic effect of clonidine. Consequently, clinicians should consider investigating combinations of these two clinically employed agents to control the pain associated with certain types of cancer.
VI. Experimental

A. Synthesis

Melting points were determined in a glass capillary on a Thomas Hoover melting point apparatus and are uncorrected. Proton nuclear magnetic resonance ($^1$H NMR) spectra were obtained on a Varian Gemini 300 MHz spectrometer and peak positions are given in parts per million (δ) downfield from TMS. Elemental analysis was performed by Atlantic Microlab Inc. (Norcross, GA), and determined values are within 0.4% of theory. Column chromatography was performed on silica gel (Kiesel gel 40, 0.040-0.063 mm, Merck) by flash chromatography. Routine thin-layer chromatography (TLC) was performed on silica gel GHLF plates (250 μ, 2.5 x 10 cm; Analtech Inc., Newark, DE). Infrared spectra were obtained on a Nicolet Avatar 360 FT-IR spectrometer.

N-(3-Chlorophenyl)guanidine Nitrate (MD-354) (21). Cyanamide (1.05 g, 33.7 mmol) was added to a solution of 3-chloroaniline (115) (2.00 g, 15.7 mmol) and concentrated HCl (1.60 mL) in absolute EtOH (15 mL). The stirred mixture was heated at reflux for 48 h. The solvent was removed under reduced pressure and the resulting oil was cooled at 0 °C. Water (15 mL) and ammonium nitrate (3.0 g) were added to give a precipitate which was collected by filtration and recrystallized from H₂O and MeOH to give 21 (1.50 g, 41%) as a white powder: mp 169-170 °C (lit. 104 mp 171-172 °C); $^1$H NMR (DMSO-d₆) δ 7.32-7.36 (m, 4H, NH), 7.40-7.50 (m, 4H, ArH), 9.80 (s, 1H, H'NH).
N-(3-Methoxyphenyl)guanidine Nitrate (26). Cyanamide (0.60 g, 15 mmol) was added to a solution of m-anisidine (117) (1.20 g, 10 mmol) and concentrated HCl (0.8 mL) in absolute EtOH (10 mL). The stirred reaction mixture was heated at reflux for 24 h. The solvent was removed under reduced pressure and the resulting oil was cooled to 0 °C. Water (5 mL) and ammonium nitrate (0.73 g) were added to give a precipitate which was collected by filtration and recrystallized from H₂O and MeOH to give 26 (0.10 g, 19%) as a white powder: mp 139-141 °C; ¹H NMR (DMSO-d₆) δ 3.76 (s, 3H, OCH₃), 6.80-6.88 (m, 3H, Ar-H), 7.31-7.37 (t, 1H, ArH), 7.52-7.56 (m, 4H, NH), 9.85 (s, 1H, NH). Anal. Calcd for (C₉H₁₁N₃O₃HNO₃) C₉H₈N₃O₃N₄.

Methyl N-(3-chlorophenyl)guanidinecarboxylate Hydrochloride (113). Method A. Methyl chloroformate (0.17 mL, 2.15 mmol) in dry THF (2 mL) was added in a dropwise manner to a solution of the free base of (3-chlorophenyl)guanidine (21) (0.50 g, 2.15 mmol) and Et₃N (0.60 mL, 4.30 mmol) in dry THF (100 mL) at 0 °C under an N₂ atmosphere. The mixture was allowed to stir at 0 °C for 30 min and then at room temperature for 16 h. The white precipitate was removed by filtration and the solvent was removed under reduced pressure to give a mixture of two products which were separated by column chromatography (silica gel, Kiesel gel 40, 0.040-0.063 mm) using CH₂Cl₂ / MeOH (4:1) as eluent. The free base of the desired product (113) was recrystallized from EtOH to give 0.01 g (2%) of a white powder: mp 128-129 °C (lit.¹⁰⁶mp 138-140 °C); ¹H NMR (CDCl₃) δ 3.63 (s, 3H, OCH₃), 7.10-7.36 (m, 4H, ArH), 7.38 (br. s, 2H, NH, NH), 8.51 (s, 1 H, NH). Anal. Calcd for (C₉H₁₀ClN₃O₂) C₉H₈N₃O₃N₄.
**Method B.** Calcium cyanamide (4.7 g, 58.7 mmol) was dissolved in H₂O (10 mL) and the solution was added to methyl chloroformate (5.04 g, 53.3 mmol) in a dropwise manner. The reaction mixture was allowed to stir for 20 min at 40-45 °C. The precipitate was removed by filtration and 3-chloroaniline hydrochloride (115) (4.38 g, 26.7 mmol) was added to the filtrate. Concentrated HCl was added to the reaction mixture to obtain a pH of 3 and the mixture was heated at reflux for 30 min. The solvent was decanted off and the solid was recrystallized from H₂O and EtOH to give 113 (0.19 g, 3%) as a white powder: mp 159-160 °C; ¹H NMR (DMSO-d₆) δ 3.78 (s, 3H, CH₃), 7.28-7.47 (m, 4H, ArH), 7.52 (br s, 2H, NH, NH), 8.81 (s, 1H, NH). Anal. Calcd for (C₉H₁₀ClN₃O₂·1.5HCl·0.25H₂O) C, H, N.

2-Amino-5-chloro-3,4-dihydroquinazoline Hydrobromide (114). Compound 114 was prepared according to a literature procedure.⁰⁷ Cyanogen bromide (0.63 mL, 1.87 mmol) was added to a solution of 122 (0.25 g, 1.57 mmol) in absolute EtOH (8 mL). The reaction mixture was allowed to stir at room temperature for 1 h and heated at reflux for 5 h. The solvent was removed under reduced pressure and H₂O (10 mL) was added to the resulting residue. The suspension was extracted with CHCl₃ (3 x 10 mL) and the combined CHCl₃ portion was washed with brine (15 mL). The extract was dried with Na₂SO₄ and the solvent was removed under reduced pressure. The product was recrystallized from absolute EtOH to give 114 (free base; 0.25 g, 88%) as a powder: mp 234-235 °C (lit⁰⁷ mp 221-223 °C); ¹H NMR (DMSO-d₆) δ 4.37 (s, 2H, CH₂N), 6.5-7.0 (m, 3H, ArH). Compound 114 (free base) was dissolved in Et₂O and the solution was saturated with HBr.
gas. The precipitate was recrystallized from absolute EtOH to give 114 (0.07 g, 25%) as a yellow powder: mp 234-235 °C (lit. mp 241-243 °C); ^1H NMR (DMSO-d_6) δ 4.5 (s, 2H, CH_2N), 6.95-7.32 (m, 3H, 3 ArH), 7.60 (br. s, 2H, NH_2), 8.33 (br. s, 1H, NH), 10.66 (br. s, 1H, "NH"); IR (KBr) 1501, 1580, 1631, 1682 cm^{-1}.

Dimethyl N,N'-(3-chlorophenyl)guanidine dicarboxylate (116). Methyl chloroformate (0.17 mL, 2.15 mmol) in dry THF (2 mL) was added in a dropwise manner to a solution of (3-chlorophenyl)guanidine (21) free base (0.50 g, 2.15 mmol) and Et_3N (0.60 mL, 4.30 mmol) in dry THF (100 mL) at 0 °C under an N_2 atmosphere. The reaction mixture was allowed to stir at 0 °C for 30 min and then at room temperature for 16 h. The white precipitate was removed by filtration and the solvent was removed under reduced pressure to give a beige solid which was recrystallized from absolute EtOH to give 116 (0.13 g, 21%) as a white powder: mp 160-161; ^1H NMR (CDCl_3) δ 3.59 (s, 3H, OCH_3), 3.75 (s, 3H, OCH_3), 7.09-7.38 (m, 4H, ArH), 9.60 (br. s, 2H, NH, NH). Anal. Calcd for (C_{11}H_{12}CIN_3O_2) C,H,N.

2-Chloro-6-nitrobenzylbromide (119). Compound 119 was prepared according to a literature procedure. N-Bromosuccinimide (8.54 g, 48 mmol) and benzoyl peroxide (0.20 g, 0.80 mmol) were added to a solution of 2-chloro-6-nitrotoluene (118) (8.32 g, 48 mmol) in CCl_4 (100 mL). The stirred mixture was heated at reflux for 26 h under a 250-Watt light bulb. The precipitate was removed by filtration and the solvent was removed under reduced pressure. The resulting solid was purified by column chromatography (hexane/EtOAc, 20:1). The crude product was recrystallized from absolute EtOH to give 119 (8.25 g, 70%) as yellow crystals: mp 50-52 °C (lit. mp 50-52 °C).
N-(2-Chloro-6-nitrobenzyl)phthalamide (120). Compound 120 was prepared according to a literature procedure.\textsuperscript{107} Potassium phthalamide (7.91 g, 10.3 mmol) was added to a stirred solution of 119 (2.35 g, 9.36 mmol) in DMF (20 mL). The exothermic reaction mixture was allowed to stir for 2 h and cooled to room temperature. Chloroform (10 mL) was added to the mixture which was then poured onto H\textsubscript{2}O/ice (55 mL). More CHCl\textsubscript{3} (20 mL) was added to the cooled mixture. The CHCl\textsubscript{3} portion was separated and washed with 0.2 N NaOH (26 mL) and brine (20 mL). The extract was dried (Na\textsubscript{2}SO\textsubscript{4}) and the solvent removed under reduced pressure to give 120 as yellow crystals. Compound 120 was used without further purification or characterization in the synthesis of 121.

2-Chloro-6-nitrobenzylamine (121). Compound 121 was prepared according to a literature procedure.\textsuperscript{107} Compound 120 was dissolved in MeOH (15 mL) and hydrazine hydrate (0.52 g, 10.3 mmol) was added. The stirred mixture was heated at reflux for 1 h. Water (10 mL) was added to the suspension and the MeOH was removed under reduced pressure. The remaining solution was acidified with concentrated HCl (10 mL) and heated at reflux for 1 h. The precipitate was removed by filtration and 3 N NaOH was added to the filtrate to obtain a pH of 12. Water (15 mL) was added to the solution and the mixture was extracted with Et\textsubscript{2}O (4 x 50 mL) and the Et\textsubscript{2}O extract was washed with brine (50 mL). The extract was dried with Na\textsubscript{2}SO\textsubscript{4}. The solvent was evaporated under reduced pressure and the crude product was purified using Kugelrohr distillation to give 121 (1.19 g, 68\%) as a yellow oil.

2-Amino-6-chlorobenzylamine (122). Compound 122 was prepared according to a literature procedure.\textsuperscript{107} Compound 121 (0.48 g, 2.57 mmol) and Raney Ni (0.71 g) in
absolute EtOH (12 mL) were placed in a Parr hydrogenator (psi = 3.5 atm) for 2 h. The Raney Ni was removed by filtration and the solvent was removed under reduced pressure. The resulting solid was dissolved in absolute EtOH (5 mL) and HCl gas was bubbled through the solution, and it was allowed to stand at 0 °C for 1 h. The precipitate was removed by filtration and recrystallized from an isopropanol, MeOH, Et2O mixture to give 122 HCl (0.42 g, 84%) as a powder: mp >240 °C (lit\textsuperscript{107} mp 263-265); \textsuperscript{1}H NMR (DMSO-d\textsubscript{6}) δ 4.13 (s, 2H, CH\textsubscript{2}), 6.59 (s, 1H, NH), 6.99-7.02 (m, 2H, ArH), 7.22-7.28 (m, 1H, ArH), 8.44 (br. s, 1H, NH). Compound 122 HCl was dissolved in H\textsubscript{2}O and 15% NaOH (2 mL) was added to obtain a pH of 13. The mixture was extracted with Et\textsubscript{2}O (3 x 40 mL) and the combined Et\textsubscript{2}O portion was dried with Na\textsubscript{2}SO\textsubscript{4}. The solvent was removed under reduced pressure to give 122 (0.25 g, 61%) as a solid. Compound 122 was used without further characterization in the synthesis of 114.

B. Behavioral studies

1. Animals

Male ICR mice (24-28g) obtained from Harlan Laboratories (Indianapolis, IN) were used throughout the study. Mice were housed in groups of five, with free access to food and water. Animals were housed in an Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC)-approved facility, and the study was approved by the Institutional Animal Care and Use Committee of Virginia Commonwealth University. Mice were allowed to adapt to the testing environment for at least 2 h prior to any
treatment. Animals were weighed on the day of the experiment(s) for the calculation of drug dosages.

2. Drugs

Clonidine hydrochloride, morphine hydrochloride, tropisetron hydrochloride, and yohimbine hydrochloride were purchased from Sigma-Aldrich Chemicals, (Milwaukee, WI). Compounds 21, 113, and 26 were synthesized as reported above. Compound 29 as its HCl salt was resynthesized as reported previously. Imiloxan hydrochloride was obtained from Tocris (Ballwin, MO). All drugs were dissolved in physiological saline (0.9% sodium chloride) and given in a total volume of 10 mL/1000 g body weight by subcutaneous (s.c.) injection to the mice.

3. Behavioral assays

a) Hot-plate assay

The method is a modification of that described by Eddy and Leimbach and Atwell and Jacobson. Mice were placed into a 10-cm-wide glass cylinder on a hot-plate (Columbus Hot-Plate Analgesia Meter) and the temperature was maintained at 55.0 ºC. A control response (6-10 s) was determined for each mouse before injection and a test latency was determined after drug administration. In order to prevent any paw damage, 30 s was used as the cutoff time. The antinociceptive response was calculated as percent maximum possible effect (% MPE), where % MPE = [(test-control)/ (cutoff time-control)] x 100. Groups of 5 to 26 mice were used for each dose and for each treatment.
The protocol for testing the antinociceptive effects of these drugs was as follows: 15 min prior to s.c. administration of drugs, baseline latency was determined for each mouse. Clonidine was administered 20 min prior to test. MD-354 (21) was administered using various (10 to 90 min) pretreatment intervals. In a combination study of MD-354 with clonidine, MD-354 was administered 25 min prior to clonidine and 45 min before the test. Compound 29 was administered 45 min prior to test. In a combination study, 29 with clonidine, 29 was administered 25 min prior to clonidine and 45 min before the test.

b) Tail-flick assay

Antinociception was assessed by the tail-flick method of D’Amour and Smith\textsuperscript{99} as modified by Dewey et al.\textsuperscript{114} using a Columbus Tail-Flick Analgesia Meter. A control latency (1.7-4.0 s) was determined for each mouse before injection, and a test latency was determined after drug administration. A cutoff time of 10 s was used to avoid tissue damage. The antinociceptive response was calculated in the same manner as in the hot-plate assay. Groups of 5 to 16 mice were used for each dose and each treatment.

The protocol for testing the antinociceptive effects of these drugs was as follows: 15 min prior to s.c. administration of drugs, baseline latency was determined for each mouse. MD-354 (21) was administered 45 min before test. In a combination study using MD-354 with morphine, MD-354 was administered 15 min before morphine and 45 min prior to the test. Compound 29 was administered 45 min before the test. In a combination study using 29 with clonidine, 29 was administered 25 min before clonidine and 45 min before the test. In a combination study using 29 and morphine, 29 was administered 15
min before morphine and 45 min before the test. Tropisetron was administered 5 min before 29 and 30 min prior to clonidine. Imiloxan was administered 10 min prior to 29 and 35 min before clonidine. Yohimbine was administered 15 min before 29 and 40 min prior to clonidine. Compound 113 was administered by using various (5 to 120 min) pretreatment intervals. In combination studies using 113 with clonidine, 113 was administered 0 to 100 min prior to clonidine and 5 to 120 min before the test. Compound 26 was administered 45 min before the test. In combination studies using 26 and clonidine, 26 was administered 25 min before clonidine and 45 min prior to the test.

c) Spontaneous activity

Mice were placed into individual Tru Scan Infrared Locomotor Activity System (Coulbourn Instruments, Allentown, PA) photocell activity cages (40 cm cube) after s.c. administration of either 0.9% saline or 29 (0.03 mg/kg). Ambulatory movement was measured by the number of times the animal interrupted the infrared beams traversing the cage for a period of 15 min. Measurements were taken 15, 30, and 45 min following drug treatment. The analysis was focused only on main measures (three main measures: total moves, move time, move distance) of activity to determine whether 29 (n = 6/dose) depressed this action relative to saline (n = 6) control or whether 29 + clonidine (n = 6/dose) potentiated the depressed action relative to clonidine (n = 6). The pretreatment time for clonidine was 5 min before the test.
d) Statistical analysis

Data were analyzed statistically by an analysis of variance (ANOVA) followed by the Newman-Keuls test for multiple post hoc comparison test. The null hypothesis was rejected at the 0.05 level. For the time-course studies, each animal was used once. Data were analyzed by one-way or a two-factor ANOVA as applicable. ED$_{50}$ values with 95% CL for behavioral data were calculated by unweighted least-square linear regression as described by Tallarida and Murray.$^{115}$
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Literature Cited


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VITA

Shawquia Elithia Young was born on May 11, 1980 to the parents of Arthur and Carolyn Young. Shawquia graduated from Hampton University in May 2002 with a Bachelor of Science degree in chemistry. In September 2002, Shawquia enrolled at Virginia Commonwealth University in the School of Pharmacy. While working on her Master of Science degree, Shawquia received a publication titled “MD-354 potentiates the antinociceptive effect of clonidine in the mouse tail-flick but not hot-plate assay” which was published in the European Journal of Pharmacology in 2004.