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Dual Mechanism Analgesia-Enhancing Agents

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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.

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Table of Contents

List of Tables

Page

List of Figures

Page

List of Schemes

Page

List of Abbreviations

Anal	Analysis
ANOVA	Analysis of variance
AR	Adrenoceptor
ATP	Adenosine triphosphate
BBB	Blood-brain barrier
br.	Broad
°C	Celsius degrees
$CaCN_2$	Calcium cyanamide
Calcd	Calculated
cAMP	3',5'-Cyclic adenosine monophosphate
CCl_4	Carbon tetrachloride
$CDCl_3$	Deuterated chloroform
CH_2Cl_2	Dichloromethane
CL	Confidence limit
CNS	Central nervous system
CNBr	Cyanogen bromide
C-terminus	Carboxyl-terminus
Cys-loop	Cysteine-loop
D_2	Dopamine D_2 receptor
D_6-DMSO	Deuterated methyl sulfoxide
DMF	Dimethylformamide
DRG	Dorsal root ganglion
ED_{50}	Median effective dose
Enk	Enkephlan
et al.	And others
Et_3N	Triethylamine
Et_2O	Diethyl ether
EtOAc	Ethyl acetate
EtOH	Ethanol
GABA	γ-Aminobutyric acid
GPCRs	G-protein coupled receptors
H_2NNH_2	Hydrazine hydrate
5-HT	5-Hydroxytryptamine
ia	Intrinsic activity
i.c.v.	Intracerebroventricular
i.e.	That is

ININ	Inhibitory interneurons
i.p.	Intraperitoneal
i.t.	Intrathecal
i.v.	Intravenous
K_i	Dissociation constant
LGIC	Ligand-gated ion channel
lit	Literature
Log P	Partition coefficient
m	Multiplet
MeOH	Methanol
mp	Melting point
MPE	Maximal possible effect
NA	Not active
nACh	Nicotinic acetylcholine receptor
$Na_2 SO_4$	Sodium sulfate
NH_4NO_3	Ammonium nitrate
nM	Nanomolar
NSAIDs	Nonsteroidal anti-inflammatory drugs
N-terminal	Amine-terminal
PAFs	Primary afferent fibers
ppm	Parts per million
s	Singlet
SAR	Structure-activity relationships
S.E.M.	Standard error of mean
THF	Tetrahydrofuran
TLC	Thin-layer chromatography
TM	Transmembrane

Abstract

DUAL MECHANISM ANALGESIA-ENHANCING AGENTS

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

Virginia Commonwealth University, 2005

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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_3 and α_{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_3/α_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 α_2-adrenoceptor agonists.[1] 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.[2,3]

Morphine was originally isolated in 1803 and was one of the first agents classified as an opiate.[1] Morphine binds at opioid receptors and more specifically, mu opioid receptors.[4] It is a potent agent and is often referred to as a narcotic analgesic.[1] This means that morphine can induce sleep and/or a state of narcosis in addition to its analgesic effects.[1] 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.[4-6]

Clonidine is an agent currently being used in regional anesthesia for the management of acute post-operative pain, labor pain, or chronic pain.[7] Clonidine binds both at α_2-adrenoceptors and imidazoline receptors.[8] It binds nonselectively at α_2-adrenoceptors, which means that it binds with similar affinity at all of the α_2-adrenoceptor subtypes (α_{2A}, α_{2B}, α_{2C}). Evidence shows that the analgesic effects of clonidine are due to its partial agonist activity at α_2-adrenoceptors.[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.[3,7] Due to its ability to lower blood pressure, clonidine is also being used to treat hypertension.[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.[9] Specifically, D_2 receptors are involved with inducing antinociception.[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_3 receptor class plays a significant role in producing analgesia. However, there are currently available only a small number of selective 5-HT_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 α_{2A}-adrenoceptor subtype.[3] Recent studies have shown that the α_{2B}-adrenoceptor subtype contributes to antinociception but is not involved in sedation.[3] 5-HT_3 and α_{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 α_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".[10] It consists of a mechanism that prevents any potential damage that can occur to the tissue.[10] 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.[10,11] 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.[9,10] 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.[10] In some cases, one mechanism can produce pain and in others, different mechanisms can coexist.[10]

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.[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.[10,12] Usually it is the common cause of major disability.[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.[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.[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.[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.[13] This latter type of stimulus is the slowest of the three in producing nociceptive responses, and the pain produced is inescapable.[13] After the noxious stimulus interacts with the peripheral tissues, the primary afferent nociceptive pathways become activated. Nociceptor C and Aδ 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.[11,14] These two types of PAFs both are nocisponsive, which respond to noxious chemical, thermal, and

mechanical stimuli.[14] The myelinated Aδ fibers elicit the first phase of pain that is rapid and sharp;[9,14] whereas the unmyelinated C fibers induce the second phase of pain which is slower and dull.[9,14] PAFs can directly activate the projection neurons that can relay this information to the brain or indirectly activate them through the excitatory interneurons.[9,14] The 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.[9,14] 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.[9,11,14] The projection neurons are inhibited by inhibiting the excitatory interneurons and simultaneously stimulating the inhibitory interneurons.[14] This activity occurs postsynaptically to the terminals of the PAFs.[9,14] 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.[9,11,14,15] 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.[11] From the former pathway, the activity is transferred through the thalamus to the somatosensory cortex.[11,14]

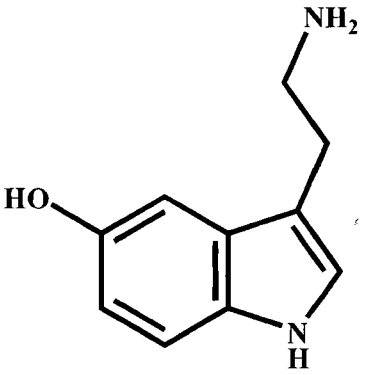
The parabrachial region is connected to other supraspinal sites such as the hippocampus, amygdala, and other regions which are involved in the nociceptive response.[9,11,14] This message is then integrated in the periaqueductal gray which plays a major role in promoting and inhibiting pronociception.[11]

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).[15] 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, α_1, α_2). Serotonin is involved in the descending serotoninergic pathway and this neurotransmitter interacts with the 5-HT receptor superfamily (5-HT_1-5-HT_7).

B. 5-HT_3 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.[16] 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-HT_1-5-HT_7).



5-HT **(1)**

All of the 5-HT receptors are G-protein coupled receptors, except 5-HT_3 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 adenyl cyclase and stimulation of phospholipase C.[16]

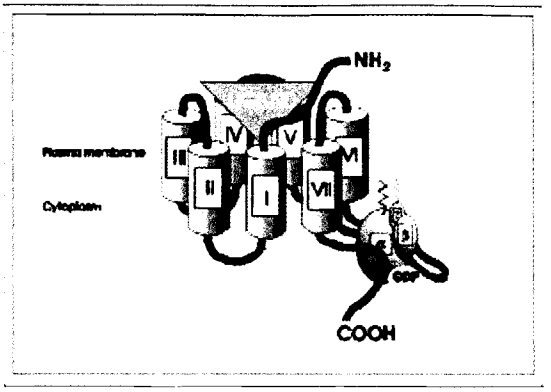


Figure 1. G-Protein coupled receptor[17]

5-HT_3 receptors are a member of the cys-loop superfamily which includes the nicotinic acetylcholine (nACh) receptors, $GABA_A$, 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.[16] In the case of 5-HT_3 receptors, 5-HT binds to the receptor and allows specific cations, such as Na^+, K^+, and Ca^{2+} to move through the lipid membrane. Activation of these receptors produces various effects including membrane depolarization, increase in intracellular Ca^{2+}, and the modulation of neurotransmitter release.[18]

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.[16,19] It is suggested that the N-terminal domain is where the ligand-binding domain is located.[16] In this domain, there are 2 cysteine residues that are linked by a disulfide bond. 5-HT_3 receptors share about 20-30% amino acid sequence identity with the nACh receptors.[16] Since the crystal structure of the ACh binding protein (AChBP) was recently determined, this facilitates the study of the 5-HT_3 ligand binding domain due to their structural similarities.

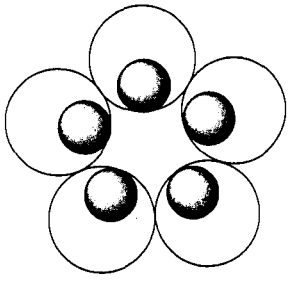


Figure 2. Quaternary structure of a LGIC receptor, and the location of the TM2 lining the pore

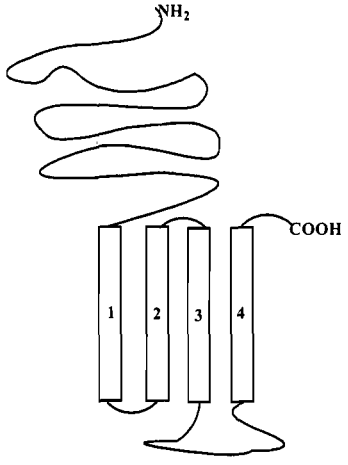


Figure 3. Topology of a LGIC receptor subunit

5-HT_3 receptors have been classified into three subunits: 5-HT_{3A}, 5-HT_{3B}, and 5-HT_{3C}.[16] However, only the 5-HT_{3A} and 5-HT_{3B} subunits have been cloned.[16] The 5-HT_{3A} 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.[16] The 5-HT_{3B} subunit has been cloned from human fetal kidney, human small intestine, and mouse and rat brain.[16] The 5-HT_{3A} subunit is expressed in heterologous

expression systems and has structural similarities with the other receptors in the cys-loop superfamily.[20,21] The protein sequence similarity between 5-HT_{3A} and the α-subunits of the other members is 22-30%.[16] 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.[16] The 5-HT_{3A} subunit consists of 487 amino acids and three potential N-glycosylation sequences that are located in the extracellular region.[16] These N-glycosylation sites could potentially be involved in the assembly of the 5-HT_3 receptor.[16] 5-HT_{3A} 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_{3A} subunit have been identified in humans.[22] 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_{3A} 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.[22] The long variant of 5-HT_{3A} consists of 510 amino acids which is 23 amino acids longer than the normal variant. It also does not form functional homomeric ion channels.[22] These additional amino acids are inserted in the extracellular loop between the TM2 and TM3 domain.[22]

The 5-HT_{3B} subunit cannot form homomeric complexes but can form heteromeric complexes with the 5-HT_{3A} subunit. This subunit has ~45% sequence identity with its 5-HT_{3A} homolog.[16] The 5-HT_{3B} subunit has no function by itself, but elicits its function when it is coexpressed with the 5-HT_{3A} subunit. This receptor subunit has 441 amino acids and

also has extracellular N-glycosylation sites as seen in the 5-HT_{3A} subunit. The 5-HT_{3B} 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.[22] When this subunit forms a heteromeric complex with the 5-HT_{3A} 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.[20] The 5-HT_{3C} subunit was just recently patented and its function is still unclear.[16,23] This receptor shows ~39% sequence identity to its 5-HT_{3A} homolog.[23] It has been suggested that the 5-HT_{3C} subunit contributes to the regulation of the 5-HT_3 receptor's responses.[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.[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.[26] In humans, the majority of 5-HT_3 receptors in the CNS are the 5-HT_{3A} subunit.[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.[20]

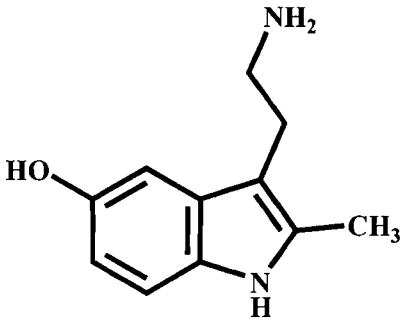
5-HT_3 ligands have become important tools in studying the 5-HT_3 receptor. For instance, 5-HT_3 antagonists are being used to determine the distribution of these receptors throughout the body. Various assays have been developed in order to differentiate between 5-HT_3 agonists and antagonists. One of 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.[27] When a 5-HT_3 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-HT_3 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-HT_3 agonists and antagonists. Another *in vivo* assay is the ferret and shrew emesis assay.[28] In this assay, cisplatin, a vomiting-inducing agent, is administered to the animal to cause emesis.[28] A 5-HT_3 antagonist will prevent this emetic response from occurring and a 5-HT_3 agonist induces emesis. Stimulation of 5-HT_3 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-HT_3 agonists produces contractions. If an agent can block these contractions it is referred to as an antagonist. In some cases, a 5-HT_3 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-HT_3 receptors and their many functions.

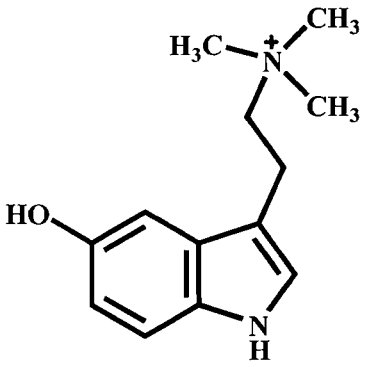
4. Ligands and Structure-Activity Relationships (SAR)

a) Agonists

Over the years a variety of drug classes have been shown to bind selectively to 5-HT_3 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-HT_3 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-HT_3 receptors nonselectively and with moderate affinity (K_i =1,000 nM).[23] 2-Methyl 5-HT (**2**) was found to display more selectivity for 5-HT_3 receptors than 5-HT but binds with slightly lower affinity (K_i =1,350 nM).[23] 2-Methyl 5-HT is one of the few currently available 5-HT_3 agonists being used. Recent investigations have shown, however, that **2** binds at 5-HT_6 receptors (K_i = 45 nM) with higher affinity than it displays for 5-HT_3 receptors.[29]



2-Methyl 5-HT (**2**)



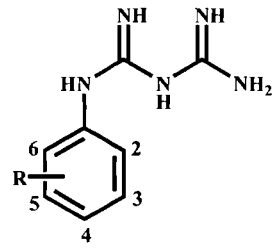
5-HTQ (**3**)

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).[23] 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.[23]

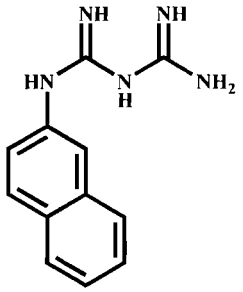
The arylbiguanides represent another class of 5-HT_3 agonists that are nontryptamines and selective for this receptor. Phenylbiguanide (PBG, **4**) was the first agent of this class to be developed and it displayed moderate affinity (K_i =1,200 nM) comparable to 5-HT. *m*-Chlorophenylbiguanide (*m*-CPBG, **5**, K_i = 17 nM) was found to be a higher affinity agent than PBG.[25] Structure-activity relationship (SAR) studies were conducted by Dukat *et al.*[25] 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).[25] The primary goal of this study was to examine the electronic and lipophilic character of these substituents and their effect on affinity.[23] 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).[30,31] 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-HT_3 Receptors.[30]



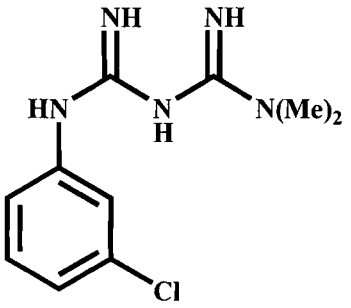
4-12 and 14-17



13

	R	K_i, nM
4	H	1200
5	3-Cl	17
6	2-Cl	62
7	4-Cl	200
8	3-NO_2	220
9	3-CH_3	780
10	4-CH_3	>10000
11	3-Cl, 4-CH_3	225
12	2-OCH_3, 5-Cl	126
13	—	12
14	3,4-di-Cl	12
15	3,5-di-Cl	1.8
16	3,4,5-tri-Cl	2.7
17	3-CF_3	700

Next, the biguanide chain was modified. Dimethylation of the terminal amine resulted in abolished affinity (**18**, K_i > 10,000 nM).[30] Various derivatives were studied and it was shown that any further modification (with one exception) of the biguanide chain also abolished affinity.[30] However, the N-(2-phenylethyl)guanidine **19**, (K_i = 40 nM) displayed high affinity.[30] More importantly, it was found that the entire biguanide chain was not necessary for binding and could be shortened; this resulted in the arylguanidines.[30]



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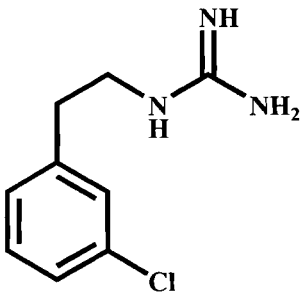
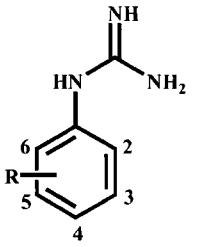


19

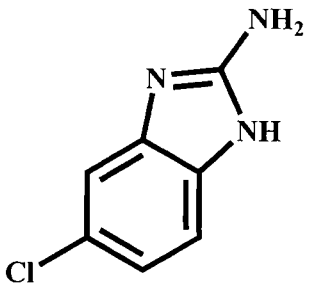
This novel class of compounds, the arylguanidines, was found to bind with affinities comparable to the arylbiguanides.[30] Between the two classes parallel structural changes led to parallel shifts in affinity.[31] SAR studies were conducted and both aryl substitution and the guanidine moiety were examined (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 nM).[30] Also, as with the arylbiguanides, high affinity was not retained when the chloro group was replaced with a trifluoromethyl group (**24**, K_i = 5700 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, and 0.7 nM, respectively). Conformationally constrained

analogs of MD-354 were also examined as possible ligands.[32] Benzimidazole **30** and quinazoline **31** were developed and it was found that only the quinazoline displayed high affinity (K_i = 725 and 34 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.[32] MD-354 (**21**) has a Log P value of -0.64 which suggests that this agent will have difficulty penetrating the BBB.[32] 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]



	R	K_i, nM
20	H	2340
21	3-Cl	35
22	2-Cl	190
23	4-Cl	320
24	3-CF_3	5700
25	3-CH_3	6520
26	3-OCH_3	1600
27	3,4-di-Cl	3.1
28	3,5-di-Cl	5.0
29	3,4,5-tri-Cl	0.7



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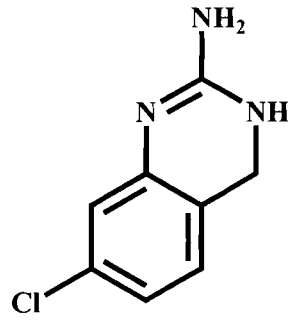
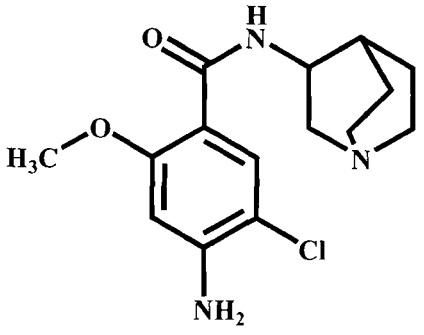


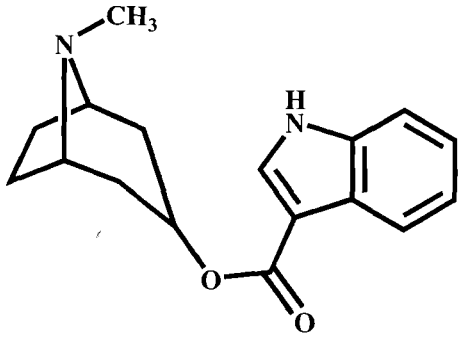
31

b) Antagonists

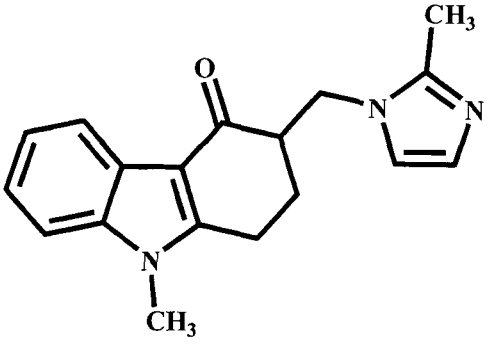
When compared with the 5-HT_3 agonists, there are definitely more available antagonists for research and on the market.[34] 5-HT_3 antagonists are currently being used clinically in the treatment of irritable bowl syndrome and as antiemetics. These antagonists include zacopride (**32**), tropisetron (**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-HT_3 receptors (K_i= 0.1-1.9 nM), but it also acts as an agonist at 5-HT_4 receptors (K_i= 1.1 nM).[35] Tropisetron (**33**) (formerly known as ICS 205-930) was one of the first clinically used antiemetics among the 5-HT_3 antagonists. It acts as an antagonist at both 5-HT_3 and 5-HT_4 receptors (K_i= 0.4-3.2 and 930 nM, respectively).[35] Both ondansetron (**34**) and granisetron (**35**) bind selectively at 5-HT_3 receptors with high affinity (K_i= 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-HT_3 receptors in the CNS.[35] These agents also are useful in evaluating the functional roles of these receptors.



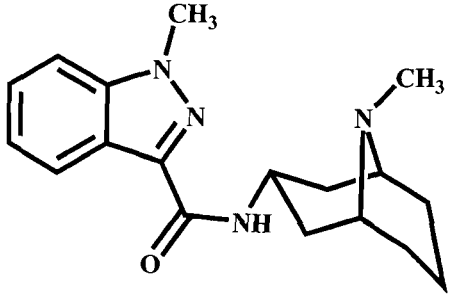
Zacopride (**32**)



Tropisetron (**33**)



Ondansetron (**34**)



Granisetron (**35**)

5-HT_3 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.[34] Ondansetron (**34**) is being used clinically to relieve nausea and vomiting associated with cancer chemotherapy, radiation, and anesthesia.[34,36] 5-HT_3 antagonists block emesis by producing antagonism of the receptors located at central sites.[36] Ondansetron (**34**) is highly effective but does not completely eliminate nausea and vomiting.[34] When compared with previous antiemetics (i.e., scopolamine), the 5-HT_3 antagonists do not cause any sedation.[34] 5-HT_3 receptors also play a role in anxiety, and it is suggested that 5-HT_3

antagonists could be used as potential anxiolytics.[23,37] In clinical studies, however, it was shown that the 5-HT_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.[37] Another area in which 5-HT_3 receptors are involved is gut motility. The stimulation of these receptors could cause diarrhea, which is commonly referred to as intestinal hypermotility.[36] This is usually associated with irritable bowel syndrome. 5-HT_3 antagonists could potentially be used in the treatment of diarrhea. On the other hand, 5-HT_3 agonists could also be used in the treatment of constipation.[23] Since it is suggested that stimulation of 5-HT_3 receptors increases intestinal motility in this disorder, it could be beneficial to use a 5-HT_3 agonist which is effective and does not induce emesis and diarrhea.[23]

As discussed earlier, 5-HT_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_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.[38,39] It was also shown that 5-HT_3 receptors located on peripheral sensory neurons are involved in inflammatory, but not mechanical or thermal, pain in animal models.[36] For example, Giordano *et al.*[40] showed that the subcutaneously (s.c.) administered 5-HT_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.[40] In this test, formalin induces inflammatory pain which is blocked by

tropisetron. Also in this study, both central and peripheral 5-HT_3 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-HT_3 receptor antagonists in thermal and mechanical pain tests supports the hypothesis that 5-HT_3 receptors do not mediate the transmission of nociceptive signals.[40] However, the antinociceptive effect produced by 5-HT_3 antagonists in the formalin-induced pain test further suggests that 5-HT_3 receptors induce a response to inflammatory pain.[40] These results also suggest that supraspinal 5-HT_3 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-HT_3 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-HT_3 receptor antagonist.[41] According to these results, it further suggests that spinal 5-HT_3 receptors induce antinociceptive responses to inflammatory pain. This analgesic activity could also be reduced by ketanserin (5-HT_2 antagonist), naloxone (opioid antagonist), and bicuculline ($GABA_A$ antagonist).[41] Because this 5-HT_3 receptor-mediated antinociception could be attenuated by various other antagonists, this suggests that the 5-HT_3 descending inhibitory system is interacting with 5-HT_2, $GABA_A$, 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-HT_3 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-HT_3 receptor.[39] 2-Methyl 5-HT and 5-HT were also analyzed in the writhing test.[39] This test was used to study visceral 5-HT_3 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.).[39] 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.[39] These results suggest that visceral 5-HT_3 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-HT_3 and 5-HT_4 receptor antagonist, this specific dose could be interacting with 5-HT_4 receptors which might be involved in this nociceptive activity.

5-HT_3 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.*,[42] 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).[42] This study further supports the concept that 5-HT_3 receptors mediate spinal analgesia. As mentioned earlier, various 5-HT_3 receptor antagonists were analyzed in the hot water bath test in rats for possible antinociceptive activity.[40] These antagonists did not

Table 3. Summary of 5-HT_3 Ligand Activity in Various Animal Nociceptive Tests.

Model	Agents	Agonist/ Antagonist	Species	Route of Admin.	Analgesic Activity	Ref.
Inflammatory pain						
Formalin test	Tropisetron	Antagonist	rats	s.c.	yes	40
	MDL-72222	Antagonist	rats	i.c.v.	no	40
	Ondansetron	Antagonist	rats	i.t.	no	40
	2-Methyl 5-HT	Agonist	rats	i.t.	yes/blocked by 5-HT_3, 5-HT_2, opioid,GABA antagonists	41
			rats	i.c.v.	no	41
Writhing test			mice	i.p.	produces no nociceptive responses alone	39
Hot plate/ Hot water bath	Tropisetron	Antagonist	rats	s.c.	no	40
	MDL-72222	Antagonist	rats	i.c.v.	no	40
	Ondansetron	Antagonist	rats	i.t.	no/blocks analgesic effect of 5-HT	40, 42
	2-Methyl 5-HT	Agonist	rats	i.t.	yes/ reduced by 5-HT_3, 5-HT_2, opioid, and GABA antagonists	41
				i.c.v	no	41
	PBG	Agonist	rats	i.t.	yes	43
Tail flick	Tropisetron	Antagonist	rats	s.c.	no	40
			rats	i.c.v.	no	40
			rats	i.t.	no/blocks i.c.v. morphine-induced analgesia	40, 46
	MDL-72222	Antagonist	rats	i.t.	no/blocks i.t. 2-Methyl 5HT	40, 42
	Zacopride	Antagonist	rats	i.t.	no	40
	2-Methyl 5-HT	Agonist	rats	i.t.	yes/ blocked by 5-HT_3, GABA, α_2 and α-adrenoceptor antagonists	42-44
	PBG	Agonist	rats	i.t.	no	43
Mechanical	Tropisetron	Antagonist	rats	s.c.	no	40
	MDL-72222	Antagonist	rats	i.c.v.	no	40
	Ondansetron	Antagonist	rats	i.t.	no	40
	2-Methyl 5-HT	Agonist	rats	i.c.v.	no	48
			rats	i.t.	no	48
	mCPBG	Agonist	rats	i.t.	yes/ blocked by 5-HT_3 antagonists	49

produce any antinociceptive activity when administered subcutaneously (s.c.) or via the i.c.v. route.[40] 5-HT produces analgesic effects through influence on the CNS, and these effects can be mimicked by 2-methyl 5-HT (**2**).[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 *et al.*[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.[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.[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.[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 *et al.*,[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.[44] The antinociceptive activity was only partially blocked by phalcofen ($GABA_B$ antagonist).[44] In earlier studies, it was suggested that 5-HT_3 receptors could mediate the release of GABA which elicits the antinociceptive activity.[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.[44]

Noradrenergic receptors also play a role in mediating 5-HT-mediated antinociception.[45] It was previously shown that i.t. administered norepinephrine and 5-HT produce a synergistic effect.[45] Sawynok *et al.*[45] showed that both 5-HT and 2-methyl 5-HT's antinociceptive activity could be reduced by phentolamine (nonselective α-AR antagonist) and yohimbine (α_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.[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.[46] It has been shown that i.t. administered morphine produces antinociception that can be blocked by tropisetron.[46] In a later study, i.t. administered tropisetron (**33**) also blocked i.c.v. administered morphine-induced antinociceptive activity.[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.[9]

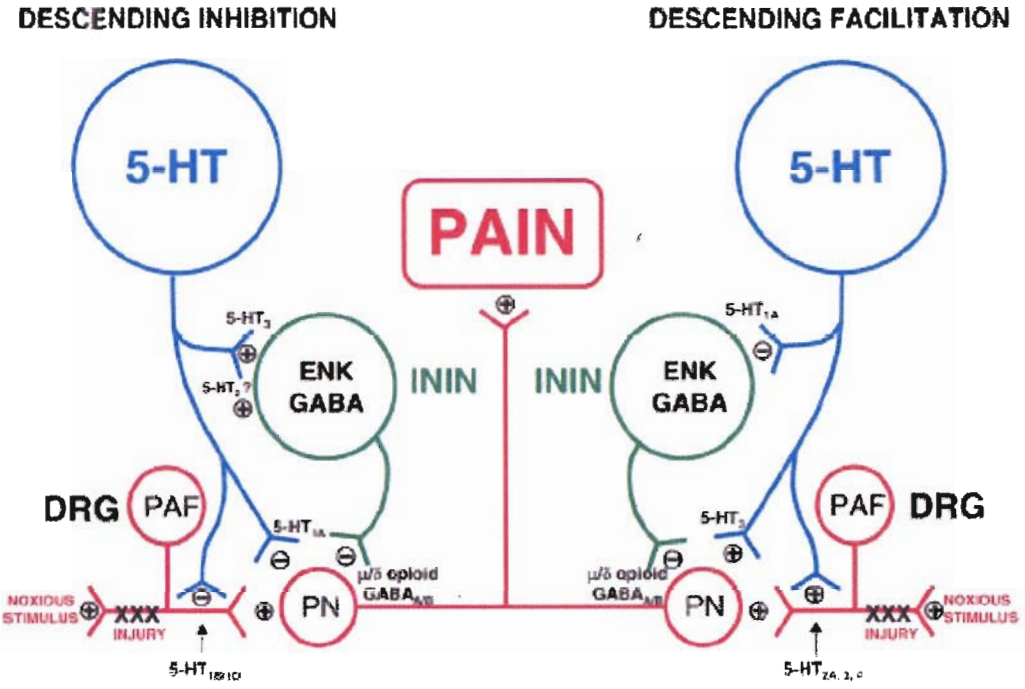


Figure 4. Schematic diagram of 5-HT serotonergic system modulating nociception.[9] This figure is used to show the involvement of the serotonergic system with other neurotransmitters.

Mechanical nociceptive tests have been used to study possible involvement of 5-HT_3 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.[48,49] As seen in certain other nociceptive tests, 5-HT_3 receptors do not seem to be conclusively involved in antinociception.[40] 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.[48] In another study, mCPBG (**5**) and 5-HT were also analyzed in the paw pressure test.[49] Both i.t. administered 5-HT and mCPBG produced a dose-dependent antinociceptive

response which was blocked by tropisetron and granisetron.[49] 5-HT produced higher antinociceptive effects than mCPBG, an agent which is more selective for 5-HT_3 receptors than 5-HT.[49] This suggests that 5-HT_3 receptors might be partially involved in producing an antinociceptive response to a mechanical stimulus.[49] 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.[50] This could also play a role in the partial antinociceptive actions of mCPBG since dopamine has been implicated in antinociception.[8,50] 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.[51] Ferber *et al.*[52] 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.[51] Muller *et al.*[53] 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.[38] In an open pilot study, 5 out of 6 patients experienced a rapid relief of pain when treated with granisetron (**35**).[38] In further studies, other 5-HT_3 receptor antagonists (i.e., ondansetron (**34**) and tropisetron (**33**)) did not consistently relieve the pain caused by a migraine attack.[38] Stratz and Muller[54] 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-HT_3 receptor antagonists can be useful in situations linked to inflammatory stimuli.

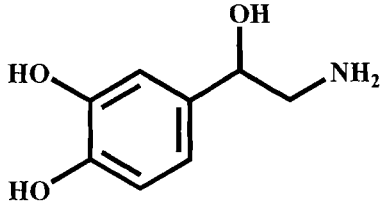
It has been shown that 5-HT_3 receptors might be involved in the modulation of nociception. The 5-HT_3 receptor agonist 2-methyl 5-HT showed analgesic activity in the inflammatory and thermal but not mechanical tests, whereas another 5-HT_3 receptor agonist, PBG, did not produce analgesia. However, the more selective 5-HT_3 receptor agonist *m*CPBG produced analgesic activity in the mechanical test. The 5-HT_3 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-HT_3 receptors in mediating nociception has been quite difficult. Inconclusive findings with 5-HT_3 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-HT_3 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-HT_3 ligand used. Additional studies with more lipophilic 5-HT_3 receptor agonists would certainly allow a clearer understanding of the involvement of 5-HT_3 receptors in pain.

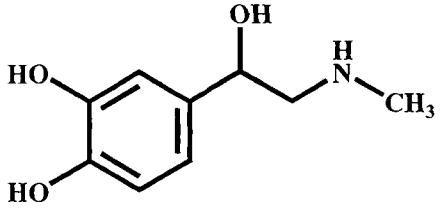
C. α_2-Adrenoceptors

1. Classification

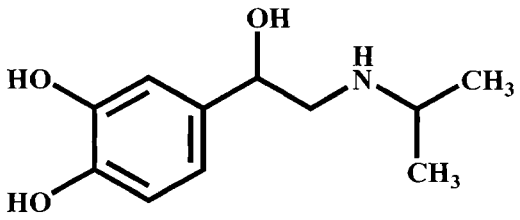
The adrenoceptors, a receptor family that has been extensively studied over the years, are membrane receptors that belong to the superfamily of G-protein coupled receptors (GPCRs). They bind the endogenous catecholamines, norepinephrine (**36**) and epinephrine (**37**), which elicit a variety of physiological effects in both the central and peripheral nervous systems.[55] Some of these effects include control of blood pressure and blood flow, neuronal modulation, digestion, respiration, reproduction, pupil dilation and contraction, and energetic metabolism.[55] Norepinephrine is released from the noradrenergic postganglionic nerve terminals, and both norepinephrine and epinephrine are secreted in the adrenal medulla.[55]



Norepinephrine (**36**)



Epinephrine (**37**)



Isoproterenol (**38**)

The adrenoceptors are divided into two broad categories, α- and β-adrenoceptors.[56] α-Adrenoceptors can be further divided into α_1- and α_2-adrenoceptors.[56] The α and β adrenoceptors were initially classified according to the rank order of potency of a series of adrenoceptor agonists.[57] For the α-adrenoceptors, epinephrine (**37**) = norepinephrine (**36**) > isoproterenol (**38**) in terms of potency, and for the β-adrenoceptors, isoproterenol > epinephrine > norepinephrine.[57] α-Adrenoceptors mediate mostly excitatory functions that include vasoconstriction, uterine musculature contraction, urethra contraction, and pupil dilation, and one inhibitory function, intestinal relaxation.[55] β-Adrenoceptors mediate mostly inhibitory functions that include vasodilation, uterine musculature relaxation, bronchodiliation, and an excitatory function, cardiac function.[55] α_2-Adrenoceptors are classified into three subtypes: α_{2A}-, α_{2B}-, and α_{2C}-adrenoceptor subtypes. As mentioned earlier, GPCRs mediate their physiological effects via second messenger systems (Figure 5). In the case of α_2-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.[57] It has been suggested that other G-protein dependent signaling pathways are mediated by activation of phospholipase D, stimulation

of phospholipase A_2 activity, and increased intracellular Ca^{2+} availability.[57,58] The α_2-adrenoceptors are coupled to $G_{i/o}$ 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 $\beta\gamma$ subunit.[58] However, it has been shown that α_2-adrenoceptors are also coupled to the stimulation of adenylyl cyclase via G_s-proteins in some cells transfected with α_2-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 α_{2A}- and α_{2C}-adrenoceptors inhibit Ca^{2+} channels and activate K^+ channels.[56]

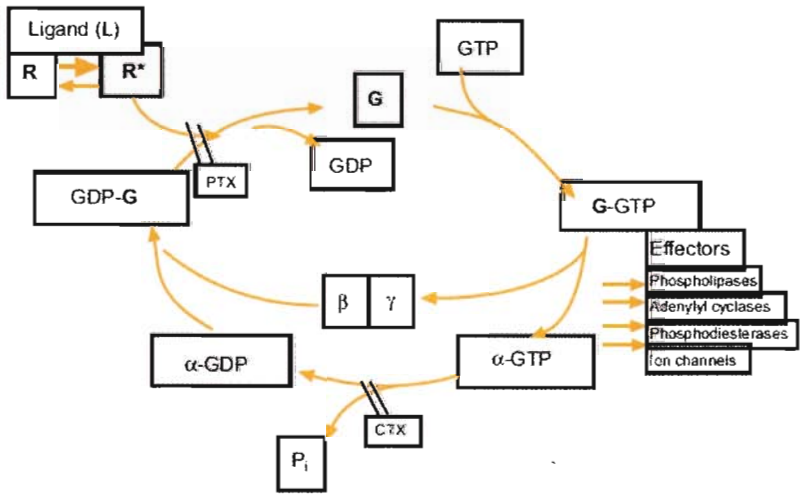


Figure 5. Schematic diagram of the G-protein cycle.[59] PTX: Pertussis toxin and CTX: cholera toxin.

2. Structure

The arrangement of the structure of α_2-adrenoceptors corresponds to a model of the protein rhodopsin, which has been extensively studied, and several subtypes have been

identified.[58] In general, α_2-adrenoceptors have seven transmembrane-spanning hydrophobic regions (TM1-TM7), with hydrophilic intracellular and extracellular loops.[8,58] The transmembrane regions are made up of 20- to 25-amino acids forming alpha helices that are embedded in the membrane.[8] The α_2-adrenoceptor subtypes share about 75% amino acid identity in their transmembrane regions.[8] The largest difference among the α_2-adrenoceptor subtypes occurs in the N-terminus and in the third intracellular loop between TM5 and TM6.[59] Among the subtypes, they share less than 25% sequence identity in the third intracellular loop.[58] The third intracellular loops of the three subtypes vary in sequence and in the number of amino acids: α_{2A}-adrenoceptors consist of 157 amino acids, α_{2B}-adrenoceptors consist of 173 amino acids, and α_{2C}-adrenoceptors consist of 148 amino acids (Table 4).[57]

Table 4. Summary of α_2-Adrenoceptor Subtype Characteristics.[59]

	α_{2A}	α_{2B}	α_{2C}
Human chromosome	10	2	4
Structure			
number of amino acids	450	450	461
transmembrane domains	7	7	7
number of amino acids in third intracellular loop	157	173	148
glycosylated	yes	no	yes
palmitoylated	yes	yes	no
Second messenger systems	$G_{i/o}$: ↓ cAMP. inhibit Ca^{2+} channels, open K^+ channels	$G_{i/o}$: ↓ cAMP	$G_{i/o}$: ↓ cAMP. inhibit Ca^{2+} channels, open K^+ channels

Interestingly, the third intracellular loop of the α_{2B}-adrenoceptor subtype contains a higher number of glutamic acid residues (13%) when compared to α_{2A}-adrenoceptors (6%) and α_{2C}-adrenoceptors (3%).[60] In this region, which is only seen in α_{2B}-adrenoceptors, there is a stretch of 12 consecutive glutamic acid residues.[60] The third intracellular loop is about two to three times longer in the α_2-adrenoceptors than in the corresponding loops of the α_1- and β-adrenoceptors.[58] The second and third intracellular loops are involved with G-protein interactions.[58] The α_{2A}-adrenoceptor subtype consists of 450 amino acids and is glycosylated on the N-terminus and palmitoylated on the C-terminus.[57,59] The α_{2B}-adrenoceptor subtype also consists of 450 amino acids and is palmitoylated on the C-terminus but is not glycosylated, whereas the α_{2C}-subtype consists of 461 amino acids and is only glycosylated on the N-terminus.[57,59] The palmitoylated cysteine residue in the α_{2A}- and α_{2B}-adrenoceptors attaches the receptor to the lipid bilayer of the cell membrane.[58] 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).[58] 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.[58]

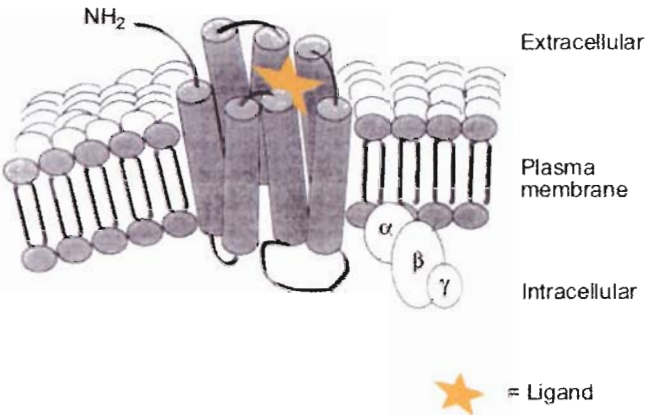
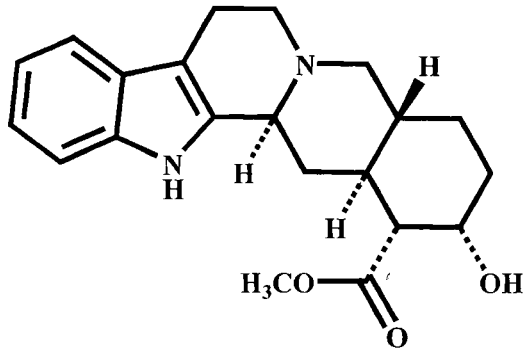


Figure 6. Model of a ligand binding to a GPCR.[58]

All of the α_2-adrenoceptor subtypes have been cloned from human, rat, mouse, and guinea pig sources.[55,58] The human α_2-adrenoceptor subtypes have been designated α_2-C10 (α_{2A}), α_2-C2 (α_{2B}), and α_2-C4 (α_{2C}). 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 α_{2A}-, chicken α_{2A}-, opossum α_{2C}- and fish α_{2C}-adrenoceptors are orthologs and have been cloned.[58] The rodent α_{2A}-adrenoceptor was initially classified as a new subtype, the α_{2D}-adrenoceptor, but is now acknowledged as the rodent homolog of the human α_{2A}-adrenoceptor subtype.[58] The difference in these two subtypes is their affinity for an α_2-adrenoceptor ligand. The rat α_{2D}-adrenoceptor subtype has a lower affinity for the nonselective α_2-adrenoceptor antagonist yohimbine (**39**) than does the human α_{2A}-adrenoceptor.[58]



Yohimbine (**39**)

3. Pharmacology

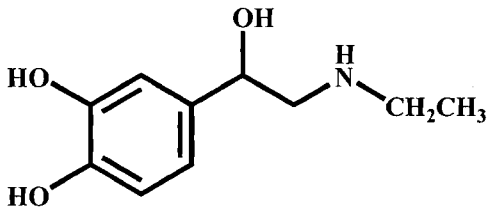
α_2-Adrenoceptors play a major role in mediating several physiological effects in the CNS. The various α_2-adrenoceptor subtypes are distributed throughout the brainstem and spinal cord. In rats and mice, α_2-adrenoceptors are localized throughout the same regions in the CNS.[61] The α_{2A}-adrenoceptor subtype is located in the brainstem, cerebral cortex, locus coeruleus, amygdala, pontine nuclei, nucleus tractus solitarii, dorsal horn, and the dorsal root ganglia.[61,62] The α_{2B}-adrenoceptor subtype is found mainly in the thalamus, and the α_{2C}-adrenoceptor subtype is found in the olfactory bulb, cerebral cortex, hippocampus, striatum, and dorsal root ganglia.[61-63] In humans, the α_{2A}-subtype is distributed in the cerebral cortex, brainstem, hippocampus, amygdala, locus coeruleus, and dorsal root ganglia.[55,64,65] The α_{2B}-adrenoceptor subtype is distributed in the thalamus, dorsal root ganglia, and throughout the spinal cord, and the α_{2C}-adrenoceptor subtype is distributed in the cerebral cortex and lumbar spinal cord.[55,64,65]

Both α_2-adrenoceptor agonists and antagonists have been analyzed to study the functions of α_2-adrenoceptors. There are various functional assays that have been used in order to characterize α_2-adrenoceptor ligands as agonists or antagonists. α_2-Adrenoceptors modulate blood pressure, therefore one *in vivo* assay that has been used is a measurement of pressor responses in a pithed rat model.[66] 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.[66] Then, arterial pressure is monitored by cannulating the right carotid artery. When an α_2-adrenoceptor agonist is administered, it causes a decrease in blood pressure (or a hypotensive effect).[66] Thus an α_2-adrenoceptor antagonist blocks this decrease in blood pressure. An alternative method to studying pressor responses is in anaesthetized rats.[67] 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, α_2-adrenoceptor agonists produce a hypotensive effect.[67] An *in vitro* assay used for α_2-adrenoceptors is contraction of the saphenous vein.[56] In this assay, an α_2-adrenoceptor ligand is administered and if it causes the saphenous vein to contract, it is referred to as an agonist.[56] α_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 α_2-adrenoceptors.

4. Ligands and SAR

a) Agonists

Currently, there are several drug classes that bind at α_2-adrenoceptor subtypes nonselectively. Both nonselective agonists and antagonists have a variety of therapeutic applications. The first drug class found to bind at α_2-adrenoceptors was the phenylethylamines.[68] This class consists of the two endogenous agonists norepinephrine (**36**, K_i = 81 nM) and epinephrine (**37**, K_i = 15 nM).[69] However, these two agonists bind nonselectively at α_1-, α_2-, and β-adrenoceptors. Increasing the steric bulk of the N-substituent to ethyl (**40**), isopropyl (**38**), or tert-butyl (**41**) eliminates affinity for α_2-adrenoceptors (K_i > 10,000).[68] Maintaining the catechol moiety is also important for



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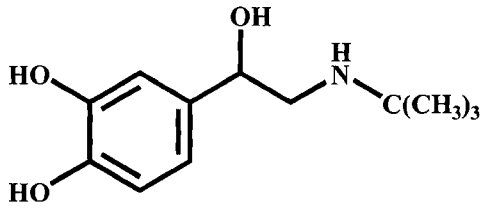
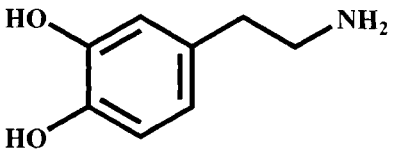


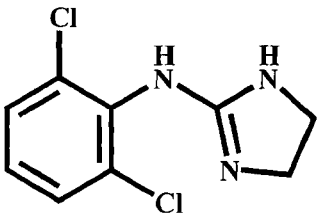
41

binding at these receptors.[68] Removal of the β-hydroxyl group usually reduces the affinity at α_2-adrenoceptors, but dopamine (**42**, K_i = 250 nM) retains high affinity.[68] The phenylethylamines have been shown to possess higher affinity for β-adrenoceptors when compared to α_2-adrenoceptors.

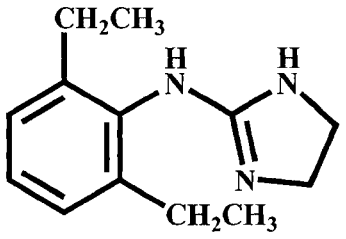


Dopamine (**42**)

The next major class of compounds that binds at α_2-adrenoceptors is the imidazolines. Most of the representative agents in this class are partial agonists, whereas others are either full agonists or antagonists.[68] In the imidazoline class, the presence of a catechol moiety results in high affinity agents but is not required for binding at α_2-adrenoceptors.[68] An important nonselective α_2-adrenoceptor agonist in this class is clonidine (**43**, K_i = 1.8 nM); **43** is an aminoimidazoline.[68,70] In general, the aminoimidazolines are highly selective for α_2-adrenoceptors and when they bear substituents at the 2- and/or 4-positions they can readily cross the blood-brain barrier.[68] An analog of clonidine, ST-91 (**44**), was also found to bind at α_2-adrenoceptors with high affinity as seen with clonidine and also behaves as an α_2-adrenoceptor agonist.[68] Since



Clonidine (**43**)

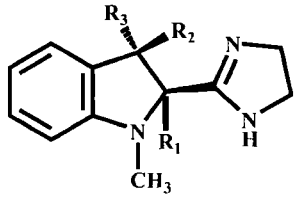


ST-91 (**44**)

clonidine (**43**) possesses such high affinity at α_2-adrenoceptors, its structure has been used as a model to develop novel ligands.[70] For example, Hlasta *et al.*[70] studied a series of indolin-2-yl (Table 5) and tetrahydroquinolin-2-yl imidazolines. It has been suggested that

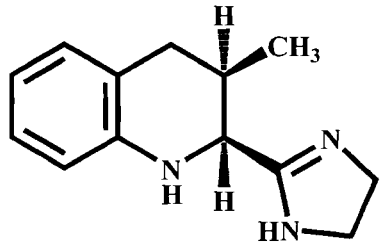
the ortho di-chloro substitution pattern of clonidine (**43**) helps to restrict the imidazoline ring in a position that is perpendicular to the 2,6-dichlorophenyl ring.[70] This conformation is supposed to be responsible for the α_2-adrenoceptor agonist activity of clonidine.[70] It was shown that the *cis*-indoline **45** (K_i = 6.9 nM) possesses high affinity that was comparable with clonidine. The *trans*-indoline **46** (K_i = 72.3 nM), which is less sterically hindered, showed a decrease in affinity at α_2-adrenoceptors.[70] Various heteroatoms were introduced into the side chain that was added to the indoline at the 2-position.[70] These additions to the indoline resulted in high affinity for these agents at α_2- adrenoceptors.[70] Ring expansion of the indolines to, for example *cis*-tetrahydroquinoline **47** (K_i >10,000 nM) resulted in elimination of affinity.[70]

Table 5. Observed Binding Affinities of Indolin-2-yl imidazolines at α_2-Adrenoceptors.[70]



45-46 and 48-53

	R_1	R_2	R_3	K_i, nM
45	H	CH_3	H	6.9
46	H	H	CH_3	72.3
48	CH_3	H	H	3.4
49	$CH_2CH{=}CH_2$	H	H	77
50	$CH_2CH_2CH_2$	H	H	260
51	CH_2OCH_3	H	H	38
52	CH_2SCH_3	H	H	13.2
53	$CH_2N(i\text{-}Pr)_2$	H	H	25.1
clonidine				1.8

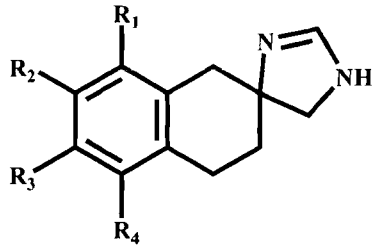


47

It has also been suggested that the structure of clonidine (**43**) has to be in a specific conformation to interact with the receptor.[71] 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.[71] This conformation prevents any resonance interaction between the aromatic portion and the imidazoline ring.[71] 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 α_{2A}-adrenoceptors with high affinity (K_i = 34 nM) and at α_{2B}-adrenoceptors with decreased affinity (K_i = 275 nM).[71] Adding a fluoro group at the R_1 position increased the binding affinity at both α_{2A}- and α_{2B}-adrenoceptors (**55**, K_i = 12 and 152 nM, respectively).[71] Chloro groups in the R_2 and R_4 positions also increased the affinity at both α_{2A}- and α_{2B}-adrenoceptors (**56**, K_i = 3 and 95 nM, respectively).[71] Adding an electron donating group in the R_1 position, a methoxy group (i.e., **57**), decreased the affinity at the α_{2A}-adrenoceptors (K_i = 51 nM) but had a similar affinity at α_{2B}-adrenoceptors (K_i = 99 nM) when compared to **56**.[71] A methoxy group in the R_2 position (i.e., **58**) increased the affinity at α_{2A}-adrenoceptors but decreased the affinity at α_{2B}-adrenoceptors (K_i= 18 and 193 nM, respectively).[71] More analogs were studied and

it was shown that this conformation might be one of the ways that clonidine (**43**) binds at both α_{2A}- and α_{2B}-adrenoceptors.[71]

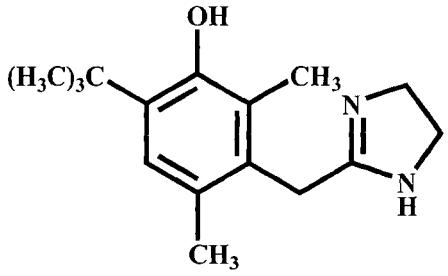
Table 6. Observed Binding Affinities of Imidazolines at α_{2A}- and α_{2B}-Adrenoceptors.[71]



					K_i, nM	
	R_1	R_2	R_3	R_4	α_{2A}	α_{2B}
54	H	H	H	H	34	275
55	F	H	H	H	12	152
56	H	Cl	H	Cl	3	95
57	CH_3O	H	H	H	51	99
58	H	CH_3O	H	H	18	193
59	H	H	CH_3O	H	87	1200
60	H	H	H	CH_3O	12	159
61	H	CH_3	H	H	7	50

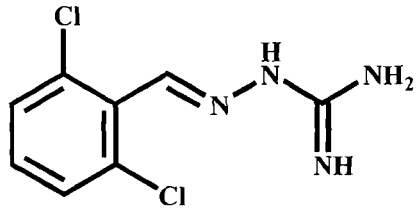
Oxymetazoline (**62**) is another example of an imidazoline but it shows selectivity among the α_2-adrenoceptor subtypes. Oxymetazoline (**62**) binds with higher affinity at α_{2A}-adrenoceptors (K_i = 5.6 nM) than at α_{2B}- and α_{2C}-adrenoceptors (K_i = 350 and 72 nM,

respectively).[69] This agent is one of the few selective α_{2A}-adrenoceptor agonists that has been developed. Although oxymetazoline **(62)** is selective among the α_2-adrenoceptor subtypes, it also binds at α_1-adrenoceptors (K_i = 29.8 nM).[69]

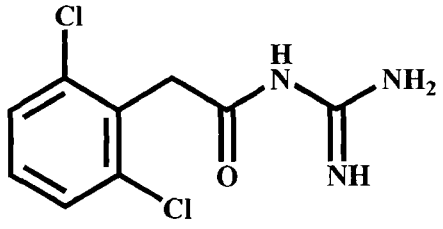


Oxymetazoline **(62)**

The substituted guanidines are a small class consisting of a couple of ligands that bind at α_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).[68] It has been shown that the substituted guanidines also bind at another receptor population, the imidazoline receptors.[68]

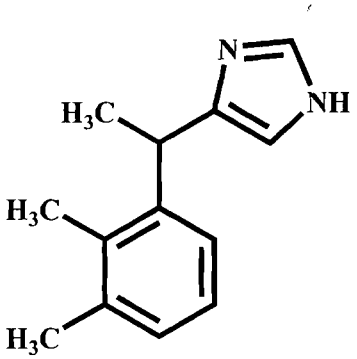


Guanabenz **(63)**



Guanfacine **(64)**

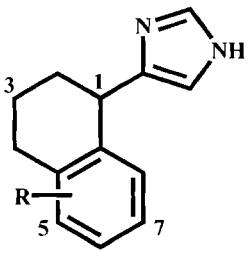
An analog of the imidazolines, medetomidine (**65**, K_i = 25 nM), is a highly potent nonselective α_2-adrenoceptor agonist.[68] A series of conformationally restricted analogs of medetomidine was developed and analyzed as possible α_2-adrenoceptor agonists.[72] For



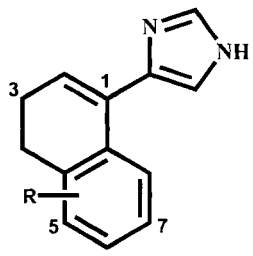
Medetomidine **(65)**

example, tetralin **67** (Table 7) displayed about 4-fold lower affinity than **65**. In this tetralin series, the 5-methoxy analog retained affinity at α_2-adrenoceptors (**66**, K_i = 98 nM).[72] Other analogs are shown in Table 7.[72] 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).[72] A methyl group (**73**, K_i = 31 nM) in the 5-position resulted in similar affinity as medetomidine (**65**).[72] However adding methyl groups in the 5- and 7-positions resulted in a decrease in affinity (**74**, K_i = 232 nM).[72] 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).[72] The 4-methylindan analog **77** (K_i = 8.8 nM) displayed enhanced affinity relative to **65**.[72]

Table 7. Observed Binding Affinities of Conformationally Restricted Analogs of Medetomidine at α_2-Adrenoceptors.[72]



66-71



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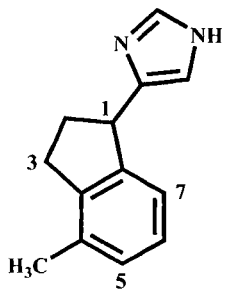
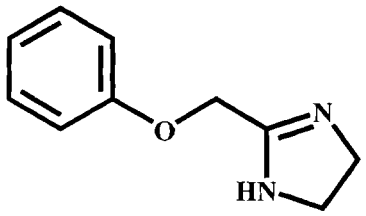


77

	R	K_i, nM
66	5-OCH_3	98
67	5-CH_3	95
68	5-OH	63
69	6-OCH_3	169
70	7-OCH_3	201
71	5,7-diCH_3	103
72	5-OCH_3	1420
73	5-CH_3	31
74	5,7-diCH_3	232
75	6-OCH_3	580
76	7-OCH_3	1570
77		8.8

In a previous study, Gentili *et al.*[73] showed that modifying the structure of imidazoline compounds resulted in a significant difference in their affinity at α_2-adrenoceptors. It was found that imidazolines **78** and **79** were antagonists at α_2-adrenoceptors and weak agonists at α_1-adrenoceptors.[73] From this study, biphenyline (**80**) was also developed and found to be selective for α_2-adrenoceptors and also behaved as an α_2-adrenoceptor agonist in *in vitro* assays.[73] Biphenyline (**80**) was obtained by introducing a phenyl ring in the *ortho* position of the 2-[1-(phenoxy)ethyl]-imidazoline basic structure.[73] It was suggested that this phenyl group might be responsible for the agonist



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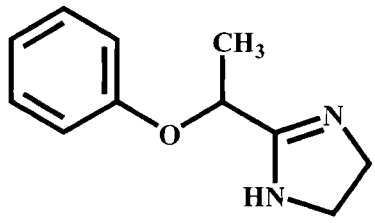

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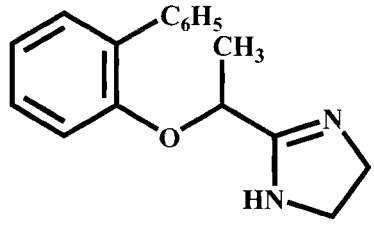
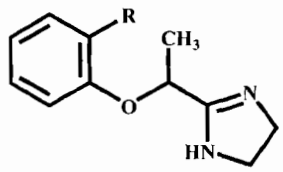
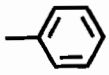
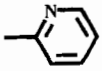
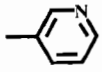
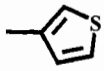
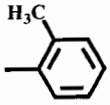
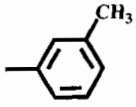
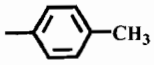
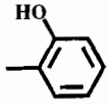
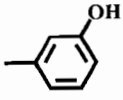
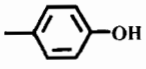
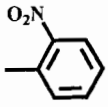


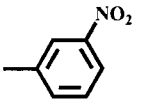
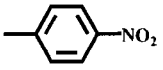
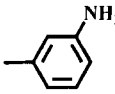
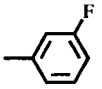
Biphenyline (**80**)

activity seen with biphenyline (**80**).[73] Various modifications were made to the phenyl ring in the *ortho* position and resulted in a series of biphenyline analogs (Table 8).[73] The affinity and intrinsic activity were measured for these biphenyline analogs as well as biphenyline (**80**).[73] Biphenyline (**80**) was found to bind with higher affinity at α_{2A}-adrenoceptors when compared to α_{2B}- and α_{2C}-adrenoceptors (K_i = 47, 501, and 199 nM, respectively).[73] It also behaved as an agonist at both α_{2A}- and α_{2C}-adrenoceptors and a partial agonist at α_{2B}-adrenoceptors.[73] Therefore a major focus for this study was to modify the phenyl group in order to develop subtype selective α_2-adrenoceptor agonists. The first approach was isosteric substitution with a pyridine or thiophene nucleus.[73] The pyridine derivatives **81** and **83** resulted in lower affinity at α_2-adrenoceptors when compared to

Table 8. Observed Binding Affinities of Biphenyline Analogs at α_2-Adrenoceptor Subtypes.[73]



	R	K_i, nM α_{2A}	α_{2B}	α_{2C}	ia α_{2A}	α_{2B}	α_{2C}
80		47	501	199	0.70	0.50	0.80
81		1202	3311	1259	NA	NA	NA
82		138	933	64	0.5	0.5	1.00
83		282	2692	1445	NA	NA	NA
84		17	214	72	0.8	0.6	0.8
85		46	417	132	0.7	NA	0.85
86		47	832	234	NA	NA	0.45
87		51	537	245	NA	NA	0.6
88		42	398	251	NA	NA	0.46
89		309	1000	1622	NA	NA	NA
90		28	355	18	NA	0.5	1.15
91		34	813	331	NA	NA	NA
92		174	1549	1230	NA	NA	NA

93 	60	661	234	NA	NA	0.6
94 	123	501	302	NA	NA	NA
95 	37	479	309	0.45	NA	0.5
96 	15	269	17	0.58	NA	1.15

ia = intrinsic activity, NA = not active (ia < 0.3)

biphenyline (**80**) and were inactive at all three subtypes.[73] However, **82** possessed higher affinity at α_2-adrenoceptors than the other pyridine derivatives and showed selectivity at α_{2C}-adrenoceptors.[73] Compound **82** behaved as an agonist at α_{2C}-adrenoceptors and a partial agonist at the other subtypes.[73] The thiophene nucleus possesses aromaticity that is comparable to that of a phenyl ring.[73] Both **84** and **85** behaved as agonists at α_{2A}- and α_{2C}-adrenoceptors and showed more selectivity in affinity for the α_{2A}-adrenoceptor.[73] 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 α_{2A}- or α_{2B}-adrenoceptors but behaved as partial agonists at α_{2C}-adrenoceptors.[73] 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 nM, ia = 1.15) that behaved as a full agonist with high affinity at α_{2C}-adrenoceptors.[73] 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

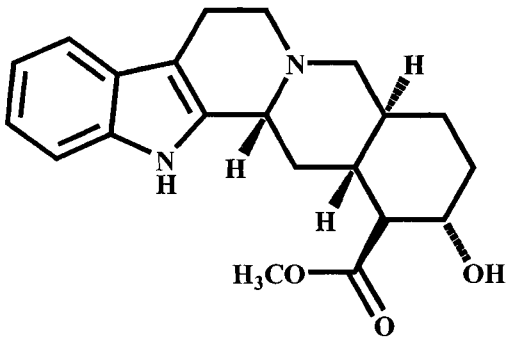
para positions (**92**, **93**, and **94**, respectively).[73] All three nitro derivatives were inactive at α_{2A}- and α_{2B}- adrenoceptors.[73] However, the *m*-nitro derivative **93** was the only nitro derivative that showed any activity at α_{2C}-adrenoceptors.[73] Since the *meta* position seemed to be optimal for agonist activity, several other substituents were introduced at this position. An amino derivative **95** possessed moderate affinity for all three α_2-adrenoceptor subtypes but behaved as a partial agonist at α_{2A}- and α_{2C}- adrenoceptors.[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 α_{2C}-adrenoceptors and as a partial agonist at α_{2A}-adrenoceptors.[73] This was the first series of α_2-adrenoceptor ligands that showed subtype selectivity for α_{2C}-adrenoceptors

Currently, there are available α_2-adrenoceptor agonists that bind nonselectively at the α_2-adrenoceptor subtypes. However, there are still only a limited number of agonists that show subtype selectivity for α_{2A}-adrenoceptors versus α_{2B}- and α_{2C}-adrenoceptor subtypes. Although these agonists show subtype selectivity among the α_2-adrenoceptor subtypes, they also bind at other receptor populations (i.e., 5-HT and α_1-adrenoceptors). Still, there is no strictly subtype-selective α_{2A}-adrenoceptor agonist that has been developed. There are also no subtype-selective α_{2B}-adrenoceptor agonists available. Recently, subtype selective α_{2C}-adrenoceptor agonists have been developed and might be used to determine the physiological effects of α_{2C}-adrenoceptors.[73] Selective α_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 α_2-adrenoceptor

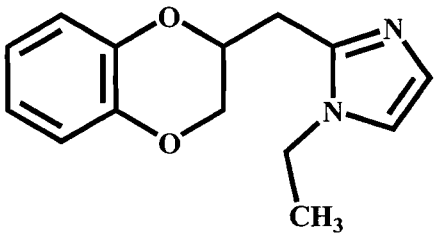
subtypes. There is an increasing clinical, and basic science, need for selective α_2-adrenoceptor subtype-selective agonists.

b) Antagonists

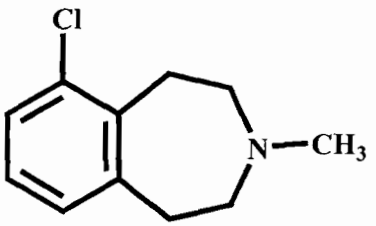
Many α_2-adrenoceptor antagonists have been developed over the years. These antagonists have been used to classify the α_2-adrenoceptor subtypes via radioligand binding studies. α_2-Adrenoceptor antagonists come from a variety of chemical classes. Some representative α_2-adrenoceptor antagonists include yohimbine (**39**), rauwolscine (**97**), imiloxan (**98**), and SK&F 86466 (**99**). Yohimbine (**39**), an indole alkaloid that has been isolated from *Pausinystlia yohimbe* bark and *Rauwolfia* root, has been used in herbal medicines for centuries.[1]



Rauwolscine (**97**)

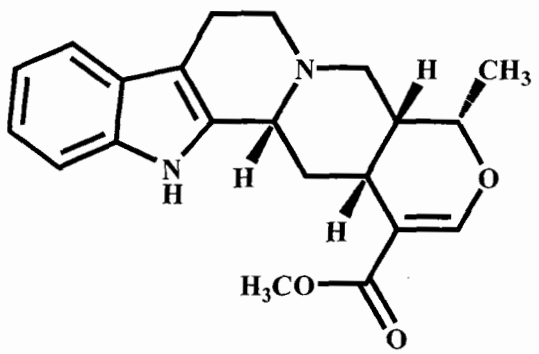


Imiloxan (**98**)

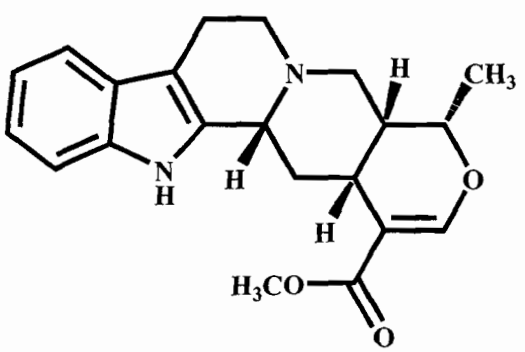


SK&F 86466 (**99**)

Yohimbine (**39**) is an α_2-adrenoceptor antagonist that binds at the α_{2A}-adrenoceptor subtype (K_i = 7.5 nM), α_{2B}-adrenoceptor subtype (K_i = 4.6 nM), and α_{2C}-adrenoceptor subtype (K_i = 2.3 nM) with similar affinity.[68] Since yohimbine (**39**) binds at α_2-adrenoceptors with such high affinity, other yohimbine derivatives were examined and characterized as α_2-adrenoceptor antagonists. Rauwolscine (**97**) is also an alkaloid that belongs to the same family as yohimbine, the yohimbanes.[68] Rauwolscine (**97**) binds at the α_2-adrenoceptor subtypes nonselectively: α_{2A}-adrenoceptor subtype (K_i = 4.6 nM), α_{2B}-adrenoceptor subtype (K_i = 4.7 nM), and α_{2C}-adrenoceptor subtype (K_i = 1.0 nM), and with an affinity comparable to that of yohimbine (**39**).[68] Several other analogs of yohimbine, raubasine (**100**) and akuammingine (**101**), are also selective α_2-adrenoceptor antagonists.[68] Both of these analogs have been isolated from the same sources as



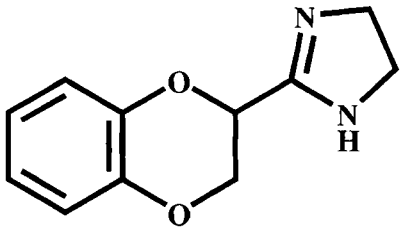
Raubasine (**100**)



Akuammingine (**101**)

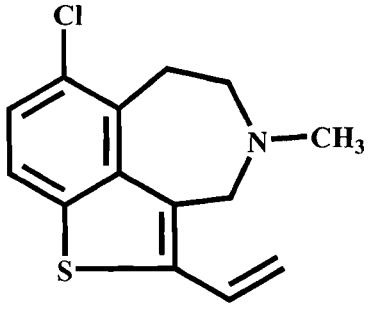
yohimbine (**39**) and are known as heteroyohimbines.[68] Raubasine (**100**) binds at all of the α_2-adrenoceptor subtypes with high affinity: α_{2A}-adrenoceptor subtype (K_i = 8.2 nM), α_{2B}-adrenoceptor subtype (K_i = 14.5 nM), and α_{2C}-adrenoceptor subtype (K_i= 5.0 nM).[68] Akuammingine (**101**) binds at each α_2-adrenoceptors with lower affinity than yohimbine (**39**): α_{2A}-adrenoceptor subtype (K_i = 106 nM), α_{2B}-adrenoceptor subtype (K_i = 116 nM), and α_{2C}-adrenoceptor subtype (K_i = 28 nM).[68]

Imiloxan (**98**) is an α_2-adrenoceptor antagonist that belongs to another class, the benzodioxans.[74] Imiloxan (**98**) is a benzodioxanyl N-ethylimidazole that binds selectively at the α_{2B}-adrenoceptor subtype (K_i = 136 nM) compared to α_{2A}- and α_{2C}-adrenoceptor (K_i = 1701 and 1056 nM respectively).[75] Imiloxan (**98**) has been used to characterize the physiological functions of α_{2B}-adrenoceptors, since it is the only available α_{2B}-adrenoceptor-selective antagonist. When imiloxan (**98**) originally was implicated as a selective α_{2B}-adrenoceptor antagonist, its binding activity was studied only at α_{2A}- and α_{2B}-adrenoceptors.[76,77] However, it has been shown that imiloxan (**98**) blocks the antinociceptive activity of the α_2-adrenoceptor agonist, ST-91 (**44**), whose actions are thought to be mediated primarily through the α_C-adrenoceptor subtype.[78] Currently, there is great controversy for which α_2-adrenoceptor subtype imiloxan (**98**) is truly selective: α_{2B}- or α_{2C}-adrenoceptors. Idazoxan (**102**), a benzodioxanyl imidazoline, is also an α_2-adrenoceptor antagonist. Unlike imiloxan (**98**), idazoxan (**102**) binds nonselectively at the α_2-adrenoceptor subtypes: α_{2A}-adrenoceptor subtype (K_i = 21 nM), α_{2B}-adrenoceptor subtype (K_i = 43 nM), and α_{2C}-adrenoceptor subtype (K_i = 35 nM).[59]

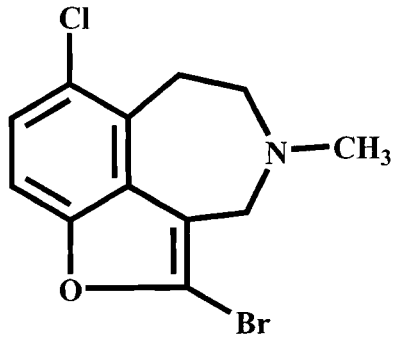


Idazoxan (**102**)

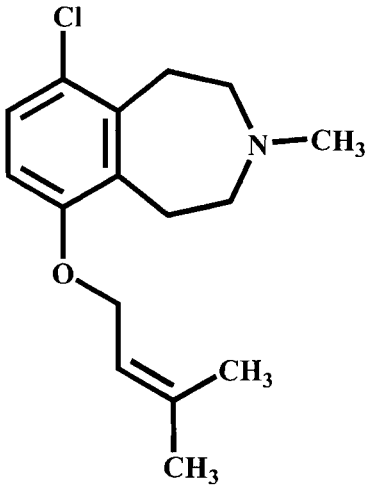
Another class of α_2-adrenoceptor antagonists are the benzazepines (Table 9).[68] SK&F 86466 (**99**) shows moderate selectivity for α_2-adrenoceptors versus α_1-adrenoceptors, but binds at the α_2-adrenoceptor subtypes nonselectively: α_{2A}-adrenoceptor subtype (K_i = 9.4 nM), α_{2B}-adrenoceptor subtype (K_i = 16 nM), and α_{2C}-adrenoceptor subtype (K_i = 20 nM).[68] SK&F 86466 (**99**) has been used to subclassify α_2-adrenoceptor physiological responses in functional assays.[68] A heterofused analog, SK&F 104856 (**103**) shows approximately 8-fold selectivity for the α_{2B}-adrenoceptor subtype (K_i = 3.4 nM), when compared to α_{2A}-adrenoceptor subtype (K_i = 24 nM) and α_{2C}-adrenoceptor subtype (K_i = 21 nM).[68] Several related 3-benzazepine derivatives have been developed; some are shown in Table 9.[68]



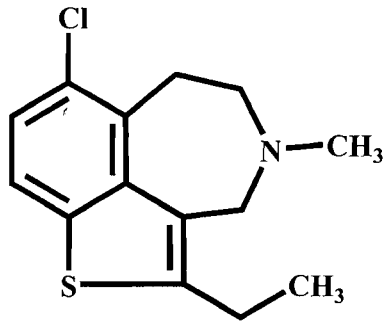
SK&F 104856 (**103**)



SK&F 105854 (**104**)



SK&F 104078 (**105**)



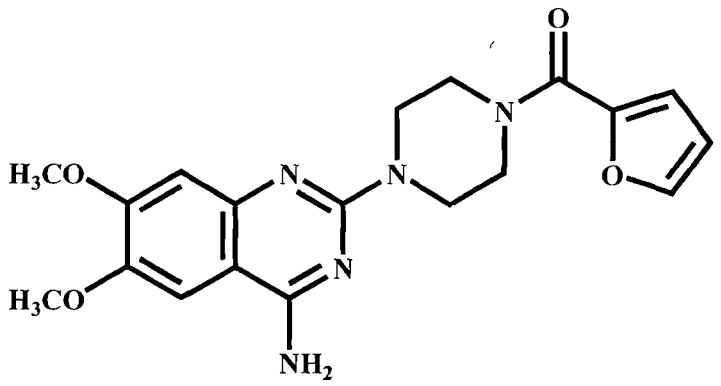
SK&F 106686 (**106**)

Table 9. Observed Binding Affinities of 3-Benzazepine Derivatives at α_2-Adrenoceptor Subtypes.[68]

	K_i, nM		
	α_{2A}	α_{2B}	α_{2C}
SK&F 86466 (**99**)	9.4	16	20
SK&F 104856 (**103**)	24	3.4	21
SK&F 105854 (**104**)	54	14	28
SK&F 104078 (**105**)	114	142	64
SK&F 106686 (**106**)	31	15	35

A well-known α_2-adrenoceptor antagonist belongs to a group, the quinazolines, that mostly behaves as α_1-adrenoceptor antagonists. Quinazolines do not discriminate between the α_1-adrenoceptors but several, including prazosin (**107**), have been shown to bind at α_2-adrenoceptors. Evidence shows that prazosin (**107**) binds selectively at both α_{2B}- and α_{2C}-

adrenoceptors with high affinity (K_i = 30.6 and 10.7 nM, respectively) when compared to α_{2A}-adrenoceptors (K_i = 302 nM).[68]



Prazosin **(107)**

In general, a number of α_2-selective antagonists have been identified, but with the exception of imiloxan (**98**), few show selectivity within the α_2-adrenoceptor family. Furthermore, it is currently unknown whether imiloxan is an α_{2B}, α_{2C} or α_{2B}/α_{2C}-selective antagonist.

c) Therapeutic Application of α_2-Adrenoceptor Agents

α_2-Adrenoceptor agonists and antagonists have a wide range of therapeutic applications. α_2-Adrenoceptor agonists have been used in the treatment of hypertension for many years.[1] This is due to the fact that α_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, α_2-

adrenoceptors and imidazoline receptors, to lower and maintain blood pressure.[79] However, some of the drawbacks of these antihypertensives are that they cause sedation, bradycardia, and mental depression.[79] α_2-Adrenoceptors are also involved with non-insulin-dependent diabetes.[68,79] Activation of α_2-adrenoceptors has been shown to inhibit the secretion of insulin from pancreatic islet cells.[68,79] Several α_2-adrenoceptor antagonists, rauwolscine (**97**) and idazoxan (**102**), enhanced glucose-induced insulin secretion in the rat.[68,79] Yohimbine (**39**) increases plasma insulin, and inhibits epinephrine-induced hyperglycemia in mice.[68,79] Therefore, blocking α_2-adrenoceptors seems to play a role in selectively enhancing glucose-stimulated insulin secretion in this type of diabetes.[79]

Another possible target of α_2-adrenoceptors' therapeutic applications is obesity. It has been shown that stimulating α_2-adrenoceptors causes an inhibition of lipolysis in isolated human adipocytes. This stimulation of α_2-adrenoceptors could possibly be used to promote weight loss.[68,79] In rodents, SK&F 86466 (**99**) can block α_2-adrenoceptors which then stimulates epinephrine-induced lipolysis.[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.[68,79] Since activation of central α_2-adrenoceptors results in sedative effects, α_2-adrenoceptor agonists have been clinically used in anesthesia.[68] Clonidine and other α_2-adrenoceptor agonists are being used as adjuncts to general anesthesia.[68,79] α_2-Adrenoceptor agonists, detomidine and medetomidine (**65**), are being used as veterinary anesthetics.[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 α_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] α_2-Adrenoceptor agonists produce antinociception through the activation of the noradrenergic system.[80] Clonidine (**43**), the most studied α_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 α_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 α_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 α_2-adrenoceptors causes hyperpolarization of neurons by opening K^+ channels, which seems to share a role in the antinociception produced by α_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**).[83] α_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**).[84] This study suggested that 5-HT-induced antinociception might be dependent on the descending noradrenergic system. In another study, Svokos *et al.*[85] 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.[85] However, i.c.v. administered yohimbine (**39**) did not attenuate the antinociceptive activity of improgan.[85] These results showed that α_2-adrenoceptors play a role in antinociception induced by histamine receptors but only the spinal α_2-adrenoceptors were involved. α_2-Adrenoceptors might also be involved in $GABA_B$ receptor-induced antinociception. Sabetkasai *et al.*[86] 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**).[86] This study further demonstrated the various systems that induce antinociception might be influenced by the noradrenergic system (Figure 7).

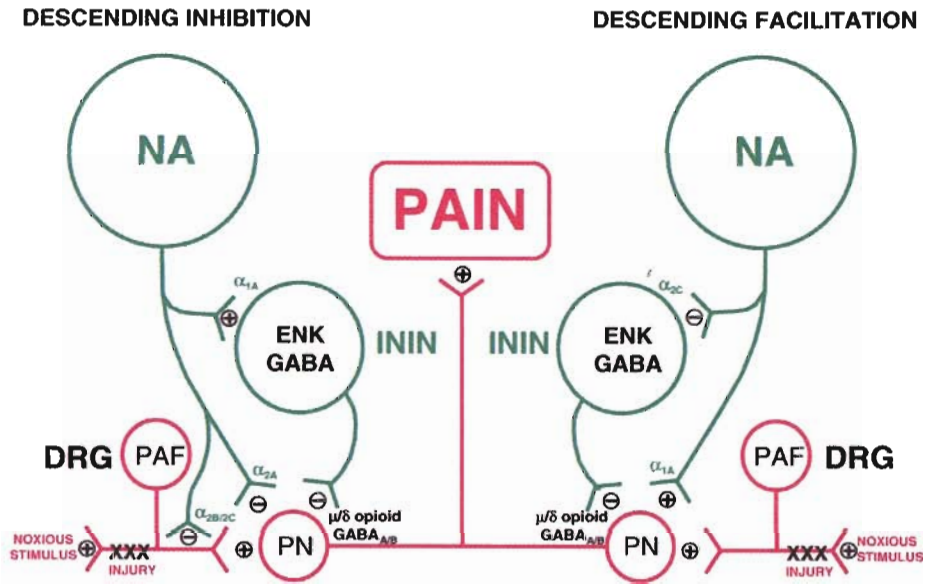
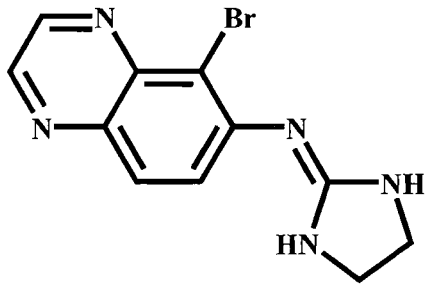


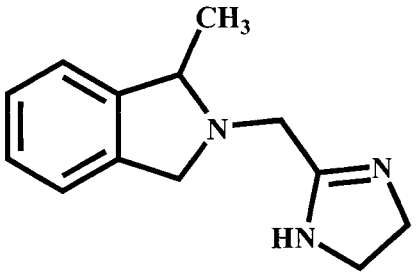
Figure 7. Schematic diagram of noradrenergic system modulating nociception.[9] This figure is used to show the involvement of the noradrenergic system with other neurotransmitters.

Thermal nociceptive tests were also used to further understand which α_2-adrenoceptor subtypes might be involved in antinociception mediated by α_2-adrenoceptors. In a study by Millan,[87] s.c. administered UK 14,304 (**108**), an α_2-adrenoceptor agonist, produced antinociceptive activity in mice in the hot-plate test. Several α_2-adrenoceptor antagonists, both nonselective or subtype selective, were used to study α_2-adrenoceptor subtype involvement.[87] The nonsubtype selective α_2-adrenoceptor antagonist idazoxan (**102**) blocked the antinociceptive activity of UK 14,304 (**108**).[87] Both BRL 44408 (**109**) and RX 821002 (**110**), α_{2A}-adrenoceptor antagonists, also blocked UK 14,304-induced antinociception.[87] However, BRL 41992 (**111**) and ARC-239 (**112**) and prazosin (**107**), selective α_{2B}- and α_{2C}-adrenoceptor antagonists, did not have any effect on the

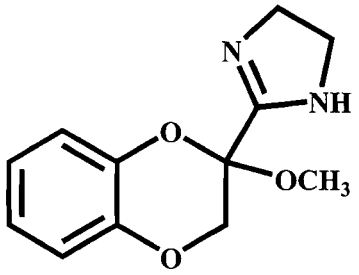
antinociceptive activity of UK 14,304.[87] This study suggested that among the supraspinal α_2-adrenoceptor subtypes, only α_{2A}-adrenoceptors are involved with antinociception induced by α_2-adrenoceptor agonists.



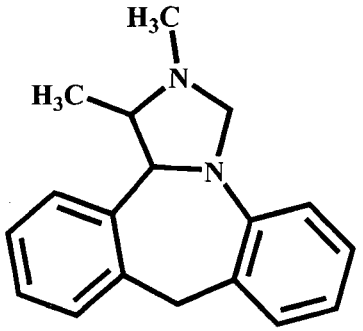
UK-14,304 (**108**)



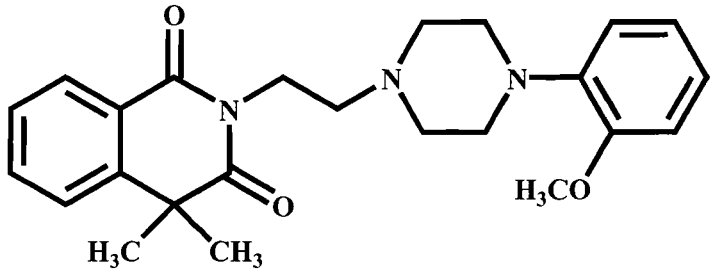
BRL 44408 (**109**)



RX 821002 (**110**)



BRL 41992 (**111**)



ARC-239 (**112**)

Figure 8. Subtype selective α_2-adrenoceptor ligands.[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 α_2-adrenoceptor sites.[81-83] However, it was also shown that the i.t.-administered α_2-adrenoceptor agonist dexmedetomidine was inactive in the hot-plate test but active in the tail-flick test.[88] 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 α_2-adrenoceptors that are located in the brain. This idea was supported by the study by Ossipov *et al.*[81] 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 α_2-adrenoceptors in the antinociception induced by spinally-administered α_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 α_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 α_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.[80] I.t. administered yohimbine (**39**) blocks the antinociceptive activity of i.t.-administered morphine.[89] This evidence further

suggests that α_2-adrenoceptors are involved in morphine-induced antinociception. However, naloxone, an opioid antagonist, did not attenuate the antinociceptive activity of clonidine (**43**).[89] Coadministration of α_2-adrenoceptor agonists and opioid agonists resulted in a greater-than-additive antinociceptive effect, or synergy.[90] These agents could be administered in lower doses than when administered separately to produce an equivalent antinociceptive effect.[90] 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.[90] 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.[89-91] 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.[90] Fairbanks and Wilcox[90] 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.[90] Similar results were shown when the mice were chronically tolerant to i.t.-administered morphine.[90] 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.[92] Evidence shows that the combination of these two analgesics can also be used in postcesarean analgesia.[93] 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.[93] This combination also reduced the opioid requirement and increased intraoperative sedation.[93]

As mentioned earlier, studies have shown that the α_{2A}-adrenoceptors are involved in antinociception. It was recently found that both α_{2B}- and α_{2C}-adrenoceptor subtypes could also play a role in mediating antinociception. Prazosin, which is selective for α_{2B}- and α_{2C}-adrenoceptors, inhibited neurotransmitter release from spinal cord preparations.[78] This supports the idea of possible roles for both α_{2B}- and α_{2C}-adrenoceptors in antinociception. Sawamura *et al.*[94] showed that activation of α_{2B}-adrenoceptors could mediate the antinociceptive activity of nitrous oxide, an adjunctive general anesthetic. In this study, null mice for the α_{2B}-adrenoceptor subtype produced a reduced or absent analgesic response to nitrous oxide.[94]

α_2-Adrenoceptor agonists have been classified as being potent analgesics. Although α_2-adrenoceptor agonists are being used therapeutically as analgesics, they still produce the undesired side effects of sedation, hypotension, and bradycardia. The use of α_2-adrenoceptor agonists in combination with other clinically known analgesics has been

studied over the years. This allows α_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 α_{2A}-adrenoceptor subtype is responsible for the hypotensive, sedative, and bradycardic actions associated with α_2-adrenoceptor agonists.[78] The development of α_2-adrenoceptor agonists that are selective for α_{2B}- and/or α_{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.[95] Also, the term "pain" is poorly defined when related to animals and their behavior.[95] Pain is a subjective and personal psychological experience that cannot be measured directly in animals since an animal cannot describe how it feels.[95] Therefore the term "pain" is usually avoided when describing the study of nociception in animal models,[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.[95] Different species and strain of animals can have a major affect on the test results and is one of the more obvious factors.[95] Another factor is that the time of day that the test is being conducted can produce a variation in results.[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.[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.[95] In thermal nociceptive tests, the stimulatory response is fixed and a standard response is defined.[95] 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.[95]

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.[95,97] The tail-flick test was developed by D'Amour and Smith[99] 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.[95] 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.[95] 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.[95,98] This idea was studied by transecting the spinal cord above the lumbar level.[95] From such studies, it was shown that transection does not block the tail-flick response.[95] 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.[95,96] The tail-flick response is induced when the temperature at the nociceptors in the skin reach a critical level.[95] The tail-flick latency depends strongly on the initial temperature of the skin which is influenced by local

blood flow.[95,96] 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.[96] Skin temperature is critical because reduction in the tail skin temperature can be interpreted as antinociception.[95] This problem can be avoided by recording the tail skin temperature and making corrections to the tail-flick latency accordingly.[95] In contrast, Lichtman *et al.*[100] 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[101] 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.[95, 97] The hot-plate test was later modified and a constant temperature of 55 °C is the temperature that is consistently used today.[95,97] 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.[97] Usually, hindpaw licking latency is used as the end-point.[97] 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.[98] One problem seen in the hot-plate test is that mild analgesics possess no, or weak, antinociceptive activity at a constant temperature of 55

°C.[97] Therefore, the increasing temperature hot-plate test was developed to solve this problem.[95,97,102] In this test, the temperature of the plate is set below the pain threshold and is gradually increased until the animal makes a response.[95] The temperature at which the response occurs is then recorded as the nociceptive endpoint.[95] 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.[95,97] This test is more sensitive than the constant temperature hot-plate test.[95,97] 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.[95,97,102]

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.[100] 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.*[30] that MD-354 (**21**) binds at 5-HT_3 receptors with high affinity (K_i = 35 nM). Additional binding studies showed that this compound also binds at α_2-adrenoceptors with high affinity.[103] MD-354 (**21**) binds selectively at α_{2B}-adrenoceptors (K_i = 25 nM) when compared to α_{2A}- (K_i = 825 nM) and α_{2C}-adrenoceptors (K_i = 140 nM).[103] As such, it might be viewed as the first example of a rather selective 5-HT_3/α_{2B}-adrenoceptor ligand. Because 5-HT_3 receptors and α_{2B}-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.[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.[103] Other doses were evaluated and it was shown that clonidine (**43**) produced a dose-dependent antinociceptive effect (Figure 9).[103]

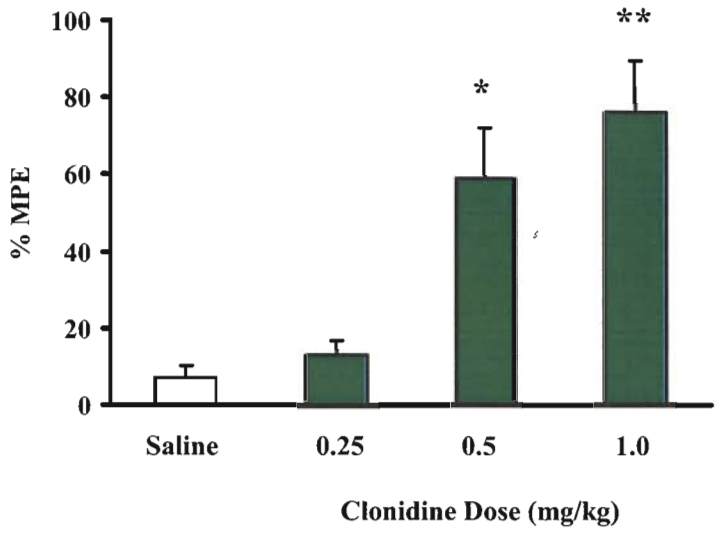


Figure 9. Antinociceptive actions of clonidine following s.c. administration in the mouse tail-flick assay. Asterisks denote significant differences compared to control group (i.e., saline); *P< 0.01 and **P< 0.001.[103]

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).[103] Results were not different using various other pretreatment times. MD-354 (**21**) was also examined as a possible antagonist of clonidine-induced antinociception.[103] 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 ED_{50} dose of clonidine (**43**) administered 20 min prior to testing.[103] Interestingly, a combination of the ED_{50} 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.[103] 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).[103]

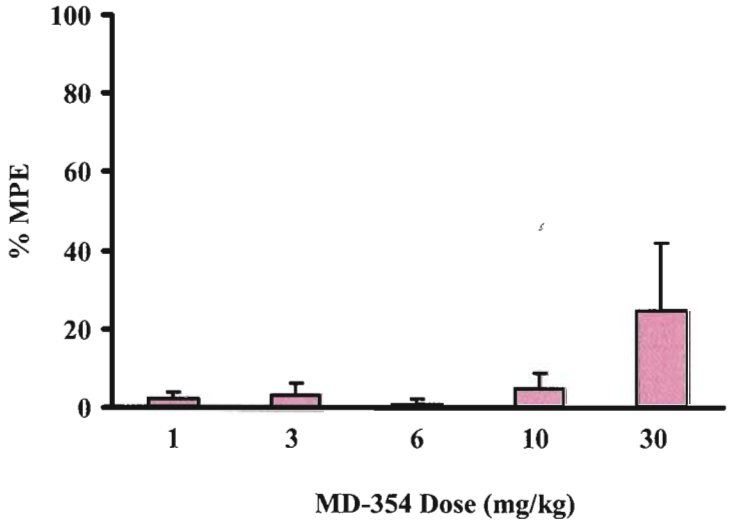


Figure 10. Effect of MD-354 doses administered 45 min prior to testing in the tail-flick assay as compared to saline control.[103]

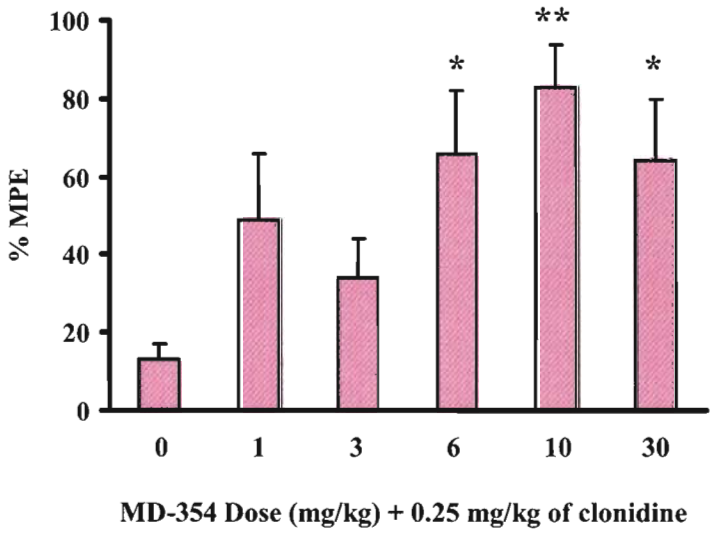
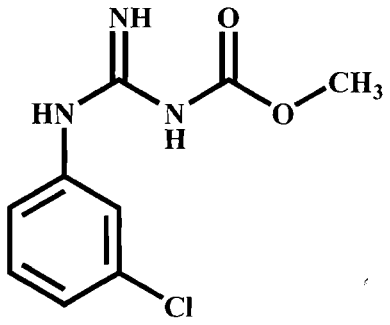


Figure 11. Potentiation of the antinociceptive actions of clonidine (0.25 mg/kg) by MD-354 in the tail-flick assay. Asterisks denote significant differences compared to control group; *P< 0.05 and **P< 0.01.[103]

Mechanistic studies were conducted to determine the mechanism behind the potentiating effect of clonidine antinociception by MD-354 in the mouse tail-flick test.[103] Pretreatment with the putative α_{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.).[103] These results suggest that neither α_{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 α_2-adrenoceptors imiloxan binds, neither α_{2B}- nor α_{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.[103] In a combination study, MD-354 (**21**) did not enhance the antinociceptive action of morphine.[103]

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 α_{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., α_{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.[32] 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.



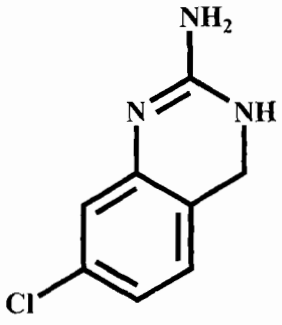
113

Next, mechanistic studies will be conducted to determine which pathway, 5-HT_3 and/or α_{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 α_{2B}-adrenoceptors (K_i = 30 nM). However, unlike MD-354 (**21**), **29** does not show subtype selectivity (α_{2A}, α_{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 α_{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 α_{2B}-adrenoceptors. The 5-HT_3 antagonist tropisetron (**33**), the putative α_{2B}-adrenoceptor antagonist imiloxan (**98**), and the α_2-nonselective adrenoceptor antagonist yohimbine (**39**) will be used to attenuate the potentiating effect of **29**. Compound **29** will

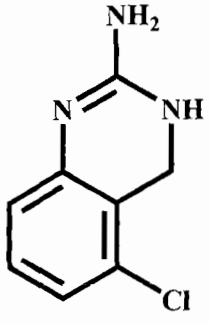
also be evaluated in the hot-plate assay to see if this compound reaches the supraspinal receptors and the results will be compared with those from MD-354 (**21**). Combination studies of **29** and morphine will be conducted to determine if **29** potentiates the antinociceptive effects of morphine. Since clonidine (**43**) has been shown to produce sedation via α_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),[30] but binds nearly equally well at the three different subpopulations of α_2-adrenoceptors (α_{2A}, α_{2B}, α_{2C}; K_i = 177, 152, and 135 nM, respectively). Furthermore, it binds with only several-fold lower affinity than **29** at each of the α_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 α_{2B}-adrenoceptors. Compound **31** has been previously prepared as a conformationally-constrained analog of MD-354.[32] 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.



31



114

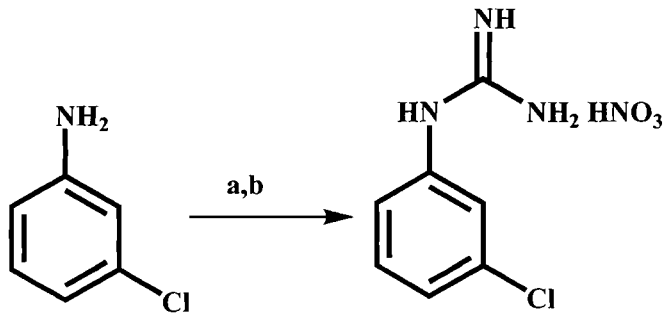
The overall focus of the studies that have been proposed is to determine the mechanism of the potentiating effect of clonidine-induced antinociception by MD-354 and MD-354-type compounds in the mouse tail-flick assay.

IV. Results and Discussion

A. Synthesis

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

The method of Kreutzberger and Tantawy[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 chromatograpy (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.

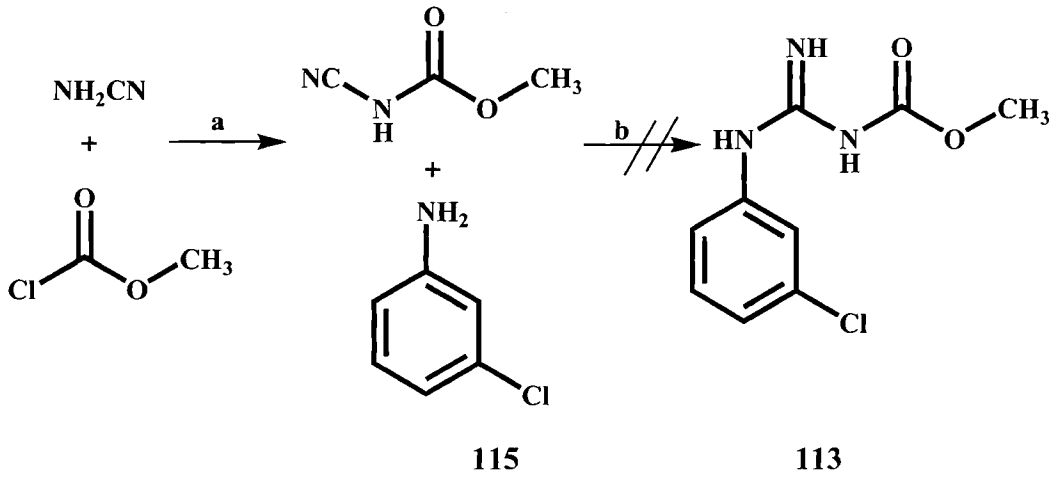


115 **21**

Scheme 1. a. NH_2CN, EtOH, concentrated HCl, reflux; b. NH_4NO_3, H_2O.

b) Carbamate analog of MD-354, **113**

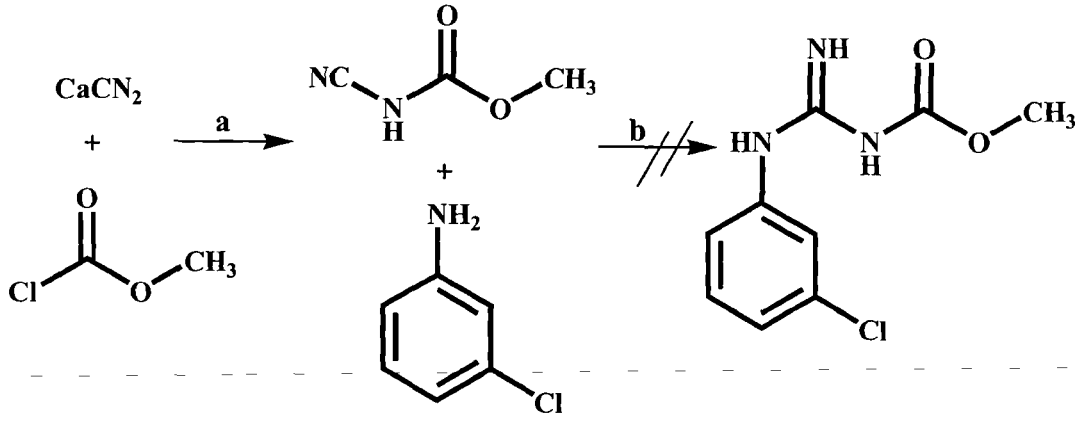
Several procedures were used in attempts to synthesize the carbamate analog of MD-354. The procedure of Goetz and Zeeh[105] (Scheme 2) was first used. Cyanamide in H_2O, methyl chloroformate, and 50% NaOH were combined and the pH was maintained between 7 and 8. An exothermic reaction was supposed to 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



Scheme 2. a. H_2O, 50% NaOH in H_2O; b. concentrated HCl, reflux

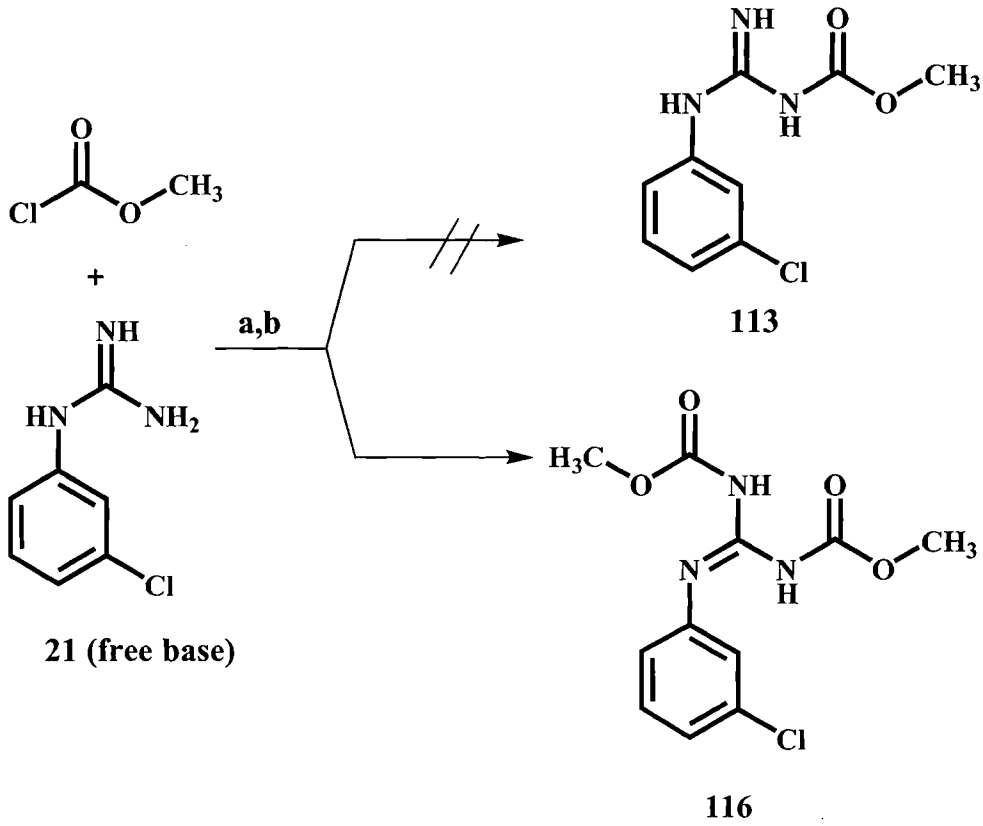
crude product (78-80 °C) did not correspond to the literature melting point of 128-129 °C. The product of this reaction was never identified.

The procedure of Khasanov[106] (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. H_2O, 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.



Scheme 4. a. THF, triethylamine, 0 °C, N_2, 30 min; b. room temperature, 16 h.

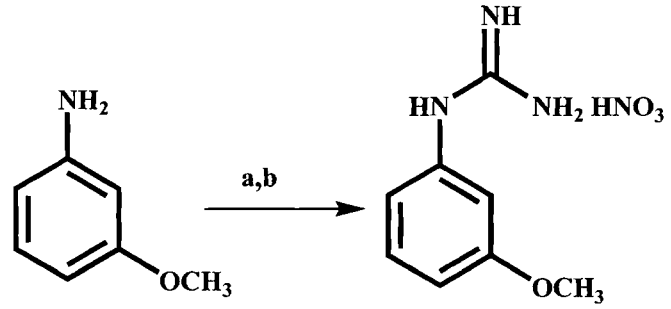
Thin-layer chromatographic analysis of the above reaction mixture indicated the formation of a second, minor product that was suspected to be the desired product. The above procedure was attempted again but the starting material was dissolved in a larger amount of THF. It was hoped that this high-dilution 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[106] to synthesize the carbamate analog of MD-354 (Scheme 3). By using an addition funnel, calcium cyanamide in H_2O 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_2O and a few drops of EtOH. The product was dried using a lyophilizer to give **113** as a white powder in low yield. The ^1H 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 chromatograpy 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_2O and a drop of MeOH to give **26** as a white powder.

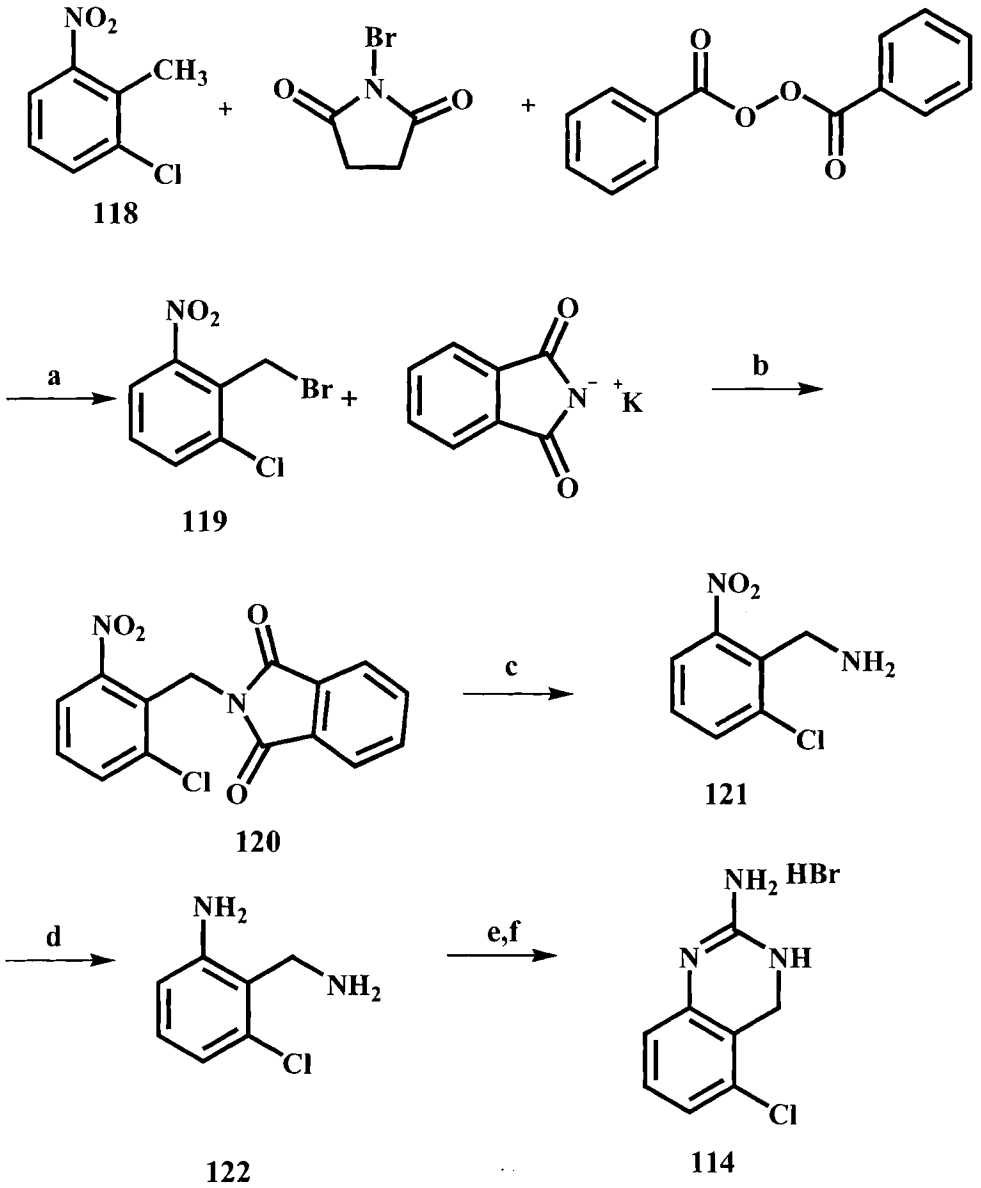


117 **26**

Scheme 5. a. NH_2CN, EtOH, concentrated HCl, reflux; b NH_4NO_3, H_2O

d) A conformationally-constrained analog of MD-354, **114**

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



Scheme 6. a. CCl_4, 250-Watt bulb, reflux; b DMF, reflux; c. H_2NNH_2, MeOH, reflux; d. Raney Nickel, EtOH, H_2; e. CNBr, EtOH, reflux; f. HBr, ether.

peroxide in carbon tetrachloride (CCl_4) 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, $CHCl_3$ was added and the mixture was poured on to H_2O/ice. The $CHCl_3$ portion was washed with 0.2 N NaOH, brine and dried with Na_2SO_4. 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 Et_2O and brine. The Et_2O extract was dried with Na_2SO_4 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 Et_2O, the solution was washed with H_2O and brine, and dried with Na_2SO_4. 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.[107] 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

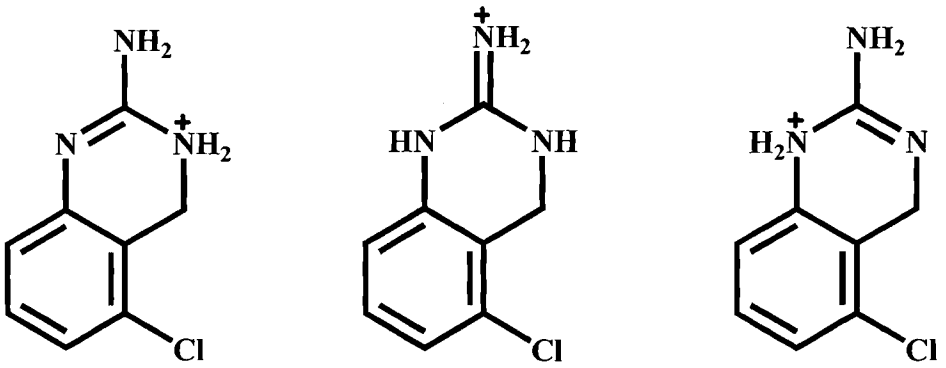
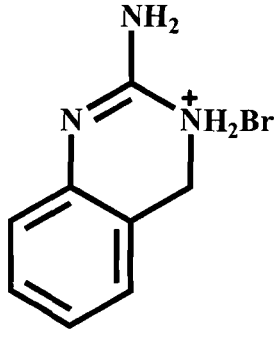


Figure 12. Compound **114** can exist as one of three tautomers.

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 H_2O 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



123

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,

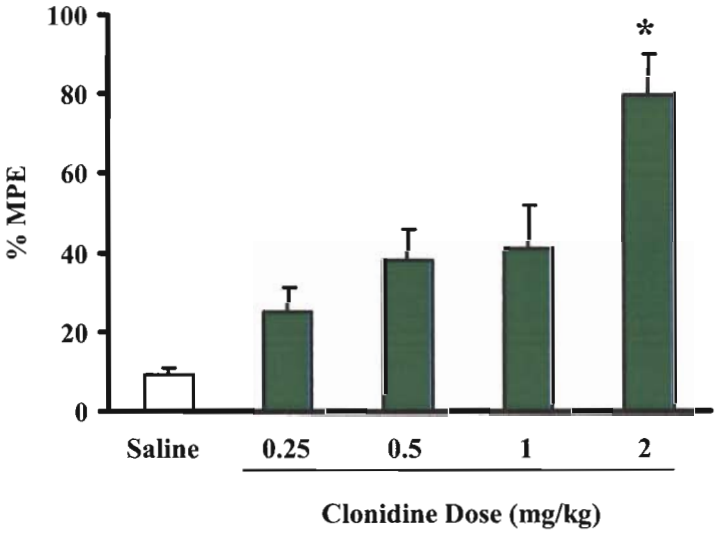


Figure 13. Antinociceptive actions (± S.E.M.) of subcutaneous clonidine in the hot-plate assay (*n* = 5-8 mice/treatment). Asterisks denote significant differences compared to control group; *P< 0.001; one-way ANOVA followed by Newman-Keuls post hoc test.

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).

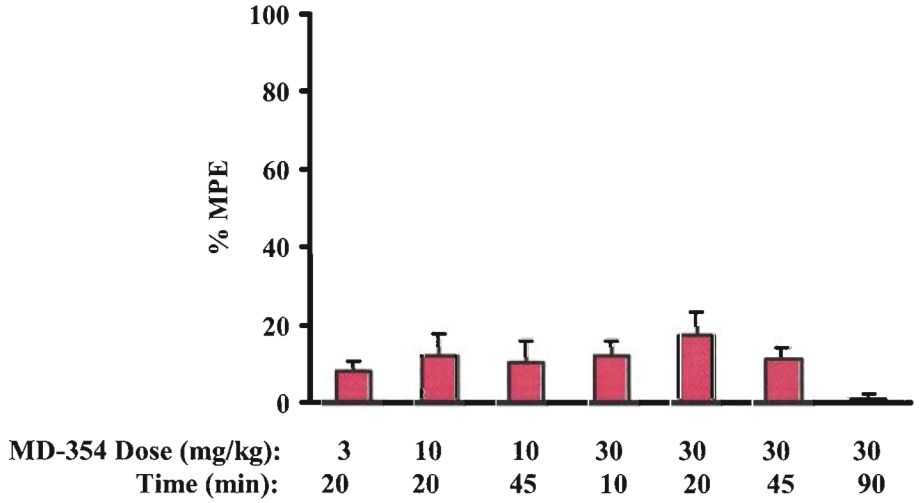


Figure 14. Effect (± S.E.M.) of subcutaneous MD-354 doses administered 20 to 90 min prior to examination in the hot-plate assay (n = 6-16 mice/treatment) as compared to saline control (8 ± 2% MPE).

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,[103] 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

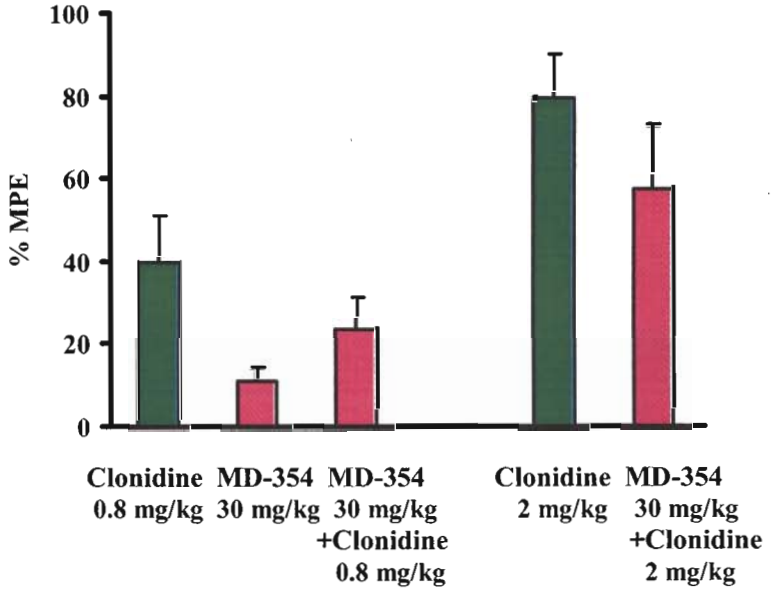


Figure 15. Effect (± S.E.M.) of subcutaneous MD-354 (30 mg/kg) administered in combination with clonidine (0.8 and 2.0 mg/kg; s.c.) in the hot-plate assay (n = 5-14 mice/treatment).

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.

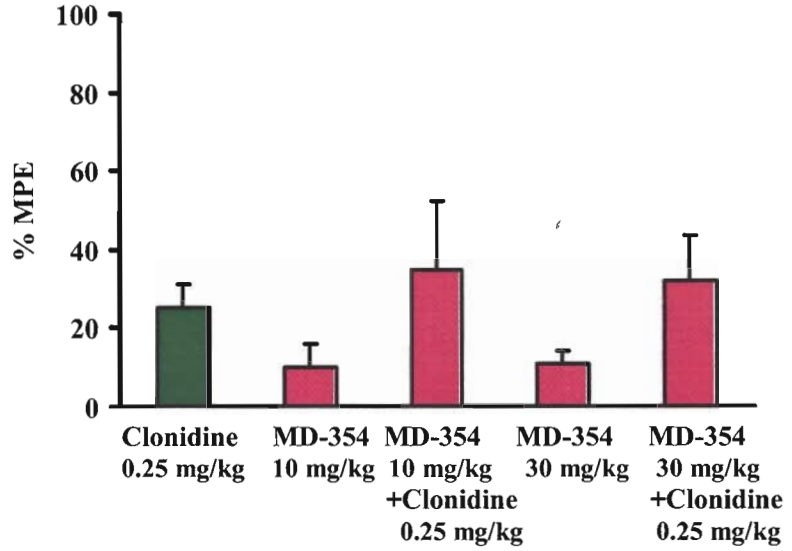


Figure 16. Effect (± S.E.M.) of subcutaneous MD-354 doses administered in combination with subcutaneous clonidine (0.25 mg/kg) in the hot-plate assay (n = 5-26 mice/treatment).

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).

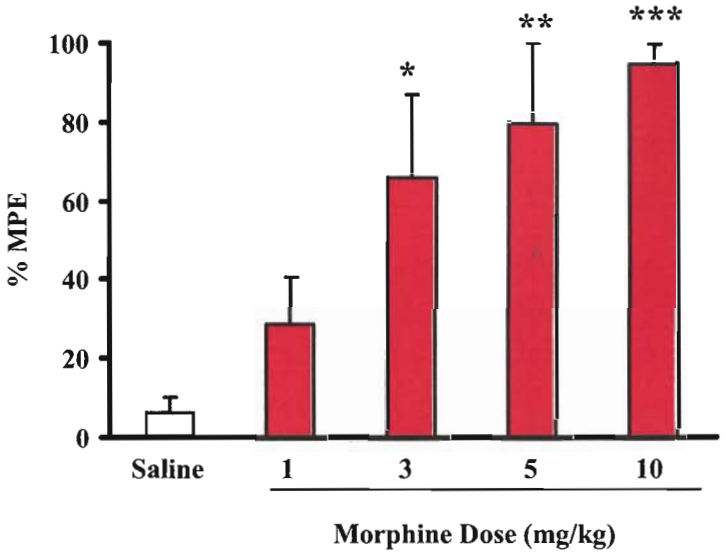


Figure 17. Antinociceptive actions (± S.E.M.) of subcutaneous morphine in the tail-flick assay (*n* = 5-8 mice/treatment). Asterisks denote significant differences compared to control group; *P< 0.05, **P<0.01, and ***P<0.001; one-way ANOVA followed by Newman-Keuls post hoc test.

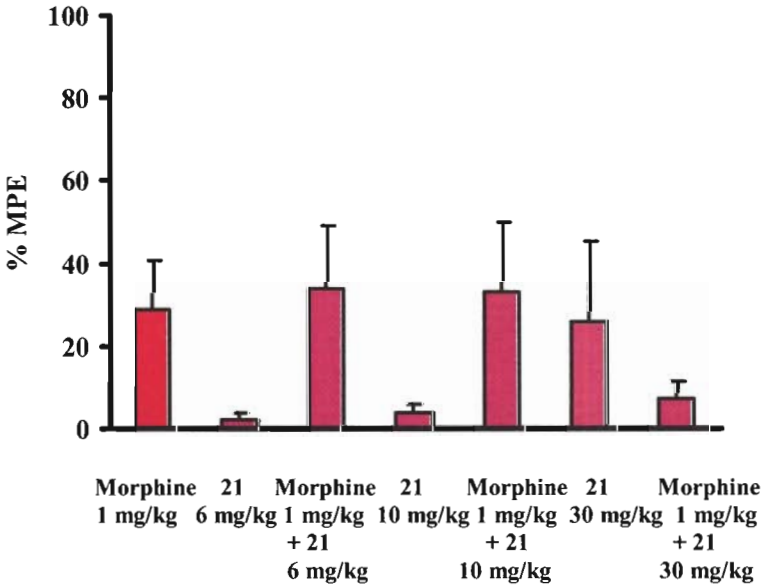


Figure 18. Effect (± S.E.M) of subcutaneous MD-354 (**21**) doses administered alone and in combination with subcutaneous morphine (1 mg/kg) in the tail-flick assay (*n* = 5-11 mice/treatment).

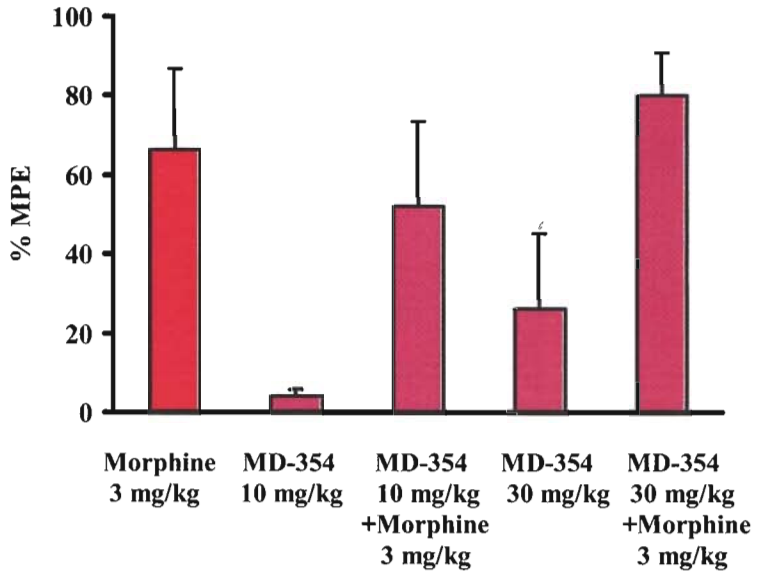


Figure 19. Effect (± S.E.M) of subcutaneous MD-354 doses administered alone and in combination with subcutaneous morphine (3 mg/kg) in the tail-flick assay (n = 5-11 mice/treatment).

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).

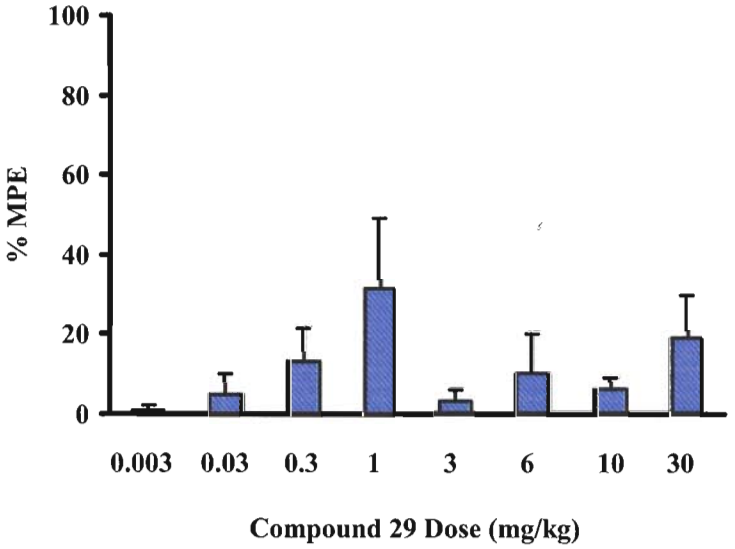


Figure 20. Effect (± S.E.M.) of subcutaneous compound **29** doses administered 45 min prior to examination in the tail-flick assay (n = 6-16 mice/treatment) as compared to saline control (6 ± 4% MPE).

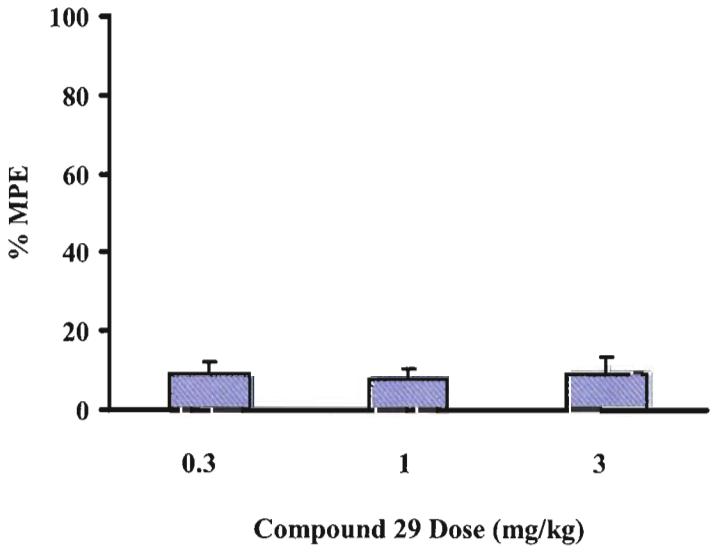


Figure 21. Effect (± S.E.M.) of subcutaneous compound **29** doses administered 45 min prior to examination in the hot-plate assay (n = 6-16 mice/treatment) as compared to saline control (8 ± 2% MPE).

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 ED_{50} 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

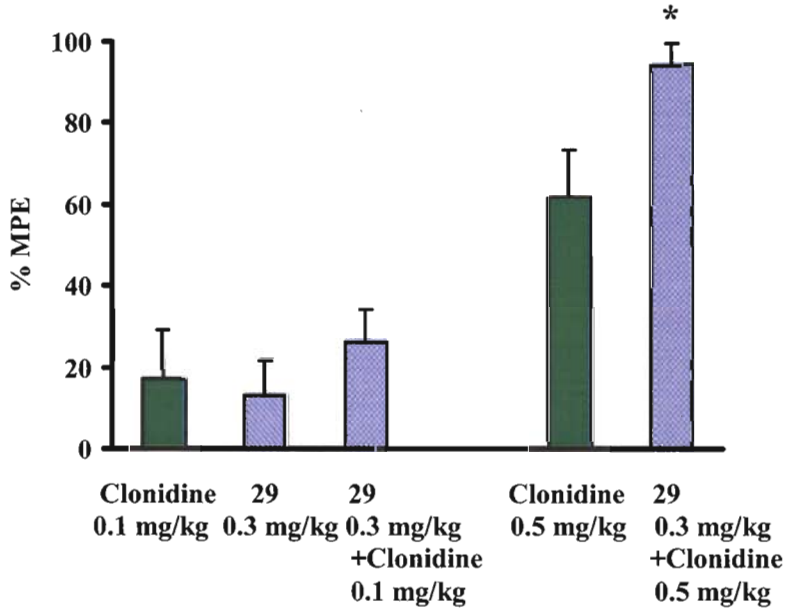


Figure 22. Effect (± S.E.M.) of subcutaneous compound **29** (0.3 mg/kg) administered alone and in combination with subcutaneous clonidine (0.1 and 0.5 mg/kg) in the tail-flick assay (n = 8 mice/treatment). Asterisks denote significant differences compared to clonidine control; *P<0.05; one-way ANOVA 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).

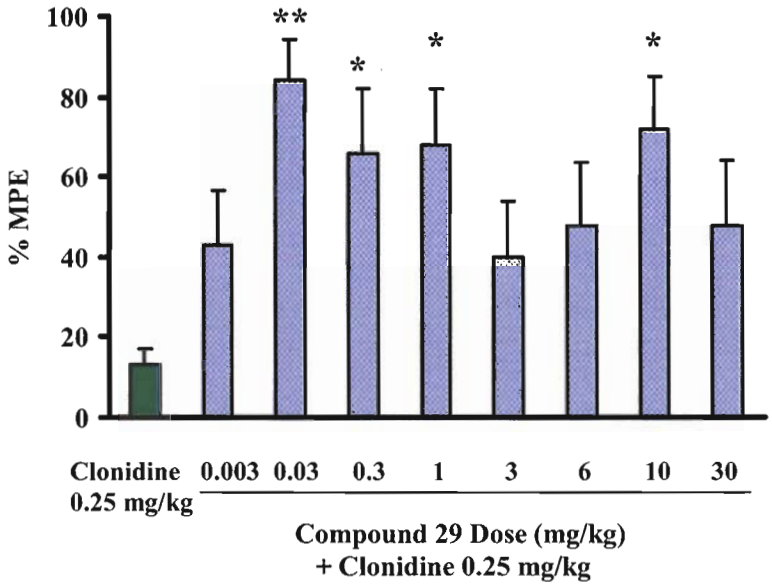


Figure 23. Potentiation of the antinociceptive actions (± S.E.M.) of subcutaneous clonidine (0.25 mg/kg) by subcutaneous compound **29** in the tail-flick assay (n = 8-9 mice/treatment). Asterisks denote significant differences compared to clonidine control; *P<0.05 and **P<0.01; one-way ANOVA followed 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_{50} 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).

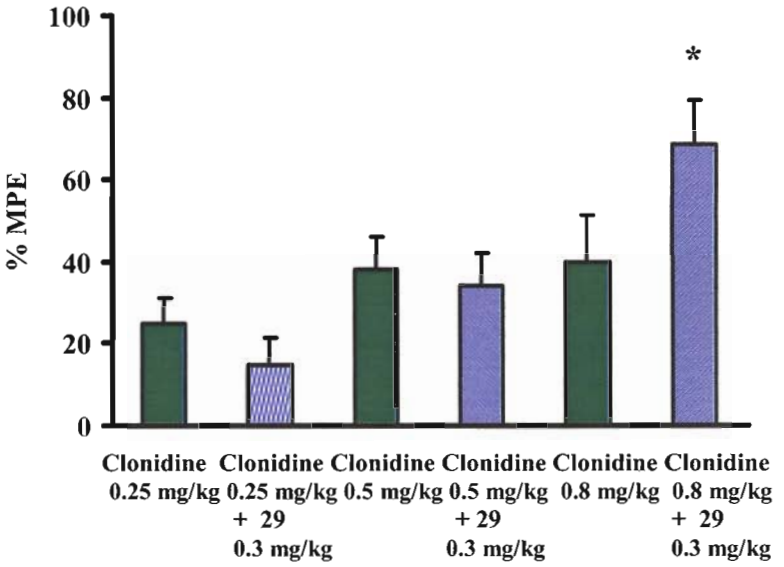


Figure 24. Effect (± S.E.M.) of **29** (0.3 mg/kg, s.c.) administered in combination with clonidine (0.25, 0.5, and 0.8 mg/kg, s.c.) in the hot-plate assay (n = 8-9 mice/treatment). Asterisks denote significant differences compared to clonidine control; *P<0.05; one-way ANOVA followed by Newman-Keuls 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 α_{2B}-adrenoceptor antagonist, the 5-HT_3 receptor antagonist tropisetron (**33**), nor yohimbine (**39**), an α_2-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).

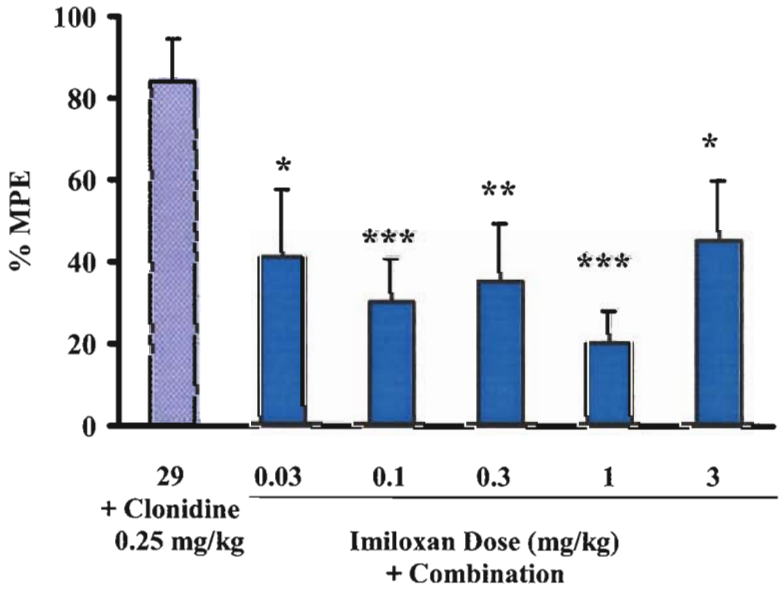


Figure 25. Effect (± S.E.M.) of subcutaneous imiloxan on the antinociceptive actions of a combination (of subcutaneous clonidine (0.25 mg/kg) and compound **29** (0.03 mg/kg)) in the tail-flick assay (n = 8-10 mice/treatment). Asterisks denote significant differences compared to the combination control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

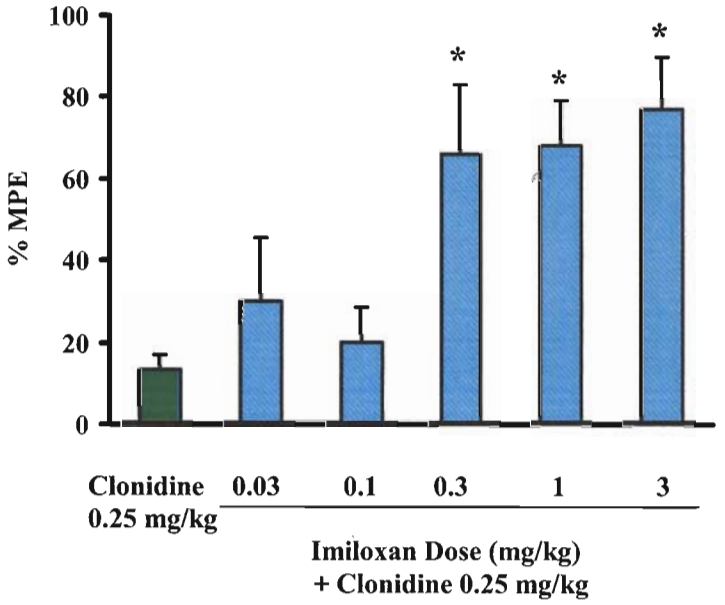


Figure 26. Potentiation of the antinociceptive actions (± S.E.M.) of subcutaneous clonidine (0.25 mg/kg) by subcutaneous imiloxan in the tail-flick assay (n = 6-15 mice/treatment). Asterisks denote significant differences compared to control group; *P<0.05; one-way ANOVA followed by Newman-Keuls post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

Pretreatment with the 5-HT_3 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**

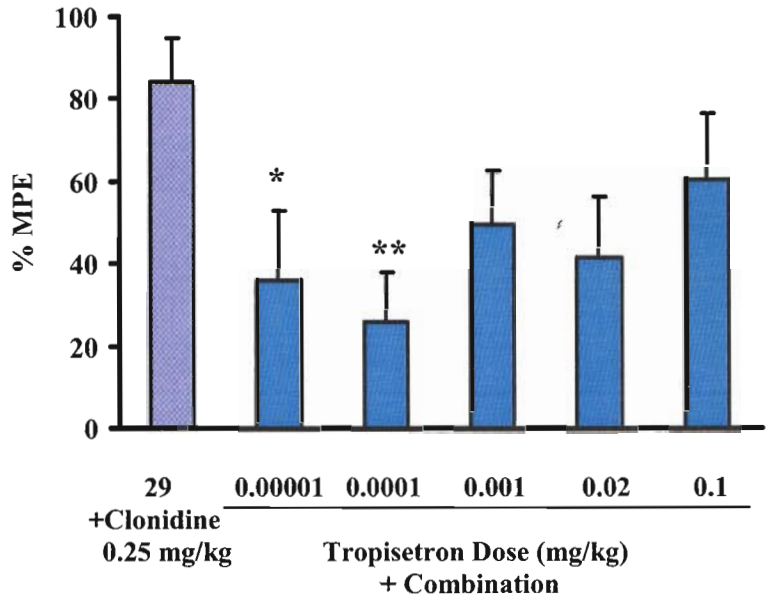


Figure 27. Effect (± S.E.M.) of subcutaneous tropisetron on the antinociceptive actions of a combination (of subcutaneous clonidine (0.25 mg/kg) and compound **29** (0.03 mg/kg)) in the tail-flick assay (n = 8-10 mice/treatment). Asterisks denote significant differences compared to the combination control group; *P<0.05 and**P<0.01; one-way ANOVA followed by Newman-Keuls post hoc test.

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 ED_{50} 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 ED_{50} 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.

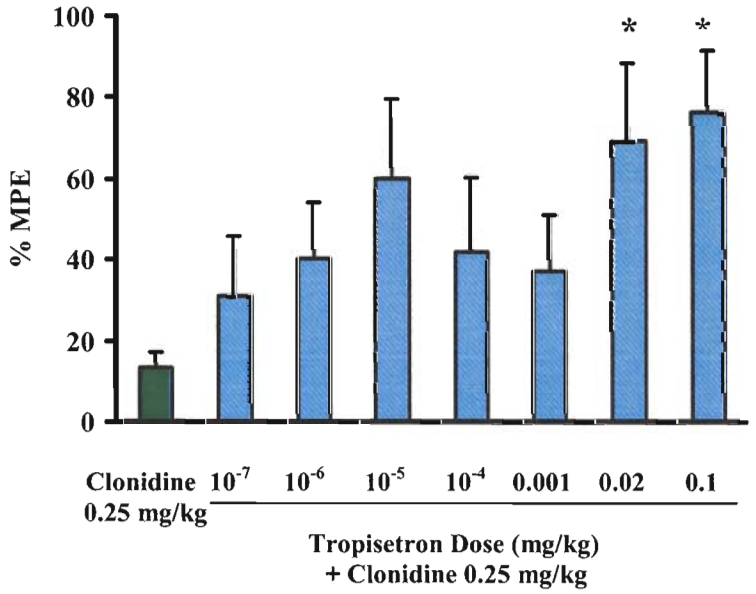


Figure 28. Potentiation of the antinociceptive actions (± S.E.M.) of subcutaneous clonidine (0.25 mg/kg) by subcutaneous tropisetron in the tail-flick assay (n = 6-8 mice/treatment). Asterisks denote significant differences compared to control group; *P<0.05; one-way ANOVA followed by Newman-Keuls post hoc test.

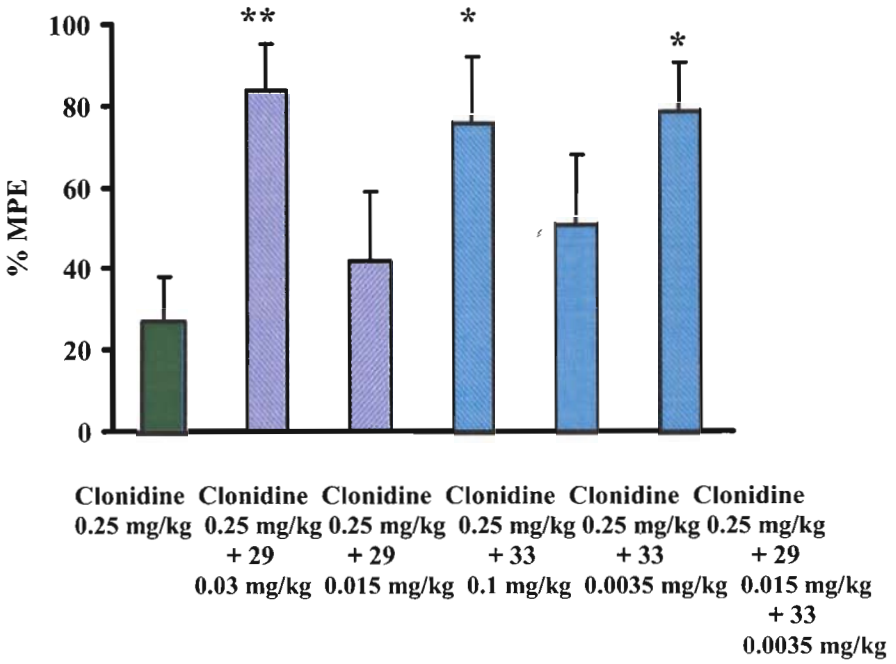


Figure 29. Effect (± S.E.M.) of tropisetron (**33**)(s.c.) and **29** (s.c.) on the antinociceptive actions of clonidine (0.25 mg/kg; s.c) and **29** (s.c.) in the tail-flick assay (n = 5-7 mice/treatment). Asterisks denote significant differences compared to control group; *P<0.01, **P<0.001; one-way 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).

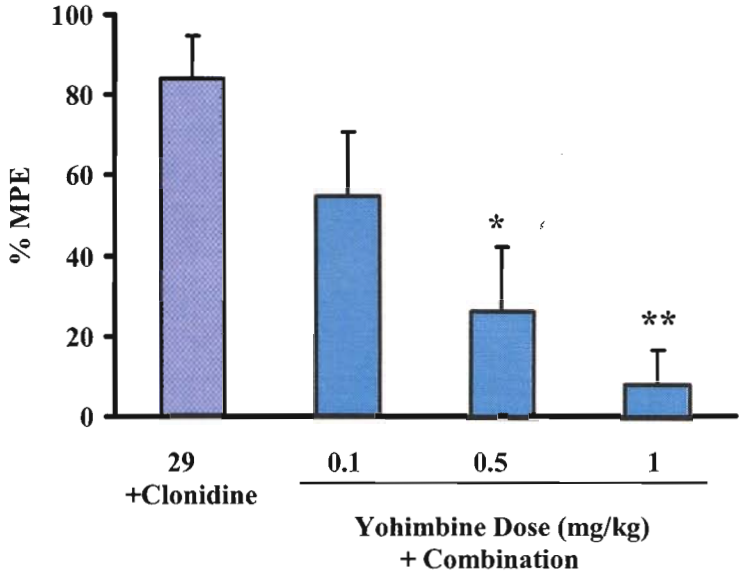


Figure 30. Effect (± S.E.M.) of subcutaneous yohimbine on the antinociceptive actions of a combination (of subcutaneous clonidine (0.25 mg/kg) and **29** (0.03 mg/kg)) in the tail-flick assay (n = 8 mice/treatment). Asterisks denote significant differences compared to control group; *P<0.05 and**P<0.01; one-way ANOVA followed by Newman-Keuls post hoc test.

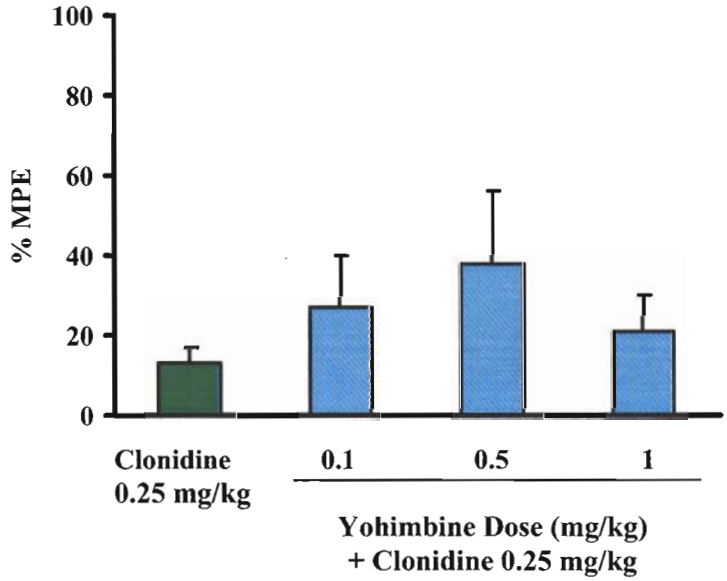


Figure 31. Potentiation of the antinociceptive actions (± S.E.M.) of subcutaneous clonidine (0.25 mg/kg) by subcutaneous yohimbine in the tail-flick assay (n = 8-16 mice/treatment).

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.[109] 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.

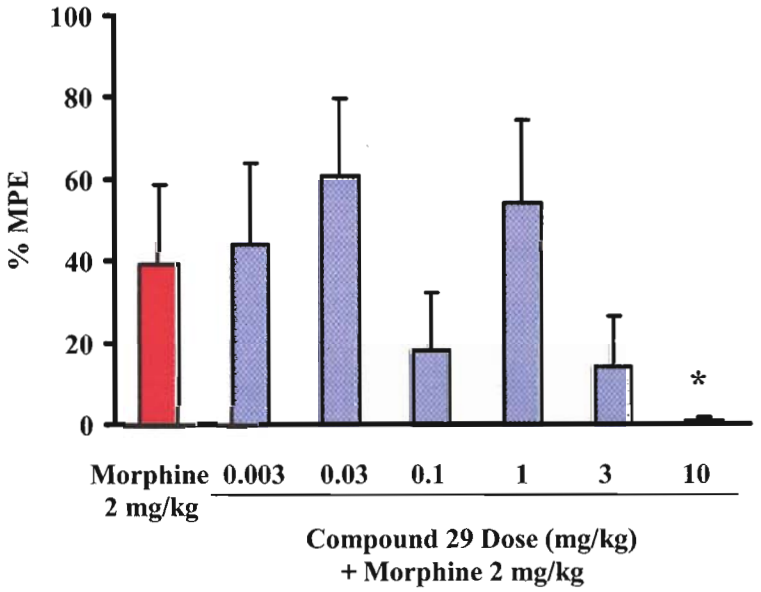


Figure 32. Effect (± S.E.M.) of subcutaneous compound **29** doses administered in combination with morphine (2 mg/kg, s.c.) in the tail-flick assay (n = 6-8 mice/treatment). Asterisks denote significant differences compared to control group; *P<0.05; one-way ANOVA followed by Newman-Keuls post hoc test.

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**).

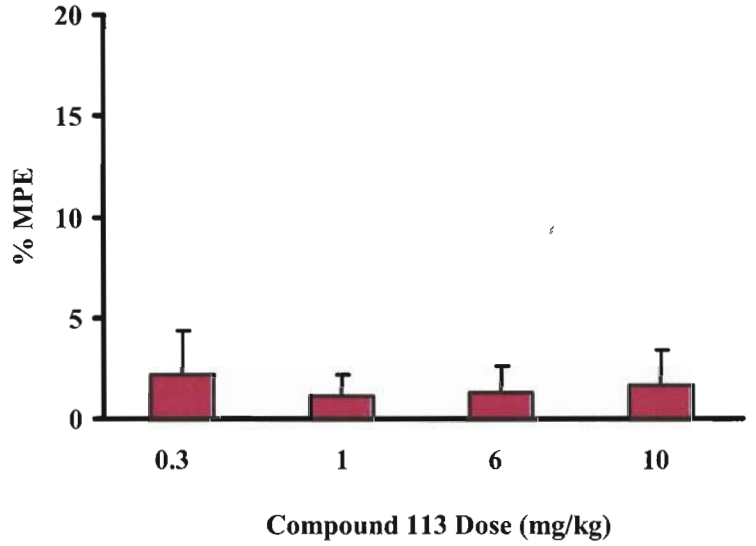


Figure 33. Effect (± S.E.M.) of **113** doses administered s.c. 45 min prior to examination in the tail-flick assay (n = 6-8 mice/treatment) as compared to saline control (6 ± 4% MPE). Note scale of y axis.

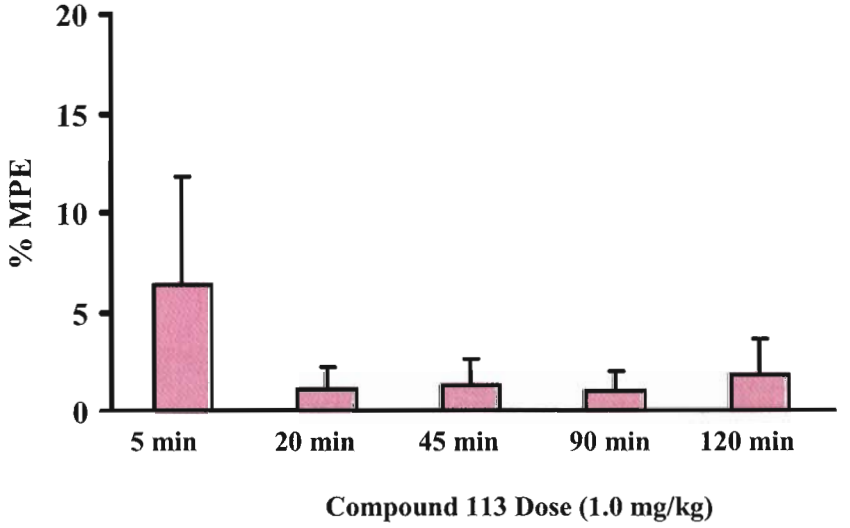


Figure 34. Effect (± S.E.M.) of a **113** dose (1 mg/kg, s.c.) administered 5 to 120 min prior to examination in the tail-flick assay (n = 6-8 mice/treatment) as compared to saline control (6 ± 4% MPE). Note scale of y axis.

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).

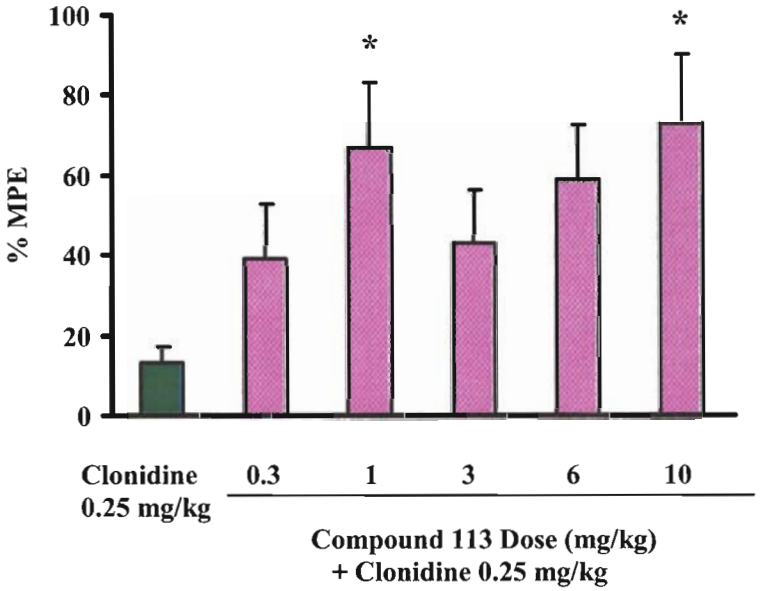


Figure 35. Potentiation of the antinociceptive actions (± S.E.M.) of clonidine (0.25 mg/kg, s.c.) by subcutaneous **113** in the tail-flick assay (n = 7-9 mice/treatment) 45 min post administration of compound **113**. Asterisks denote significant differences compared to control group; *P<0.05; one-way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

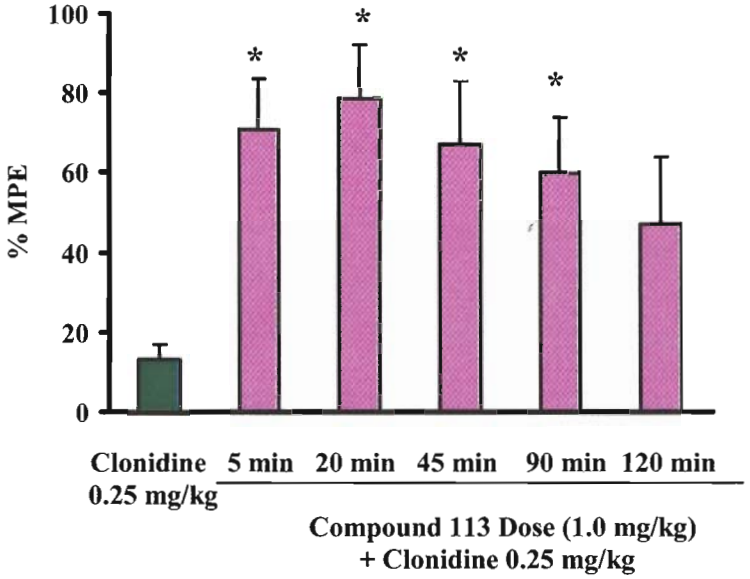


Figure 36. Potentiation of the antinociceptive actions (± S.E.M.) of clonidine (0.25 mg/kg, s.c.) by **113** (1.0 mg/kg, s.c.) when administered 5 to 120 min prior to examination in the tail-flick assay (*n* = 8-9 mice/treatment). Asterisks denote significant differences compared to control group; *P<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.

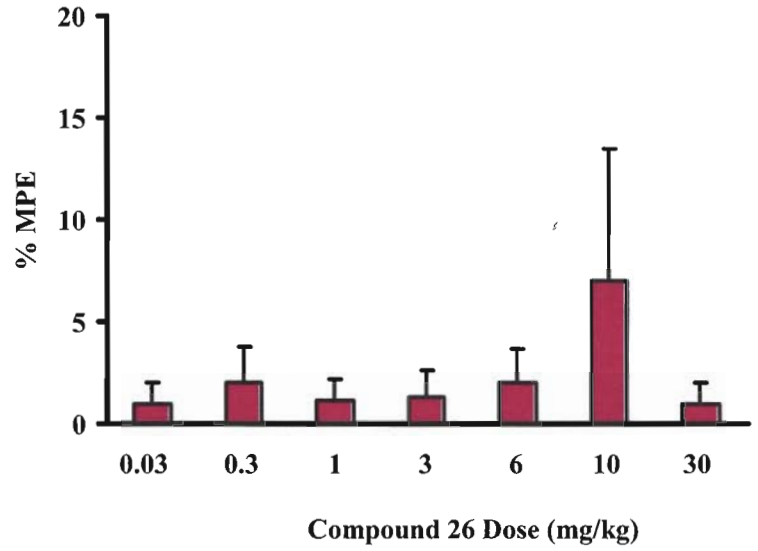


Figure 37. Effect (± S.E.M.) of subcutaneous **26** doses administered 45 min prior to examination in the tail-flick assay (n = 5-7 mice/treatment) as compared to saline control (6 ± 4% MPE). Note scale of y axis.

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).

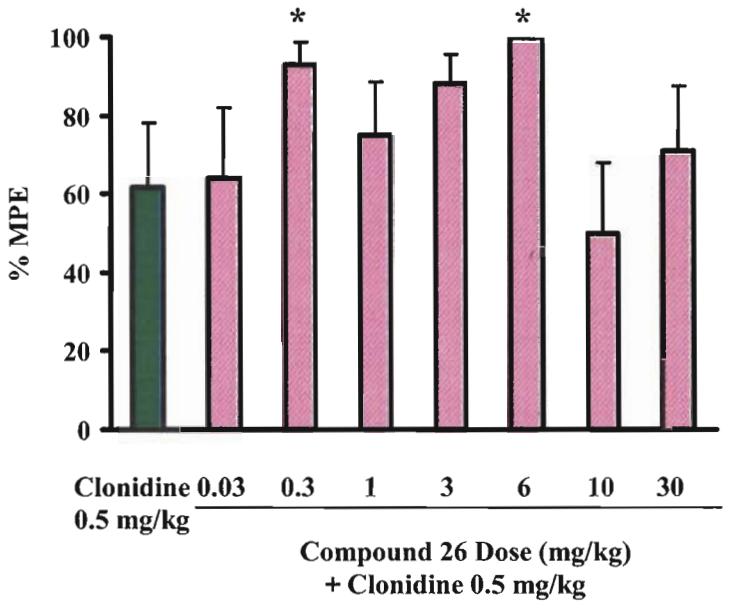


Figure 38. Potentiation (± S.E.M.) of the antinociceptive actions of clonidine (0.5 mg/kg, s.c.) by subcutaneous **26** in the tail-flick assay (n = 7 mice/treatment). Asterisks denote significant differences compared to control group; *P<0.05; one-way ANOVA followed by Newman-Keuls post hoc test.

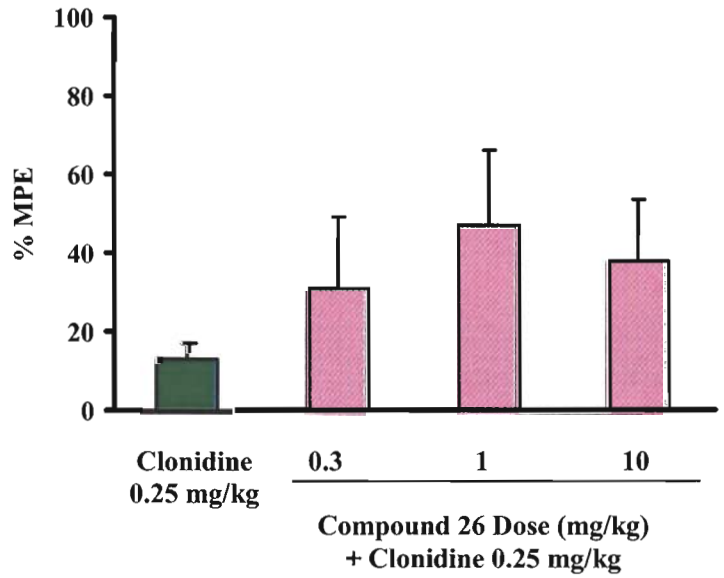


Figure 39. Effect (± S.E.M.) of subcutaneous **26** doses administered in combination with clonidine (0.25 mg/kg, s.c.) in the tail-flick assay (n = 7 mice/treatment).

2. Discussion

MD-354 (**21**) binds selectively both to 5-HT_3 receptors and α_{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_{50} = 0.8 mg/kg). The results obtained (Figure 13) were consistent with those reported in the literature.[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,[95] whereas the hot-plate assay involves supraspinal receptors.[98] It has been suggested that supraspinal 5-HT_3 receptors might not be involved with spinal analgesia.[41] This was supported by a study showing that the nonselective 5-HT_3 receptor agonist, 2-methyl-5-HT did not produce

antinociception when administered directly into the brain.[41] Therefore, if supraspinal 5-HT_3 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-HT_3 supraspinal receptors. Another possibility could be that there is a low density of α_{2B}-adrenoceptors present in the brain, and/or that MD-354 may not reach these receptors. Since there is only a small number of supraspinal α_{2B}-adrenoceptors[61-63] and supraspinal 5-HT_3 receptors,[41] 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,[32] indicating that it might have difficulty penetrating the blood-brain barrier.

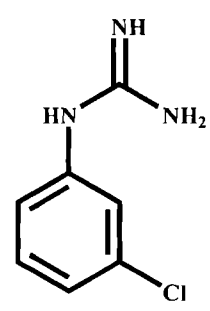
The carbamate derivative of MD-354, **113**, was synthesized and examined to study this factor. In theory, the more lipophilic **113** should more readily penetrate the blood-brain barrier than MD-354 (**21**), and be subsequently hydrolyzed by brain tissue esterases to MD-354. Compound **113** produced an effect similar to that of MD-354. As reported for MD-354,[103] 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).[103] 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**.

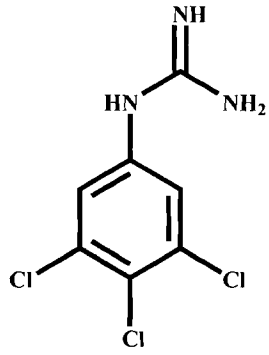
Next, mechanistic studies were conducted to help determine which pathway, 5-HT_3 and/or α_{2B}-adrenoceptors, is responsible for the potentiation of clonidine analgesia by

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 α_{2B}-adrenoceptors. However, unlike MD-354, **29** does not show subtype selectivity for α_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 α_{2B}-adrenoceptors primarily mediated the potentiating effect of

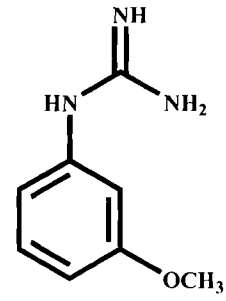
Table 10. Binding Data for MD-354 (**21**), **29**, and **26** at 5-HT_3 and α_2-Adrenoceptors.[103,111]



MD-354 (21)



29



26

K_i (nM)

		Adrenoceptors		
	5-HT_3	α_{2A}	α_{2B}	α_{2C}
MD-354	35 ± 5	825 ±160	25 ± 5	140 ± 40
29	0.7 ± 0.1	32 ± 3	30 ± 5	30 ± 5
26	1600 ± 300	177 ± 6	152 ± 12	135 ± 6

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 α_2-adrenoceptors cannot be ruled out. Compound **29** also has a higher affinity for α_{2A}- and α_{2C}-adrenoceptor subtypes than MD-354 (Table 10). Compound **29** is about 25 times more potent than MD-354 in binding at α_{2A}-adrenoceptors and 5 times more potent in binding at α_{2C}-adrenoceptors. Since it has been shown that the α_{2A}-

adrenoceptor subtype plays a role in antinociception, the increase in potency might also involve the activation of α_{2A}-adrenoceptors. It might also be noted that **29** is more lipophilic than MD-354 (**21**);[32] 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 liphophilic enough to cross the blood-brain barrier and reach supraspinal sites. Compound **29** possesses higher affinity for 5-HT_3 receptors, α_{2A}- and α_{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 α_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 α_{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 ED_{50} 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-HT_3 antagonist tropisetron (doses 0.00001 and 0.0001 mg/kg), the α_{2B}-adrenoceptor antagonist imiloxan (doses 0.03 to 3.0 mg/kg), and the nonselective α_2-adrenoceptor antagonist yohimbine (doses 0.5 and 1.0 mg/kg). The non-selective α_2-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 α_2-adrenoceptor mechanism,[3] unavailability of α_2-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-HT_3 and α_{2B}-adrenoceptors. The α_{2B}/α_{2C}-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 α_{2B}/α_{2C}-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 α_2-adrenoceptor subtypes. It has been shown that both α_{2A}- and α_{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 α_{2A}-adrenoceptors than before and could possibly potentiate its antinociceptive actions. Also, α_{2A}-adrenoceptors mediate the sedative effects caused by clonidine.[78] Therefore, if more α_{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 α_{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-HT_3 agonist mechanism; this is consistent with the observation that the effect of clonidine/**29** is blocked by the 5-HT_3 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-HT_3 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 α_2-adrenoceptor and 5-HT_3 receptor mechanism. Although the arylguanidines might behave as α_2-adrenoceptor agonists (or partial agonists), the specific α_2-adrenoceptor subtype(s) involved remains to be elucidated. Several of the studies argue in favor of a role for 5-HT_3 receptors; but here, it is unclear whether the agents act via a 5-HT_3 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-HT_3 and α_2-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-HT_3 and 5-HT_4 antagonist, another explanation is that tropisetron is blocking both 5-HT_3 and 5-HT_4 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).[9,15] Serotonin, by interacting with 5-HT_3 receptors, can also modulate the release of other neurotransmitters and neuropeptides (i.e., dopamine, acetylcholine, GABA, substance P, cholecystokinin).[52] Another possibility could be that α_2-adrenoceptor antagonists block the antinociception mediated by 5-HT_3 receptors because the two systems are functionally related.

The effect of the ED_{50} 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 ED_{50} 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-HT_3 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-HT_3 and/or α_{2B}-adrenoceptor antagonists show that the potentiating effect of **29** with clonidine might involve a biphasic mechanism. This means that at extremely low doses, 5-HT_3 receptors might be involved and that at higher doses, α_{2B}-adrenoceptors could be involved in the action of **29**. It might be noted that 5-HT_3

receptor ligands commonly show potent actions (i.e., at sub-mg/kg doses) in a variety of pharmacological assays.[42]

Clonidine (**43**), a nonselective α_2-adrenoceptor agonist, potentiates the antinociceptive actions of morphine but the exact adrenoceptor population responsible is still unknown.[90] Since the potentiation of clonidine by **29** might involve α_2-adrenoceptors via an agonist mechanism, **29** was also examined in combination with morphine in the tail-flick assay. In theory, if the potentiating effect of **29** involves α_2-adrenoceptors as with clonidine, then **29** should potentiate the antinociceptive actions of morphine. Compound **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 **29** but the effect was not statistically significant. In contrast, doses of 0.1 and 3 mg/kg of **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 **29** blocked the actions of morphine and these results were statistically significant (Figure 32). The studies of **29** in combination with morphine can be explained in several ways. As mentioned earlier, co-administration of α_2-adrenoceptor agonists and opioid agonists produce a synergistic effect.[90] These results suggest that certain doses of **29** possibly potentiate morphine's antinociception via an α_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_3 antagonist tropisetron.[46] Consequently, any adrenergically-mediated potentiating effects of **29** might be counteracted by the antagonist actions of **29** at 5-HT_3 receptors if **29** is a 5-HT_3 partial agonist. These results

from the **29**/morphine studies suggest that **29** could be a 5-HT_3 antagonist and an α_2-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-HT_3 receptor antagonist mechanism, the difference in activity could be due to the fact that **29** binds with a 50-fold higher affinity at 5-HT_3 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 ED_{50} 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 α_2-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-HT_3/α_{2B}-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-HT_3 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-HT_3 receptor antagonist tropisetron, the α_{2B}-adrenoceptor antagonist imiloxan, and the α_2-adrenoceptor antagonist yohimbine. This shows that both 5-HT_3 and α_2-adrenoceptors are involved in the potentiation of clonidine antinociception by **29**. Compound **26**, an analog of MD-354 that lacks affinity at 5-HT_3 receptors, did not potentiate the effect of an inactive dose of clonidine in the tail-flick assay. However, **26** potentiated the effect of the ED_{50} 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-HT_3 and α_2-adrenoceptors has been established. At least some arylguanidines 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-HT_3 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 H_2O 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-d_6) 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 H_2O and MeOH to give **26** (0.10 g, 19%) as a white powder: mp 139-141 °C; ^{1}H NMR (DMSO-d_6) δ 3.76 (s, 3H, OCH_3), 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 ($C_8H_{11}N_3O \cdot HNO_3$) C,H,N.

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 Et_3N (0.60 mL, 4.30 mmol) in dry THF (100 mL) at 0 °C under an N_2 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 CH_2Cl_2 / 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.[106]mp 138-140 °C); ^{1}H NMR ($CDCl_3$) δ 3.63 (s, 3H, OCH_3), 7.10-7.36 (m, 4H, ArH), 7.38 (br. s, 2H, NH, NH), 8.51 (s, 1 H, NH). Anal. Calcd for ($C_9H_{10}ClN_3O_2$) C,H,N.

Method B. Calcium cyanamide (4.7 g, 58.7 mmol) was dissolved in H_2O (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 H_2O and EtOH to give **113** (0.19 g, 3%) as a white powder: mp 159-160 °C; ^{1}H NMR (DMSO-d_6) δ 3.78 (s, 3H, CH_3), 7.28-7.47 (m, 4H, ArH), 7.52 (br s, 2H, NH, NH), 8.81 (s, 1H, NH). Anal. Calcd for ($C_9H_{10}ClN_3O_2 \cdot 1.5HCl \cdot 0.25H_2O$) C,H,N.

2-Amino-5-chloro-3,4-dihydroquinazoline Hydrobromide (114). Compound **114** was prepared according to a literature procedure.[107] 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 H_2O (10 mL) was added to the resulting residue. The suspension was extracted with $CHCl_3$ (3 x 10 mL) and the combined $CHCl_3$ portion was washed with brine (15 mL). The extract was dried with Na_2SO_4 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 (lit[107] mp 221-223 °C); ^{1}H NMR (DMSO-d_6) δ 4.37 (s, 2H, CH_2N), 6.5-7.0 (m, 3H, ArH). Compound **114 (free base)** was dissolved in Et_2O 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[107]mp 241-243 °C); ^{1}H 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)guanidinedicarboxylate (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; ^{1}H 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 ($C_{11}H_{12}ClN_3O_4$) C,H,N.

2-Chloro-6-nitrobenzylbromide (119). Compound **119** was prepared according to a literature procedure.[107] 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.[107] mp 50-52 °C).

N-(2-Chloro-6-nitrobenzyl)phthalamide (120). Compound **120** was prepared according to a literature procedure.[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_2O/ice (55 mL). More $CHCl_3$ (20 mL) was added to the cooled mixture. The $CHCl_3$ portion was separated and washed with 0.2 N NaOH (26 mL) and brine (20 mL). The extract was dried (Na_2SO_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.[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_2O (4 x 50 mL) and the Et_2O extract was washed with brine (50 mL). The extract was dried with Na_2SO_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.[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, Et_2O mixture to give **122 HCl** (0.42 g, 84%) as a powder: mp >240 °C (lit[107] mp 263-265); ^{1}H NMR (DMSO-d_6) δ 4.13 (s, 2H, CH_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_2O and 15% NaOH (2 mL) was added to obtain a pH of 13. The mixture was extracted with Et_2O (3 x 40 mL) and the combined Et_2O portion was dried with Na_2SO_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.[30] 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[113] and Atwell and Jacobson.[112] 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 °C. 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[99] as modified by Dewey et al.[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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