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# NOVEL ANALOGS OF *m*-CHLOROPHENYLGUANIDINE AS 5-$HT_3$ RECEPTOR LIGANDS

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

by

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## List of Abbreviations

| | |
|---|---|
| 2-NP | 1-(2-Naphthyl)piperazine |
| 5-HT | 5-Hydroxytryptamine/Serotonin |
| 5-HTQ | N,N,N-Trimethyl-5-hydroxytryptamine |
| AC | Adenylate cyclase |
| AChBP | Acetylcholine binding protein |
| AcOH | Acetic acid |
| ANOVA | Analysis of variance |
| BBB | Blood-brain barrier |
| BCC | Bond correction charges |
| CMR | Complete molar refraction |
| CNS | Central nervous system |
| DMF | *N*,*N*-Dimethylformamide |
| DMF-DMA | *N*,*N*-Dimethylformamide dimethylacetyl |
| $EC_{50}$ | Activation concentration (half-maximal effect) |
| ECD | Extracellular domain |
| EL | Extracellular loop |
| epmr | Equipotent molar ratio |
| EtOAc | Ethyl acetate |
| EtOH | Ethanol |
| FDA | Food and Drug Administration |
| FST | Forced swim test |
| GABA | γ-Aminobutyric acid |
| GI | Gastrointestinal tract |
| GOLD | Genetic optimization of ligand docking |
| GPCR | G-protein coupled receptor |
| HPLC | High performance liquid chromatography |
| IACUC | Institutional Animal Care and Use Committee |
| IBS | Irritable bowel syndrome |
| $IC_{50}$ | Inhibition concentration (half-maximal effect) |
| i.d. | Inside diameter |
| IL | Intracellular loop |
| ip | Intraperitoneal |
| *i*-PrOH | Isopropanol |
| IUPHAR | International Union of Pharmacology |
| iv | Intravenous |
| $K_i$ | Dissociation constant |

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| | |
|---|---|
| KO | Knock-out |
| l. | Length |
| LGIC | Ligand-gated ion channel |
| LSD | (+)Lysergic acid diethylamide |
| M (M1-M4) | Transmembrane-spanning helix |
| mCPBG | *meta*-Chlorophenylbigudanide |
| mCPG | *meta*-Chlorophenylguanidine (MD-354) |
| MCVH | Medical College of Virginia Hospital |
| MD-354 | *meta*-Chlorophenylguanidine |
| MOPAC | Molecular orbital package |
| MR | Molar refraction |
| MV | Molar volume |
| nACh | nicotinic acetylcholine |
| NAMD | Nanoscale molecular dynamics |
| NIH | National Institutes of Health |
| NMQ | *N*-Methylquipazine |
| NTS | Nucleus tractus solitaries |
| NVE | Number of valence electrons |
| PBG | Phenylbiguanide |
| Pc | Parachor |
| PDB | Protein data bank |
| PG | Phenylguanidine |
| PLC | Phospholipase C |
| po | Per os (orally) |
| QSAR | Quantitative structure-activity relationships |
| SAFIR | Structure-affinity relationships |
| SAR | Structure-activity relationships |
| sc | Subcutaneous |
| SCWRL | Side-chain assignment with rotamer library |
| SEM | Standard error of the mean |
| SERT | Serotonin reuptake transporter |
| SNRI | Serotonin-norepinephrine reuptake inhibitor |
| SSRI | Selective serotonin reuptake inhibitor |
| TBAB | Tetrabutylammonium bromide |
| TCA | Tricyclic antidepressant |
| THF | Tetrahydrofuran |
| TLC | Thin layer chromatography |
| TM | Transmembrane |
| TMS | Tetramethylsilane |
| TST | Tail suspension test |
| WT | Wild-type |

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**Abstract**

NOVEL ANALOGS OF *m*-CHLOROPHENYLGUANIDINE AS 5-$HT_3$ RECEPTOR LIGANDS

By Katie Elizabeth Alix

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

Virginia Commonwealth University, 2009

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

Serotonin receptors play a variety of functional roles in the body. Some indications and treatment claims for one of the classes of serotonin receptors, the 5-$HT_3$ receptor family, include: anxiety, depression, chemotherapy- and radiation-induced emesis, constipation, irritable bowel syndrome, pain, drug addiction, and satiety control.

A 5-$HT_3$ receptor partial agonist, MD-354, served as a lead compound in the development of new 5-$HT_3$ receptor ligands. Using halogenated analogs the study investigated their effect on binding to the 5-$HT_3$ receptor. Conformationally-constrained analogs (quinazolines) were shown to be a novel class of 5-$HT_3$ receptor antagonists. The

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log P values were determined for several analogs, and indicated that these ligands should be able to penetrate the blood-brain barrier. A homology model of the 5-$HT_3$ receptor was built and the docking modes were assessed for these two series. Quinazolines were investigated for antidepressant properties using the mouse tail suspension test, and were shown to possess antidepressant-like activity.

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## I. Introduction

Serotonin (5-HT) research in the central nervous system (CNS) has come a long way since serotonin was first proposed to be a neurotransmitter.[1] After the identification of the first two serotonin receptors in 1957,[2] the list has expanded several times to include seven major families and their subtypes. Most serotonin receptors are G-protein coupled-receptors (GPCRs); however, one family of serotonin receptors consists of ligand-gated ion-channel (LGIC) receptors—the 5-$HT_3$ receptor family.

Ligand-gated ion-channels are fast acting. Once a ligand activates the receptor, an agonist, binds to the receptor; the receptor is depolarized and ions quickly travel through the pore, changing the synaptic polarity. 5-$HT_3$ receptors are generally co-localized on nerve endings with other neurotransmitter receptors. The change in synaptic polarity from activation of 5-$HT_3$ receptors can stimulate the release of nearby neurotransmitters (i.e., dopamine, norepinephrine, glutamate, acetylcholine, γ-aminobutyric acid, and serotonin).[3,4] Since 5-$HT_3$ receptors can stimulate the release of so many neurotransmitters it seems to have the potential to be involved in several different functions.

Most notably, 5-$HT_3$ receptors are known for their antagonists, most of which are clinically available as anti-emetics. These drugs are effective in the treatment of chemotherapy- and radiation-induced emesis.[5] From the investigations into treating



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emesis, other 5-$HT_3$ receptor antagonists have been identified to be useful in the treatment of irritable bowel syndrome (IBS).[5] The 5-$HT_3$ receptors have also been implicated in depression, pain modulation, the reward pathway, schizophrenia, and anxiety.[5,6] No known therapeutic function has been identified for 5-$HT_3$ receptor agonists.

*m*-Chlorophenylguanidine (mCPG; MD-354) is a partial agonist at the 5-$HT_3$ receptor, it behaves as an agonist in some assays and as antagonist in other assays; but binds with high affinity to the 5-$HT_3$ receptor ($K_i$ = 35 nM).[7,8] Because of the partial agonist characteristic, it would be valuable to develop novel analogs to explore which aspects are necessary for binding to the 5-$HT_3$ receptor as well as ways to improve binding affinity.

The 5-$HT_3$ receptors are located in the CNS as well as in the periphery. In order to study the effects of the 5-$HT_3$ receptors in the CNS, agents must be able to get into the brain and cross the blood-brain barrier (BBB). MD-354 has a log P value of -0.64 suggesting it may not be able to easily penetrate the BBB;[9] thus, developing analogs that can cross the BBB would be necessary for studying 5-$HT_3$ receptors in the CNS.

In drug development, molecular modeling has proven to be a powerful tool. Since the structure of the 5-$HT_3$ receptor has yet to be determined by X-ray crystallography, homology models of the receptor can be built from other ligand-gated ion-channels of the Cys-loop family whose structure has been determined to identify possible binding modes of known agents and aid in the design of new agents.

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From data compiled since 1960, depression affects up to 38% of cancer patients.[10,11] The idea of being able to treat the emesis from chemotherapy as well as the depression with one drug is an inviting possibility. Both 5-$HT_3$ receptor antagonists and agonists have been shown to behave as antidepressants in the forced swim and tail suspension tests in rodents.[12-17] This suggests that a variety of classes of 5-$HT_3$ receptor agents are capable of having the same interactions with the 5-$HT_3$ receptor, an analysis of selected agents for antidepressant-like properties may yield a novel class of antidepressants.

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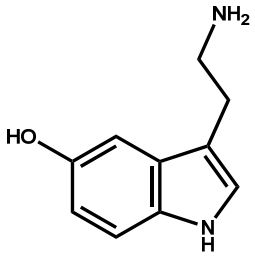
## II. Background:

### A. History of Serotonin

Evidence for the existence of serotonin (5-hydroxytryptamine; 5-HT; **1**) (Figure 1) was detected as early as 1868 as a vasoconstrictor.[18] However, its isolation did not occur for more than another half century. Independently, Erspamer and Vialli[19] isolated enteramine in the late 1930s from enterochromaffin cells in the gut and Rapport et al.[20] isolated serotonin as a beef serum vasoconstrictor substance in 1948. In 1949, Rapport determined the structure of serotonin as 5-hydroxytryptamine.[21] But it was not until 1952 that Erspamer and Asero discovered that enteramine and serotonin were one and the same.[22]




**1**

**Figure 1.** The structure of serotonin (5-HT).

The central nervous system (CNS) story of serotonin began in 1953 when Twarog and Page discovered serotonin in dog, rat, and rabbit brains;[23] later, in 1954, Twarog



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proposed serotonin to be a neurotransmitter.[1] Independently Woolley and Shaw,[24] and Gaddum and Hameed[25] proposed that the action of 5-HT in the brain and in the gut could be antagonized by the hallucinogen LSD ((+)-lysergic acid diethylamide).

Serotonin has been implicated in a number of different processes in both the periphery and central nervous systems and has been shown to play a role in alcoholism and drug abuse, cognitive disorders, depression, anxiety, schizophrenia, chemotherapy- and radiation-induced emesis, constipation, irritable bowel syndrome, migraines, fibromyalgia, pain, obesity, appetite disorders, and autism.[6]

### B. Classification of 5-HT Receptors

The first definitive receptors for 5-HT were identified by Gaddum and Picarelli in 1957.[2] These receptors were originally called D- and M-receptors because they were antagonized by dibenzyline and morphine, respectively. In 1979 Peroutka and Snyder used $^{14}C$ and $^{3}H$ techniques to determine 5-HT receptor subtypes in vitro.[26] A receptor classification scheme was proposed by the Nomenclature Committee of the International Union of Pharmacology (IUPHAR) in 1992.[27] Table 1 provides the general criteria for receptor classification as provided by IUPHAR.[28] Receptors are classified by operational (drug-binding), structural (sequence of the receptor), and transductional (receptor-effector coupling) characteristics. Upper case lettering is used when the receptor has been identified in whole cells while lower case lettering is used when identifying recombinant receptors.

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To date there are seven major families of serotonin receptors (5-$HT_1$-5-$HT_7$). All, but the 5-$HT_3$ (5-HT3A-5-HT3E) receptors, belong to the G-protein coupled-receptor (GPCR) superfamily. The 5-$HT_3$ receptors belong to the Cys-loop ligand-gated ion-channel (LGIC) receptor superfamily. The 5-$HT_1$ (5-$HT_{1A}$, 5-$HT_{1B}$, 5-$HT_{1D}$, 5-$ht_{1e}$, 5-$HT_{1F}$) receptors inhibit adenylate cyclase (AC); the 5-$HT_2$ (5-$HT_{2A}$, 5-$HT_{2B}$, 5-$HT_{2C}$) receptors stimulate phospholipase C (PLC) production. The 5-$HT_4$, 5-$HT_6$, 5-$HT_7$ (5-$HT_{7A}$, 5-$HT_{7B}$, 5-$HT_{7D}$) receptors stimulate adenylate cyclase while a second messenger system for activation of the 5-$ht_{5a}$ and 5-$ht_{5b}$ receptors has yet to be defined.[6,28-30]

**Table 1.** Criteria for receptor characterization.[28]

| Criteria | Definition |
|---|---|
| Operational | |
| a. Selective agonists | Agonists showing high selectivity for a particular receptor compared to their potencies at other receptors need to be identified. |
| b. Selective antagonists | Receptor-blocking drugs are needed that can block the actions of agonists by blocking the receptor. |
| c. Ligand-binding affinities | Dissociation constants for ligands in binding studies should correlate with data from functional studies. |
| Structural | |
| d. Molecular structure | Amino acid sequence of the receptor protein provides evidence for receptor identity; relative homologies can be used for classification and defining families and subfamilies. |
| Transductional | |
| e. Intracellular transduction mechanisms | Information that further defines a superfamily and any information that may be indicative of the nature of the intracellular protein structure. |

### 1. G-Protein Coupled-Receptors

GPCRs are characterized by seven transmembrane-spanning helicies (TM1-TM7), three intracellular loops (IL1-IL3), and three extracellular loops (EL1-EL3) (as reviewed

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by Lagerström and Schiöth).[31] The amine terminus is extracellular and the carboxyl terminus is intracellular. One of the intracellular loops, usually IL2 or IL3, is coupled to a heterotrimeric G-protein, which upon ligand binding dissociates to a βγ subunit and an α subunit to activate second messenger systems (e.g., PLC and AC). There are many other GPCRs including the adrenergic receptors and rhodopsin receptors.

### 2. Ligand-Gated Ion-Channel Receptors

5-$HT_3$-type receptors are ligand-gated ion-channel receptors and are part of the Cys-loop superfamily.[32] Structurally, these receptors are pentamers with each subunit (Figure 2) consisting of a very large extracellular domain (ECD) containing the amine terminus and the ligand-binding domain, four transmembrane-spanning helicies (M1-M4), a short intracellular loop connecting M1 to M2, a short extracellular loop connecting M2 to M3, an intracellular loop between M3 and M4, and an extracellular carboxyl terminus.[33,34] The M2 helix of each subunit makes up the inner lining of the cationic pore. Upon binding of an agonist, the receptor depolarizes and allows the influx of cations through the pore. This change in synaptic polarity may stimulate the release of other nearby neurotransmitters. The long ECD that is associated with the amine terminus consists of six putative loops (A-F); loops A, B, and C make up the principle side of the binding interface and loops D, E, and F make up the complementary side of the binding interface (Figure 3).[35] Incidentally, the binding site for agonists and competitive antagonists lies at the interface of two protomers. On the C loop there are two cysteines separated by 13 amino acids; they form a disulfide linkage and thus a Cys-loop. Other

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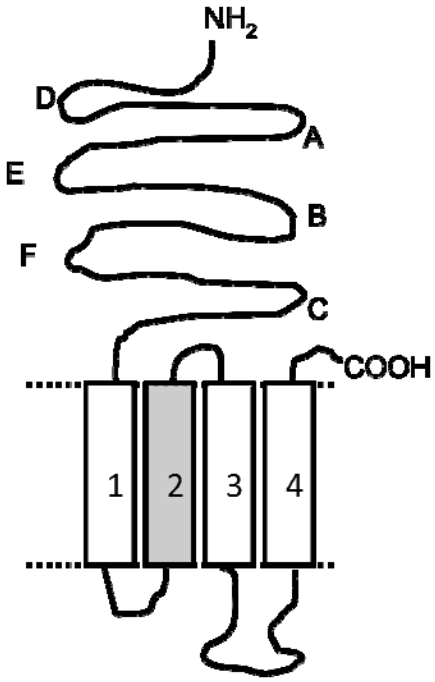


**Figure 2.** Schematic representation of a single protomer of a LGIC receptor.

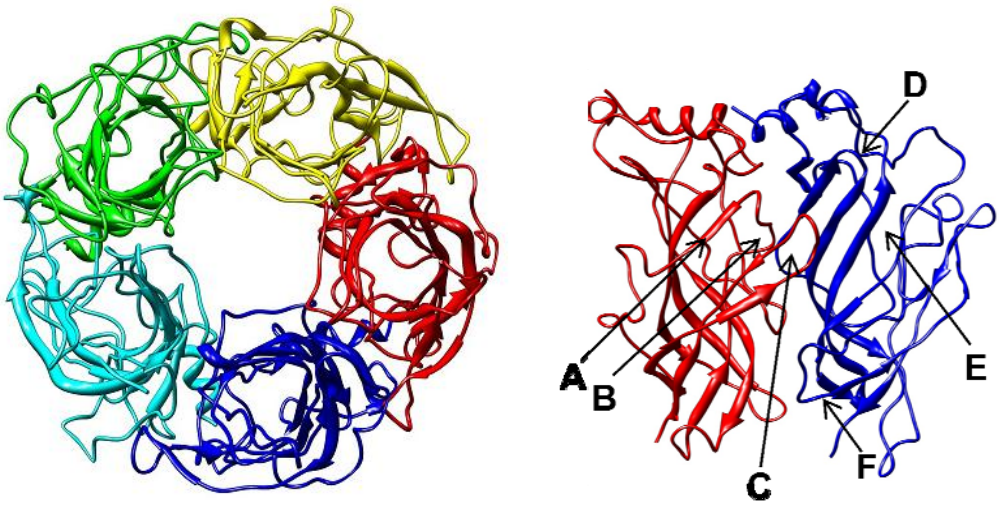


**Figure 3.** Bottom veiw of the homopentameric h5-HT3A ECD (left). Side view of the ligand binding domain; loops A, B, and C are on one protomer ("principle subunit"; red) and loops D, E, and F are on the adjacent protomer ("complementary subunit"; blue) (right).

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LGIC receptors include nicotinic acetylcholine (nACh), γ-aminobutyric acid type A ($GABA_A$), and glycine receptors.[5,6] There is a 22-30% sequence homology between the 5-HT3A receptor subunit sequence and the α-subunits of other members of the LGIC family.[36] Ortells and Lunt proposed that 5-$HT_3$ receptors are a possible common ancestor of all LGIC receptors.[37] To date, there are no X-ray crystal structures of 5-$HT_3$ receptors; however, various acetylcholine binding proteins' structures have been identified by X-ray crystallography.[38-40] The structure of a nicotinic acetylcholine receptor has been derived using electron microscopy.[41] Also, the α1 subunit ECD of a mouse nACh receptor bound to α-bungarotoxin has been crystallized and the structure determined.[42] Since the 5-$HT_3$ receptor subunits share features with nACh receptors (i.e., protein sequence homology between 5-HT3A receptor subunit sequences and the α subunit of other LGIC receptors is 22-30%),[36] homology models for 5-$HT_3$ receptors can be developed based on the crystal structures available.[38-42] There have been some reports of chimeric receptors between different members of the LGIC family.[35,43]

## C. 5-$HT_3$ Receptors

### 1. Classification

5-$HT_3$ receptors were identified as the original M receptor but were later renamed 5-$HT_3$ receptors by Bradley et al.[44] The 5-HT3A receptor subtype was initially cloned by Maricq et al. in 1991 from NCB-20 murine hybridoma cells.[45] The first human 5-$HT_3$ receptors were cloned from the hippocampus, amygdala, and colon.[46,47] The human *HTR3A* gene is located on chromosome 11 and each receptor subunit consists of 478

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amino acids.[48] Species orthologs of the 5-HT3A receptor have also been identified in the rat,[47] mouse,[44] dog,[49] guinea pig,[50] and ferret.[51] There are a few splice variants of the 5-HT3A receptor subunits: 5-HT3AS (i.e., short), h5-HT3AT (i.e., short, truncated) and h5-HT3AL (i.e., long).[52] The 5-HT3AS receptor subunit is found in mouse, rat, and guinea pig, and has six fewer amino acids in the loop between M3 and M4.[44,47,50] The h5-HT3AT receptor subunit, found in humans, only has 238 amino acids and a single transmembrane helix (M1).[52] The h5-HT3AL receptor subunit, found in humans, has an additional 32 amino acids in the loop between M2 and M3.[52] These isoforms do not form functional homomeric receptors but form functional heteromeric receptors with the native h5-HT3A receptor subunits.[52] Receptors containing h5-HT3AT subunits have decelerated desensitization and therefore, a larger response to $Ca^{2+}$ fluxes.[52] Those receptors containing h5-HT3AL subunits accelerate desensitization and have reduced $Ca^{2+}$ fluxes.[52] The 5-HT3B receptor subtype was isolated in 1999 by screening human genomic sequence data by Davies et al.[53] It shares 41% amino acid sequence identity with h5-HT3A receptors and is also located on chromosome 11, and each subunit has 441 amino acids.[53,54] Rat and mouse orthologs of the 5-HT3B receptor have also been identified.[55] The 5-HT3C, 5-HT3D, 5-HT3E receptor subtypes were cloned by Niesler et al.[56] and Karnovsky et al.[57] independently in 2003. The 5-HT3D receptor subtype as predicted by Niesler et al.[56] lacks a signal peptide and most of the amine terminus ECD including the Cys-loop; not surprisingly, each subunit only has 279 amino acids. The 5-HT3C and 5-HT3E receptor subtypes share 36% and 39% amino acid sequence homology with h5-HT3A receptors,[57] and have 447 and 471 amino acids, respectively. All three of these

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subtypes are located on chromosome 3 and were originally thought to be exclusive to humans. 5-HT3C and 5-HT3E have since been found in chimp and dog.[58]

### 2. Structure and Distribution

Only the 5-HT3A receptor subtype forms homomeric receptors; the remainder of the subtypes form functional heteromeric receptors with 5-HT3A subunits.[53,54,59] The composition and stoichiometry of native heteromeric 5-$HT_3$ receptors has yet to be determined.[60,61] When h5-HT3A and h5-HT3B receptor subunits were epitope-tagged and expressed heterologously in tsA-201 cells they formed heteromeric receptors containing three 5-HT3B and two 5-HT3A receptor subunits with the order of B-B-A-B-A.[62] This stoichiometry was determined by atomic force microscopy. However, the heteromeric 5-HT3AB receptors have different biophysical properties than the homomeric 5-HT3A receptors.[53,54] The pharmacological profile of the heteromeric receptors containing 5-HT3C, 5HT3D, or 5-HT3E subunits in combination with 5-HT3A subunits is similar to homomeric 5-HT3A receptors or heteromeric 5-HT3AB receptors[59,61] but the biophysical properties have yet to be determined. *HTR3A-C* genes are expressed both centrally and in the periphery, whereas *HTR3D* and *HTR3E* genes are only expressed in the gastrointestinal (GI) tract.[61] In addition, 5-HT3C-E receptors appear to be absent in rodents.[61] Within the CNS there are two main regions in which 5-$HT_3$ receptors are expressed: the dorsal vagal complex and the forebrain.[63-65] The dorsal vagal complex contains the nucleus tractus solitarius (NTS), area postrema, and dorsal motor nucleus of the vagus nerve. These are important for the vomiting reflex.[64] Within the forebrain,

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human 5-$HT_3$ receptor recognition sites have been located in the caudate nucleus, putamen, hippocampus, and amygdala.[66-68] The caudate nucleus and putamen are involved in learning while the hippocampus and amygdala (part of the limbic system) are involved in memory processing.[60]

### 3. Pharmacology and Biological Functions

Several different in vivo and in vitro assays are employed for the identification of agonists and antagonists at the 5-$HT_3$ receptors. The von Bezold-Jarisch reflex is one of the most prominent in vivo assays used.[69] Upon administration of an agonist there is a cardiopulmonary reflex in which bradycardia is the most prominent response (the other two responses of the reflex are apnea and hypotension). An antagonist would be any agent that blocks the response of the agonist. Another in vivo assay used is the ferret or shrew emesis assay.[70] Cisplatin is given to the animal to induce vomiting; an antagonist would block this effect, while an agonist would induce vomiting when administered alone. After an application of cantharidin to human skin a blister forms. When 5-HT is applied to the base of the blister it causes pain which can be reversed by 5-$HT_3$ receptor antagonists.[71] In vitro, the most commonly used assay is contraction of guinea pig ileum.[2] An agonist produces contraction while an antagonist would block the contraction of the agonist. Other in vitro assays include the rabbit vagus nerve (5-$HT_3$ receptor agonists produce an increase in the amplitude of C-fiber action potentials), the isolated rabbit heart (release of noradrenaline and acetylcholine by 5-$HT_3$ receptor agonists), and the uptake of [$^{14}$C]guanidinium in NG108-15 cells (5-$HT_3$ receptor agonists stimulate ion

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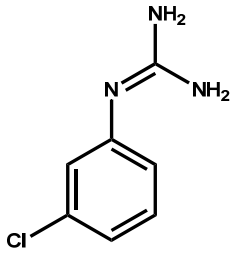
accumulation).[71] Some agents have been shown to be agonists in one assay while antagonists in another assay. For example, *m*-chlorophenylguanidine (mCPG; MD-354; **2**; Figure 4) behaves as an agonist in the von Bezold-Jarisch assay[7] and emesis assays in shrews,[8] but at higher doses behaves as an antagonist in the cisplatin-induced emesis assay.[8] A novel functional assay was developed based on micromechanical measurement of membrane receptor binding.[72] The deflection of microcantilevers is dependent on molecular binding and thus radiolabelled ligands are unnecessary.[73] These particular microcantilevers are modified with a membrane preparation containing 5-HT3AS receptors and bend on the application of the 5-$HT_3$ receptor agonist 5-HT or the 5-$HT_3$ receptor antagonist MDL-72222.[72] Agonists and competitive antagonists compete for the binding on these membrane preparations.




**2**

**Figure 4.** The structure of *m*-chlorophenylguanidine (mCPG; MD-354).

5-$HT_3$ receptors are concentrated in regions of the brain that are involved in the vomiting reflex, anxiety and depression, pain processing, and the reward system.[5,6] The receptors are predominantly found in presynaptic regions with the exception of the hippocampus in which they are postsynaptic.[5] This localization on nerve endings

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contributes to 5-$HT_3$ receptors modulating the release of neurotransmitters.[5] Activation of 5-$HT_3$ receptors modulates the release of various neurotransmitters and neuropeptides including: dopamine, norepinephrine, glutamate, acetylcholine, GABA, and even serotonin.[3,4] Presynaptic activation provides for the influx of $Ca^{2+}$, whereas postsynaptic activation (i.e., in the hippocampus) provides for the influx of $K^+$ and $Na^+$.[22]

There are six 5-$HT_3$ receptor antagonists (Figure 5) on the global clinical market and five are effective in the treatment of chemotherapy-induced and radiation-induced emesis (i.e., ondansetron (**3**), granisetron (**4**), tropisetron (**5**), dolasetron (**6**), and palonosetron (**7**)).[5] These agents are ineffective against motion sickness and apomorphine-induced emesis.[6,74] They induce a complete blockade of both central and peripheral 5-$HT_3$ receptors. The sixth available 5-$HT_3$ receptor antagonist on the market is alosetron (**8**) which is used for the treatment of irritable bowel syndrome (IBS).[5] Recently, the application for the approval by the FDA of cilansetron was withdrawn, but it is a 5-$HT_3$ receptor antagonist also used in the treatment of IBS.[75]

5-$HT_3$ receptors have been implicated in IBS and constipation. In diarrhea-prominent IBS, the 5-$HT_3$ receptor antagonist, ondansetron (**3**), has been shown to alleviate symptoms but can also cause excessive constipation.[76] It is thought that 5-$HT_3$ receptor partial agonists would be able to alleviate the symptoms without causing side-effects.[76] Constipation, which is normally treated by laxatives that cause side-effects, can also be treated by 5-$HT_3$ receptor agonists.[77]

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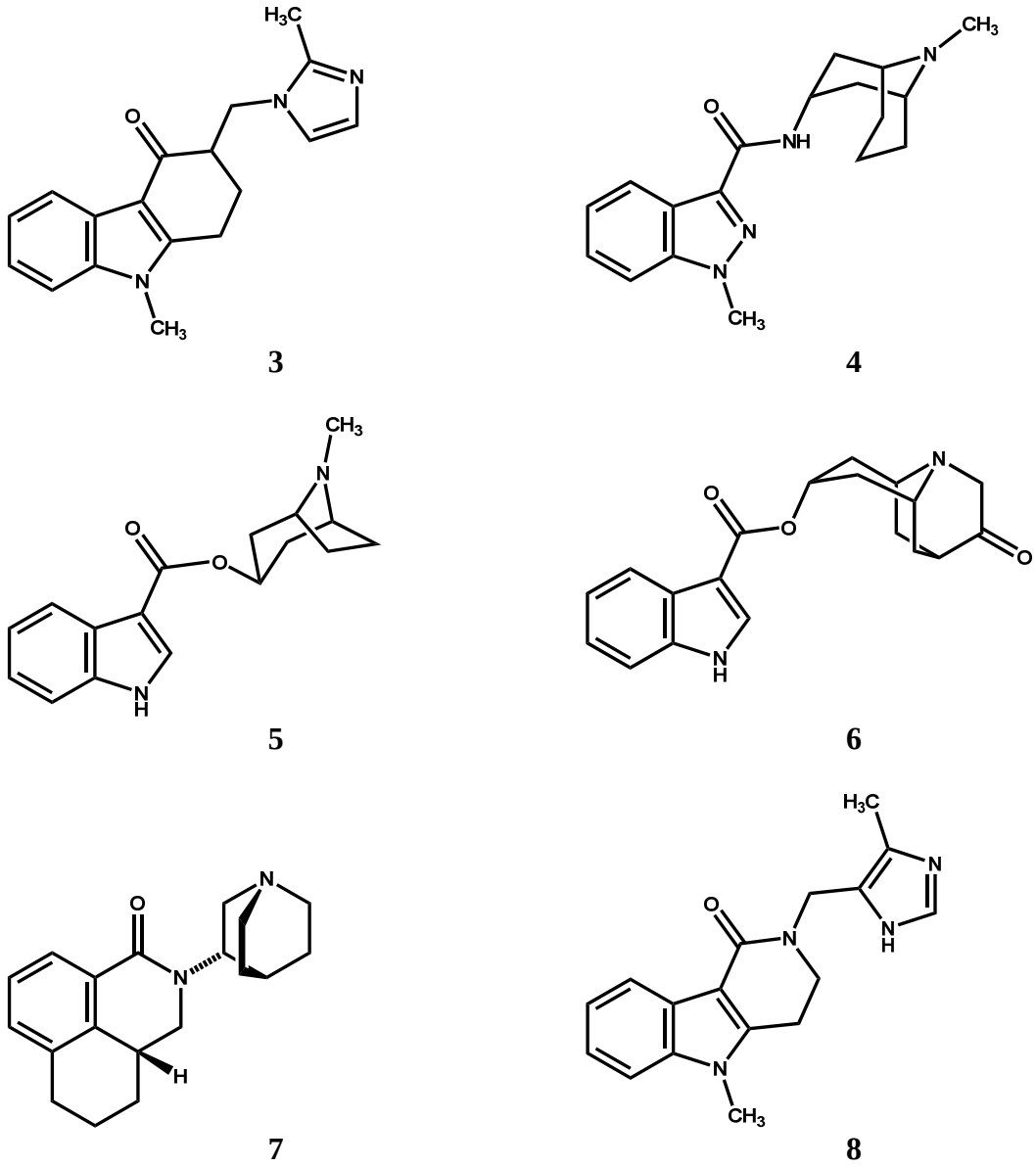


**Figure 5.** Clinically available 5-$HT_3$ receptor antagonists: ondansetron (**3**), granisetron (**4**), tropisetron (**5**), dolasetron (**6**), palonosetron (**7**), and alosetron (**8**).

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5-$HT_3$ receptors are located in the dorsal horn as well as in other regions involved in pain modulation.[78] Stimulation of these receptors produces an antinociceptive effect.[5] MD-354 (**2**), a 5-$HT_3$ receptor partial agonist, has been shown to selectively potentiate the antinociceptive action of clonidine in the mouse tail-flick assay without potentiating clonidine's sedative effects.[79]

In the last decade, 5-$HT_3$ receptors have also been implicated in depression.[80] Ondansetron (**3**), a 5-$HT_3$ receptor antagonist, has been shown to be active as an antidepressant in both the tail suspension and forced swim tests (for a detailed description see Section 7) in mice (Swiss 0.01 – 0.1 μg/kg, ip; albino, 0.25 – 2 mg/kg, ip).[15,17] In earlier studies, however, ondansetron (**3**) (0.01 μg/kg, ip) had been shown to be inactive in the mouse forced swim test by itself but potentiates the sub-active doses of the antidepressants fluoxetine (16 mg/kg), citalopram (16 mg/kg), and fluvoxamine (8 mg/kg).[16] In another study, the 5-$HT_3$ receptor antagonist bemesetron (**9**) (Figure 10) has been shown to be active by itself (3 mg/kg, sc) and at inactive doses (1 mg/kg, sc) in combination with inactive doses of ketamine (12.5-25 mg/kg, sc) in the mouse tail suspension test (C57BL/6J/Han).[13] The exact involvement of 5-$HT_3$ receptors in depression has yet to be identified. However, Bhatnager et al.[81] demonstrated that there are differences between wild-type (WT) and 5-$HT_3$ receptor knock-out (KO) male and female mice in the forced swim and the defensive withdrawal tests (light-dark test). In the mouse forced swim test, KO females exhibited increased behavioral indices of depression type behavior in comparison to WT females, thus showing that 5-$HT_3$ receptors do play a

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role in depression. In addition, single nucleotide polymorphisms on the *HTR3B* gene have been linked with major depression.[82]

5-$HT_3$ receptor antagonists may also be effective in influencing the reward system of drug abuse because of the modulation of dopamine.[5] Other possible clinical implications for 5-$HT_3$ receptor antagonists include cognitive functions, schizophrenia, satiety, and anxiety.[5]

**4. Agonists**

Since serotonin (**1**; 5-$HT_3$ $K_i \approx 1000$ nM) is a tryptamine, the first ligands studied were other tryptamine analogs. Methylation of the indole nucleus of 5-HT (e.g., 2-methyl-5-hydroxytryptamine; **10**; $K_i \approx 1300$ nM) (Figure 6) was found to result in an agent that was less potent but more selective than 5-HT as a 5-$HT_3$ receptor agonist.[83,84] However, **10** is rapidly metabolized and has difficulty crossing the blood-brain barrier (BBB).[85] Methylation of the terminal amine was also examined.[86,87] *N*-Monomethyl-5-HT was shown to be active in the depolarization of superior cervical ganglion cells (it has an equipotent molar ratio (epmr) of 1.3 relative to 5-HT) while *N,N*-dimethyl— (**11**) and *N,N,N*-trimethyl—5-HT (5-HTQ; **12**) were found to have increased activity (empr of 0.08 and 0.01, respectively).[87] Similarly, in binding affinity assays, **11** and **12** were found to bind with enhanced affinity ($K_i \approx 280$ nM and 75 nM, respectively) relative to 5-HT.[86] 5-HTQ (**12**) has a permanent positive charge that was well tolerated by 5-$HT_3$ receptors ($K_i \approx 75$ nM), but 5-HTQ does not readily cross the BBB.[86] Longer terminal amine substituents and locking the terminal amine into a ring abolished affinity.[86]

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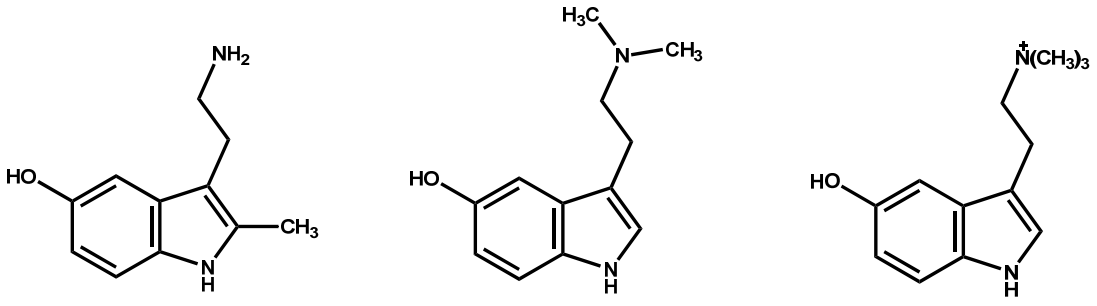
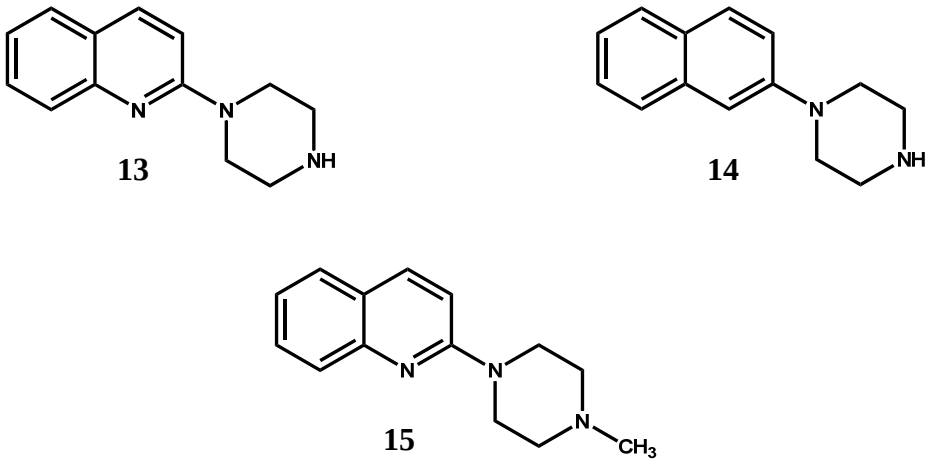



**10** **11** **12**

**Figure 6.** The structure of methylated analogs of 5-HT.

Another of the first agonists investigated were the arylpiperazines (Figure 7).[7,88] Quipazine (2-(1-piperazinyl)quinoline; **13**; $K_i \approx 3$ nM) was the first of this class to demonstrate 5-$HT_3$ receptor binding.[89] Quipazine (**13**) was used as a lead compound to develop two general series: bicyclic and monocyclic arylpiperazines. Phenylpiperazine itself binds with low affinity ($K_i \approx 3000$ nM) at 5-$HT_3$ receptors.[7] The 3-position of phenylpiperazine was shown to be important for binding as demonstrated by the impro-




**13** **14** **15**

**Figure 7.** The structure of a few arylpiperazine analogs.

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ved affinity of 3-chlorophenylpiperazine ($K_i \approx 62$ nM).[7] However, the corresponding 3-$CF_3$ analog displayed decreased affinity ($K_i \approx 1390$ nM).[7] The affinity of 3-chlorophenylpiperazine could be mimicked by its naphthyl analog 1-(2-naphthyl)piperazine (2-NP; **14**; $K_i \approx 32$ nM).[88] Methylation of the terminal piperazine nitrogen atom of quipazine further increased binding affinity and selectivity (NMQ; **15**; $K_i \approx 2$ nM).[88] Tricyclic derivatives have also been studied.[90-92] Quite a few arylpiperazines have been shown to bind at 5-$HT_3$ receptors; some have been shown to be agonists and some have been shown to be antagonists.[83] However, none of them have been shown to be highly selective for 5-$HT_3$ receptors because they also show affinity for other serotonin receptors[93,94] and the serotonin reuptake transporter (SERT).[95]

The first non-tryptamine, non-arylpiperazine agonist ligand that demonstrated selectivity for 5-$HT_3$ receptors was phenylbiguanide (PBG; **16**; $K_i \approx 1000$ nM) (Figure 8); despite its low affinity, PBG was shown to be more selective than early arylpiperazines.[7,87] PBG is a selective low affinity agonist and arylpiperazines are higher affinity non-selective agents. Although there was no evidence suggesting that PBG was binding in the same manner as arylpiperazines, the assumption that they use the same aromatic site served as the starting point for the investigation of novel analogs of PBG. If they bind in a similar manner, then it might be possible to use the structure-affinity relationships (SAFIR) of arylpiperazines to enhance the affinity of the arylbiguanides. Two novel arylbiguanides were looked at initially, 1-(3-chlorophenyl)biguanide (mCPBG; **17**; $K_i \approx 17$ nM) and 1-(2-naphthyl)biguanide (**18**; $K_i \approx 12$ nM).[7,93] Both have

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high affinities. mCPBG not only displayed partial agonist characteristics in functional studies (i.e., rabbit bladder preparation and von Bezold-Jarisch reflex)[7] but was shown that in a competition for binding to 5-$HT_3$ receptors mCPBG inhibited [$^3$H]GR67330 with 100 times higher affinity than 5-HT (**1**) or PBG (**16**).[96] PBG and mCPBG have been two of the most widely used 5-$HT_3$ receptor agonists even though they may not penetrate the BBB.[97] Bachy et al.[97] found that both PBG and mCPBG displayed poor brain penetration in mice because they did not displace the binding of [$^3$H]granisetron in an ex vivo binding experiment. In addition, Rahman et al.[9] showed that both possess low octanol/water partition coefficients. In contrast, Kilpatrick and Rogers[98] have shown that mCPBG displaces [$^3$H]GR65630 in rat entorhinal cortex in an ex vivo binding study. Moreover, Steward et al.[99] have used [$^3$H]mCPBG to label 5-$HT_3$ receptor recognition sites in rat brain, which would also indicate that mCPBG does cross the BBB. More selective agonists have since been developed (e.g., 2,3,5-tri-Cl-PBG, $K_i$ = 0.4 nM)[100]; however, mCPBG is still commonly used. A wide variety of substituents on the phenylbiguanide ring have been investigated but will not be discussed here.[7,83,101]

Using a deconstruction-reconstruction-elaboration approach, the elements needed for binding of mCPBG (**17**) at 5-$HT_3$ receptors were evaluated.[7,100] In the deconstruction-reconstruction-elaboration approach all substituents from a ligand that bind well to a particular receptor are removed and then the ligand is reconstructed by re-introducing each of the original substituents individually.[102] This approach allows for each substituent to be evaluated for its effect on binding affinity and/or receptor selectivity.[102] The

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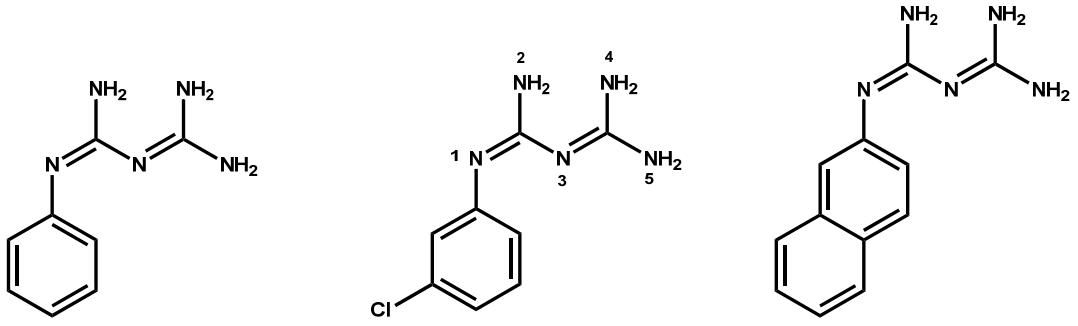
resulting ligand is elaborated on to explore new substituents and their role on binding affinity and/or receptor selectivity.[102] During the elaboration process N,N-Dimethylation of the terminal amine of the biguanide moiety was found to abolish affinity.[7] Replacing the N2 nitrogen atom (Figure 8) of mCPBG with an oxygen atom greatly decreased affinity.[7] Furthermore, removal of the N4 nitrogen atom abolished affinity.[7] The N-(2-phenylethyl)guanidine analog binds with high affinity ($K_i \approx 40$ nM), indicating that the




**16** **17** **18**

**Figure 8.** The structure of phenylbiguanide analogs.

biguanide moiety may not be required for binding.[7] Upon further deconstruction of mCPBG, the biguanide function was shortened to a guanidine yielding a new class of 5-$HT_3$ receptor agonists—the phenylguanidines (PG). The first member of this class was 3-chlorophenylguanidine (mCPG; MD-354; **2**; $K_i \approx 35$ nM) which has been shown to also be an agonist in the von Bezold-Jarisch assay and the rabbit bladder prepartion.[7] Various aryl substituents have been investigated by our laboratory (Table 2).[7,100] Parallel changes in the structures of PBGs and PGs led to parallel shifts in affinity suggesting that the two

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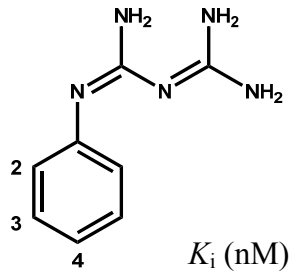
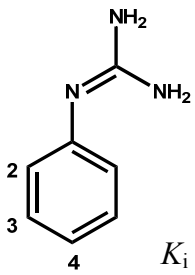
series bind in a similar manner.[100] For example, removal of the 3-chloro group to give the parent PG (**27**) reduced affinity ($K_i \approx 2340$ nM); likewise removal of the 3-Cl group from mCPBG (**17**) to generate PBG (**16**) decreased affinity ($K_i \approx 1000$ nM). Positional changes of the chloro group (i.e., 2-Cl and 4-Cl) also decreased affinity (**19-20**, **28-29**), but not as

**Table 2.** Binding affinities of several arylbiguanides and arylguanidines.[7,8,101]




| | $K_i$ (nM) | R | | $K_i$ (nM) |
|---|---|---|---|---|
| **16** | ≈1,000 | H | **27** | 2,340 |
| **17** | 17 | 3-Cl | **2** | 35 |
| **19** | 62 | 2-Cl | **28** | 190 |
| **20** | 200 | 4-Cl | **29** | 320 |
| **21** | 220 | 3-$NO_2$ | **30** | 85 |
| **22** | 780 | 3-$CH_3$ | **31** | 6,520 |
| **23** | 700 | 3-$CF_3$ | **32** | 5,700 |
| **24** | 12 | 3,4-di-Cl | **33** | 3.1 |
| **25** | 1.8 | 3,5-di-Cl | **34** | 5 |
| **26** | 2.7 | 3,4,5-tri-Cl | **35** | 0.7 |

dramatically as removal of the chloro group. Replacing the 3-chloro group with an electron-donating group (i.e., 3-methyl; **22**, **31**) or a better electron-withdrawing group (i.e., 3-$CF_3$: **23**, **32)** resulted in a decrease in affinity as shown in Table 2. Replacing the 3-chloro group with the electron-withdrawing group 3-$NO_2$ (i.e., **21**, **30**) resulted in slightly decreased affinity. Multiple chloro groups (i.e., 3,4-di-Cl, 3,5-di-Cl, 3,4,5-tri-Cl) increased affinity (**24-26**, **33-35**).

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Based on the binding profile of several arylguanidines at 5-$HT_3$ receptors a pharmacophore model was developed. A few pharmacophore models for agonists and partial agonists have been developed but these were for other classes of 5-$HT_3$ receptor ligands.[103,104] Dukat developed the current working pharmacophore for arylguanidines and arylbiguanides (Figure 9).[83] The three main components of the working pharmacophore for arylguanidines and arylbiguanides are i) an aromatic ring, ii) an adjacent nitrogen atom, N1, to the ring, and iii) a terminal amine; with distances

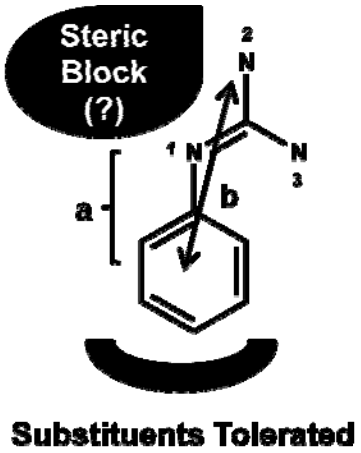


**Figure 9.** Dukat's pharmacophore for the binding of arylguanidines and arylbiguanides at 5-$HT_3$ receptors. [83]

between the aromatic ring centroid and the adjacent nitrogen 2.7 Å (a) and the distance between the centroid and the terminal amine between 4.5 and 4.9 Å (b). Substitution at the *meta* and *para* positions are tolerated and can enhance affinity depending on their lipophilic and electronic character. Steric bulk does not seem to be tolerated near the N1 nitrogen atom.

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### 5. Antagonists

In the late 1970s, Fozard reported metoclopramide and cocaine (which was also reported by Gaddum and Picarelli)[2] to be weak antagonists of the 5-HT-M receptor.[105] In 1983, bemesetron (MDL-72222; **9**) was first synthesized as an analog of cocaine.[106] Shortly after in 1985, workers at Sandoz identified tropisetron (ICS 205-930; **5**) as a 5-$HT_3$ receptor antagonist.[84] Since then, several hundred other compounds have been developed as 5-$HT_3$ receptor antagonists with a wide range of classifications.[107-109] The two overarching classes are the benzamides or benzoate esters, and 6,5-heteroaromatics.

Benzoate esters (i.e., bemesetron; **9**; Figure 10) and benzamides (i.e., zacopride; **37**; Figure 10) were developed, as mentioned earlier, as analogs of cocaine.[105] Starting with benzoate esters, the endo isomer was found to be more active than the exo isomer.[106] Substitutions at the 3-position were favored over the 4-position, and a 3,5-di substituted analog (e.g., bemesetron; **9**; $pA_2$ = 9.3 rabbit heart) was found to be more active than just the 3-substituted analog (as reviewed by King).[108] However, the 3,5-dichlorobenzamide analog was reported to be more potent than the 3,5-dichlorobenzoate analog.[110] Expanding further, it was found that 2-$OCH_3$, and 4-$NH_2$ groups increased potency significantly leading to zacopride (**37**; Bezold-Jarish, $ID_{50}$ 0.7 μg/kg i.v.) and renzapride (**38**) (Figure 9) (as reviewed by King).[108]

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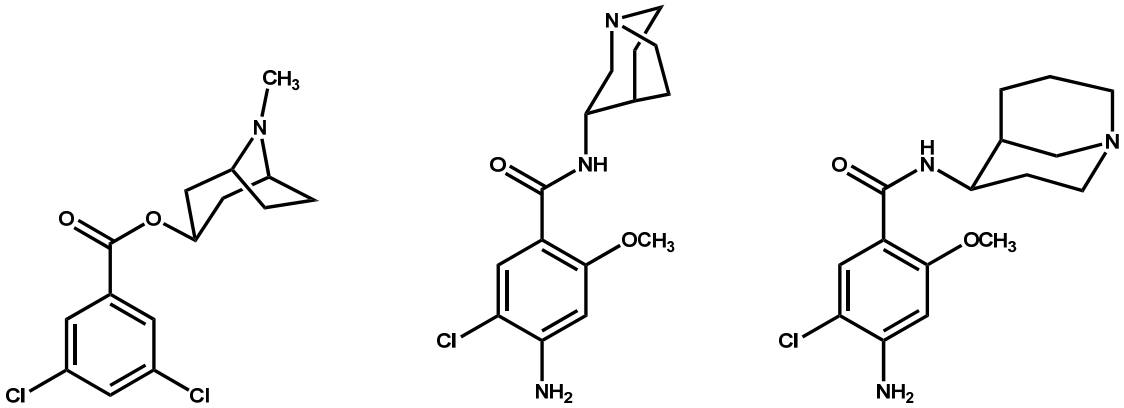


**9** **37** **38**

**Figure 10.** The structure of a few benzoate ester and benzamide 5-$HT_3$ receptor antagonists.

The largest class, 6,5-heteroaromatics, includes the carbazole ondansetron (**3**; $K_i \approx$ 7.59-7.76 nM), the indole tropisetron (**5**; $K_i \approx$ 3.80-5.50 nM) and the indazole granisetron (**4**; $K_i \approx$ 2.34-3.24 nM**)**.[111] Richardson et al. identified tropisetron (**5**) as the first highly potent 5-$HT_3$ receptor antagonist that includes an indole ring and an endo(tropane) side chain.[84] Variations on the side chain led to the development of dolasetron (**6**).[108] Modifications of the indole ring to indazole produced compounds similar to granisetron (**4**).[108] In the indazole series, the 1-$CH_3$ analog showed increased potency unlike with the indole series.[108] In the indolylpropanone series, substitutions of the terminal amine with –$N(CH_3)_2$ (p$A_2$ = 6.5, rat vagus nerve) and –imidazole (p$A_2$ = 7.61, rat vagus nerve) were shown to be active; while –pyrazole and –triazole were inactive.[112] Substitutions at the 2- and 4-position of the imidazole of an imidazolyl indolylpropanone were investigated to prevent interactions with Cytochrome P450.[112] The methyl group at the 2-position of the imidzole was shown to be the most potent N-linked imidazolyl indolylpropanone

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investigated so far in the Bezold-Jarisch assay.[112] Conformationally constraining the carbonyl to the indole ring to form an N-linked imidazolylmethyl tetrahydrocarbazolone provided a more potent analog (i.e., ondansetron; **3**; p$A_2$ = 8.6 rat vagus nerve) than the unconstrained analog (i.e., the 2-$CH_3$ imidazolyl indolylpropanone analog; p$A_2$ = 8.0 rat vagus nerve).[112] Ondansetron (**3**) had enhanced oral activity ($ED_{50}$ = 7 μg/kg, po) in the rat and a long duration of action (>3 h at 30 μg/kg, iv) in the Bezold-Jarisch assay in the cat.[112] Unusually, both enantiomers of ondansetron had similar activity in vitro ((S)-enantiomer p$A_2$ = 8.55; (R)-enantiomer p$A_2$ = 8.95, rat vagus nerve).[112] In determining whether a N-linked imidazole was preferred, several C-linked imidazole analogs were investigated (i.e., GR65630; p$A_2$ = 9.9; alosetron; **8**; p$A_2$ = 10.2, rat vagus nerve).[112] This class is of particular interest since it includes all but one of the clinically available and highly selective antagonists for 5-$HT_3$ receptors (Figure 5). The remaining clinically available agent is a 6,6,6-heteroaromatic compound (i.e., palonosetron; **7**) (Figure 5).

### 6. Quantitative Structure-Activity Relationships

The concept of quantitative structure-activity relationships (QSAR) was developed by Hansch on the basis that biological activity is a function of chemical structure.[113,114] By structure, Hansch considered the lipophilic, electronic, and steric effects of a molecule. A Hansch analysis is performed by inspecting the linear regression of the biological activity (e.g., p$K_i$) and various structural parameters (e.g., σ, π, MR, L, B1, B5) (Table 3). Examples of lipophilic parameters include partition coefficients (log P) and the substituent's hydrophobicity constant (e.g., π).[115] Electronic parameters

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include Hammet constants (i.e., $\sigma_m$ and $\sigma_p$),[115] dissociation constants,[115] and dipole moments.[116] Steric parameters include Taft's substituent constant ($E_s$),[117] Verloop parameters (e.g., L, B1, B5),[118,119] and molar refraction (MR).[115] Molar refraction and complete molar refraction (CMR) can also be considered a polarizability parameter along with the number of valence electrons (NVE),[120] the parachor constant (Pc),[121] and the polarizability values.[120]

**Table 3.** Definition of various Hansch analysis parameters.

| Parameter | Definition |
|---|---|
| $\pi$ | Hydrophobicity constant[a] |
| $\sigma_m$ | Hammett constant, electronic[a] |
| L | Length between substituent and parent compound[b] |
| B1 | Shortest width of the substituent[b] |
| B5 | Longest width of the substituent[c] |
| MR | Molar refraction of the substituent;[a] $\left[\left(n^2-1\right)/\left(n^2+2\right)\right]*\left[MW/\rho\right]$ |
| Vol | Solvent accessible volume[d] |
| NVE | Number of valence electrons;[e] H = 1; C = 4; N = 5; O = 6, Halogen = 7 |
| CMR | Complete molar refraction[f] |
| MV | Molar volume;[g] $MW/\rho$ |
| Pc | Parachor based on surface tension and MV;[g] $\gamma^{1/4}\left(MW/\rho\right)$ |
| Polarizability | Polarizability of the compound[g] |

[a]Hansch, et al.[115]
[b]Verloop, et al. [118]
[c]Tipker and Verloop[119]
[d]Measured using Chimera
[e]Verma et al.[120]
[f]Calculated using ChemDraw
[g]Calculated using ChemSketch

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Comparative molecular field analysis (CoMFA) is another method of QSAR analysis. A 2001 study by Dukat et al. on the binding of arylguanidines and arylbiguanides was conducted using 33 compounds spanning a 10,000-fold $K_i$ range.[100] The relationship between parallel substituted arylguanidines and arylbiguanides was nearly linear (i.e., $r = 0.932$, $n = 9$), so it was considered acceptable to use a combination of these two classes of agents in the same study.[100] The results showed that binding to 5-$HT_3$ receptors was sensitive to the electronic character of the 3-position and that the lipophilic character of the 4-positon might also contribute to binding. However, these compounds are not co-planar making the two *meta*-positions different; with a sample size this small ($n = 33$) the investigators were unable to determine which *meta*-position is involved in binding.[100] In CoMFA studies the $q^2$ value is the predictability of a model based on cross-validation,[122] and a good $q^2$ value is considered to be greater than 0.6.[123] The $r^2$ value is the fit of the binding affinities; the squared correlation coefficient is usually high when the ligands are well aligned. The $q^2$ value for this CoMFA study was 0.584 and the $r^2$ value was 0.851.[100] Based on the results 79.5% steric and 20.5% electronic effects impacted the binding at 5-$HT_3$ receptors.[100]

In 2003, Glennon et al.[124] performed a QSAR study on the binding of arylguanidines and arylbiguanides at 5-$HT_3$ receptors. As part of this study the authors found no correlation between affinity and $\pi_3$, but that there is some relationship between affinity and $\sigma_m$ for the 3-monosubstituted arylguanidines.[124] As for the 4-monosubstituted arylguanidines it was found to be the opposite of the 3-monosubstituted compounds;

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there was a relationship between affinity and the hydrophobicity constant $\pi_4$, but not between affinity and the electronic constant $\sigma_p$.[124] Overall, the electron withdrawing nature of the 3-position substituent and the lipophilic nature of the 4-position substituent contribute to the affinity of arylguanidines.[124] In addition, the polarizability of the arylguanidines seems to play a role in the affinity of these ligands for 5-$HT_3$ receptors.[124]

### 7. Behavioral Assays for Antidepressants

Because a portion of the work to be described herein involves the possible antidepressant actions of 5-$HT_3$ receptor ligands, it is appropriate to describe a few assays that have been used previously to evaluate possible antidepressant activity and are applicable to the current investigation.

In the late 1970's Porsolt developed a behavioral assay for the screening of antidepressants—the forced swimming test (FST).[125,126] This test is based on the observation that a mouse placed in water swims (perceivably to escape) then alternates to a period of immobility. Antidepressants decrease the amount of time the mouse spends immobile and increase the amount of time the mouse spends swimming. However, there are a number of issues with the FST that make it less than ideal. Since the water is approximately at room temperature (23-25 °C) and the mouse's body is quite small, hypothermia can occur.[127] With this test, there is an inherent need for the animals to be able to swim; some genetically altered mice have decreased motor function and cannot swim.[128] The FST has also been shown to be ineffective in certain strains of mice (i.e., NMRI, C57BL/6J, and DBA2) for almost all known types of antidepressants.[129] In

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addition, the FST has been shown to display inconsistent results regarding selective serotonin reuptake inhibitors (SSRIs).[130]

The mouse tail suspension test (TST) is a behavioral assay developed by Steru et al.[131] based on the observation that a mouse suspended by its tail will exhibit two types of alternating behaviors: a waiting phase characterized by immobility or passive swaying, and a searching phase characterized by running motions, body jerks, and body torsions in which the mouse attempts to catch its tail. This test, similar to the FST, is based on changes in immobility time.[126] A mouse given an antidepressant will exhibit lower immobility times than a mouse given saline; the mouse will spend more time in the searching phase. With the TST, the mice become immobile more quickly over the course of the test; in the FST, immobility generally occurs in the last half of the test, but in the TST immobility can occur at any point.[128] However, immobility periods are shorter in duration over the course of the TST.[128] Occasionally, false positives are encountered if the drug given causes an overall increase in motor activity and which can be interpreted as decreased immobility time (see below for greater detail; "Locomotor activity"). The TST also is more sensitive to lower doses of drugs.[131]

In the few studies reported to date, the 5-$HT_3$ receptor antagonists ondansetron (**3**) and bemesetron (**9**) have been shown to be active in these types of behavioral tests for antidepressants leading investigators to believe that 5-$HT_3$ receptors may be involved in depression.[13,15,17] However, in two of the four FST assays, ondansetron was found to be inactive (Table 4). Bemesetron has only been examined in the TST. In one investigation,

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the 5-$HT_3$ receptor agonist, SR 57227A, was studied both in mouse and rat FST and was found to be effective at high doses.[14] Table 4 summarizes the 5-$HT_3$ receptor ligands that have been tested for antidepressant activity in rodents.

**8. Locomotor Activity Assay**

The TST and FST are only indicative of antidepressant activity. Decreased immobility time may also occur when the overall movements are increased. This increase in generalized motion can give a false positive result in the TST and FST. Using a locomotor activity assay, the overall motion can be investigated in relation to vehicle (i.e., saline). This test is conducted in square, transparent chambers surrounded by infrared photo detectors. These photo detectors are located near the floor of the chamber (~ 1.75 cm above the floor), as well as, ~ 6.5 cm above the floor of the chamber. Using the breaks in the infrared beams, a computer program identifies precisely where the mouse is located in the chamber and the type of motion. If the compound in question has increased general motion relative to the vehicle, it may indicate that the decreased immobility times of the FST or TST are not because of an antidepressive effect but to an overall increase in movement.

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**Table 4.** 5-$HT_3$ receptor ligands assessed in antidepressant assays.

| Compound | Species | Strain | Assay | Route of Admini-stration[a] | Antidep-ressant Activity | Doses Examined | Active Dose | Ref |
|---|---|---|---|---|---|---|---|---|
| Ondansetron | Mice | Swiss | FST | ip | Yes | 0.005-1000 µg/kg | 0.01-0.1 µg/kg | 15 |
| | Mice | Swiss | TST | ip | Yes | 0.005-1000 µg/kg | 0.1-1 µg/kg | 15 |
| | Mice | Swiss | FST | ip | No | 0.01 µg/kg | none | 16 |
| | Mice | Albino | FST | ip | Yes | 0.25-2 mg/kg | 0.25-2 mg/kg | 17 |
| | Mice | Swiss | FST | ip | No | 0.01-0.1 µg/kg | none | 12 |
| Bemesetron | Mice | C57BL/6J/Han | TST | sc | Yes | 0.3-3 mg/kg | 3 mg/kg | 13 |
| SR 57227A | Mice | ICR | FST | ip | Yes | 1-30 mg/kg | 3-30 mg/kg | 14 |
| | Mice | ICR | FST | po | Yes | 1-30 mg/kg | 10-30 mg/kg | 14 |
| | Rat | Sprague-Dawley | FST | ip | Yes | 1-30 mg/kg | 10-30 mg/kg | 14 |
| | Rat | Sprague-Dawley | FST | po | Yes | 1-30 mg/kg | 10-30 mg/kg | 14 |

[a] Intraperitoneal (ip), Subcutaneous (sc), Per os (po).

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## III. Specific Aims and Rationale

The arylguanidine series of 5-$HT_3$ receptor ligands has been examined by Dukat and co-workers[9,100,124] in the past to determine the type of substituents favored for binding. MD-354 (**2**) is a bi-planar molecule where the guanidine function is skewed relative to the aromatic ring; therefore, the *meta*-positions are non-equivalent. Several series of compounds will be synthesized to investigate the role of *meta*-position substituents in binding. One series will investigate the size of the substituent while another will investigate which *meta*-position is important.

Based on prior QSAR studies (see Background), steric and electronic effects at the 3-position, but not lipophilic effects at the 3-position, can influence the fit (i.e., affinity) of the ligand in the 5-$HT_3$ receptor binding pocket. These previous studies indicated *meta*-position substituents are important for binding. Since the 3-Cl analog (MD-354, **2**; $K_i$ = 32 nM), which bears an electron-withdrawing substituent, binds with high affinity, but analogs with electron-donating groups, as seen with the 3-$CH_3$ (**31**; $K_i$ = 6520 nM) and 3-$OCH_3$ (**39**; $K_i$ = 1600 nM) analogs, bind with reduced affinity, it might be concluded that electronic effects play a significant role.[124] However, the 3-$CF_3$ analog (**32**; $K_i$ = 5700 nM), an analog containing a stronger electron-withdrawing substituent than a chloro group, binds with reduced affinity relative to MD-354 (**2**) which may indicate that the possibility of steric interactions or bulk limitation.[124] It may be the size



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or the shape of the $–CF_3$ group that detracts from binding. The –Cl substituent has a spherical shape whereas a $–CF_3$ substituent does not; its shape may not be tolerated by the receptor. The $–CF_3$ group occupies a slightly larger volume of space than the –Cl group (Table 5) which may also not be tolerated by the receptor. One of the aims of this project is to synthesize other 3-halogenated analogs of MD-354 (**2**) and to examine their electronic and steric effects on binding affinity. Fluoro, bromo, and iodo groups are electron-withdrawing groups and together with the –Cl and$–CF_3$ groups, provide a range of electron-withdrawing substituents of graded size and relatively similar electron-withdrawing capability (Table 5). Examination of the –F, –Br, and –I analogs (i.e., **42-44**), in comparison with the –Cl (**2**) and $–CF_3$ (**32**) compounds (as well as with the parent unsubstituted molecule **27**), will provide information about the importance of the steric and electronic contribution of 3-position substituents to affinity. With the halogen analogs, substituent size can be varied while keeping shape and electronic character relatively constant (Figure 11). Therefore, compounds **42-44** will be synthesized and examined for comparison with **2**, **27**, and **32**.

A Hansch analysis of 3-substituted analogs might assist in determining which structural features may be important for binding at $5\text{-}HT_3$ receptors. The relationship between $pK_i$ and various parameters (i.e., π, σ, L, B1, B5, MR, complete molar refraction (CMR), volume, molar volume (MV), NVE, Pc, and polarizability) will be investigated through a Hansch analysis. Correlation coefficients from linear regression analysis would indicate the possible significance of those parameters.

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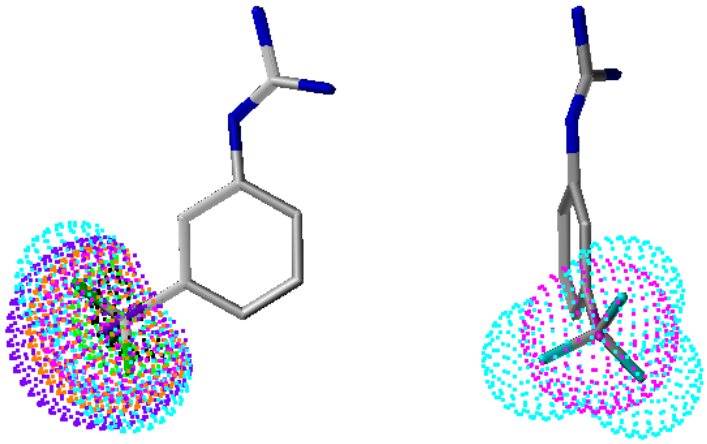
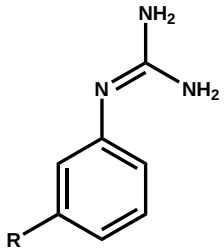


**Figure 11.** Superimposition of the Van der Waals surface of 3-$CF_3$ (cyan), 3-I (purple), 3-Br (orange), 3-Cl (pink), 3-F (green), and 3-H (black) PGs (left). Superimposition of the Van der Waals surface of 3-$CF_3$ (cyan) and 3-Cl (pink) PGs (right.)

**Table 5.** Electronic, lipophilic, and steric effects of 3-substituted arylguanidines.




| | R | $\pi^a$ | $\sigma^a$ | Volume[b] | $K_i$ (nM)[c] |
|---|---|---|---|---|---|
| **27** | H | 0.00 | 0.00 | 121.7 | 2,340 |
| **2** | Cl | 0.71 | 0.37 | 144.8 | 35 |
| **30** | $NO_2$ | -0.28 | 0.71 | 143.9 | 85 |
| **31** | $CH_3$ | 0.56 | -0.07 | 138.3 | 6,520 |
| **32** | $CF_3$ | 0.88 | 0.43 | 150.5 | 5,700 |
| **39** | $OCH_3$ | -0.02 | 0.12 | 145.6 | 1,600 |
| **40** | CN | -0.57 | 0.56 | 139.2 | 123 |
| **41** | OH | -0.67 | 0.12 | 126.8 | 2020 |
| **42** | F | 0.14 | 0.34 | 125.6 | |
| **43** | Br | 0.86 | 0.35 | 165.6 | |
| **44** | I | 1.12 | 0.35 | 197.7 | |

[a] π and σ values as reported by Hansch et al.[115]
[b] Volume is the solvent accessible volume measured using Chimera.
[c] Results from a previous study.[7,100]

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Since MD-354 is bi-planar, it can exist in multiple conformations. In two of the possible rotamers, the substitutents can reside at either one of two *meta*-positions (i.e., the 3- or 5-positions) (Figure 12). By constraining the guanidine function, it can be determined which of the two *meta*-positions is more important for binding (Figure 13). Constraining the guanidine to a five-membered ring (e.g., as with **45**) sharply decreased binding affinity ($K_i$ = 725 nM) relative to MD-354 (**2**; $K_i$ = 35 nM).[9] Previous literature reported the synthesis of what was thought to be 2-amino-7-chloro-3,4-dihydroquinazoline (**46**);[9] this was later shown to actually be 2-amino-6-chloro-3,4-dihydroquinazoline (**47**).[132] In addition, 2-amino-5-chloro-3,4-dihydroquinazoline (**48**) has been synthesized previously.[133] Since 2-amino-7-chloro-3,4-dihydroquinazoline (**46**) has not been synthesized previously, it will be targeted for synthesis by this investigation to complete the series.

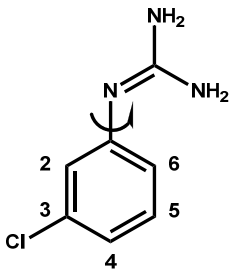


**Figure 12.** Representation of a rotatable bond in MD-354 (**2**).

Increasing the ring size constraining the guanidine allows for an increase in the angle of the aromatic and guanidine planes, so the seven-membered constrained analog

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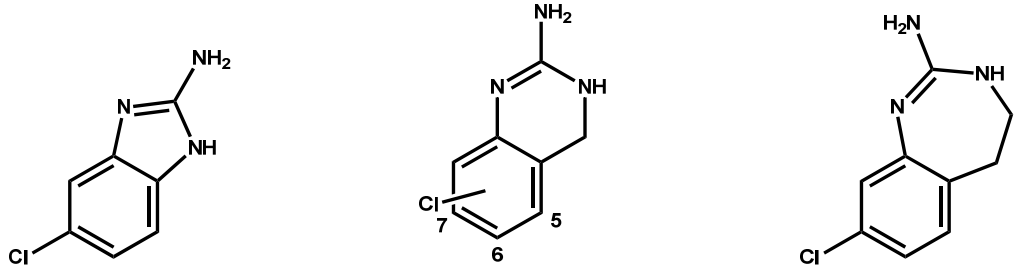


**45** **46** 7-Cl **49**
**47** 6-Cl
**48** 5-Cl

**Figure 13.** Structure of conformationally-constrained analogs of MD-354.

(2-amino-8-chloro-1,3-benzodiazepine; **49**; Figure 13) will also be synthesized. In addition, the different ring sizes allow for small adjustments in the centroid-to-amine distance which has been shown to be important for 5-$HT_3$ receptor binding (Figure 9) (MD-354 centroid-to-amine distance was previously calculated to range from 3.7 to 4.9 Å; **45** centroid-to-amine distance was previously calculated to be 4.5 Å; **46-48** centroid-to-amine distance was previously calculated to be 4.9 Å).[9] For the purpose of this study, the ligands were built in Sybyl 7.3, given new charges and geometries calculated with molecular orbital package (MOPAC), and the aromatic centroid to primary amine distances were measured (Table 6). The aromatic centroid to primary amine (N2) distance of MD-354 (**2**) is calculated to be 5.1 Å. The aromatic centroid to primary amine distance of **45** is calculated to be 4.5 Å. The aromatic centroid to primary amine distance for **46-48** is calculated to be 5.0 Å and the aromatic centroid to primary amine distance of **49** is calculated to be 5.1 Å. Other methods of investigating the skewness of these constrained analogs are to examine the height above the plane, the plane angle and the

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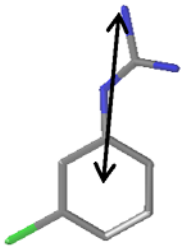
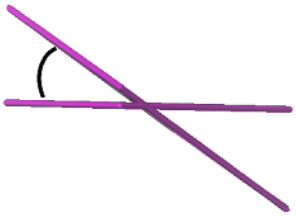
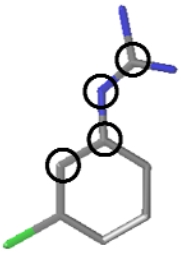
torsion angle (Table 6). The height above the plane for the nitrogen atoms indicate how much out of the plane of the aromatic ring the guanidine is. Since the guanidine function is in a separate plane from the aromatic ring the angle of these planes can also be calculated. The torsion angle is the twist of the molecule while going from the guanidine portion to the aromatic ring.

**Table 6.** Measurements of biplanarity of MD-354 and its constrained analogs.

| Compound | Amine height above plane | Aromatic centroid to primary amine distance | Plane angle | Torsion angle |
|---|---|---|---|---|
| **2** | 0.21683 Å | 5.122 Å | 34.91° | 149.9° |
| **45** | 0.00327 Å | 4.537 Å | 0.06° | 180.0° |
| **46 - 48** | 0.16662 Å | 5.042 Å | 8.48° | 189.9° |
| **49** | 0.24949 Å | 5.133 Å | 7.13° | 184.4° |

Another goal of this work will be to confirm whether or not 5-$HT_3$ receptor ligands produce an antidepressant effect in animals. Depression has a life-time prevalence of 17% in the United States.[134] Current treatments for depression are fully effective for 30% of patients.[135] The antidepressants currently available (i.e., tricyclic antidepressants (TCAs), selective serotonin reuptake inhibitors (SSRIs), and serotonin-norepinephrine reuptake inhibitors (SNRIs)) have a long onset of action (usually two or more weeks).[136,137] These drawbacks indicate a need for a new understanding on how to treat

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depression. Although studies are scant, two 5-$HT_3$ receptor antagonists (i.e., ondansetron and bemesetron) and the 5-$HT_3$ receptor agonist (i.e., SR 57227A) have been shown to behave as potential antidepressants in animal studies (Table 4). If 5-$HT_3$ receptors play a role in depression then different structural classes of 5-$HT_3$ receptor agents or different receptor interactions may be able to elicit the same response. To determine if the novel series of quinazoline ligands behaves in the same manner, several analogs will be tested using the mouse tail suspension test for antidepressant activity. The parent compound, MD-354 (**2**), will also be tested. Tricyclic antidepressants (i.e., desipramine and imipramine), an SSRI (i.e., fluoxetine), and a 5-$HT_3$ receptor antagonist (i.e., ondansetron) will be used as controls. Statistical analysis will be performed to determine the statistical significance of any decreases in immobility time relative to saline using a one-way analysis of variance (ANOVA) test. Overall locomotor activity will be examined at any doses of those drugs that display significantly lower immobility times than saline to determine if the effect is because of hyperactivity or not.

A recurring issue with most 5-$HT_3$ receptor ligands is their inability to cross the BBB. Compounds with log P values ranging from 1.5 to 2.5 generally can penetrate the BBB.[138] MD-354 (**2**), PG (**27**), 3,4,5-tri-Cl-PG (**35**) and mCPBG (**17**) have been shown to have log P values of -0.64, -1.32, 1.16, and -0.38, respectively, by the shake-flask method of measuring the concentration of drug in the aqueous layer of an aqueous 1-octanol solution, which would indicate that these compounds should not be able to readily cross the BBB.[9] Supporting this, Bachy et al.[91] showed that PBG and mCPBG

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were unable to displace [$^3$H]granisetron in an ex vivo study indicating that arylbiguanides probably do not cross the BBB. In contrast, Kilpatrick and Rogers[98] showed that mCPBG was able to displace [$^3$H]GR65630 in rat entorhinal cortex ex vivo and Steward et al.[99] used [$^3$H]mCPBG to label 5-$HT_3$ receptor recognition sites in the rat brain. These last two studies indicate that the arylbiguanide mCPBG does cross the BBB. Because the data are controversial, a new method is needed to determine if arylguanidines and arylbiguanides cross the BBB. Using ChemDraw to predict log P values, the predicted log P value for mCPBG is 1.28, which indicates that it is slightly too low for mCPBG to easily cross the BBB, supporting the shake-flask data. Whereas, the predicted log P value for MD-354 is 1.43, which is closer to the threshold for easily crossing the BBB. Both of these predicted values are much higher than the shake-flask data. The determination of log P values using the shake-flask method is time consuming, requires a large amount of sample, and is subject to errors (i.e., sample impurities, inability to detect the sample, dissociation, decomposition, and stable emulsion formation).[139] In addition, very high and very low partition compounds cannot be measured through the shake flask method.[139]

Another method of determining the log P value is through the relative retention times using high performance liquid chromatography (HPLC) which requires much smaller quantities of sample and provides for better detection. The retention times of several compounds (i.e., imidazole, acetanilide, benzophenone, naphthalene, and diphenylamine) are determined relative to the retention time of uracil for various concentrations of aqueous acetonitrile. Values for the log of the capacity factor ($k'$) are

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calculated for each of these compounds at varying concentrations of acetonitrile and water ($\log k' = \log\left[\left(t_R - t_0\right)/t_0\right]$).[140-142] For each compound, a plot of $\log k'$ versus the concentration of acetonitrile is used to calculate the $\log k_w^{'}$ (i.e., the log of the capacity factor in 100% water).[140-142] These $\log k_w^{'}$ values can be plotted against experimental log P values from the literature to construct a standard curve. By identifying the $\log k_w^{'}$ value of unknown compounds the log P value can be determined using the standard curve obtained from the known compounds. This method will be implemented to determine the log P values of mCPBG and several arylguanidines.

Different homology models of the h5-HT3A receptor can be built through various modes of construction. Each program uses different methods for developing models. Using the Biopolymer package of Sybyl, residues of a template can be mutated to the sequence of the target receptor. This is followed by optimizing the side-chain orientation with SCRWL. Another method is to use an alignment of the template and the sequence of the target, and allowing the program Modeller to generate multiple models at one time. By using docking programs (e.g., GOLD and AutoDock) and different standards, the models can be validated by mutagenesis data. Since arylguanidines have not been generally studied as a class of 5-$HT_3$ receptor ligands, their possible binding modes have yet to be determined. In the present study, 5-$HT_3$ receptor models will be constructed and docking studies performed with both GOLD and AutoDock. Based on the docking poses

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of MD-354 and 5-HT, new ligands might be developed which encompass structural features that are beneficial for each.

To summarize, the specific aims of the present work are:

a) to synthesize various halogen-substituted arylguanidines,
b) to determine the structural features important for binding through a Hansch analysis,
c) to synthesize conformationally constrained arylguanidines,
d) to evaluate the possible antidepressant activity of selected agents through the mouse tail suspension test,
e) to determine the log P value of various arylguanidines, and
f) to construct and validate a homology model of the 5-$HT_3$ receptor.

Overall, the aims of these studies are the rational design and synthesis of novel 5-$HT_3$ receptor agents that enter the brain, and an initiation of their pharmacological action.

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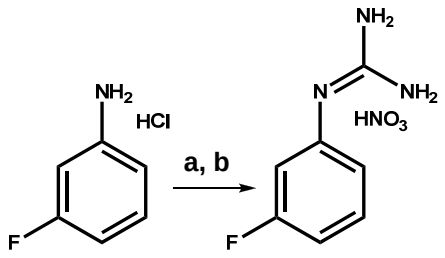
## IV. Results and Discussion

### A. Halogen Series

#### 1. Synthesis of *N*-(3-Fluorophenyl)guanidine Nitrate (42)

*N*-(3-Fluorophenyl)guanidine nitrate (**42**), was synthesized in the same manner as MD-354 (**2**) (Scheme 1).[143] *N*-(3-Bromophenyl)guanidine nitrate (**43**) and *N*-(iodophenyl)guanidine nitrate (**44**) analogs were previously synthesized in our laboratory[132] as nitrate salts and were available for our studies. The hydrochloride salt of the aniline was allowed to react with cyanamide in ethanol and heated at reflux. The hydrochloride salt of the arylguanidine was converted to the nitrate salt (i.e., **42**) with ammonium nitrate. The structure was confirmed by IR spectrometry, $^{1}$H NMR spectrometry, and elemental analysis for C, H, N.

**Scheme 1.**[a]




**50** **42**

[a]Reagents and conditions: a. $H_2NCN$, EtOH, reflux; b. $NH_4NO_3$, $H_2O$.



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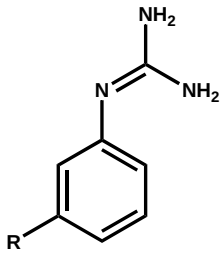
**2. Binding Studies**

The three new arylguanidine analogs (**42-44**) were evaluated and MD-354 (**2**) was re-evaluated for binding affinity at recombinant mouse 5-HT3A receptors (Table 7) using [$^3$H]LY 278,584. The –Br (i.e., **43**) and –I (i.e., **44**) analogs were found to bind with the highest affinity. MD-354 (**2**) was found to bind with a somewhat lower affinity than previously reported for binding affinity at cloned human 5-HT3AB native receptors.[7] Based on these results it can be concluded that the size of the electron-withdrawing substituent possibly plays a role in how well these compounds bind to the 5-$HT_3$ receptor since compounds with smaller *meta*-substituents (i.e., –H (**27**; $K_i$ = 2340 nM) and –F (**42**)) bind with decreased affinity and compounds with larger *meta*-substituents (i.e., –Br (**43**) and –I (**44**)) bind with increased affinity compared to the parent compound, MD-354 (**2**).

**Table 7.** Binding affinity of halogen series at the mouse 5-HT3A receptors.




| | R | $K_i$ (nM) | (SEM) |
|---|---|---|---|
| **42** | F | 1420 | (105) |
| **2** | Cl | 166 | (16) |
| **43** | Br | 81 | (10) |
| **44** | I | 40 | (5) |

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This is the reverse of what was expected and suggests that *meta*-substituents larger in size than a chloro group are tolerated by the receptor. Since the $–CF_3$ analog binds with lower affinity at the 5-$HT_3$ receptors than any of the mono-halogenated analogs, but takes up less volume than –Br and –I it may be concluded that it is the shape and not necessarily the size of the $–CF_3$ group that interferes with binding. The halogen substituents are spherical but the $–CF_3$ substituent is not; a more spherical shape might be more tolerated. It might be noted that for the simple halogenated analogs shown in Table 7, affinity increases as the lipophilicity (i.e., π value; see Table 5) increases. Likewise, affinity increases as substituent volume (see Table 5) increases. For example, there is a relationship between p*K*i and π ($r$ = 0.997, $n$ = 4; Figure 14) as well as with volume ($r$ = 0.920, $n$ = 4; Figure 14). This trend holds if phenylguanidine **27** is included ($r$ = 0.998, $n$ =5; $r$ = 0.934, $n$ = 5; Figure 14). However, there are too few observations to draw any reliable conclusions.

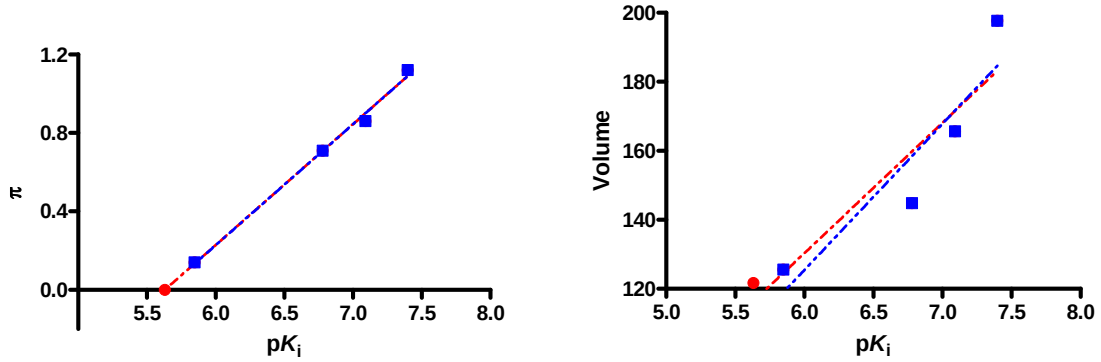


**Figure 14.** Linear regression plots of p$K_i$ versus π (left) and volume (right). The analyses including the unsubstituted compound **27** are shown in red, excluding the unsubstituted compound are shown in blue.

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**3. Hansch Analysis**

A Hansch analysis was performed on the 3-position monosubstituted arylguanidines to better identify electronic and steric effects that might be influencing their binding to the 5-$HT_3$ receptor. The analysis was performed on the parameters (i.e., π, σ, L, B1, B5, MR, CMR, volume, MV, NVE, Pc, and polarizability) (Table 8) versus affinity (i.e., p$K_i$). A linear regression analysis (Table 9) of each of these individual parameters using GraphPad Prism against p$K_i$ revealed statistically significant correlations ($p < 0.05$) with the electronic factor σ ($n = 11$, $r = 0.730$, $F = 10.25$; eq 2, Table 9; Figure 15), polarizability ($n = 11$, $r = 0.704$, $F = 8.86$; eq 12, Table 9; Figure 16), solvent accessible volume ($n = 11$, $r = 0.656$, $F = 6.80$; eq 7, Table 9; Figure 16), and complete molar refraction (CMR) ($n = 11$, $r = 0.670$, $F = 7.31$; eq 9, Table 9; Figure 17). These linear regression analyses indicate that the electronic effect of the substituent, the polarizability of the molecule, the solvent accessible volume of the substituent, or the complete molar refraction of the molecule may play a role in how well the arylguanidines bind to the 5-$HT_3$ receptor.

Internal correlations between parameters can lead to collinearity. Multicollinearity may be or is a problem for these compounds with volume and polarizability ($r = 0.742$, $n = 11$), CMR and polarizability ($r = 0.757$, $n = 11$), and volume and CMR ($r = 0.704$, $n = 11$)—since these three parameters are intercorrelated with each other it is expected that there is a problem with multicollinearity.

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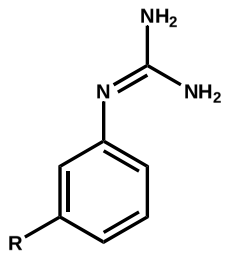
**Table 8.** Hansch analysis of the 5-$HT_3$ receptor binding of 3-substituted arylguanidines.




| | R | $pK_i$[a] | $\pi$[b] | $\sigma_m$[b] | L[b] | B1[b] | B5[b] | MR[b] | Vol[b] | NVE[b] | CMR[b] | MV[b] | Pc[b] | Polarizability[b] |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **27** | H | 5.631 | 0.00 | 0.00 | 2.1 | 1.0 | 1.0 | 1.03 | 122 | 52 | 4.08 | 115 | 304 | 15.51 |
| **2** | Cl | 6.780 | 0.71 | 0.37 | 3.5 | 1.8 | 1.8 | 6.03 | 145 | 58 | 4.57 | 124 | 333 | 17.33 |
| **30** | $NO_2$ | 7.071 | -0.28 | 0.71 | 3.4 | 1.7 | 2.4 | 7.36 | 144 | 68 | 4.69 | 120 | 349 | 17.75 |
| **31** | $CH_3$ | 5.186 | 0.56 | -0.07 | 3.0 | 1.5 | 2.0 | 5.65 | 138 | 58 | 4.54 | 130 | 335 | 17.26 |
| **32** | $CF_3$ | 5.613 | 0.88 | 0.43 | 3.3 | 2.0 | 2.6 | 5.02 | 151 | 76 | 4.59 | 145 | 358 | 17.39 |
| **39** | $OCH_3$ | 5.796 | -0.02 | 0.12 | 4.0 | 1.4 | 3.1 | 7.87 | 146 | 64 | 4.70 | 137 | 354 | 17.81 |
| **40** | CN | 6.910 | -0.57 | 0.56 | 4.2 | 1.6 | 1.6 | 6.33 | 139 | 60 | 4.56 | 128 | 350 | 18.10 |
| **41** | OH | 5.695 | -0.67 | 0.12 | 2.7 | 1.4 | 1.9 | 2.85 | 127 | 58 | 4.15 | 112 | 310 | 15.85 |
| **42** | F | 5.484 | 0.14 | 0.34 | 2.7 | 1.4 | 1.4 | 0.92 | 126 | 58 | 4.10 | 118 | 304 | 15.46 |
| **43** | Br | 7.092 | 0.86 | 0.39 | 3.8 | 2.0 | 2.0 | 8.88 | 166 | 58 | 4.86 | 127 | 347 | 18.51 |
| **44** | I | 7.398 | 1.12 | 0.35 | 4.2 | 2.2 | 2.2 | 13.9 | 198 | 58 | 5.39 | 132 | 367 | 20.53 |

[a] Binding data from Tables 5 and 7.[7,100]
[b]See Table 4 for description of parameters.

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**Table 9.** Linear regression analysis results ($n$ = 11).

| Equation | $pK_i$ = | $r$ |
|---|---|---|
| 1 | $0.1467(\pm0.2599)\pi - 0.6721(\pm1.642)$ | 0.185 |
| 2 | $0.2230(\pm0.06967)\sigma - 1.098(\pm0.4401)$ | 0.730 |
| 3 | $2.583(\pm1.381)MR - 10.77(\pm8.724)$ | 0.529 |
| 4 | $0.2399(\pm0.4358)L + 1.333(\pm2.753)$ | 0.181 |
| 5 | $0.3214(\pm0.1425)B1 - 0.2826(\pm0.9004)$ | 0.601 |
| 6 | $1.085(\pm0.7941)B5 - 3.850(\pm5.017)$ | 0.414 |
| 7 | $18.07(\pm6.932)volume + 32.04(\pm43.80)$ | 0.656 |
| 8 | $-0.2806(\pm2.779)NVE + 62.49(\pm17.56)$ | 0.034 |
| 9 | $0.3263(\pm0.1207)CMR + 2.519(\pm0.7624)$ | 0.670 |
| 10 | $0.1198(\pm4.232)MV + 125.5(\pm26.74)$ | 0.009 |
| 11 | $14.82(\pm8.242)Pc + 244.3(\pm52.07)$ | 0.514 |
| 12 | $1.334(\pm0.4484)polarizability + 9.036(\pm2.833)$ | 0.704 |

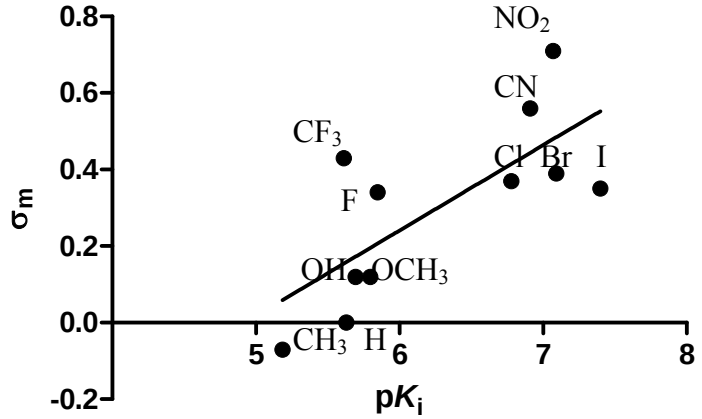


**Figure 15.** Linear regression plot of $pK_i$ versus $\sigma_m$ ($r$ = 0.730; eq 2; Table 9).

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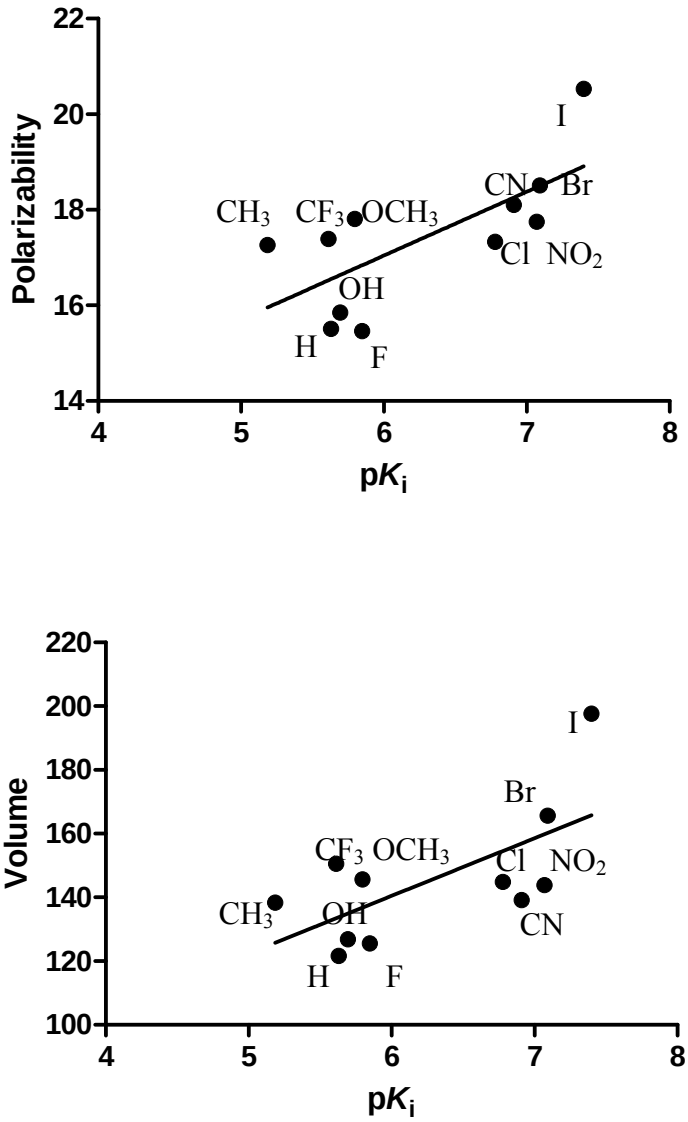


**Figure 16.** Linear regression plots of p$K_i$ versus polarizability ($r$ = 0.704; eq 12; Table 9) (top) and p$K_i$ versus volume ($r$ = 0.656; eq 7; Table 9) (bottom).

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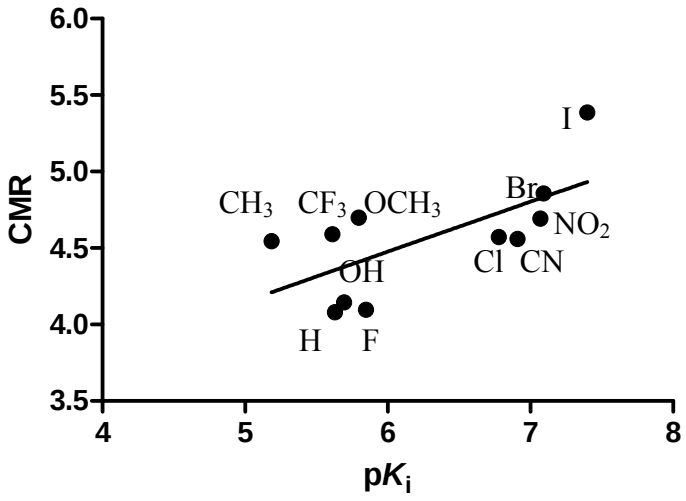


**Figure 17.** Linear regression plot of p$K_i$ versus CMR ($r$ = 0.670; eq 9; Table 9).

In order to obtain statistically relevant results for each parameter analyzed, data on at least six compounds are required. Using compounds **2**, **27**, **30-32**, **39-44**, there are only eleven compounds and, therefore, only one variable can be investigated with statistical significance. Despite lacking statistical rigor, inclusion of a second variable might provide some clues as to which variables are simultaneously affecting binding affinity. A multiple linear regression analysis was performed using GraphPad's InStat program on σ, volume, CMR, and polarizability versus p$K_i$ (Table 10). There is no internal correlation between volume ($r$ = 0.851, $n$ = 11), CMR ($r$ = 0.855, $n$ = 11), or polarizability ($r$ = 0.867, $n$ = 11) and σ; since they are independent of each other, any relationship of σ and the other three parameters may show an actual relationship together with the binding affinity. There is a good correlation ($p < 0.01$) with σ and polarizability

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versus p$K_i$ ($n$ = 11, $r$ = 0.867, $F$ = 12.05; eq 15, Table 10), σ and volume versus p$K_i$ ($n$ = 11, $r$ = 0.851, $F$ = 10.54; eq 16, Table 10), and σ and CMR versus p$K_i$ ($n$ = 11, $r$ = 0.855, $F$ = 10.86; eq14, Table 10). Based on the results of these analyses, the electron-withdrawing nature and the size, polarizability, or volume of the substituent at the 3-position impact binding affinity. Since there are multiple linear regression correlations, the later three parameters are impacting binding affinity and not just one individual parameter.

**Table 10.** Multiple linear regression analysis results ($n$ = 11).

| Equation | p$K_i$ = | $r$ |
|---|---|---|
| 13 | -2.010*CMR* + 0.9141*polarizability* – 0.4960 | 0.757 |
| 14 | 1.749*σ* + 1.118*CMR* + 0.6070 | 0.855 |
| 15 | 1.648*σ* + 0.3036*poloarizability* + 0.4594 | 0.867 |
| 16 | 1.846*σ* + 0.01917*volume* + 2.893 | 0.851 |
| 17 | -0.005657*volume* + 0.4817*polarizability* – 1.320 | 0.742 |
| 18 | -0.001501*volume* + 1.578*CMR* – 0.7439 | 0.704 |

In agreement with Glennon et al.,[124] this study has shown with different compounds that the electronic character at the 3-position is important for binding arylguanidines to the 5-$HT_3$ receptor. Dukat et al.[100] has shown previously that electronic effects account for nearly 20% and that steric effects account for nearly 80% of binding of arylguanidines and arylbiguanides at the 5-$HT_3$ receptor. This study is in agreement with Dukat and co-worker's[100] study as shown by the correlation between σ and binding affinity and it has been shown that the volume and complete molar refraction (CMR), both of which can be viewed as steric factors, provide statistically significant correlations

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with binding affinity. In addition, the polarizability of the molecule, as shown by the polarizability factor and CMR, also display significant correlations. In this study the volume may be acting as a surrogate for the polarizability or the polarizability as a surrogate for the volume. One way to examine this possibility would be to synthesize and include in future analyses a compound with a substituent that has relatively low volume and relatively high polarizability (e.g., (3-cyclohexylphenyl)guanidine has a substituent volume of 201.3 and a polarizability of 25.46 or 2-(3-(prop-1-ynyl)phenyl)guanidine has a substituent volume of 154.1 and a polarizability of 20.92).

**4. Log P Analysis**

The retention times on a nitrile reversed-phase high performance liquid chromatography (HPLC) column of uracil, imidazole, acetanilide, benzophenone, naphthalene, and diphenylamine were determined in triplicate at various concentrations of aqueous acetonitrile (i.e., 40%, 50%, 60%, 70% acetonitrile). From the retention times of the above standards relative to uracil (i.e., $t_R - t_0$; where $t_R$ = retention time of the standard and $t_0$ = retention time of uracil), the log of the capacity factor (i.e., $\log k' = \log\left[\left(t_R - t_0\right)/t_0\right]$) was determined for each of the standards. A plot of $\log k'$ versus the concentration of acetonitrile was constructed (shown for benzophenone in Figure 18) and a linear regression analysis performed. Using the equation for each plot the lines for each standard could be extrapolated and the log of the capacity factor at 0% acetonitrile could be determined ($\log k'_w$). The $\log k'_w$ values for each of the standards

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(i.e., imidazole, $\log k_w^{'}$= -0.88; acetanilide, $\log k_w^{'}$= 0.06; benzophenone, $\log k_w^{'}$= 1.18; naphthalene, $\log k_w^{'}$= 1.06; and diphenylamine, $\log k_w^{'}$= 1.38) were plotted against the literature shake-flask log P values (log P = -0.08, 1.16, 3.30, 3.18, 3.50; respectively) giving a standard curve for this particular column (Figure 19). A linear regression analysis of this data calculated an equation for the line of $\log P = 1.660 \log k_w^{'} + 1.285$ ($r$ = 0.996, $F$ = 346.4).

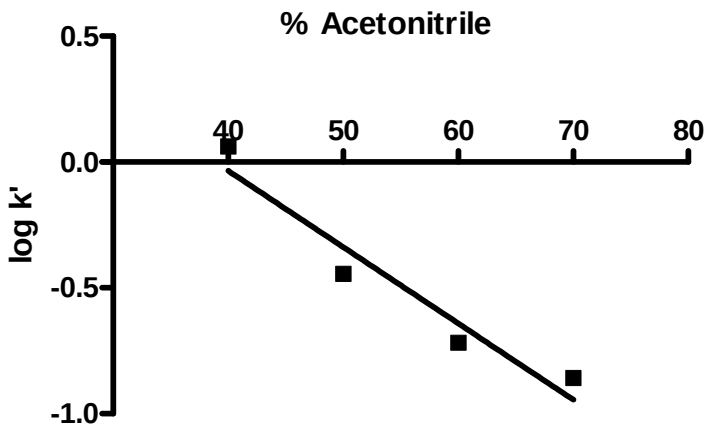


**Figure 18.** Plot of $\log k^{'}$ of benzophenone versus concentration of aqueous $CH_3CN$.

The retention times of PG (**27**), MD-354 (**2**), 3-FPG (**42**), 3-BrPG (**43**), 3-IPG (**44**), 3,4,5-tri-Cl-PG (**35**), and mCPBG (**17**) were determined at various concentrations of aqueous acetonitrile (i.e., 55%, 65%, 75% acetonitrile). Based on the retention times relative to uracil, the log of the capacity factor was calculated for each of the compounds. The capacity factors were plotted against the concentration of acetonitrile and a linear

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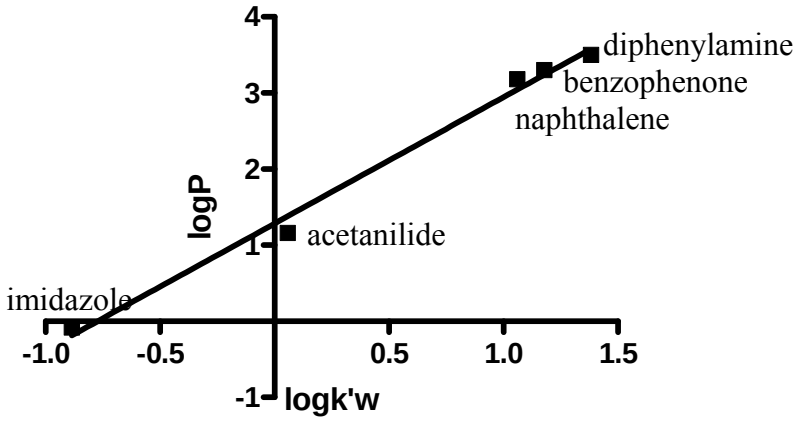


**Figure 19.** Plot of literature log P values from shake-flask data[144] versus $\log k_w^{'}$ values.

regression analysis was performed (Figure 20). Based on this analysis the line from each plot could be extrapolated and the capacity factors at 0% acetonitrile (i.e., $\log k_w^{'}$) could be determined (Table 11). As long as the same general range of concentration of acetonitrile was used the exact concentrations need not be the same with the standards and unknowns since the true value of interest is found based on the extrapolation to 0% acetonitrile—as if the compound was eluted in water only. The $\log k^{'}$ value at 0% acetonitrile (i.e., $\log k_w^{'}$) cannot actually be determined since running the HPLC column in pure water is ill-advised since it may destroy the column. Based on the capacity factors determined for 0% acetonitrile, the log P values for each of these compounds was calculated based on the standard curve (Table 11).

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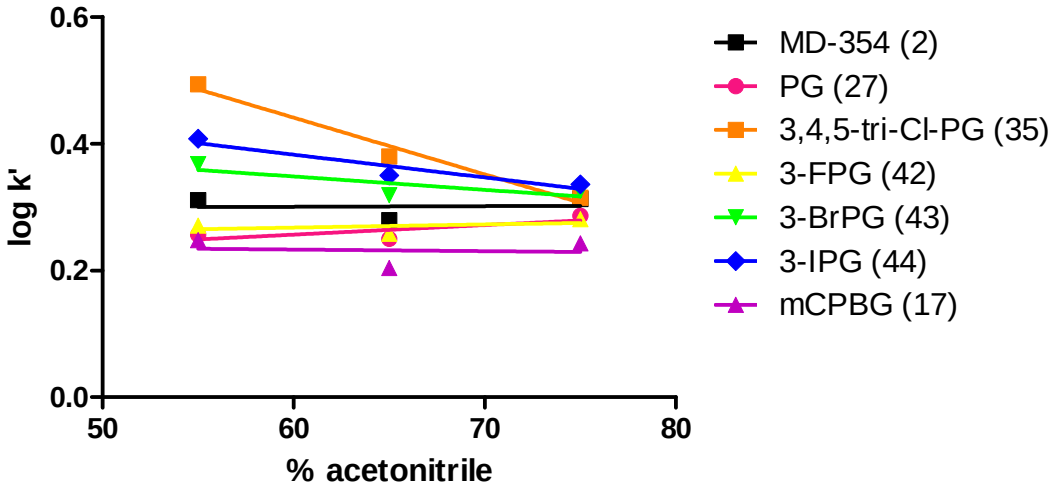


**Figure 20.** Plot of $\log k'$ of various arylguanidines and mCPBG versus concentration of aqueous $CH_3CN$.

**Table 11.** Capacity factors ($\log k'_w$) determined by linear regression and calculated/ determined log P values for the selected arylguanidines and an arylbiguanide.

| Compound | $\log k'_w$ | Predicted log P[a] | Actual log P (shake-flask)[b] | Actual log P (HPLC) |
|---|---|---|---|---|
| **27** | 0.1665 ± 0.0794 | 0.87 | -1.32 | 1.56 |
| **2** | 0.2945 ± 0.1248 | 1.43 | -0.64 | 1.77 |
| **42** | 0.2375 ± 0.0681 | 1.03 | -- | 1.68 |
| **43** | 0.4742 ± 0.1059 | 1.70 | -- | 2.07 |
| **44** | 0.5987 ± 0.0832 | 2.22 | -- | 2.28 |
| **35** | 0.9781 ± 0.0927 | 2.54 | 1.16 | 2.91 |
| **17** | 0.2479 ± 0.1570 | 1.28 | -0.38 | 1.70 |

[a] Predicted using ChemDraw.
[b] Results from a previously published study.[9]

As mentioned earlier, compounds with log P values ranging from 1.5 to 2.5 should readily penetrate the BBB.[138] Based on these results, most of these arylguanidines should be able to cross the BBB. The results obtained by relative retention times using

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HPLC were consistent with the predicted log P values from ChemDraw ($r$ = 0.957) and with the previous studies indicating that mCPBG (**17**) crosses the BBB.[98,99] The study by Bachy et al.[97] that indicated mCPBG could not cross the BBB used a different radioligand (i.e., [$^3$H]granisetron versus [$^3$H]GR65630), a larger dose (i.e., 2 and 10 mg/kg versus 0.1-1.0 mg/kg), a longer time between injection and removal of the brains (i.e., 30 min versus 5 min), and a different species (i.e., mouse versus rat). Any of these differences may account for the difference in results between the two laboratories. Furthermore, given the evidence that mCPBG (**17**) can penetrate the BBB through ex vivo inhibition and radioligand binding, it would seem likely that the halogenated arylguanidines **2**, **42**, **43**, and **44** should also be capable of such. This method of determining log P values seems to be more accurate when compared to the predicted values than the shake-flask method.

**5. Molecular Modeling**

Homology models were constructed by two different methods (i.e, Method A and Method B). In both cases the models were developed from an alignment between the N-terminal domain of the h5-HT3A sequence and the sequence of three AChBP's, the N-terminal domain of a nACh receptor α1 subunit bound to α-bungarotoxin, the N-terminal domains of three different $GABA_A$ receptor subunits, the N-terminal domains of two different $GABA_C$ receptor subunits, the N-terminal domain of two different glycine receptor subunits, and the N-terminal domain of the mouse 5-HT3A receptor. This alignment was performed using ClustalX to identify conserved residues within the

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different LGICs; specifically W90, W183, Y234, and the two cysteines forming the Cys-loop.

Method A. Using Sybyl's (version 7.3) Biopolymer package, the α1 subunit of a nACh receptor (PDB 2qc1)[42] was mutated to the sequence of the h5-HT3A receptor using the conserved residue alignment to identify where insertions and deletions were needed. This single subunit was minimized using the Amber force field and Amber charges. The program, SCWRL, was used to optimize side chain orientations. ProCheck was run on the single subunit to create a Ramachandran plot (Figure 21) to identify amino acid residues in most favored (red field), additionally allowed (dark yellow), generously allowed (lighter yellow), and disallowed (white) regions. The Ramachandran plot indicated that 86.8% of amino acid residues were in the most favored regions, 12.6% of residues were in the additionally allowed regions, and only 0.5% of residues were in the disallowed regions. The one amino acid shown in the disallowed region is facing into the pore and does not interact with the binding pocket so it has no impact on the docking. Using the backbone of an AChBP (PDB 1i9b)[38] for orientation in space of two protomers, since it is a pentameric structure, a dimer of the h5-HT3A protomer was constructed.

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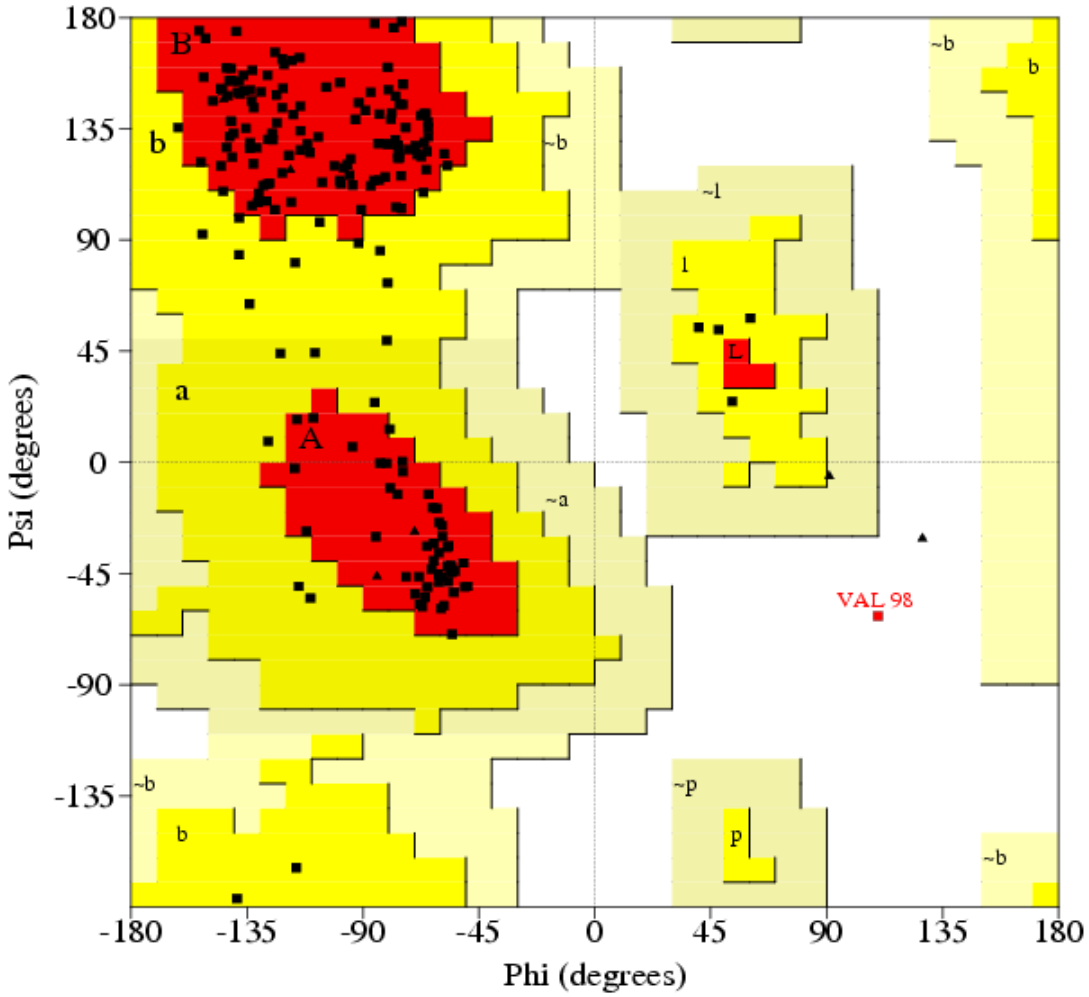


**Figure 21.** Ramachadran plot of a single subunit of the h5-HT3A receptor model developed using Sybyl's Biopolymer package (Method A).

The ligand 5-HT (**1**) was built in Sybyl 7.3 and given AM1-BCC charges and geometries from MOPAC. AM1-BCC charges are quantum mechanical charges that have bond correction charges (BCC) that allow for accurate charges of small molecules within the Amber force field. Using Gold 3.1, 5-HT was docked with 20 genetic algorithm runs in a 15 Å sphere around W183 to the interface between the two protomers. The proposed

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binding mode of 5-HT (Figure 22) was consistent with the literature as the aliphatic amine is situated in the binding pocket in a way that it is directed towards the aromatic rings of W183 and Y234 and possibily capable of forming the cation-π bond as reported in the literature.[101,145,146]

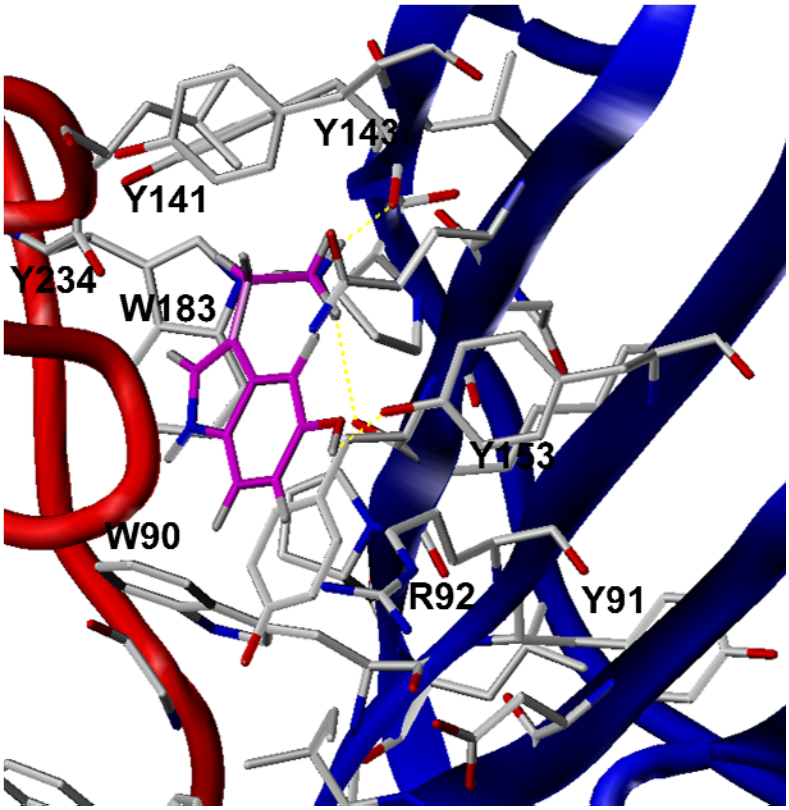


**Figure 22.** Proposed binding mode of serotonin (5-HT; **1**) in the h5-HT3A receptor model developed using Sybyl's Biopolymer package (Method A). 5-HT (**1**) is in magenta and the side chains of residues within 5 Å of the ligand are shown.

Since the proposed binding mode of 5-HT is relatively similar to that in the literature,[101,145,146] MD-354 (**2**) was built and docked to the receptor model in the same manner as 5-HT (Figure 23). The proposed binding mode of MD-354 is similar to 5-HT

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in that the guanidine portion of MD-354 overlaps with the aliphatic amine of 5-HT and is possibly interacting with W183, Y234, and Y143.

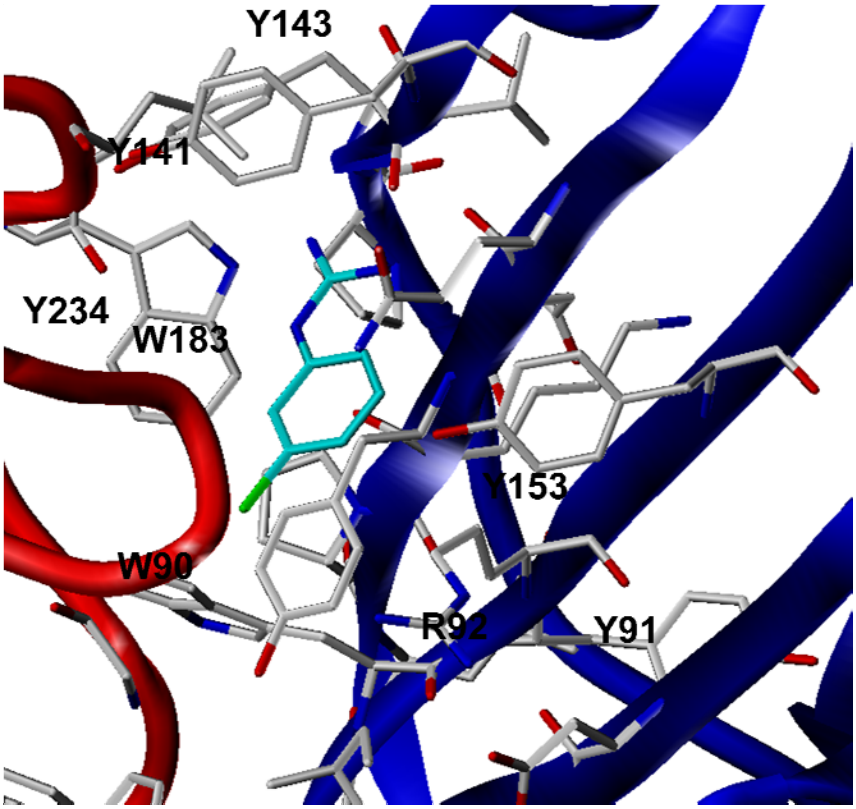


**Figure 23.** Proposed binding mode of MD-354 (**2**) to the h5-HT3A receptor using Sybyl's Biopolymer package (Method A). MD-354 (**2**) is in cyan and the side chains of residues within 5 Å of the ligand are shown.

The overlap of MD-354 (**2**) and 5-HT (**1**) is depicted in Figure 24; based on these proposed binding modes to this model, a new ligand was designed taking into account features both from 5-HT and MD-354. From the overlap of 5-HT and MD-354, it appears that the addition of a hydroxyl group to the aromatic ring of MD-354 in the 4-position

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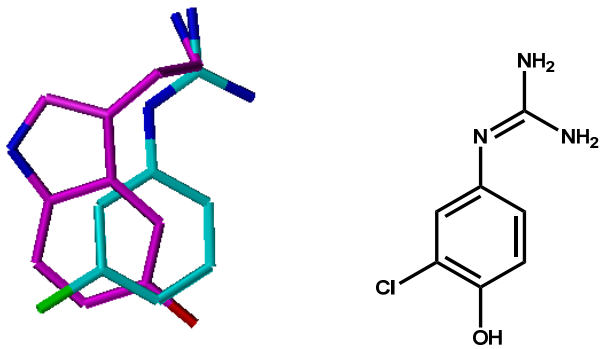
would enhance binding (Figure 24). The hydroxyl group of this designed ligand may be able to interact with Y153.




**51**

**Figure 24.** Overlap of 5-HT (**1**) and MD-354 (**2**) from the proposed binding modes to the h5-HT3A receptor developed using Sybyl's Biopolymer package (Method A) (left). The designed ligand *N*-(3-chloro-4-hydroxyphenyl)guanidine (right).

Method B. The homology model constructed through Sybyl (Method A) is crude and rigid, so another method for developing a homology model was explored. An alignment of AChBP (PDB 1i9b)[38], α1 nACh (2qc1)[42], and h5-HT3A based on the conserved residue alignment was used to create ten initial pentameric models from five separate random seeds using the program Modeller 9.3. The program then generated ten loop models from each of the initial models, to create a total of 100 unique models. These models were given Kollman charges then minimized using NAMD, first with the backbone constrained then with the backbone unconstrained. Using AutoDock4, the ligand 5-HT (**1**), with the charges and geometries generated by MOPAC, was docked to

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each of the five interfaces of each of the models with 20 genetic algorithm runs in a 40 Å x 40 Å x 40 Å region around the C-loop (Figure 25). Based on poses consistent with the

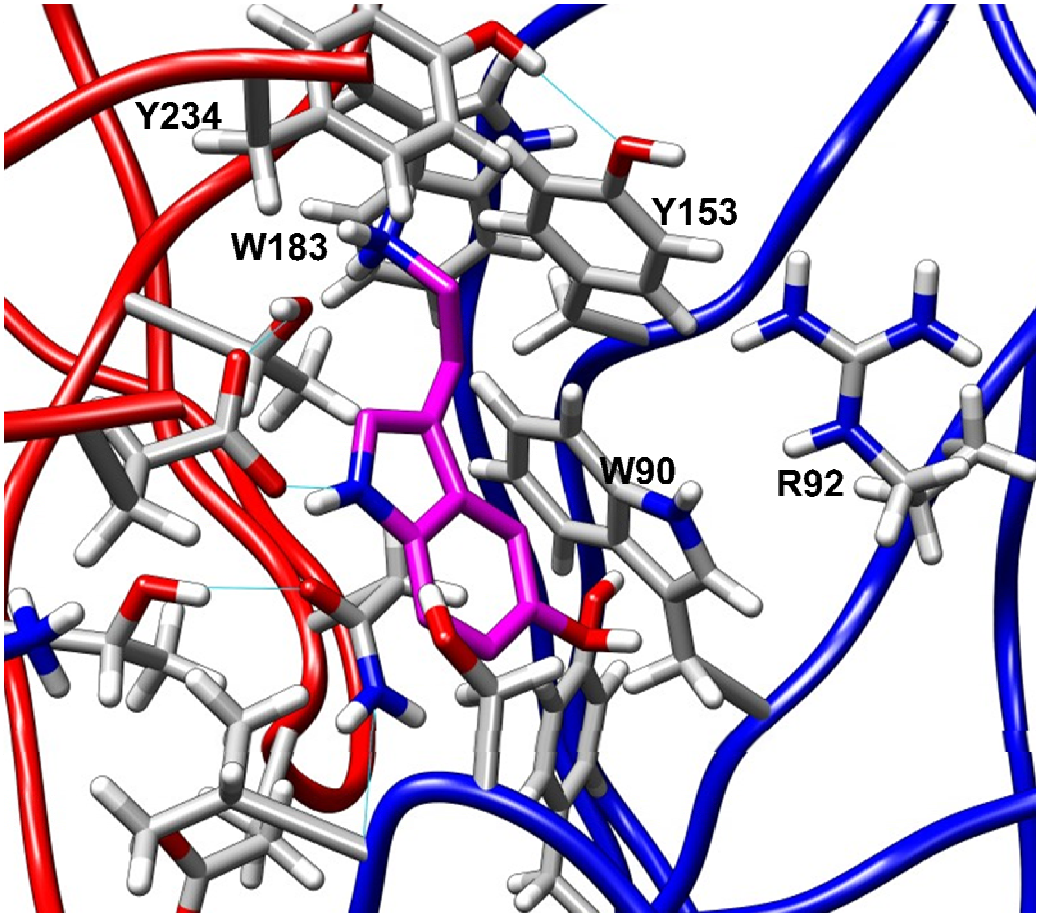


**Figure 25.** Proposed binding mode of 5-HT (**1**) in the h5-HT3A receptor agonist model developed using Modeller (Method B). 5-HT (**1**) is in magenta and the side chains of residues within 5 Å of the ligand are shown.

literature for 5-HT[145,146] and the scoring function of AutoDock, one model was identified as the best model for agonists. ProCheck generated a Ramachandran plot in which 67.8% of residues were in most favored regions, 24.1% were in additionally allowed regions,

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4.5% were in generously allowed regions, and 3.6% were in disallowed regions (Figure 26). Since multiple loop models were made by slight energy adjustments, the side chain conformations were not optimized using SCWRL; thus, there are more residues in the generously allowed and disallowed regions than with the model developed with Sybyl's Biopolymer package (i.e., Method A).

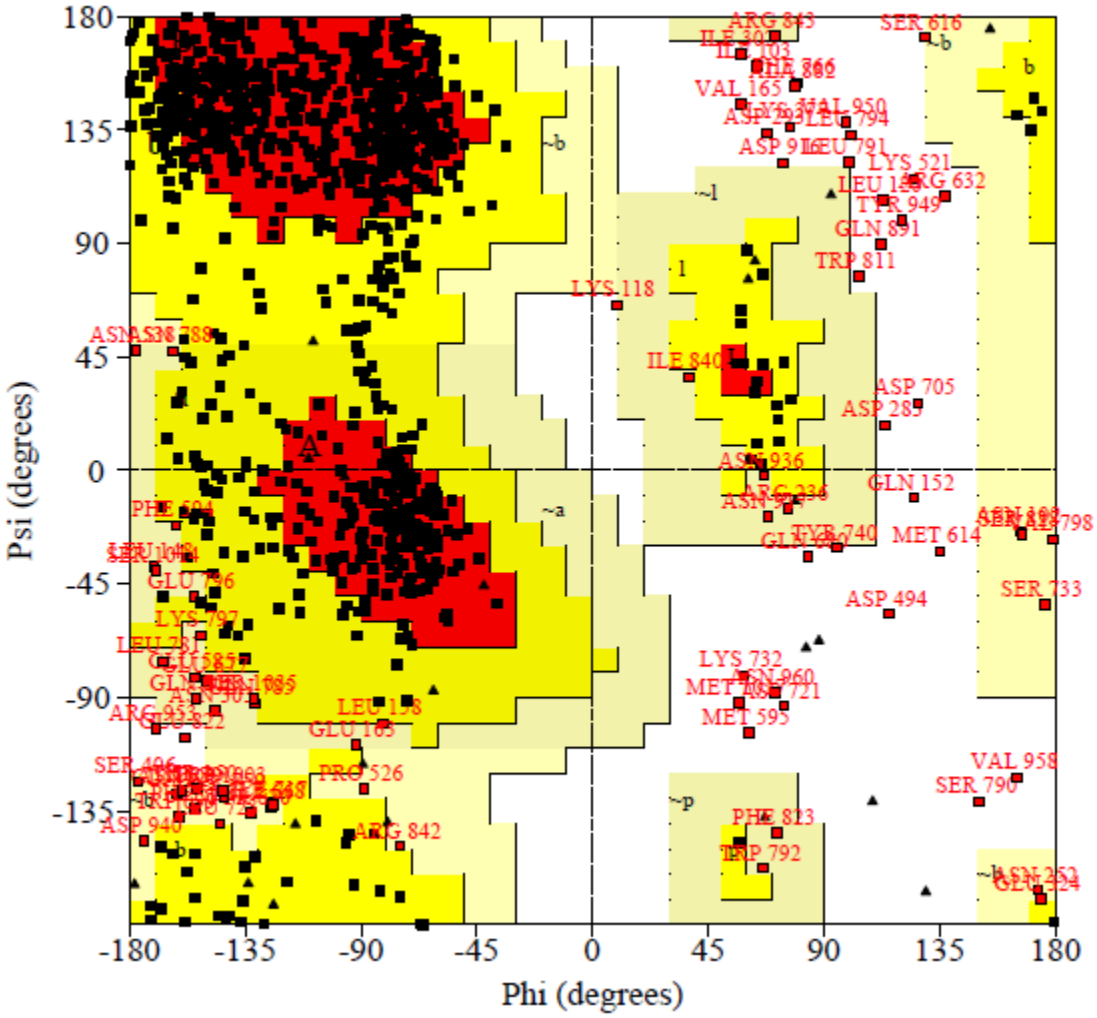


**Figure 26.** Ramachadran plot of the h5-HT3A receptor agonist model developed using Modeller (Method B).

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Serotonin's indole ring is perpendicular to W90 and the aliphatic amine is situated between Y234 and W183 forming a possible cation-π bond. This pose is consistent with poses proposed by Reeves et al.[146] The indole amine is in close proximity to the hydroxyl group of Y153 and may form a H-bond.

Since this model was validated by docking 5-HT, MD-354 (**2**) was built and docked in the same manner as 5-HT. In the model, MD-354 docks in a manner in which the aromatic rings of 5-HT and MD-354 are in the same region of the binding pocket as are the aliphatic amine of 5-HT and the guanidine of MD-354 (Figure 27). The guanidine portion of MD-354 presumably forms a cation-π bond with W183 and Y234. The aromatic ring portion of MD-354 also may form a π-π interaction with Y153. However, the designed ligand (i.e., **51**) from the model developed using Method A (Figure 24) is no longer valid. The overlap using the model developed using Method B indicates a slightly different overlap of MD-354 and 5-HT. In the model developed using Method B, the overlap is such that the –OH group from 5-HT overlaps with the open *meta*-position of MD-354.

The newly synthesized halogen analogs were built and docked to this model following the same parameters. MD-354 (**2**), 3-BrPG (**43**), 3-IPG (**44**) overlapped very well; however, the aromatic ring and guanidine portion of 3-FPG (**42**) overlapped with the other halogenated analogs but the fluoro substituent was situated on the opposite side of the ring (Figure 28). This slight change may explain why **42** binds with decreased

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affinity relative to MD-354, 3-BrPG, and 3-IPG. In fact, 3-FPG binds more closely to 5-HT, and both have lower binding affinities than MD-354, 3-BrPG, and 3-IPG.

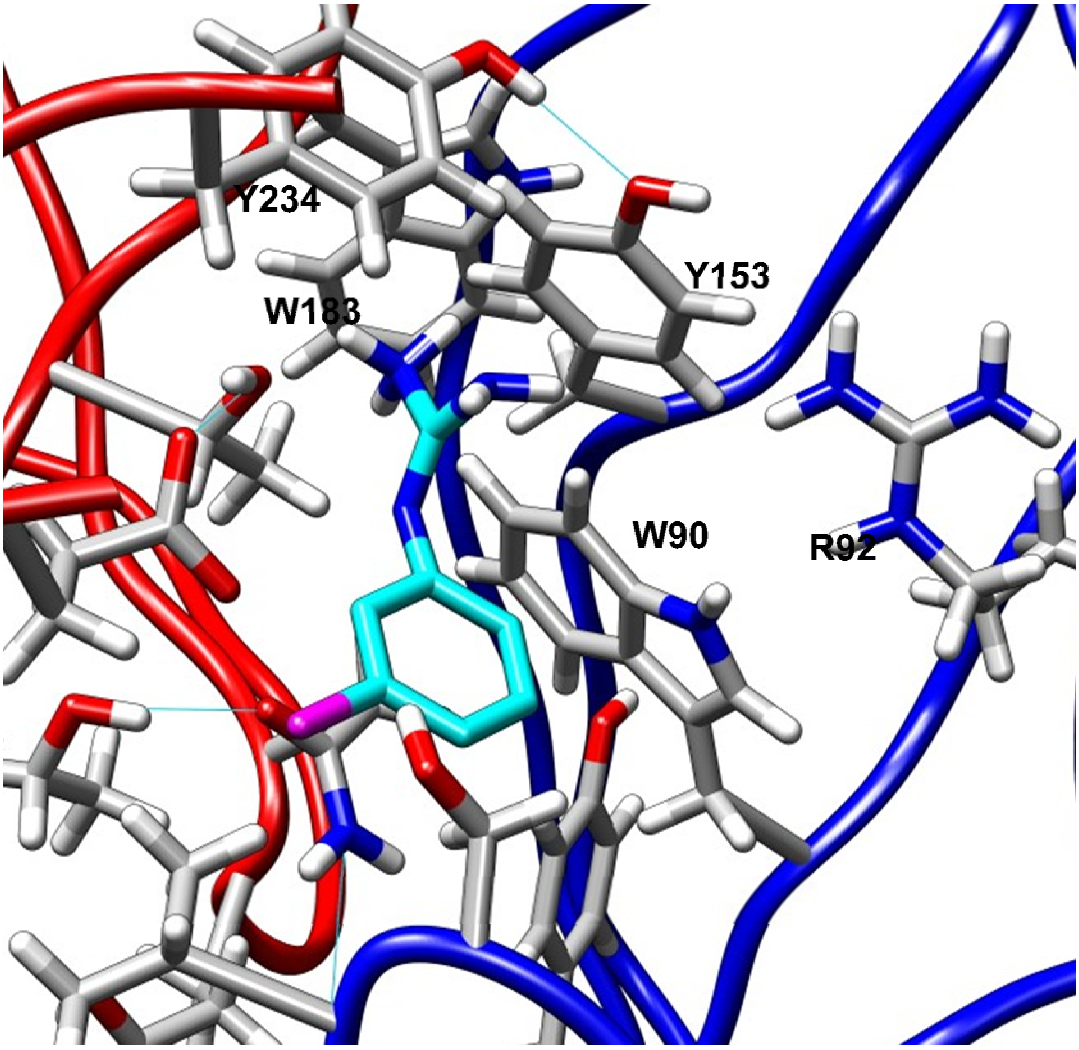


**Figure 27.** Proposed binding mode of MD-354 (**2**) to the h5-HT3A receptor agonist model developed using Modeller (Method B). MD-354 is shown in cyan and the side chains of residues within 5 Å of the ligand are shown.

The fluoro substituent affects the aromatic ring system more than the other halogen substituents. It is very electronegative and has orbitals similar in size to those of

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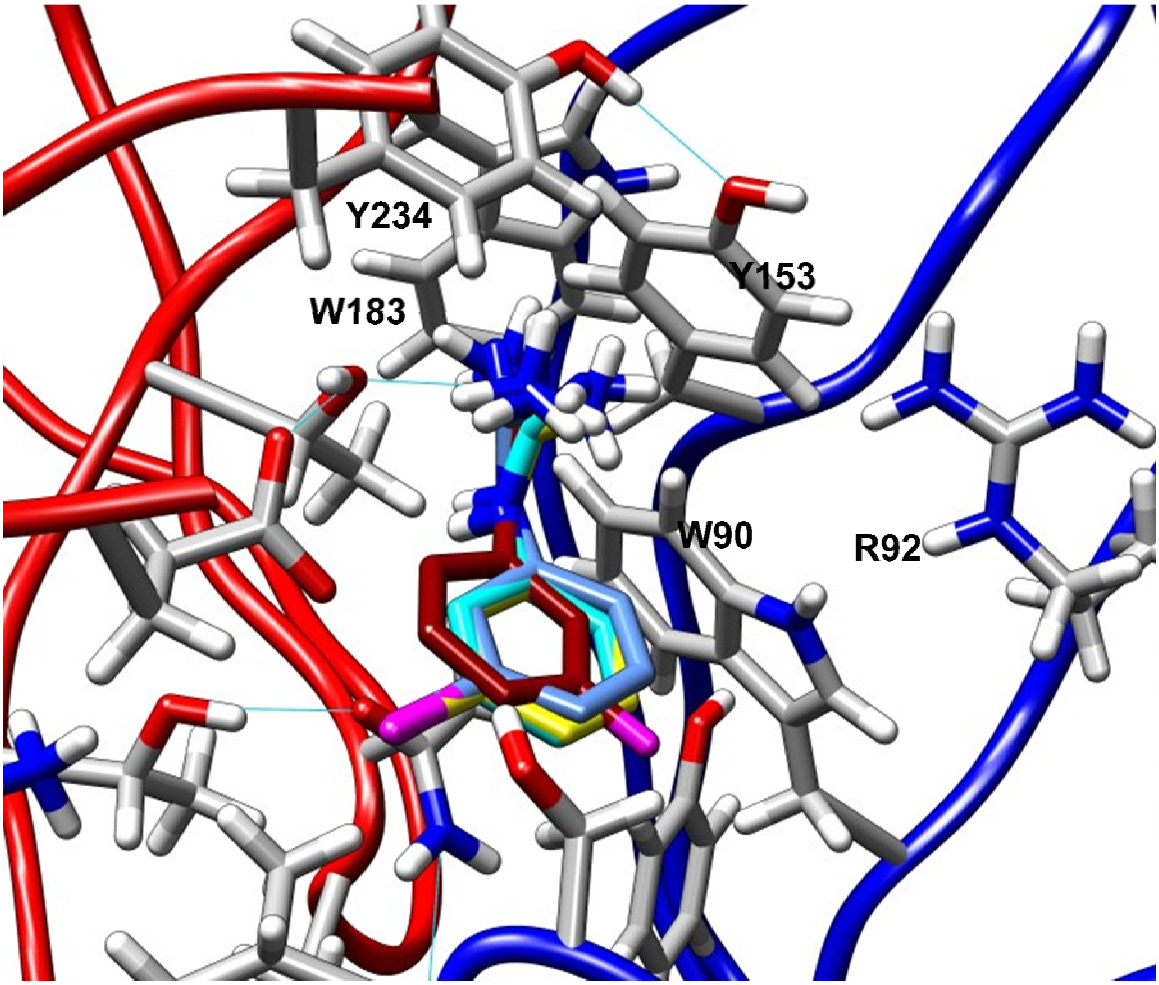


**Figure 28.** Proposed binding mode of MD-354 (cyan), 3-FPG (red), 3-BrPG (blue), 3-IPG (yellow) to the h5-HT3A receptor agonist model developed using Modeller (Method B).

carbon.[147] These two factors make the C—F bond the most energetic bond in which carbon can participate.[147] Because of the difference in electronegativity, the dipole moment that is created may contribute to the compound's ability to engage in intermolecular interactions since the physical properties and chemical reactivities are greatly affected. In addition, it has been shown through X-ray crystal structure data that

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the fluoro group is actually closer in size sterically to an oxygen atom than a hydrogen atom.[148] The 3-OH substituted phenylguanidine (**41**; $K_i$ = 2020 nM)[100] has been shown to have binding affinity slightly lower than 3-FPG (**42**; *K*i = 1420 nM). Based on these data, the size of the substituent needs to be larger than a –F group or a –OH group to bind well to the 5-$HT_3$ receptors. Overall, the 3-sustituted analogs analyzed earlier also follow this trend (Table 8).

Very recent mutagenesis studies have suggested that E129 is involved in the hydrogen-bond with the –OH in 5-HT.[149] In the models created here E129 does not face into the binding pocket. This does not mean that the model is incorrect; however, it is just a model. Mutagenesis of E129 to A, D, G, H, N, K, or Q either decreased the $pEC_{50}$ value or there was no response. This may have also been because the mutations are not allowing the receptor binding pocket to form correctly. The model generated using Modeller (i.e., Method B) is a more reliable model than the model generated using Sybyl (i.e., Method A) because it has allowed for flexibility in not only building the receptor model but also in the docking algorithms. The rigidity of the model generated using Sybyl (i.e., Method A) does not allow the ligands to dock to the receptor as closely as those proposed in the literature.

**6. Designed Ligand**

The ligand designed on the basis of the Method A modeling studies (e.g., see Figure 22), *N*-(3-chloro-4-hydroxyphenyl)guanidine (**51**) (Figure 24), was synthesized by

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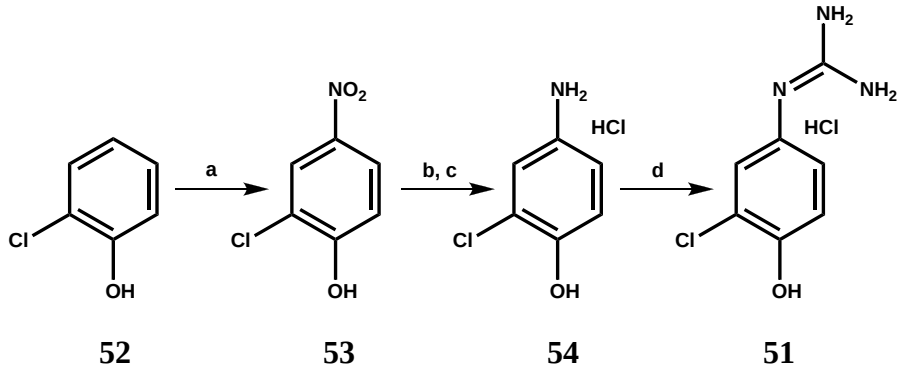
the method outlined in Scheme 2. The target compound is a known compound.[150] The nitro intermediate (**53**) was obtained by sonnication of 2-chlorophenol (**52**) with nitric acid (9% aqueous).[151] The nitro group was then reduced using sodium hydrosulfite and aqueous sodium hydroxide.[152] An ethanolic solution of the HCl salt of aniline **54** was heated at reflux with cyanamide (50% aqueous) to give **51**.[150] The structure was confirmed by IR, $^1$H NMR, and melting point.

**Scheme 2.**[a]




[a] Reagents and conditions: a. $HNO_3$, 1,2-dichloroethane, sonnication; b. $Na_2S_2O_4$, NaOH, $H_2O$, reflux; c. HCl, EtOAc; d. $NH_2CN$, EtOH, reflux.

## B. Conformationally-Constrained Analogs

### 1. Synthesis of 2-Amino-7-chloro-3,4-dihydroquinazoline (46)

The synthesis of 2-amino-7-chloro-3,4-dihydroquinazoline (**46**) was performed as described in Scheme 3. This was a novel compound at the time of its synthesis, and its method of preparation followed a literature procedure for the synthesis of 2-(alkylamino)-5,6- and 6,7-dihydroxy-3,4-dihydroquinazolines.[153] S-Methylisothiourea sulfate, sodium

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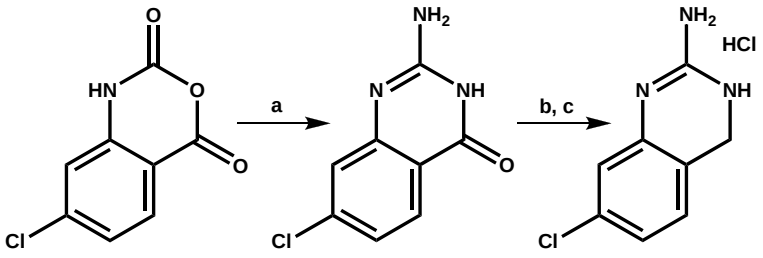
carbonate, and 4-chloroisotoic anhydride (**55**) were heated at reflux in 80% aqueous acetonitrile. After cooling the reaction mixture to room temperature, 2-amino-7-chloroquinazolin-4-(3*H*)-one (**56**) precipitated from the solution and was collected by filtration. This intermediate (i.e., **56**) was reduced using borane in tetrahydrofuran and the HCl salt **46** was prepared. The structural assignment is consistent with IR, $^1$H NMR, and elemental analysis.

**Scheme 3.**[a]




**55** **56** **46**

[a] Reagents and conditions: a. S-methylisothiourea sulfate, $Na_2CO_3$, $CH_3CN$ (80%), reflux; b. $BH_3$-THF, reflux, c. HCl

**2. Synthesis of 2-Amino-6-chloro-3,4-dihydroquinazoline (47)**

2-Amino-6-chloro-3,4-dihydroquinazoline (**47**) was re-synthesized for this study by the route outlined in Scheme 3 except that 5-chloroisotoic anhydride (**57**) was used in place of 4-chloroisotoic anhydride (**55**). It was found that the product yield was much higher (84% versus 19%) and the reaction time much shorter (6 h versus 20 h) using aqueous acetonitrile (80%) rather than 1,4-dioxane, which was used previously for the

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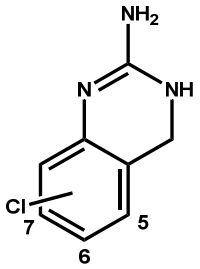
synthesis of 2-amino-6-chloroquinazolin-4-(3*H*)-one (**58**).[132] The physicochemical and spectral character of **47** was consistent with an authentic sample prepared earlier in this laboratory.[132] A comparison of **46** and **47** on TLC showed little differences in $R_f$ values regardless of eluant system (i.e., $H_2O$, $CH_3OH$, $CH_2Cl_2$:$CH_3OH$:$NH_4OH$ 9:1:0.1).

### 3. Binding Studies

The new quinazoline analog **46** was evaluated, and the quinazoline analog **47** was re-evaluated for binding at the mouse 5-HT3A receptor. Compound **47** was found to bind with the highest affinity out of the three quinazoline analogs (Table 12). This is interesting since it would be the equivalent of 4-chlorophenylguanidine (**29**; $K_i$ = 320 nM) and thus indicating that neither *meta*-position is important for binding at the 5-$HT_3$

**Table 12.** Binding affinity of quinazoline series at 5-$HT_3$ receptors.




| Compound | -Cl position | $K_i$ nM[a] NG108-15 GR65630 | $K_i$ nM[b] HEK293 Granisetron | $K_i$ nM (SEM)[c] NIH3T3 LY 278,584 |
|---|---|---|---|---|
| **48** | 5 | | 1148 | 1325 (257) |
| **47** | 6 | 34 | 123 | 80 (11) |
| **46** | 7 | | | 1975 (168) |

[a]NG108-15 mouse/rat recombinant receptors express 5-HT3A; Previously reported by Rahman et al.[9]
[b]HEK293 human native receptors express 5-HT3AB; Dr. M. White (personal communication).
[c]NIH3T3 mouse recombinant receptors express 5-HT3A; $K_i$ values were generously provided by the National Institute of Mental Health's Psychoactive Drug Screening Program, Contract # NO1MH32004 (NIMH PDSP)[154]

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receptor. But, since it has been shown that one of the *meta*-positions is important for the binding of arylguanidines (see previous section on halogen analogs), these constrained analogs might not bind in the same manner as MD-354 (**2**). The three sets of binding data presented in Table 12 were obtained from three different laboratories. The discrepancies in the binding data may be contributed to the different types of cells used (i.e., NG105-15, HEK293, NIH3T3) and thus variations in the population type of 5-$HT_3$ receptors (i.e., homomeric mouse 5-HT3A or heteromeric human 5-HT3AB). Or it may be the result of the radioligand used (i.e., GR65630, granisetron, and LY 278,584).

Analogs **47** and **48** were submitted for functional assay and were identified as antagonists for 5-$HT_3$ receptors using whole cell patch-clamp experiments. MD-354 (**2**), **47**, and **48** were investigated using this functional assay. MD-354 was found to be an agonist in this assay with an $EC_{50}$ value of 3.24 (± 0.55) μM. Quinazolines **47** and **48** were found to be antagonists with $IC_{50}$ values of 4.39 (± 0.78) and 16.53 (± 5.18) μM, respectively (Dr. M. Schulte, personal communication, unpublished data). This functional data indicates that this quinazoline series is a novel class of 5-$HT_3$ receptor antagonists.

**4. Log P Analysis**

An analysis of the log P values for the quinazoline series of compounds was conducted in the same manner as with the arylguanidine series (Figure 29). Because these are positional isomers, it might be expected that their log P values would be very similar. The predicted log P value for each is 1.79 (Table 13). Constraining the guanidine by

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introducing a methylene bridge between the terminal amine and the aromatic ring makes the compound more lipophilic than MD-354 (**2**; predicted log P = 1.43; Table 11) so that it should more readily cross the BBB. The actual log P values as determined using HPLC for the three quinazolines are nearly identical; **46** had a log P value of 1.933, **47** has a log P value of 1.859, and **48** had a log P value of 1.889 (Table 13).

**Table 13.** Capacity factors ($\log k_w^{'}$) determined by linear regression and calculated/ determined log P values for the quinazolines.

| Compound | Position of Cl | $\log k_w^{'}$ | Predicted log P[a] | log P (HPLC) |
|---|---|---|---|---|
| **48** | 5 | 0.4388 ± 0.01562 | 1.79 | 1.89 |
| **47** | 6 | 0.4175 ± 0.00654 | 1.79 | 1.86 |
| **46** | 7 | 0.4712 ± 0.03175 | 1.79 | 1.93 |

[a] Predicted using ChemDraw.

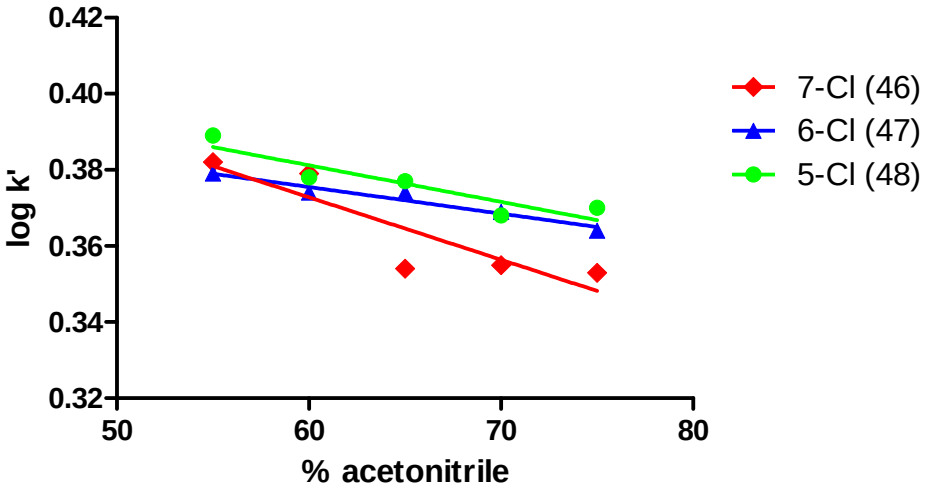


**Figure 29.** Linear regression analysis from the $\log k^{'}$ values of **46**, **47**, **48** at various concentrations of acetonitrile for the quinzaoline analogs.

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The log P values determined for the quinazoline analogs (i.e., approximately 1.9) fall within the range of log P values that are expected to cross the BBB (i.e., 1.5-2.5). Since they should be able to cross the BBB they should be able to act at centrally-located 5-$HT_3$ receptors.

**5. Behavioral Studies**

**a. Tail Suspension Test**

The partial agonist MD-354 (**2**) and quinazoline analogs **46-48** were tested for antidepressant activity using the mouse tail suspension assay (n = 8-11 mice/dose). Tricyclic antidepressants (i.e., desipramine and imipramine; 20 mg/kg), an SSRI (i.e., fluoxetine; 20 mg/kg), and a 5-$HT_3$ receptor antagonist (i.e., ondansetron; 0.1 μg/kg) were given by ip injection thirty minutes before the test and were used as controls. The doses of the controls (i.e., TCAs, SSRI, and ondansetron) and the length of the test were chosen based on literature studies.[15,155] The tests were video recorded and the immobility times for each mouse were measured in triplicate. A one-way analysis of variance (ANOVA) showed that all of the standards displayed statistically lower immobility times when compared with saline (Figure 30). The strain of mice used in this study was the ICR strain; this strain has not typically been used in literature studies since ICR mice tend to be more active than other strains. In fact, van der Hayden et al.[156] and Nomura et al.[157] have shown imipramine to be inactive in ICR mice (i.e., 3, 10, 30 mg/kg and 10 mg/kg,

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respectively). Both studies used automated devices to determine immobility rather than manual observation that was used in the present study.

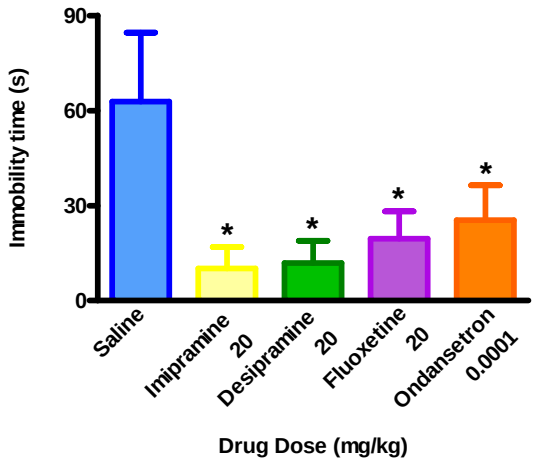


**Figure 30.** Effect (± SEM) of standards (ip) on duration of immbolity in the mouse tail suspension test ($n$ = 8-11 mice/treatment). The significance of the effect was evaluated with a one-way ANOVA test ($F_{4,41} = 3.256$, $p < 0.05$), Newman-Keuls post-hoc test ($p < 0.05$).

The quinazoline analogs (i.e., **46** and **47**) as HCl salts and MD-354 as a nitrate salt, were injected ip thirty minutes before the test at doses ranging from 0.1 to 30 mg/kg. The quinazoline analog (i.e., **48**) as HBr salt was injected ip thirty minutes before the test at doses ranging from 0.1 to 10 mg/kg. The 6-Cl and 7-Cl analogs (i.e., **47** and **46**, respectively) exhibited statistically lowered immobility times when compared to saline (Figure 31). Using a one-way ANOVA the 6-Cl analog (i.e., **47**; $K_i$ = 80 nM) exhibited statistically significant lowered immobility times at the 1.0 and 3.0 mg/kg doses ($F_{6,58} = 3.232$, $p < 0.01$; Dunnett's post-hoc test $p < 0.01$, $p < 0.05$, respectively). Using a one-

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way ANOVA the 7-Cl analog (i.e., **46**; $K_i$ = 1975 nM) exhibited statistically significant lowered immobility times at the 0.1, 0.3, and 1.0 mg/kg doses ($F_{6,63}$ = 5.036, $p < 0.01$; Dunnett's post-hoc test $p < 0.05$, 0.1 and 0.3 mg/kg doses; $p < 0.01$, 1.0 mg/kg dose). The

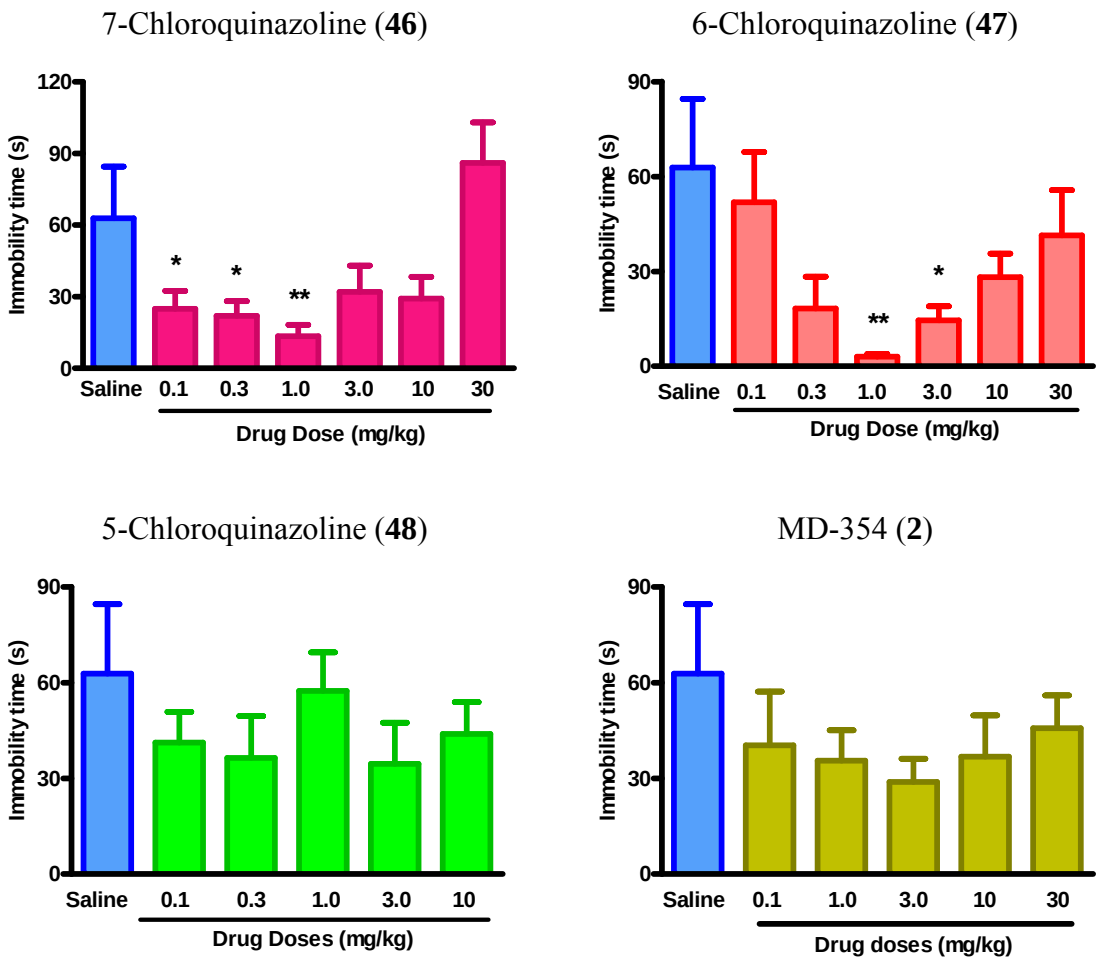


**Figure 31.** Effect (± SEM) of **46** (top left), **47** (top right), **48** (bottom left), and **2** (bottom right) (ip) on duration of immobility in the mouse tail suspension test ($n$ = 8-10 mice/treatment). The significance of the effects were evaluated using one-way ANOVA tests. For **46** ($F_{6,63}$ = 5.036, $p < 0.01$), Dunnett's post-hoc test (** $p < 0.01$, * $p < 0.05$). For **47** ($F_{6,58}$ = 3.232, $p < 0.01$), Dunnett's post-hoc test (** $p < 0.01$, * $p < 0.05$).

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5-Cl analog (i.e., **48**; $K_i$ = 1148 nM) did not exhibit significantly lowered immobility time for any of the doses examined (Figure 31). MD-354 (**2**; $K_i$ = 166 nM) exhibited reduced immobility times which were not significantly different than saline (Figure 31). MD-354 and **48** produce a saline-like effect in the mouse tail suspension test. The log P values determined for all four of these compounds suggest that they can easily cross the BBB. The decreased immobility time is the result of these compounds acting at a central receptor. Since these compounds were given by ip injection (i.e., injection into the body cavity) and some doses display a significant effect it can be assumed that they are crossing the BBB and acting at a centrally located receptor as the log P data suggested from the HPLC analysis.

As shown in Figure 31, 6-chloroquinazoline **47** seems to behave as antidepressant at 1.0 and 3.0 mg/kg doses. But the immobility time, while still reduced, is higher at doses greater than 3.0 mg/kg and less than 1.0 mg/kg resulting in a U-shaped dose-response curve. This curve can also been seen with the 7-chloroquinazoline analog (**46**) and MD-354 (**2**) but it is not as pronounced. The U-shape dose-response curve is characteristic for 5-$HT_3$ receptor ligands in biological assays.[5] With regards to agonists, this type of dose-response curve can be explained by desensitization of the receptor. However, with regards to antagonists the underlying mechanism has yet to be explained. One possible explanation is that 5-$HT_3$ receptor antagonists may bind differently at the homomeric 5-HT3A receptors and heteromeric 5-HT3AB receptors.[5,158]

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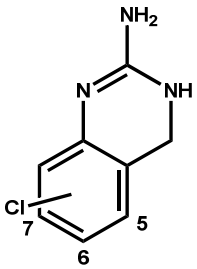
The 7-Cl analog (**46**) does not bind with high affinity ($K_i$ = 1975 nM) to 5-$HT_3$ receptors but displays lower immobility scores than saline; this may indicate that it acts via a non-5-$HT_3$ receptor mechanism to produce this effect. During the course of this investigation Peters et al.[159,160] reported the synthesis of a number of cyclic guanidines, including **46-48**, through high throughput screening methods. They showed that some bind both to 5-$ht_{5a}$ and 5-$HT_7$ receptors (Table 14). Peters et al. report prompted us to test our analogs for binding affinity at 5-$ht_{5a}$ and 5-$HT_7$ receptors. We have found that the 7-Cl analog binds at 5-$ht_{5a}$ and the 5-$HT_7$ receptors ($K_i$ = 302 and 310 nM, respectively)[154] with 5-fold higher affinity than at 5-$HT_3$ receptors. Interestingly, the 5-$HT_7$ receptor has

**Table 14.** Binding profile of selected cyclic guanidines at 5-$HT_3$, 5-$ht_{5a}$, and 5-$HT_7$ receptors.




| Compound | R | 5-$HT_3$ | | 5-$ht_{5a}$ | | 5-$HT_7$ | |
|---|---|---|---|---|---|---|---|
| **48** | 5-Cl | 1325 ± 257 | 1051[b] | 155[a] | 99[b] | 1109[a] | 793[b] |
| **47** | 6-Cl | 80 ± 11[a] | — | 357 ± 38[a] | 807[b] | 1701 ± 242[a] | — |
| **46** | 7-Cl | 1975 ± 168[a] | — | 302 ± 48[a] | 347[b] | 310 ± 43[a] | — |

[a]$K_i$ values were generously provided by the National Institute of Mental Health's Psychoactive Drug Screening Program, Contract # NO1MH32004 (NIMH PDSP)[154]
[b]Peters et al.[159]

been implicated in depression.[161] A recent review by Mnie-Filali et al. showed that 5-$HT_7$ receptor KO mice display antidepressant-like behavior in the FST and TST relative to

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WT mice.[161] In addition, the administration of the 5-$HT_7$ receptor antagonist SB-269970 also decreased immobility time.[161] Therefore, the 5-$HT_7$ receptor mechanism in the antidepressant action of **46** cannot be excluded. The 5-$ht_{5a}$ receptor's function is not fully understood because of the lack of selective agents. However, since it is located only in the CNS, primarily in the cerebral cortex, hippocampus, and cerebellum, it has been postulated that this receptor may be involved in schizophrenia or mood disorders.[162,163] Based on this information **47** could be exhibiting its antidepressant effect through a 5-$ht_{5a}$ or 5-$HT_7$ receptor mechanism. Interestingly, the 5-chloroquinazoline **48** seems to be more selective for the 5-$ht_{5a}$ receptor over the 5-$HT_3$ and 5-$HT_7$ receptors. Optimization of this analog by Peters et al. has led to even more analogs that have increased binding affinity for 5-$ht_{5a}$ receptors (e.g., 2-amino-5-chloro-4-methyl-3,4-dihydroquinazoline; 5-$ht_{5a}$ $K_i$ = 5.1 nM).[159,160] The 5-Cl-4-$CH_3$ analog was also shown to be a competitive 5-$ht_{5a}$ receptor antagonist through the [$^{35}$S]GTPγS assay with a $pA_2$ value of 8.52.[159] Most of their analogs had no more than a 4-fold selectivity over the 5-$HT_7$ receptors but because of the similar localization of the 5-$ht_{5a}$ and 5-$HT_7$ receptors in the brain, Peters et al. continued to examine this series.[159] Their overall goal was to develop a pharmacological tool for behavioral studies to examine the role of the 5-$ht_{5a}$ receptor's antipsychotic-like effects.[160] Although the involvement of a 5-$HT_7$ receptor mechanism in the antidepressant activity of **46** cannot be excluded at this time, it is unlikely to undermine the antidepressant activity of these quinazoline analogs because **47** binds with more than 20-fold lower affinity at 5-$HT_7$ receptors versus 5-$HT_3$ receptors.

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**b. Locomotor Activity Assay**

Since hypermobility may also explain lowered immobility times relative to saline, the effective tail suspension test doses (**46**, 0.3 mg/kg; **47**, 1.0 and 3.0 mg/kg) were tested against saline for hyperactivity using the locomotor activity assay. The doses were injected ip with no pre-treatment time and their effect on locomotor activity was examined over 45 minutes. This time encompasses the 30 minutes pretreatment from the tail suspension test, the six minute tail suspension test, and nine minutes after the tail suspension test would be over; so any movements that have increased relative to saline during this time would have been increased during the antidepressant assay.

Using a one-way ANOVA there was no significant difference between any of the doses and saline for the 45 minute duration for total movement episodes, total movement time, total movement distance, number of jumps or average velocity (Figure 32). These parameters are usually used in the identification of stimulants. Stimulants generally increase the movement time, distance, and velocity but decrease the number of movement episodes (i.e., the drug increases the duration of motor activity but decreases the number of stops).[164] There was also no difference from saline for the retraced local movements, retraced local movement episodes (3 repeated local movements), and these movements'

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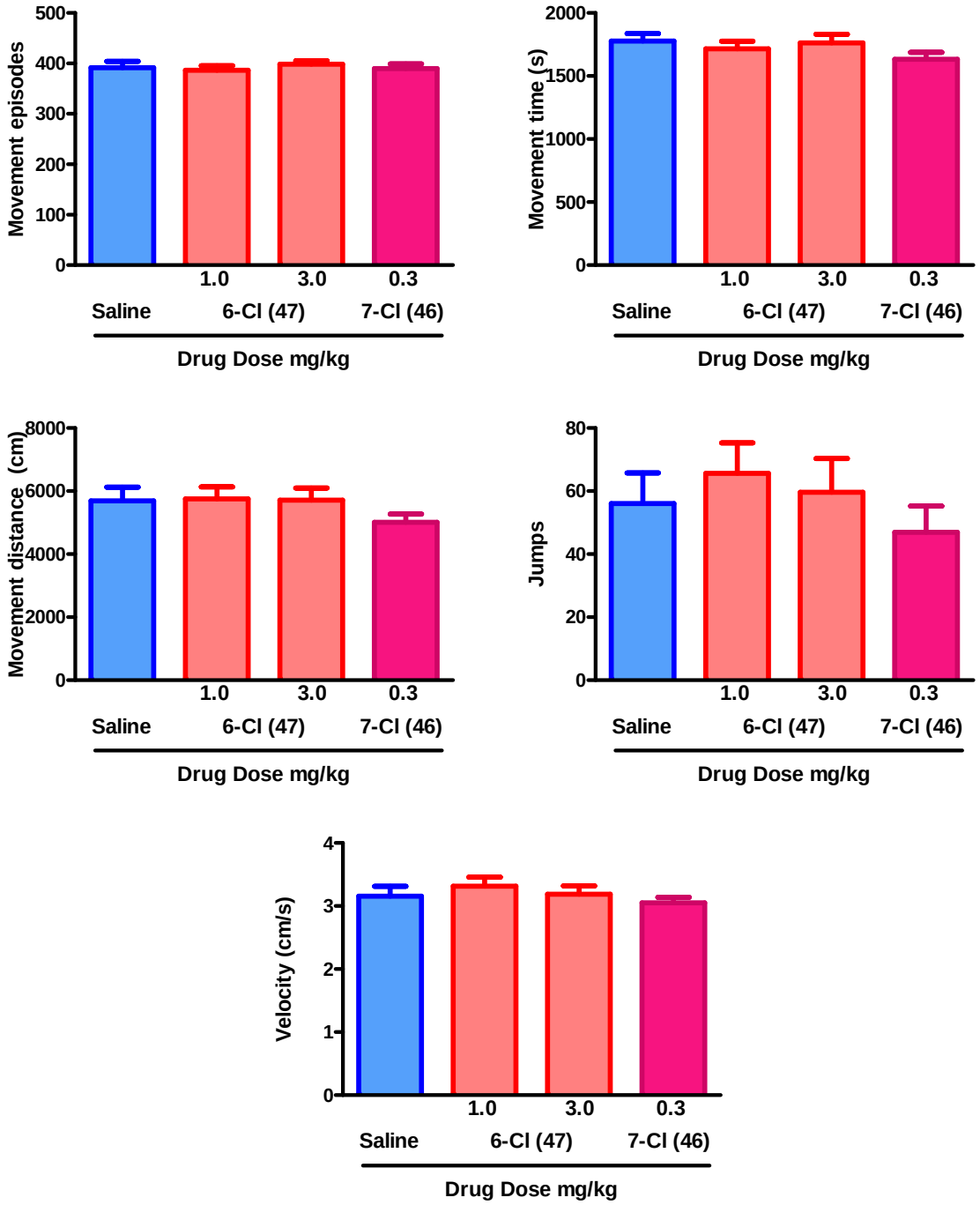


**Figure 32.** Effect (± SEM) of **47** (1.0 and 3.0 mg/kg) and **46** (0.3 mg/kg) on total number of movement episodes, total movement time, total movement distance, total number of jumps, and average velocity (*n* = 12-13 mice/treatment).

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time which may indicate exploratory behavior (Figure 33). The term local refers to the fact that the animal's movement does not produce a large enough change in location from its starting point (i.e., less than ±1.499 beam spaces from the starting point). As with all of the other parameters examined there was no difference using a one-way ANOVA between the doses evaluated and saline for margin distance traveled, time spent in the margin, center distance traveled, time spent in the center, number of center entries, and the number of vertical entries (Figure 34). Increased margin activity or decreased central activity is generally viewed as an anxiogenic-like effect. Vertical entries may indicate the "nonspecific excitability level" of the animal.

The 5-$HT_3$ receptor antagonist ondansetron (**3**) and the 5-$HT_3$ receptor partial agonist MD-354 (**2**) showed no effect on locomotor activity in previous studies.[15,132] Based on those results, there was no reason to expect a change in locomotor activity for the quinazoline analogs **46** and **47** unless these compounds cause hyperactivity. Since there was no difference in activity from saline using a one-way ANOVA with any of the doses examined, the lowered immobility scores from the tail suspension test should be from an antidepressant-like action of the compounds and not from hyperactivity. However, **46** displayed a decreased number of center entries, a decreased amount of time spent in the center of the arena, and a decreased distance traveled in the center of the

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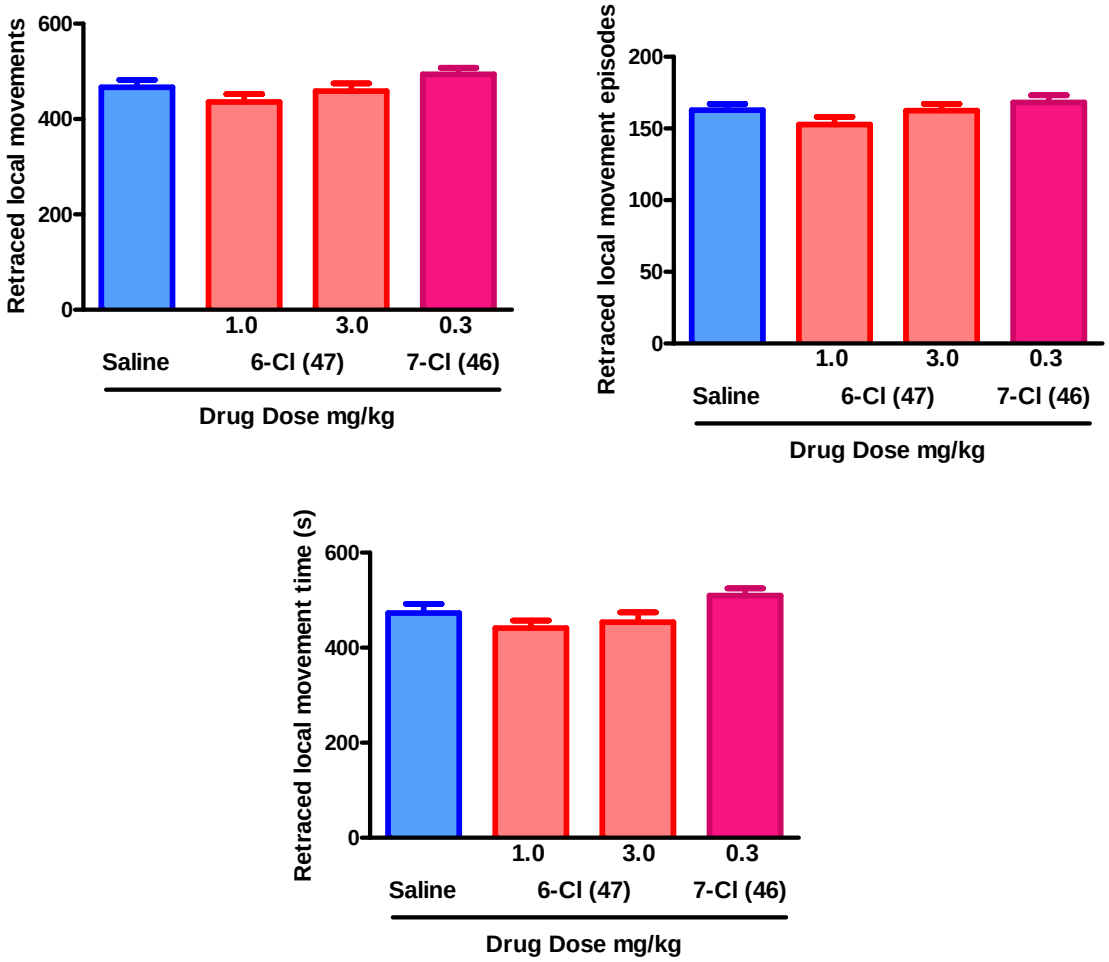


**Figure 33.** Effect (± SEM) of **47** (1.0 and 3.0 mg/kg) and **46** (0.3 mg/kg) on total number of retraced local movements, total number of retraced local movement episodes, and total time spent on retraced local movements (*n* = 12-13 mice/treatment).

arena, but they were not found to be significantly different from saline using a one-way ANOVA. A significant decrease in center activities may have indicated anxiogenic-like properties. Because these parameters were not significantly different than saline using a one-way ANOVA, this may be because of variation and not a true example of anxiogenic properties. If it is because of anxiogenic properties, it is most likely the result of acute

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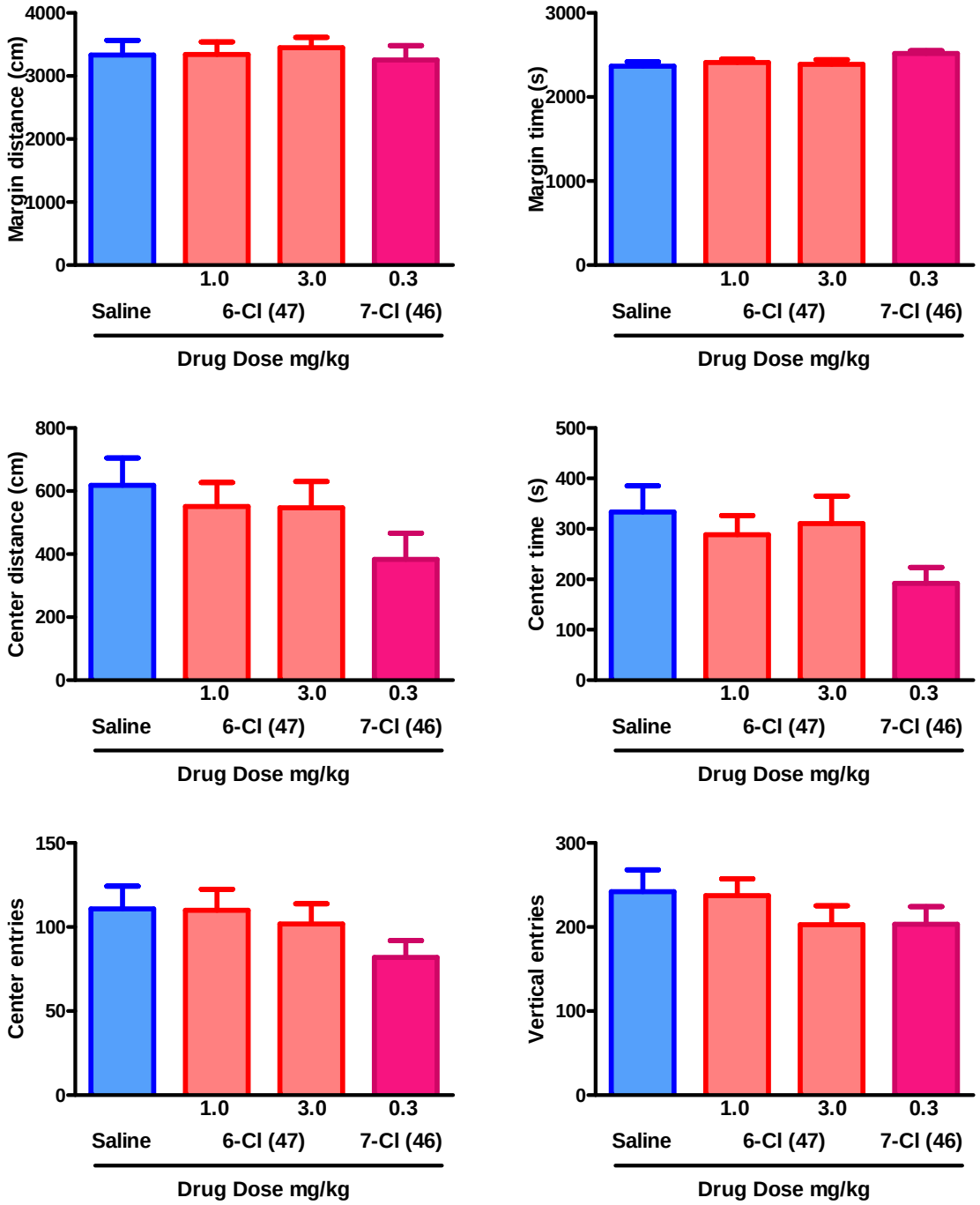


**Figure 34.** Effect (± SEM) of **47** (1.0 and 3.0 mg/kg) and **46** (0.3 mg/kg) on total distance traveled in the margin, total time spent in the margin, total distance traveled in the center, total time spent in the center, the number of center entries, and the number of vertical entries ($n$ = 12-13 mice/treatment).

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administration; there have been reports indicating that some antidepressants increase anxiety levels when administered acutely.[165,166]

### 6. Molecular Modeling

Since the constrained analogs **47** and **48** were found to be antagonists for the 5-$HT_3$ receptor, it would be of value to identify the docking poses of these constrained analogs to a 5-$HT_3$ receptor homology model. Using the same set of models generated using Modeller (Method B) and AutoDock4, the 5-$HT_3$ receptor anatagonist granisetron (**4**), with the charges and geometries generated by MOPAC, was docked to each of the five interfaces of each of the 100 models with 20 genetic algorithms in a 40 Å x 40 Å x 40 Å region around the C-loop. A different model than the model identified by the agonist 5-HT, was identified through the proposed binding mode of the antagonist granisetron. This proposed binding mode is similar to that in the literature for granisetron (Figure 35).[167,168] The azabicyclic ring is located towards W90 and F226, while the aromatic ring is located towards W183 and Y234. ProCheck was used to create a Ramachadran plot of the pentamer; it was found that 64.8% of residues were in favored regions, 28% in additionally allowed regions, 4.8% in generously allowed regions and 2.5% in disallowed regions (Figure 36). Since the loop models are generated by small changes in energies, the side chains were not optimized with SCRWL leading to residues found in the generously allowed and disallowed regions as seen with the 5-$HT_3$ receptor agonist model also developed by Modeller. In addition, AutoDock allowed for flexibility

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in the backbone atoms during the docking process to allow for the best possible interactions.

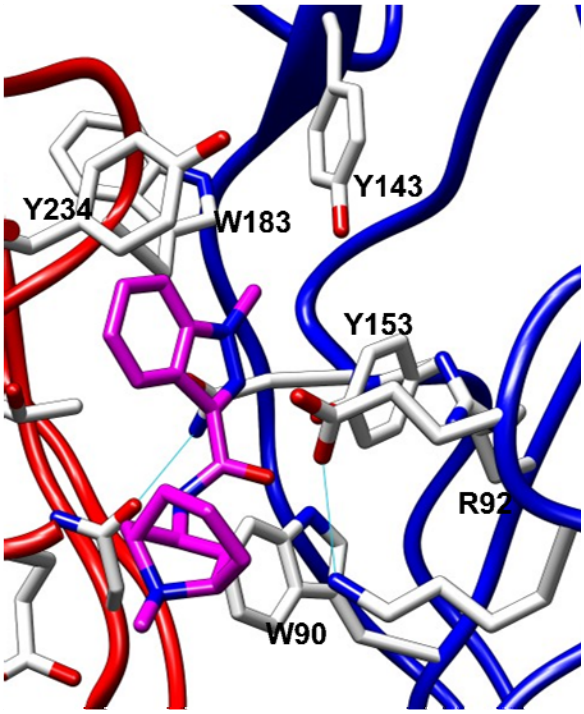


**Figure 35.** Proposed binding mode of granisetron (**4**) to the h5-HT3A receptor antagonist model developed using Modeller (Method B). Granisetron is shown in magenta and residues within 5 Å are shown.

Since this antagonist model could be validated by granisetron (**4**), the quinazoline analogs **46-48** and the 5-$HT_3$ receptor partial agonist MD-354 (**2**) were built and given geometries and charges according to MOPAC. They were docked to the antagonist validated receptor model using AutoDock and the same parameters as before. The

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guanidine portion of the quinazolines is oriented towards W90 instead of towards W183 as seen with the MD-354 (Figure 37). An overlap of MD-354 (**2**) and the highest binding affinity analog, 6-Cl quinazoline **47**, is shown in Figure 38.

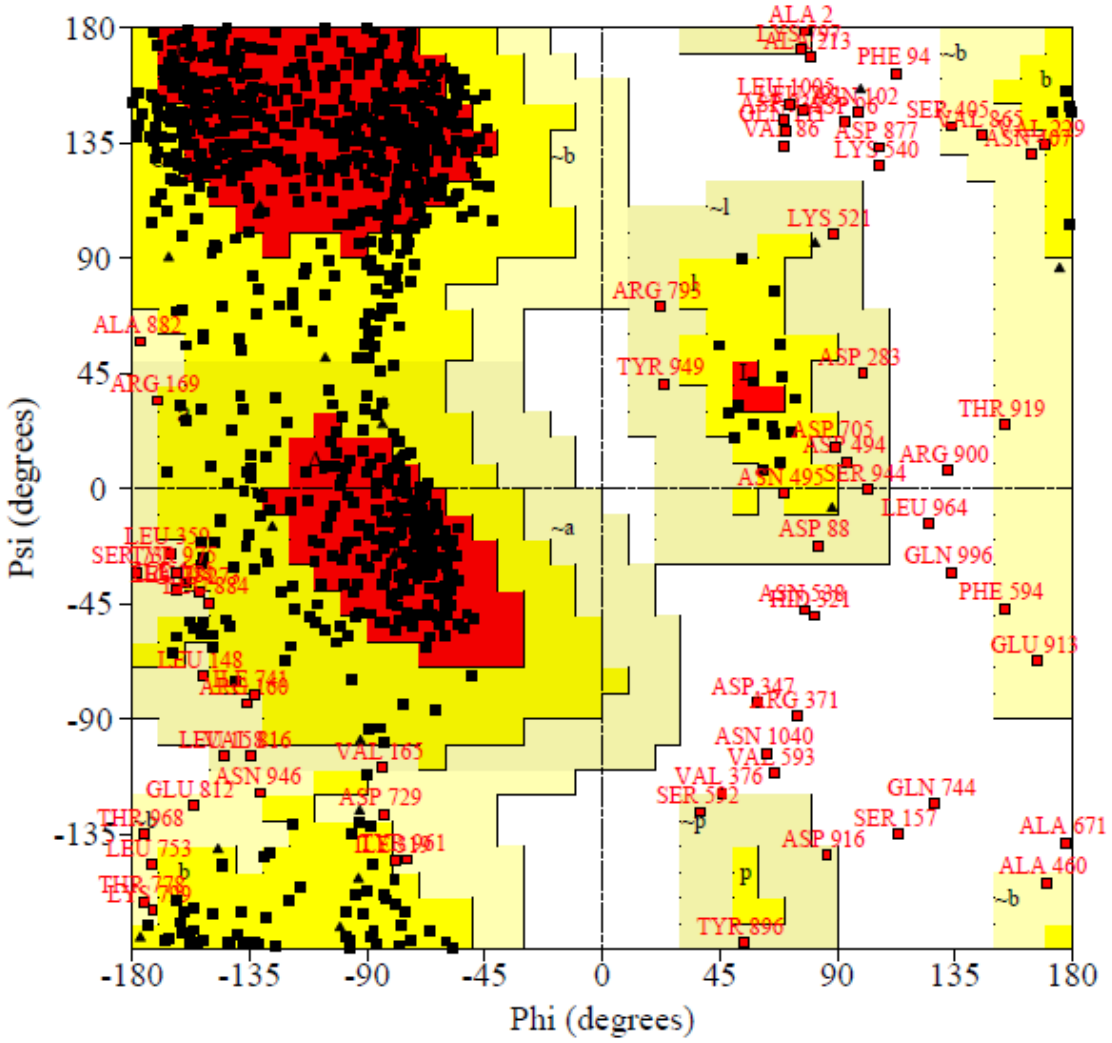


**Figure 36.** Ramachadran plot of the h5-HT3A receptor antagonist model developed using Modeller (Method B).

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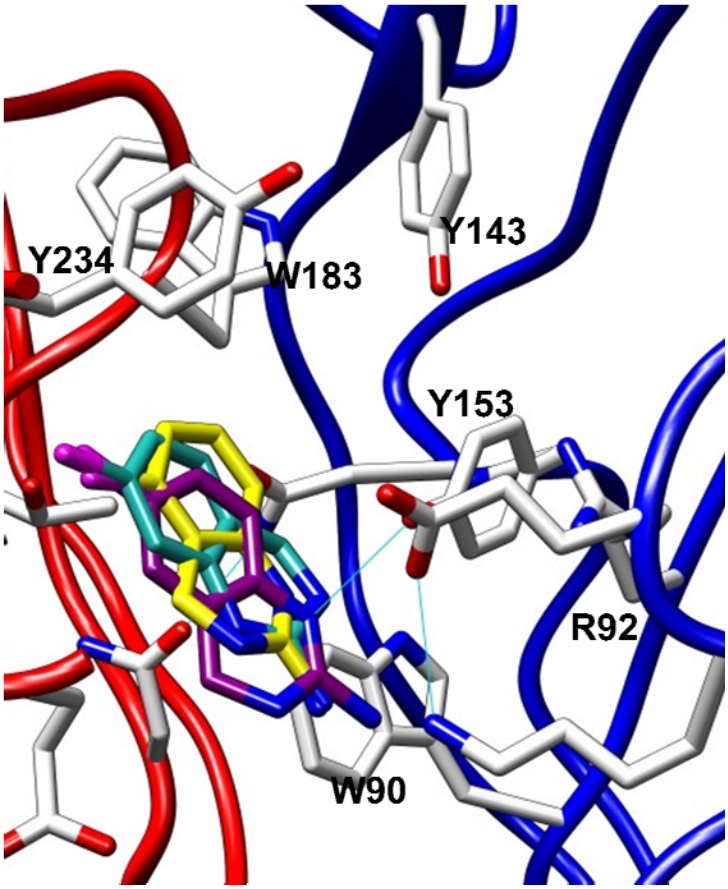


**Figure 37.** Proposed binding mode of the quinazoline analogs **46-48** to the h5-HT3A receptor anatagonist model developed using Modeller (Method B). The 5-Cl analog (**48**) is shown in cyan, the 7-Cl analog (**46**) is shown in yellow, and the 6-Cl analog (**47**) is shown in purple. Residues within 5 Å are also shown.

Based on the structures of the quinazolines and MD-354, it was assumed that the quinazolines should be able to bind overlapping MD-354. However, based on the proposed binding modes, the quinazoline analogs do not bind in the same manner as the partial agonist MD-354. But bind in the opposite direction as MD-354 (Figure 38). The

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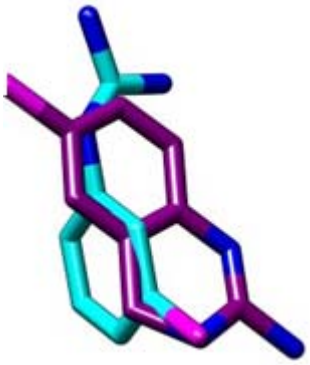

**Figure 38.** Overlap of the proposed binding modes of MD-354 (**2**; cyan) and the highest binding affinity analog, 6-Cl quinazoline **47** (purple).

guanidine of the quinazolines may be interacting with W90 instead of W183 as seen with the proposed binding mode of MD-354. These changes in proposed binding modes may explain why the quinazolines are antagonists and MD-354 is merely a partial agonist. The constrained guanidine cannot make the same interactions (W183 and Y234) with its nitrogen atoms as the free guanidine of MD-354. This may also explain why neither of the two rotameric constrained analogs of MD-354 (i.e., **46** and **48**) bind with the same affinity as MD-354.

### 7. Synthesis of 2-Amino-8-chloro-1,3-benzodiazepine (49)

The synthesis of this constrained analog of MD-354 (**2**) would allow for a longer centroid-to-amine distance, closer to that of MD-354, and a larger height of the primary amine above the plane of the aromatic ring (Table 6). The first attempted synthesis followed the reaction outlined in Scheme 4. The benzoic acid **59** was reduced to the benzyl alcohol **60**.[169] An attempt was made to convert the benzyl alcohol to the benzyl

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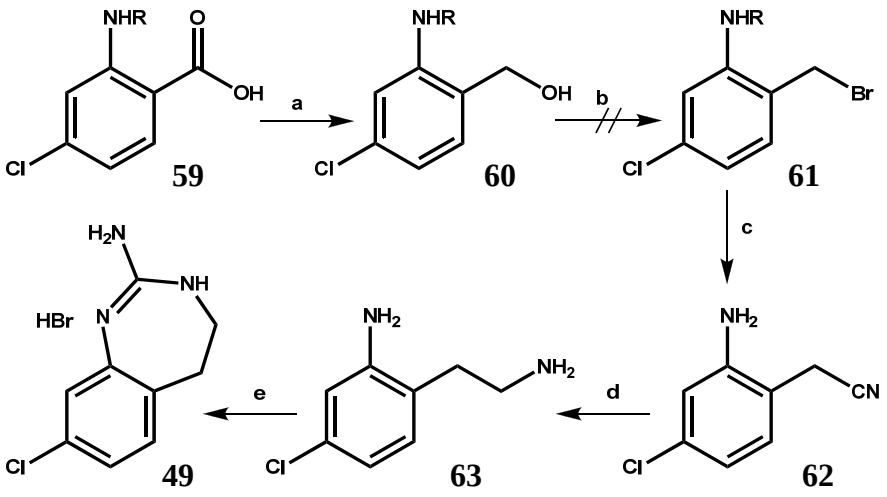
bromide **61**.[170] However, the benzyl bromide was unable to be isolated and characterized with and without first protecting the amine (i.e., $(Boc)_2O$ or trifluoroacetic anhydride).

**Scheme 4.**[a]




R = H, Boc, TFA

[a]Reagents and conditions: a. $LiAlH_4$, THF (R = H); b. $PBr_3$, $CH_2Cl_2$ or $Et_2O$; c. NaCN, isopropanol, $H_2O$, d. Raney Ni, e. BrCN, EtOH

The second route of synthesis involved the opening of the oxindole ring **64** to obtain the carboxylic acid **65**.[171] Under the conditions described in Scheme 5 the desired ring-opened intermediate was not isolated. Either it was formed, and then recyclized to the starting material (i.e., **64**), as the use of acid is typically employed to prepare oxindoles from the 2-aminophenylacetic acids,[172] or only tautomer **66** of the starting material was formed which then reverts back to starting material. The NMR and IR

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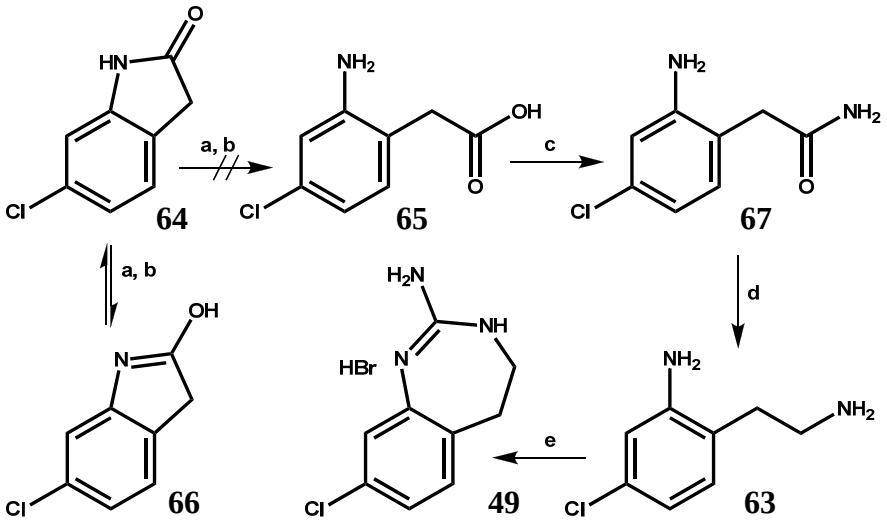
analysis of the product from the reaction could not conclusively be identified as either **65** or **66** but was different from **64** on TLC.

**Scheme 5.**[a]




[a]Reagents and conditions: a. NaOH, $H_2O$, reflux; b. HCl, $H_2O$; c. carbonyldiimidazole, $NH_3$, $CH_3OH$, d. $BH_3$-THF, e. BrCN, EtOH

The third route of synthesis involved the conversion of nitrotoluene **68** to benzaldehyde **69** with DMF-DMA,[173] then converting the benzaldehyde to a nitrostyrene **70** with nitromethane[174] as outlined in Scheme 6. The benzaldehyde **69** could not be purified from bi-products formed during the reaction.

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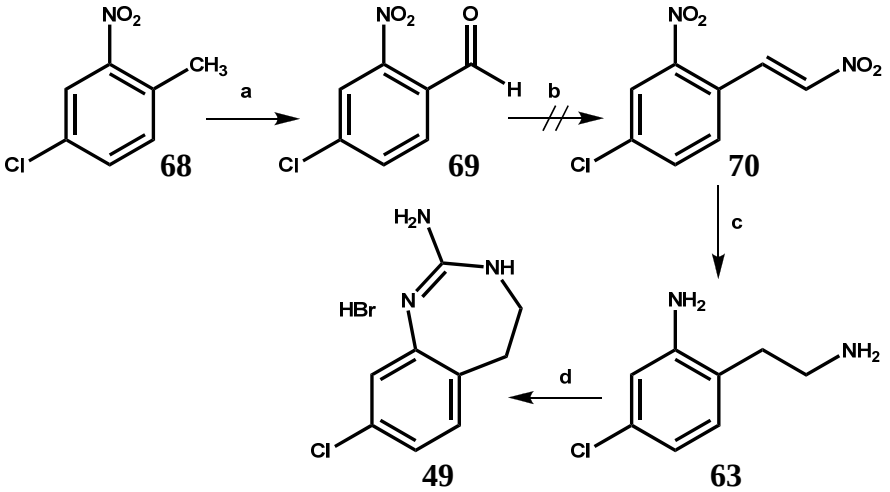
**Scheme 6.**[a]




[a]Reagents and conditions: a. 1. DMF-DMA, 140 °C, 2. $NaIO_4$; b. $CH_3NO_2$, $NH_4OAc$, reflux, c. $SnCl_2$, HCl, d. BrCN, EtOH

The fourth route of synthesis attempted to convert benzoic acid **71** to nitrophenylethanone **73** going through intermediate **72** as outlined in Scheme 7.[175] However, only the intermediate **72** was formed and isolated; the nitrophenylethanone **73** was not. It appears that the intermediate does not react as easily with the nitromethane as suggested in the literature.

The fifth route of synthesis involved the reaction of nitrotoluene **68** with N-bromosuccinamide and benzoyl peroxide to form benzyl bromide **75** (Scheme 8).[176] The benzyl bromide was slightly more polar than the starting material and has a very similar melting point making separation difficult. An attempt to convert benzyl bromide **75** to (nitrophenyl)acetonitrile **76** using hydrogen cyanide formed in situ from sodium cyanide

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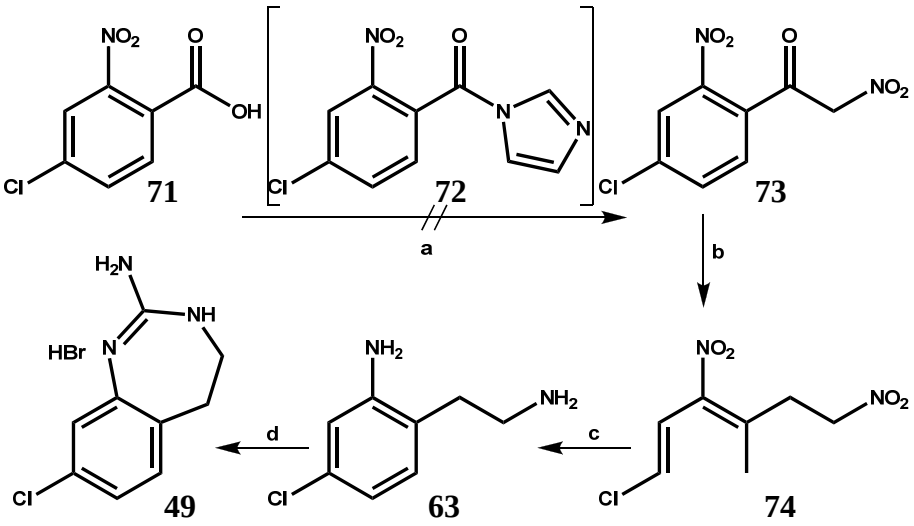
**Scheme 7.**[a]




[a]Reagents and conditions: a. carbonyldiimidazole, $K_2CO_3$, THF, $CH_3NO_2$, reflux; b. $BH_3$-THF; c. $SnCl_2$, HCl; d. BrCN, EtOH

and strong acid resulted in the formation of a dimer complex (i.e., **77**). This type of dimerization was seen by Kalir and Mualem with the reaction of 2-nitrobenzylbromide using this reaction.[177] Another attempt to convert the benzyl bromide **75** to (nitrophenyl)acetonitrile **76** was made using liquid-liquid phase transfer catalysis. Sodium cyanide ionizes in water into a sodium ion and cyanide ion. The cyanide reacts with **75** to form **76**, and then quickly reacts with another equivalent of **75** to form 2,3-bis(4-chloro-2-nitrophenyl)propanenitrile (**77**). Adjusting the pH to be acidic to increase the ionization of the cyanide completely halted the reaction. Using solid-liquid phase transfer catalysis with potassium cyanide, 18-crown-6 ether, in acetonitrile still produced **77** and not **76** as desired.

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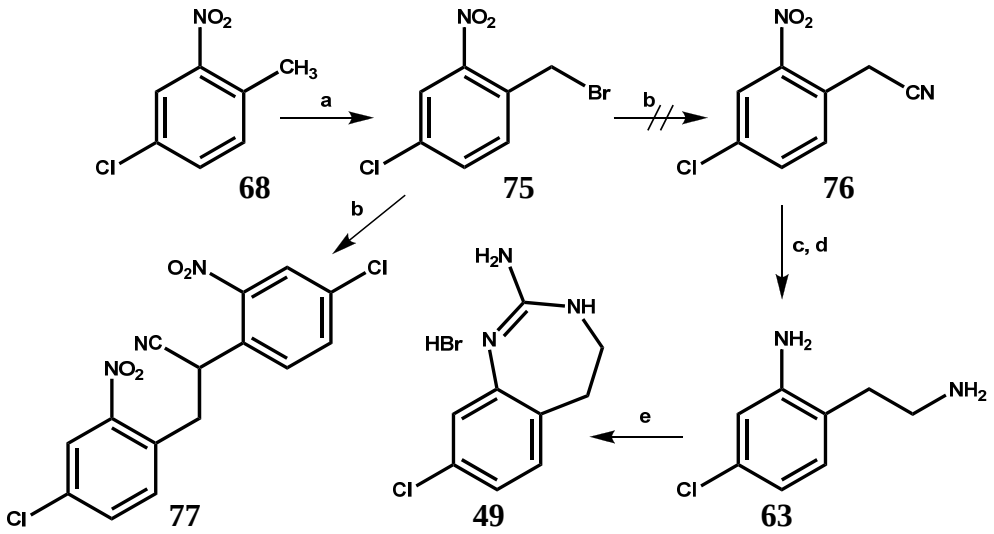
**Scheme 8.**[a]




Reagents and conditions: a. N-bromosuccinamide, benzoyl peroxide, $CCl_4$, light, reflux; b. NaCN, $H_2O$, TBAB, $CH_2Cl_2$; c. $BH_3$-$(CH_3)_2S$, reflux; d. Raney Ni; e. BrCN, EtOH, reflux

Given that the height of the amine above the plane and the aromatic centroid to primary amine distances of **49** are closer to that of MD-354 (**2**) relative to the other constrained analogs (i.e., **45-48**), **49** may bind with enhanced affinity. Since the synthesis of **49** was unsuccessful, binding data is not available. If **49** binds with higher affinity than **46**, the longer amine to centroid distance and/or the out-of-plane character of **49** might explain the difference in binding affinities.

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## V. Conclusions

Two novel series of MD-354 (**2**) analogs were synthesized as part of this investigation. The intended purpose of evaluating these series was to identify *what* characteristics of the *meta*-position are necessary for binding to the 5-$HT_3$ receptor and *which meta*-position is important for binding.

The first series of analogs developed was the mono-substituted halogen analogs of MD-354. By purely examining the binding affinity of PG (**27**), 3-FPG (**42**), MD-354 (**2**), 3-BrPG (**43**), and 3-IPG (**44**) it appears that smaller substituents do not bind very well at the 5-$HT_3$ receptors. The receptor may need a substituent at the 3-position of a particular size or larger to have high affinity; the –H or –F groups may be too small to fit this requirement. However, the 3-$CF_3$ analog binds with even lower affinity than PG or 3-FPG and is much larger in terms of solvent accessible volume than any of the halogenated analogs, suggesting that something more than just size plays a role in binding affinity. A Hansch analysis indicated that size in the form of volume (i.e., surface accessible volume and CMR), polarizability (i.e., CMR and polarizability) and electronic character (i.e., $\pi$) are important components in binding affinity. However, volume and polarizability are intercorrelated and can therefore act as surrogates for each other. But a multiple linear regression analysis has shown that $\pi$ and either CMR, volume, or polarizability impact binding affinity. All of this information is consistent with a prior



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QSAR analysis[100,124] of arylguanidines and supports the current pharmacophore for arylguanidines and arylbiguanides[83] that at one of the *meta*-positions an electron-withdrawing group of a certain volume or polarizability is needed for optimal binding affinity.

Two distinct homology models of the h5-HT3A receptor were developed by different methods (i.e., Method A and Method B) and validated by docking the 5-$HT_3$ receptor agonist serotonin. Upon validation of the models, the docking of the halogenated analogs to a homology model (Method B) of the h5-HT3A receptor was investigated. The results suggested that these analogs bind in the same manner as MD-354. However, 3-FPG (**42**) is suggested to dock in an orientation in which the fluorine atom does not overlap with the other halogens but is on the opposite side of the aromatic ring. This can be explained by the effects of a fluorine atom attached to an aromatic ring. The electronegativity of the fluorine atom might affect the electronic character of not only the ring but also of the guanidine.

Based on the proposed docking poses of MD-354 and serotonin (Method A) a new analog was designed and synthesized, (3-chloro-4-hydroxyphenyl)guanidine (**51**). The guanidine portion should be able to interact with W183 and Y234 as the aliphatic amine in serotonin does and the hydroxyl group should be able to interact with Y153. The –Cl group from this compound should be able to sit in the same pocket as the –Cl group from MD-354. In theory **51** should bind with higher affinity than 5-HT or MD-354 because it should be able to interact with the receptor similar to both 5-HT and MD-354.

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The second series of analogs developed were constrained analogs of MD-354 (**2**); in which the guanidine was constrained by a methylene group to form a series of quinazolines (i.e., **46-48**). This series was initially developed to determine which *meta*-position was important for binding affinity. However, binding data at the 5-$HT_3$ receptor of these three analogs indicated that neither position was important. Since it has been shown previously that there is an electronic effect for a *meta*-substituent (*vide supra*), at least one *meta*-position is important and necessary for binding to the 5-$HT_3$ receptor if the compounds are binding in the same manner. Based on this data, the quinazoline series does not seem to be binding to the 5-$HT_3$ receptor in the same manner as the arylguanidines. A functional assay has also shown that some of these quinazolines are antagonists for the 5-$HT_3$ receptor (**47**; $IC_{50}$ = 4.39 ± 0.78 μM and **48**; $IC_{50}$ = 16.53 ± 5.18 μM; Dr. M. Schulte, personal communication, unpublished data).

Molecular modeling of the possible binding modes of the quinazolines to a homology model (Method B) of the h5-HT3A receptor validated by the docking of the 5-$HT_3$ receptor antagonist granisetron, indicates that the quinazolines do not bind in the same manner as MD-354. The quinazolines seem to bind upside-down relative to MD-354 in which their guanidine portion interacts with W90 and not W183. Since this series has been shown to be functionally different than MD-354 and does not seem to bind in the same manner, it should be considered a novel class of 5-$HT_3$ receptor ligands.

Some of the newly synthesized analogs (e.g., **43**, **44**, and **47**) have higher binding affinity for the 5-$HT_3$ receptors than MD-354. But, having higher binding affinity had no

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effect on the ability of the compound to cross the BBB. The evidence for mCPBG (**17**) to penetrate the BBB has been controversial. A newer method of determining partition coefficients was employed using HPLC. With this method, mCPBG was shown to have a log P value consistent with its being able to cross the BBB. The log P values determined for PG (**27**), 3-FPG (**42**), MD-354 (**2**), 3-BrPG (**43**), 3-IPG (**44**), 3,4,5-tri-Cl-PG (**35**), 5-Cl quinazoline **48**, 6-Cl quinazoline **47**, and 7-Cl quinazoline **46** also indicate that they should be able to readily cross the BBB.

Considering the log P values of the quinazoline series indicates that these compounds should be able to readily cross the BBB and interact with central 5-$HT_3$ receptors and, considering that this is a novel series of 5-$HT_3$ receptor ligands, their ability to treat depression was evaluated with the mouse tail suspension test. Results from the study indicated that some of the doses of two of the quinazolines (1.0 and 3.0 mg/kg of **47** and 0.1, 0.3, and 1.0 mg/kg of **46**) decrease immobility time relative to saline. Whereas, the other quinazoline analog (i.e., **48**) and MD-354 (**2**) did not statistically decrease immobility time. Furthermore, the lowered immobility scores are not because of increased movements of the mice as shown by saline-like effects on the locomotor parameters assessed. However, the lack of selectivity for the 5-$HT_3$ receptor may indicate that the antidepressive action seen may or may not be because of a 5-$HT_3$ receptor mechanism. If the antidepressive action that is observed is not because of a 5-$HT_3$ receptor mechanism, then these compounds being antagonists is not relevant. But, if the antidepressive action that is observed is because of a 5-$HT_3$ receptor mechanism, then

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these compounds are behaving similarly to the other 5-$HT_3$ receptor antagonists, ondansetron and bemesetron; if that is the case, the 5-$HT_3$ receptor agonist SR 57227A may be desensitizing the receptor and producing its antidepressive effect. If the antidepressant action of the agonist SR 57227A is from desensitization it may explain why MD-354, a partial agonist, is inactive and that the antidepressant action is because of an antagonist effect at the 5-$HT_3$ receptors. However, since 5-$HT_3$ receptors have been implicated in anxiety, the SR 57227A could be anxiogenic and the lowered immobility scores due to tremors from anxiety.

In conclusion, two novel series of MD-354 analogs were synthesized. The first series, halogen analogs, was used to determine that an electronic effect at a *meta*-position is essential for binding. Furthermore, the poloarizability or the volume of the substituent is important as well for the binding of arylguanidines and arylbiguanides. It is most likely a combination of both the electronic effect and the polarizability, or the electronic effect and the volume, that affect binding together rather than just one individually. While the second series, quinazoline analogs, was developed to determine which *meta*-position was important, it has instead been shown to be a novel class of 5-$HT_3$ receptor ligands. Two of the analogs (i.e., **46** and **47**) have been shown to behave as antidepressants in the mouse tail suspension test. They may or may not be acting through a 5-$HT_3$ receptor mechanism since **46** binds with higher affinity to the 5-$ht_5$ and 5-$HT_7$ receptors and **47** only has a 4-fold selectivity over the 5-$ht_5$ receptor. Overall the current pharmacophore for arylguanidines and arylbiguanides has been validated for unconstrained

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arylguanidines. And a novel class of 5-$HT_3$ receptor ligands, which are conformationally-constrained arylguanidines, has been developed which do not fit the pharmacophore for arylguanidines and arylbiguanides.

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## VI. Experimental

### A. Synthesis

Melting points were taken on one of two melting point apparatuses in glass capillary tubes and are uncorrected. Unless otherwise noted melting points were taken on a Thomas-Hoover melting point apparatus. When noted, the melting points were taken on a Büchi B-545 melting point apparatus. $^{1}$H NMR spectra were recorded with a Varian EM-390 spectrometer with tetramethylsilane (TMS) as an internal standard. Peak positions are given in parts per million (δ). Infrared spectra were obtained on a Nicolet Avatar 360 FT-IR spectrophotometer. Microanalyses were performed by Atlantic Microlab Inc. (Norcross, GA) for the indicated elements and results are within 0.4% of calculated values. Chromatographic separations were performed on silica gel columns (Silica Gel 62, 60-200 mesh, Sigma-Aldrich). Flash chromatography was performed on a CombiFlash Companion/TS (Teledyne Isco Inc. Lincoln, NE). Reactions were monitored by thin-layer chromatography (TLC) on silica gel GHLF plates (250 μ, 2.5 X 10 cm; Analtech Inc. Neward, DE).

***N*-(3-Fluorophenyl)guanidine Nitrate (42).** Compound **42** was prepared according to a literature procedure.[143] A solution of 3-fluoroaniline hydrochloride (**50**) (1.00 g, 6.78 mmol) and cyanamide (0.38 g, 9.04 mmol) in abs EtOH (5 mL) was heated at reflux for 6



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h. The solvent was removed under reduced pressure to give a pale yellow oil. The crude product was dissolved in $H_2O$ (2 mL) and $NH_4NO_3$ (1.00 g, 12.5 mmol) was added in excess. The solvent was removed under reduced pressure and the residue was crystallized from $H_2O$. The light brown crystals were collected by filtration and washed with cold $Et_2O$ (3 x 5 mL) to yield 0.81 g (55%) of **42**: mp 145-146 °C ($H_2O$), 146-147 °C (EtOH) (lit[178] 165 °C, EtOH); IR (KBr, $cm^{-1}$): 3342, 3330, 3195, 1669, 1597, 1493, 1369, 1143; $^1H$ NMR (DMSO-$d_6$) δ 7.21-7.04 (m, 3H, ArH), 7.53-7.45 (m, 5H, ArH, $NH_2$, ex with $D_2O$), 9.72 (s, 1H, NH, ex with $D_2O$). Anal. calcd for $C_7H_8FN_3 \cdot HNO_3$: C, 38.89; H, 4.20; N, 25.92 Found: C, 39.08; H, 4.15; N, 25.89.

**2-Amino-7-chloro-3,4-dihyroquinazoline, Hydrochloride (46).** Compound **46** was prepared using a literature procedure for a similar compound.[153] $BH_3$-THF complex (12.2 mL, 1 M, 12.2 mmol) was added in a dropwise manner to **56** (0.60 g, 3.06 mmol) under a $N_2$ atmosphere. The orange mixture was heated at reflux for 2.5 h. After cooling the reaction mixture to room temperature, a 6 N solution of HCl (3 mL) was added in a dropwise manner releasing gas. Then a 6 N solution of NaOH (12 mL) was added in a dropwise manner to basify the reaction mixture. The solution was concentrated under vacuum, and hot $CHCl_3$ (3 x 10 mL) was added. The solid at the interface was collected and dried under reduced pressure for 4 h to give a white solid 0.15 g, (24%): mp 183-185 °C; $^1H$ NMR (DMSO-$\delta_6$): 3.45 (br s, 1H, NH, $D_2O$ ex), 4.30 (s, 2H, $CH_2$), 6.12 (br s, 1H, NH, $D_2O$ ex), 6.60 (s, 1H, ArH), 6.73 (d, 1H, ArH), 6.89 (d, 1H, ArH). A solution of the crude product (0.13g, 0.72 mmol) in abs EtOH was cooled in an ice/water bath.

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Concentrated HCl was added in a dropwise manner to a pH ≈ 3. The solvent was removed under reduced pressure and the process repeated twice more to give a white solid. The solid was recrystallized from hot EtOH to give a white solid 0.08 g (53%) of **46**: mp 249-251°C; IR (KBr, $cm^{-1}$): 3300, 3190, 2979, 2927, 2855, 1700, 1618, 1493, 1091; $^1$H NMR (DMSO- $\delta_6$): 4.45 (s, 2H, $CH_2$), 7.07 (d, 1H, ArH), 7.17 (dd, 1H, ArH), 7.25 (d, 1H, ArH), 7.77 (s, 2H, $NH_2$, 1H $D_2O$ ex), 8.63 (s, 1H, NH, $D_2O$ ex), 11.02 (s, 1H, NH, $D_2O$ ex). Anal calcd for $C_8H_8N_3Cl \cdot HCl \cdot 0.25\ H_2O$: C, 43.17; H, 4.30; N, 18.88 Found C, 43.37; H, 4.07; N, 18.55.

**2-Amino-6-chloro-3,4-dihyroquinazoline, Hydrochloride (47).** Compound **47** was prepared according to a literature procedure for a similar compound.[153] $BH_3$-THF complex (11.6 mL, 1 M, 11.6 mmol) was added in a dropwise manner to **58** (0.65 g, 3.33 mmol) under a $N_2$ atmosphere. The orange mixture was heated at reflux for 2 h. After cooling the reaction mixture to room temperature, a 6 N solution of HCl (3 mL) was added in a dropwise manner releasing gas. Then a 6 N solution of NaOH (12 mL) was added to basify the mixture. The solution was concentrated under vacuum, and extracted with hot $CHCl_3$ (20 mL x 3). The solvent was removed under reduced pressure to give a white solid 0.28 g (46%): mp 227-230 °C (lit[132] 225-230 °C, EtOH). A solution of the crude product (0.28 g, 1.51 mmol) in abs EtOH was cooled in an ice/water bath. Concentrated HCl was added in a dropwise manner to pH ≈ 3. The solvent was removed under reduced pressure and the process repeated twice more to give a white solid. The

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solid was recrystallized from EtOH to afford a white crystalline solid 0.26 g (80%) of **47**: mp 237-240°C (lit[132] 237-238 °C, EtOH).

***N*-(3-Chloro-4-hydroxyphenyl)guanidine, Hydrochloride (51).** Compound **51** was prepared according to a literature procedure.[150] A solution of **54** (0.13 g, 0.72 mmol) and cyanamide (0.046 g, 1.08 mmol, 50% aq solution) in abs EtOH was heated at reflux for 18 h. After allowing the purple solution to cool to room temperature, it was cooled further to -25 °C (in the freezer). A purple precipitate formed and was collected by filtration and washed with cold EtOH. The solid was dried under reduced pressure for 2 h over toluene to give **51** (0.080 g, 0.36 mmol, 50%) as a purple solid: mp 239-241 °C, 251-254 °C (Büchi melting point apparatus) (lit[150] 244-246 °C dec, EtOH); $^1$H (DMSO-$d_6$): 2.53 (s, 1H, OH), 7.05 (s, 1H, ArH), 7.26-7.40 (d, 2H, ArH); IR (KBr, cm$^{-1}$) 3370, 1287.

**2-Chloro-4-nitrophenol (53).** Compound **53** was prepared according to a literature procedure.[151] Nitric acid (10.9 mL $H_2O$, 9% wt) was added in a dropwise manner to a solution of 2-chlorophenol (**52**) (0.50 g, 3.89 mmol) in 1,2-dichloroethane (12.5 mL). The reaction mixture was sonnicated for 3 h, then washed with $H_2O$ (3 x 25 mL) and extracted with $CH_2Cl_2$ (3 x 25 mL). The combined organic portion was dried ($Na_2SO_4$), and the solvent was evaporated under reduced pressure to give a yellow, oily residue. Purification by flash chromatography on a silica gel column using hexanes/EtOAc (9:1) as eluent gave a crude yellow solid. The product was recrystallized from petroleum ether

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to afford yellow crystals 0.24 g (35%) of **55**: mp 106-108 °C (lit[152] 106-110 °C, $H_2O$ and AcOH).

**3-Chloro-4-hydroxyaniline, Hydrochloride (54).** The free base of compound **54** was prepared according to a literature procedure.[152] Sodium hydrosulfite (1.20 g, 6.91 mmol) was added in ten equal portions (0.12 g) over 10 min to a solution of **53** (0.30 g, 1.73 mmol) in $H_2O$ (2 mL with 0.83 g, 20.74 mmol NaOH) heated at reflux. The reaction mixture was heated at reflux until the red color disappeared (approximately 20-30 min). After cooling to room temperature, a precipitate formed and was filtered then washed with cold $H_2O$. The filtrate was extracted with $Et_2O$ (3 x 15 mL), the organic layer was dried ($Na_2SO_4$), and the solvent was removed under reduced pressure to afford a gray powder 0.13 g (50%) (which turned brown over time) of the free base of **54**: mp 149-151 °C (lit[152] 150-151 °C, $H_2O$). The free base (0.125g, 0.87 mmol) was dissolved in EtOAc, cooled in an ice/water bath, and charged with $N_2$ (g). HCl (g) was bubbled in for 10 min. After 30 min of standing on ice the suspension was filtered to give **54** (0.13 g, 83%) as a pale gray solid: mp > 250 °C.

**2-Amino-7-chloro-quinazolin-4-(3*H*)-one (56).** Compound **56** was prepared according to a literature procedure for a similar compound.[153] S-Methylisothiurea sulfate (1.4 g, 5.06 mmol) and $Na_2CO_3$ (0.58 g, 5.47 mmol) were added to a solution of 4-chloroisatoic anhydride (**55**) (1.00 g, 5.06 mmol) in $CH_3CN$ (24 mL, 80%) and heated at reflux for 5 h. The reaction mixture was allowed to cool to room temperature over 30 min and the precipitate was collected by filtration. The solid was washed with $CH_3CN$ (80%, 3 x 25

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mL) then dried under reduced pressure over toluene for 16 h to give **56** (0.64 g, 65%) as a pale pink solid: mp > 300 °C (lit[179] > 300 °C); IR (KBr, $cm^{-1}$) 3401, 2133, 1597, 1442, 1101; $^1$H NMR (DMSO-$\delta_6$): 6.58 (br s, 2H, $NH_2$, $D_2O$ ex), 7.11 (dd, 1H, ArH), 7.21 (d, 1H, ArH), 7.87 (d, 1H, ArH), 11.10 (br s, 1H, NH, $D_2O$ ex).

**2-Amino-6-chloro-quinazolin-4-(3*H*)-one (58).** This compound was prepared according to a literature procedure for a similar compound.[153] 5-Chloroisatoic anhydride (**57**) (1.00 g, 5.06 mmol) was dissolved in $CH_3CN$ (24 mL, 80%), then S-methylisothiourea sulfate (1.4 g, 5.06 mmol) and $Na_2CO_3$ (0.58 g, 5.47 mmol) were added to the solution. The resulting solution was heated at reflux for 6 h. The reaction was allowed to cool to room temperature over 0.5 h. The suspension was filtered and washed with $CH_3CN$ (80%, 25 mL x 3). The solid was dried under reduced pressure for 16 h to give a pale pink solid 0.67 g (67%): mp >300 °C (lit[9] >300 °C).

**2-Amino-4-chlorobenzylalcohol (60, R = H).** Compound **60**, R = H, was prepared according to a literature procedure.[169] A suspension of $LiAlH_4$ (0.33 g, 8.74 mmol) in anhydrous THF (50 mL) was cooled to 0 °C in an ice/water bath under $N_2$ atmosphere. 2-Amino-4-chlorobenzoic acid (**59**, R = H) (1.0 g, 5.83 mmol) in THF (25 mL) was added in a dropwise manner. The mixture was allowed to stir in an ice/water bath for 30 min, room temperature for 30 min, then heated at reflux for 40 min. The mixture was cooled to 0 °C in an ice/water bath and an aqueous solution of THF (THF:$H_2O$, 2:1) was added. The precipitate was removed by filtration and washed with THF (3 x 10 mL). The filtrate was dried ($MgSO_4$) and the solvent was removed under reduced pressure. The resulting

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solid was purified by column chromatography (hexanes:EtOAc, 2:1; hexanes:EtOAc, 1:1; EtOAc). The crude product was recrystallized from *i*-PrOH to give white crystals 0.80 g (87%) of the title compound: mp 139-140 °C (Lit[169] 140-142 °C).

**2-Amino-4-chlorobenzylbromide (61, R = H). Method A.** Compound **61** (R = H) was attempted to be prepared according to the literature procedure for a similar compound.[180] Phosphorous tribromide (0.09 g, 0.33 mmol) was added to a suspension of **60** (R = H) (0.10 g, 0.63 mmol) in $Et_2O$ (3 mL) while stirring in an ice/water bath. The solution was stirred for 1 h then $NH_4Cl$ (saturated, 5 mL) was added and gas evolved. Water (5 mL) was added, and the reaction mixture was extracted with $Et_2O$ (3 x 5 mL). The $Et_2O$ layer was washed with brine (5 mL) and dried ($MgSO_4$). The solvent was removed under reduce pressure. Compound **61** (R = H) could not be isolated.

**Method B.** Compound **61** (R = H) was attempted to be prepared according to the literature procedure for a similar compound.[181] Phosphorous tribromide (0.34 g, 1.27 mmol) was added to a stirred solution of **60** (R = H) (0.10 g, 0.63 mmol) in $CH_2Cl_2$ (10 mL). The solution was stirred at room temperature for 4 h then quenched by the addition of $H_2O$ (10 mL). The reaction mixture was extracted with $CH_2Cl_2$ (3 x 10 mL), washed with brine (10 mL), and dried ($MgSO_4$). The solvent was removed under reduced pressure. Compound **61** could not be isolated.

**2-(2-Amino-4-chlorophenyl)acetic Acid (65).** Attempts were made to prepare this compound according to a literature procedure.[171] 6-Chlorooxindole (0.20 g, 1.19 mmol)

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was suspended in 4 M NaOH (3 mL). The mixture was heated at reflux for 4.5 h, then cooled to room temperature. The precipitate was removed by filtration and washed with $Et_2O$. The solid was dissolved in $H_2O$ (5 mL) and 6 N HCl was added in a dropwise manner while the solution was allowed to stir in an ice bath until a precipitate formed. The pale brown precipitate was collected: mp 195-197 °C. The filtrate was basified with 6 N NaOH and extracted with $Et_2O$ (3 x 20 mL) and dried ($Na_2SO_4$). The solvent was removed to give a pale brown solid: mp 192-195 °C. The melting points of both solids matched the melting point of the starting material **64**: mp 195-199 °C; $^1$H NMR (DMSO-$\delta_6$) 3.44-3.54 (t, 2H), 3.94 (br s), 6.84 (s, 1H), 6.97-7.00 (d, 1H), 7.21-7.24 (d, 1H), 10.57 (s, 1H); IR (KBr, cm$^{-1}$) 3370, 1555, 1483, 1390, 1091. The literature[171] melting point for **65**: 251-254 °C.

**4-Chloro-2-nitrobenzaldedhyde (69).** Compound **69** was prepared according to a literature procedure.[173] A solution of **68** (2.5 g, 12.2 mmol) and DMF-DMA (4.36 g, 36.6 mmol) was allowed to stir in a 135 °C (oil bath) for 11 h then cooled to room temperature. The reaction mixture was added in a dropwise manner to a solution of $NaIO_4$ (7.8 g, 36.5 mmol) in $H_2O$ (25 mL) and DMF (12.5 mL). After 3 h the suspension was filtered and the precipitate washed with toluene. The layers were separated and the organic portion was extracted with $H_2O$ (3 x 15 mL) and solvent was removed under reduced pressure. The resulting oil was purified by column chromatography (hexanes:EtOAc, 5:1). The starting material **68** was recovered (1.4 g) and compound **69** was precipitated from the column fractions using hexanes to give 0.12 g (5%): mp 60-61

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°C (lit[182] 50-60 °C, lit[183] 67-68 °C); $^1$H NMR (DMSO-$d_6$) δ 7.95-8.05 (m, 2H, ArH), 8.31 (s, 1H, ArH), 10.19 (s, 1H, OH).

**4-Chloro-2-nitro-1-(2-nitrovinyl)benzene (70).** Compound **70** was attempted to be synthesized by a literature procedure for a similar compound.[174] Ammonium acetate (0.02 g, 0.30 mmol) was added to a solution of **69** (0.10 g, 0.54 mmol) in $CH_3NO_2$ (1.0 mL). The reaction mixture was heated at reflux for 5.5 h then allowed to cool to room temperature. The solution was concentrated under reduced pressure and the crude product was triturated in cold $CH_3OH$ (1 mL) and the solid removed by filtration. The filtrate was concentrated under reduced pressure. Neither column chromatography (hexanes:EtOAc, 4:1) nor Kugelrohr distillation could purify the product.

**(4-Chloro-2-nitrophenyl)(1*H*-imidazol-1-yl)methanone (72).** Compound **72** was synthesized according to a literature procedure.[175] Carbonyldiimidazole (1.2 g, 7.5 mmol) was added to a suspension of **71** (1.5 g, 7.5 mmol) in THF (10 mL); $CH_3NO_2$ (2.0 mL) and $K_2CO_3$ (1.1 g, 8.2 mmol) were added in THF (8 mL). The reaction mixture was allowed to stir and heated at reflux for 4 h then allowed to cool to room temperature and the solvent removed under reduced pressure. The oily residue was dissolved in EtOAc (25 mL) and extracted with $H_2O$ (3 x 25 mL), washed with brine (10 mL), and dried ($Na_2SO_4$). The solvent was removed under reduced pressure and the residue was purified by column chromatography (EtOAc:$CH_3OH$, 9:1) to give a solid compound **72**: mp 139-140 °C; $^1$H NMR (DMSO-$d_6$) δ 7.18 (s, 1H), 7.73 (s, 1H), 8.04-8.23 (m, 3H), 8.505 (s, 1H).

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**4-Chloro-2-nitrobenzylbromide (75)**. This compound was prepared according to a literature procedure.[184] Benzoyl peroxide (0.54 g, 2.2 mmol) was slowly added to a stirred solution of 4-chloro-2-nitrotoluene (**68**) (22.3 g, 130.0 mmol) and *N*-bromosuccinamide (23.1 g, 130.0 mmol) in $CCl_4$ (200 mL). The reaction mixture was heated at reflux for 4 h, then irradiated with a 250 W light bulb for an additional 65 h. After cooling to room temperature the suspension was filtered and the filtrate was washed with $H_2O$ (2 x 50 mL). The organic portion was dried ($MgSO_4$) and the solvent was removed under reduced pressure to give a brown oil. Crystals formed upon the edition of $Et_2O$ and petroleum ether. The remaining oil was purified by column chromatography (hexanes:EtOAc, 30:1) to give yellow crystals 14.5 g (45%): mp 38-39 °C (lit[184] 40-41°C, hexanes).

**2,3-Bis(4-chloro-2-nitrophenyl)propanenitrile (77). Method A.** Compound **77** was prepared according to a literature procedure for a similar compound.[177] Sodium cyanide (0.37 g, 7.5 mmol) was dissolved in anhydrous DMSO (2.4 mL) under $N_2$ atmosphere at 10-15 °C. Concentrated $H_2SO_4$ (0.25 g, 2.5 mmol) was added by pipette and the mixture was allowed to stir for 10 min. A solution of **75** (0.63 g, 2.5 mmol) in anhydrous DMSO (1.1 mL) was added at -5 °C. The frozen suspension was allowed to warm to 5 °C. A blue precipitate formed then the solution turned brown. The solution was allowed to stir at 5 °C for 3 h. The reaction mixture was poured into ice water (25 mL). The suspension was extracted with EtOAc (3 x 15 mL). The organic portion was washed with brine (15 mL), dried ($Na_2SO_4$) and the solvent removed under reduced pressure. A crude brown oily

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solid (0.50 g) was recrystallized from a mixture of $Et_2O$ and $CH_3OH$ to give 0.1 g of a beige solid: mp 155-157 °C; $^1$H NMR ($CDCl_3$) δ 3.45-3.51 (d, 1H), 3.78-3.82 (d, 1H), 5.12-5.16 (t, 1H), 7.29 (s, 2H), 7.42 (d, 1H), 7.63-7.73 (m, 3H), 8.08 (s, 2H).

**Method B.** Sodium cyanide (23.48 g, 490 mmol) was dissolved in $H_2O$ (133 mL). Compound **75** (12 g, 49.01 mmol) was added as a solution in $CH_2Cl_2$ (30 mL) with tetrabutylammonium bromide (0.05 g). The biphasic solution was allowed to vigorously stir for 30 min then separated. The organic portion was washed with $H_2O$ (3 x 100 mL), dried ($Na_2SO_4$), and the solvent evaporated. The crude product was separated by flash chromatography (hexanes:EtOAc, 10:1; hexanes:EtOAc, 4:1, hexanes:EtOAc, 1:1). The collected fractions solvent was removed under reduced pressure and the product was recrystallized from a mixture of hexanes and $Et_2O$ to give a pale, yellow solid: mp 149-150 °C.

**Method C.** Sodium cyanide (1.0 g) was dissolved in $H_2O$ (5 mL). Hydrobromic acid was added in a dropwise manner until approximately pH 1-2. Compound **75** (0.1 g) was added as a solution in $CH_2Cl_2$ (10 mL). Addition of tetrabutylammonium bromide did not initiate the reaction.

**Method D.** Potassium cyanide (0.2 g) was suspended in acetonitrile (2 mL) and 18-crown-6 (~0.05 g) was added. Compound **75** (0.05 g) was added and the solution immediately turned blue then dark brown. After 1 h the starting material was consumed

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and TLC comparison of the product was consistent with products from both Methods A and B.

### B. Log P Analysis

High-performance liquid-chromatography was performed on a Varian ProStar (Model 210, Varian Inc. Palo Alto, CA). Compounds were dissolved in aqueous acetonitrile (~0.7 mg/mL; ~85%). Each solution was filtered a total of four times; twice through two separate 0.45 μm Teflon filters. An ammonium acetate buffer was made using HPLC-grade water (Spectrum, Gardena, CA) and ammonium hydroxide (28% $NH_3$ in $H_2O$; Sigma-Aldrich) and acetic acid (99.5%; Sigma-Aldrich), the pH was measured using a pH meter and the pH was adjusted to 6.93-7.40. A Microsorb-MV 100 CN column (particle size 5 μm, 4.6 mm i.d. x 250 mm l.; Varian Inc,. Palo Alto, CA), was equilibrated with HPLC-grade aceonitrile and the ammonium acetate buffer solution until a standard baseline and constant pressure were achieved. The column was set to run in isocratic mode for 30 min and detect at a wavelength of 254 nm.

#### 1. Standards

A sample (30 μL) of one of the standards (i.e., acetanilide, benzophenone, naphthalene, diphenylamine, imidazole) or uracil was injected into a 20 μL loop and the retention time from the elution peak was recorded. At the end of 30 min cycle another 30 μL sample was run. This was repeated a third time then the column was washed by running it without a sample in graduated mode 80% to 20% acetonitrile. This process of

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running three samples at one concentration of acetonitrile then washing the column was repeated for each sample at each concentration of acetonitrile used (40%, 50%, 60%, and 70%).

The average retention time for each standard and uracil was calculated for each concentration of acetonitrile (40%, 50%, 60%, and 70%). The relative retention time (RRT) to uracil was calculated for each compound at each concentration of acetonitrile by subtracting the average retention time of uracil from the average retention time of the standard (See Appendix A).

The log of the capacity factor ($\log k'$) was calculated for each compound at each concentration of acetonitrile by taking the log of RRT divided by the average retention time of uracil (See Appendix A).

The $\log k'$ values were plotted on the y-axis and the concentration of acetonitrile was plotted on the x-axis separately for each standard. Linear regression analyses were performed using GraphPad Prism (GraphPad Software Inc. La Jolla, CA) to give a linear equation for each compound. The $\log k'_w$ value is the y-intercept or the value when the concentration of acetonitrile is 0%.

The $\log k'_w$ values for each standard were plotted on the x-axis and their experimental log P values from a shake-flask experiment were plotted on the y-axis.

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Linear regression analysis of this plot gave a standard curve equation (e.g., log P = 1.660 ($\log k_w^{'}$) + 1.285).

**2. Halogen Series**

The average retention time for each compound in the halogen series (i.e., **27**, **2**, **42-44**, **35**, **17**) was calculated for each concentration of acetonitrile (55%, 65%, and 75%). The relative retention time (RRT) to uracil was calculated for each compound at each concentration of acetonitrile by subtracting the average retention time of uracil from the average retention time of each compound tested. The log capacity factor ($\log k^{'}$) was calculated for each compound at each concentration of acetonitrile by taking the log of the RRT divided by the average retention time of uracil (See Appendix A).

The $\log k^{'}$ values were plotted on the y-axis and the concentration of acetonitrile was plotted on the x-axis separately for each compound. Linear regression analyses were performed using GraphPad Prism to give a linear equation for each compound. The $\log k_w^{'}$ value is the y-intercept or the value when the concentration of acetonitrile is 0%. By substituting their $\log k_w^{'}$ values into the standard curve equation the log P values were obtained.

**3. Quinazoline Series**

The average retention time for each compound in the quinazoline series (i.e., **46-48**) was calculated for each concentration of acetonitrile (55%, 60%, 65%, 70% and

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75%). The relative retention time (RRT) to uracil was calculated for each compound at each concentration of acetonitrile by subtracting the average retention time of uracil from the average retention time of each compound tested. The log capacity factor ($\log k'$) was calculated for each compound at each concentration of acetonitrile by taking the log of the RRT divided by the average retention time of uracil (See Appendix A).

The $\log k'$ values were plotted on the y-axis and the concentration of acetonitrile was plotted on the x-axis separately for each compound. Linear regression analyses were performed using GraphPad Prism to give a linear equation for each compound. The $\log k'_w$ value is the y-intercept or the value when the concentration of acetonitrile is 0%. By substituting their $\log k'_w$ values into the standard curve equation the log P values were obtained.

## C. Behavioral Studies

### 1. Animals

Male ICR mice (19-30 g) were used throughout the study (Harlan Laboratories, Indianapolis, IN). Mice were housed in groups of 5-6 in solid-bottom plastic cages in a temperature (~22 °C)- and humidity (~50%)-controlled room. A standard 12:12 h dark:light cycle (lights on at 0700 h) was used, and food and water were available *ad lib.* The experiments were conducted according to standards set by the Institutional Animal Care and Use Committee (IACUC) of Virginia Commonwealth University and the NIH Guide for Care and Use of Laboratory Animals. Mice were allowed to adapt to the testing

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environment at least 1 h prior to any treatment, and weighed 30 min prior to the start of the experiments.

**2. Drugs**

Ondansetron hydrochloride (Zofran®, Lot CO99723; GlaxoSmithKline) was purchased from MCVH-Pharmacy. Fluoxetine hydrochloride (Prozac®, Batch 4A/80352; Eli Lilly) was purchased from Tocris. Desipramine hydrochloride (Norpramin®, Lot 86H0942; Aventis Pharm) and imipramine hydrochloride (Tofranil®, Lot 27H1380; Novartis) were purchased from Sigma. Compound **2** was used as a nitrate salt, compounds **46** and **47** were used as HCl salts, and compound **48** was used as an HBr salt; these four compounds were synthesized in our laboratory. Solutions were prepared fresh daily; all drugs were dissolved in 0.9% saline and administered to mice in a total volume of 10 mL/kg body weight by intraperitoneal (ip) injections. One exception was that solutions of compound **48** were not made fresh daily but were refrigerated overnight and used over a two-day period since very little of this compound was in stock and the synthesis is more extensive than the other quinazoline analogs.[133]

**3. Tail Suspension Test**

The mice were allowed to adapt to the room and to a white noise generator (to block out ambient noises from the surrounding hallways) for at least 2 h prior to the test; tests were conducted between 1000 and 1500 h. Mice, naïve to the test apparatus, were suspended ~1.5 cm from the tip of their tail with industrial grade Duct Tape to a bar 60

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cm above a flat bench. The experimentor/rater was unaware of the experimental conditions (drug, drug dose) for individual animals. A random number table was used to determine the order the drugs were administered to the mice.[185] The mice were treated with either saline (30 min pre-injection time), imipramine (20 mg/kg; 30 min pre-injection time), desipramine (20 mg/kg; 30 min pre-injection time), fluoxetine (20 mg/kg, 30 min pre-injection time), ondansetron (0.1 μg/kg, 30 min pre-injection time), MD-354 (0.1, 1.0, 3.0, 10, or 30 mg/kg; 30 min pre-injection time), **46** (0.1, 0.3, 1.0, 3.0, 10, or 30 mg/kg; 30 min pre-injection time), **47** (0.1, 0.3, 1.0, 3.0, 10, or 30 mg/kg; 30 min pre-injection time), or **48** (0.1, 0.3, 1.0, 3.0, or 10 mg/kg; 30 min pre-injection time). Drug doses and pre-injection times for the standard agents were identical with what has been previously reported.[15,155] The mice were only tested once and each dose of test agent was studied in 8-11 mice ($n$ = 8-11 mice/treatment). The six minute suspension test was captured by video recording either with a Logitech Quickcam Pro for Notebooks or Nikon Coolpix S210 and Pinnacle USB-video capture device. The immobility time for each mouse was determined by viewing each mouse's 6 min trial and recording the immobility time; each trial was reviewed and scored in triplicate and the mean of the three scores was used. A mouse was considered immobile when it was making no active escape movements but included passive swaying. A mouse was considered mobile when it was making running motions, body jerks, or attempting to catch its tail.

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### 4. Locomotor Activity Assay

The mice were allowed to adapt to the room for at least 1 h prior to the test; tests were conducted between 0800 and 1630 h. Mice, naïve to the test apparatus, were placed in individual TruScan Activity System (Coulbourn Instruments, Allentown, PA) photocell arena chambers (model E63-10; 26 cm x 26 cm x 39 cm). The walls of the chamber were transparent and surrounded by two rings of infrared photodetectors (model E63-12). Each ring contained an array of 16 x 16 infrared detectors (spaced 1.524 cm (0.6 in) apart). These rings were interfaced to a computer for the monitoring of the coordinates of each mouse's location. A random number table was used to determine the order the drugs were administered.[185] The mice were treated with saline (0 min pre-injection time), **46** (0.3 mg/kg; 0 min pre-injection time), or **47** (1.0 or 3.0 mg/kg; 0 min pre-injection time). The mice were only tested once and each dose of test agent was studied in 12-13 mice ($n$ = 12-13 mice/treatment). The behavioral analysis examined 14 measures of activity: movement episodes, movement time (s), movement distance (cm), jumps, retraced local movements, retraced local movement episodes, retraced local movement time (s), margin distance traveled (cm), margin time (s), center arena entries, center distance (cm), center time (s), velocity (cm/s), and vertical plane entries.

### 5. Statistical Analysis

Data for immobility times and for each measure of locomotor activity were analyzed statistically by a one-way analysis of variance (ANOVA) followed by either the Dunnett's t-test or the Newman-Keuls post-hoc comparison test using GraphPad Prism

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(GraphPad Software Inc. La Jolla, CA). A Grubb's test for outliers was used to identify outliers with an $\alpha = 0.05$.

### D. Molecular Modeling

The homology model studies were performed on a Silicon Graphics workstation using Sybyl (Version 7.3.3; Tripos Inc. St. Louis, MO), Modeller (Version 9.3; University of California San Francisco, San Francisco, CA), Chimera (Version 1, Build 2422; University of California San Francisco, San Francisco, CA), GOLD (Version 3.1; Cambridge Crystallographic Data Centre, Cambridge, UK), and AutoDock (Version 4; Scripps Research Institute, La Jolla, CA). All ligands were built in Sybyl and given AM1 charges and geometries based on MOPAC. The alignment of several LGICs and AChBPs was performed in Clustal X[186] using the following accession numbers: 47519841 (5-HT3A; *Homo sapiens*), 7305175 (5-HT3A; *Mus musculus*), 38327554 ($GABA_A$ α1; *Homo sapiens*), 4503863 ($GABA_A$ β1; *Homo sapiens*), 34734071 ($GABA_A$ δ; *Homo sapiens*), 12314059 ($GABA_A$ ρ2; *Homo sapiens*), 119372310 (Gly α1; *Homo sapiens*), and 1346173 (Gly β; *Homo sapiens*). PDBs used for alignment are 1i9b (AChBP; *Lymnaea stagnalis*), 2bg9 (nAChR; *Torpedo marmorata*), 2bj0 (AChBP; *Bulinus truncates*), 2byn (AChBP; *Aplysia californica*), and 2qc1 (nAChR α1 and α-bungarotoxin; *Bungarus multicinctus*).

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## Appendix A

HPLC Data for Standards in Log P Analysis

| Compound | | Acetonitrile 40% | 50% | 60% | 70% |
|---|---|---|---|---|---|
| Uracil | RT | 3.973 | 4.032 | 4.136 | 4.034 |
| Acetanilide | RT | 5.360 | 4.745 | 4.664 | 4.541 |
| | RRT | 1.387 | 0.713 | 0.528 | 0.507 |
| | $\log k'$ | -0.457 | -0.753 | -0.894 | -0.900 |
| Benzophenone | RT | 8.551 | 5.478 | 4.928 | 4.593 |
| | RRT | 4.578 | 1.446 | 0.792 | 0.559 |
| | $\log k'$ | 0.062 | -0.445 | -0.718 | -0.858 |
| Naphthalene | RT | 9.119 | 6.256 | 5.054 | 5.016 |
| | RRT | 5.146 | 2.224 | 0.918 | 0.982 |
| | $\log k'$ | 0.112 | -0.258 | -0.654 | -0.613 |
| Diphenylamine | RT | 10.467 | 6.502 | 4.750 | 4.954 |
| | RRT | 6.494 | 2.470 | 0.614 | 0.920 |
| | $\log k'$ | 0.213 | -0.213 | -0.828 | -0.642 |
| Imidazole | RT | 4.859 | 4.924 | 5.577 | 5.226 |
| | RRT | 0.886 | 0.892 | 1.441 | 1.192 |
| | $\log k'$ | -0.652 | -0.655 | -0.458 | -0.529 |

HPLC Data for Halogen Series in Log P Analysis

| Compound | | Acetonitrile 55% | 65% | 75% |
|---|---|---|---|---|
| Uracil | RT | 3.314 | 3.391 | 3.466 |
| **27** | RT | 10.093 | 9.833 | 10.596 |
| | RRT | 6.779 | 6.441 | 7.130 |
| | $\log k'$ | 0.311 | 0.279 | 0.313 |
| **2** | RT | 9.286 | 9.423 | 10.168 |
| | RRT | 5.972 | 6.032 | 6.703 |
| | $\log k'$ | 0.256 | 0.250 | 0.286 |

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| | | | | |
|---|---|---|---|---|
| **42** | RT | 9.498 | 9.529 | 10.083 |
| | RRT | 6.184 | 6.138 | 6.618 |
| | $\log k'$ | 0.271 | 0.258 | 0.281 |
| **43** | RT | 11.039 | 10.468 | 10.801 |
| | RRT | 7.725 | 7.077 | 7.336 |
| | $\log k'$ | 0.368 | 0.319 | 0.326 |
| **44** | RT | 11.792 | 10.989 | 10.980 |
| | RRT | 8.478 | 7.598 | 7.514 |
| | $\log k'$ | 0.408 | 0.350 | 0.336 |
| **35** | RT | 13.646 | 11.518 | 10.630 |
| | RRT | 10.332 | 8.127 | 7.164 |
| | $\log k'$ | 0.494 | 0.380 | 0.315 |
| Uracil | RT | 3.045 | 3.239 | 3.380 |
| **17** | RT | 8.439 | 8.417 | 9.299 |
| | RRT | 5.394 | 5.178 | 5.919 |
| | $\log k'$ | 0.248 | 0.204 | 0.243 |

HPLC Data for Quinazoline Series in Log P Analysis

| | | Acetonitrile | | | | |
|---|---|---|---|---|---|---|
| Compound | | 55% | 60% | 65% | 70% | 75% |
| Uracil | RT | 3.314 | 3.209 | 3.391 | 3.435 | 3.466 |
| **46** | RT | 11.297 | 10.893 | 11.059 | 11.207 | 11.276 |
| | RRT | 7.983 | 7.684 | 7.667 | 7.772 | 7.810 |
| | $\log k'$ | 0.382 | 0.379 | 0.354 | 0.355 | 0.353 |
| **47** | RT | 11.253 | 10.801 | 11.424 | 11.471 | 11.481 |
| | RRT | 7.939 | 7.593 | 8.033 | 8.036 | 8.016 |
| | $\log k'$ | 0.379 | 0.374 | 0.374 | 0.369 | 0.364 |
| **48** | RT | 11.431 | 10.867 | 11.463 | 11.441 | 11.588 |
| | RRT | 8.117 | 7.658 | 8.071 | 8.007 | 8.122 |
| | $\log k'$ | 0.389 | 0.378 | 0.377 | 0.368 | 0.370 |

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**Vita**

Katie Elizabeth Alix was born to Michael and Linda Ownby August 15, 1984 in Landstuhl, Germany. She received her Bachelor of Science in Biochemistry and completed the equivalent work for a Bachelor of Art in Chemistry from Virginia Tech in May 2006. During her undergraduate career she authored a paper in the Journal of Biological Chemistry in 2005 entitled, "A Methanocaldococcus jannaschii archaeal signature gene encodes for a 5-formaminoimidazole-4-carboxamid-1-b-d-ribofuranosyl 5'-monophosphate synthetase: a new enzyme in purine biosynthesis." After receiving her bachelor degrees she subsequently enrolled in Virginia Commonwealth University's School of Pharmacy Medicinal Chemistry graduate program.

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