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A Dissertation

Entitled

Structure-activity relationships for a series of M5 muscarinic receptor modulators

by

Paluri Sai Shantanu Rao

Submitted to the Graduate Faculty as partial fulfillment of the requirements

for the Doctor of Philosophy Degree in Medicinal Chemistry

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December 2011

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An Abstract of

Structure-activity relationships for a series of M₅ muscarinic receptor modulators

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Submitted to the Graduate Faculty as partial fulfillment of the requirements for the Doctor of Philosophy Degree in Medicinal Chemistry

The University of Toledo December 2011

The National Institute on Drug Abuse (NIDA) defines drug addiction as a chronic, often relapsing brain disease that causes compulsive drug seeking and use, despite harmful consequences to the addicted individual and to those around him or her. The mesolimbic dopaminergic system is the key circuitry involved in the rewarding properties associated with drug of abuse. Dopaminergic neurons with cell bodies in the ventral tegmental area (VTA) of the midbrain project towards the limbic forebrain, chiefly to the nucleus accumbens (NA).^[1, 2]

Several studies have indicated that M_5 muscarinic receptors are expressed on the dopaminergic neurons in the VTA and are involved in establishing the drug rewarding properties in the mice.^[32, 33] Moreover, the M_5 receptor knockout mice ($M_5^{-/-}$) studies showed a decreased dependence on the drug of abuse.^[3] Given the evidence of M_5 muscarinic receptor involvement in the development of drug dependence, antagonists targeting these receptors present a unique tool to tackle the problem of drug addiction.

Preliminary studies identified the fumarate salt of 2-(4-methoxy-1,2,5-thiadiazol-3-yloxy)-N,N-dimethylethanamine, compound 1, as a promising lead compound for its ability to inhibit the stimulation of carbachol, a known muscarinic agonist, at the M_5

muscarinic receptors in a dose dependent manner. Therefore, based on compound 1 and other known compounds exhibiting selectivity for muscarinic M_5 receptor subtype namely, amiodarone^[36], VU0238429^[37], VU0365114 and VU0400265^[38], a general scaffold for M_5 muscarinic antagonists was designed.

Subsequently, a retro-synthetic pathway was designed in order to synthesize a series of compounds with possible antagonistic action at the M_5 muscarinic receptors. Following synthesis, the compounds were characterized for their activity at the M_5 muscarinic receptor using the phosphatidyl inositol (PI) turnover assay.^[4] In order to determine the antagonistic properties of the synthesized compounds, the effects of CDD compounds (1µM and 100µM) on the EC₈₀ concentration of acetylcholine were also studied. The studies identified a few compounds that were able to alter the response of acetylcholine at M_5 muscarinic receptors and helped characterized structure activity relationships for a M_5 muscarinic receptor antagonist. These studies form the groundwork for future efforts focused on developing selective muscarinic antagonists for the treatment of drug abuse.

Dedication

This work is dedicated to my parents, brother and other family members. Their support and love has given me strength to achieve goals which, otherwise, my limited abilities could never have aimed for. Sai, my spiritual guru, has been the quintessential beacon of inspiration and provided the will power to develop confidence and positive thinking.

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Friends from my high school, college and UT are an extremely important part of my life. They have stood by me when times were tough and their support gave me the courage to pursue my dreams. I would also like to thank my dear uncle, Dr. Srikanth K. Varanasi, for always being there for me. He has always treated me like his son and I'm fortunate to have received his endless support.

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Chapter One

Introduction

In 1914, Henry Dale^[5] successfully isolated naturally occurring acetylcholine from ergot extract.^[6] Acetylcholine acts as a neurotransmitter in the central nervous system (CNS) and the peripheral nervous system of humans. Acetylcholine (ACh) acts via its interaction with two distinct classes of receptors, the muscarinic and nicotinic acetylcholine receptors. The nomenclature is based on the natural compounds that interact with these receptors, muscarine and nicotine respectively.

The muscarinic acetylcholine receptors (mAChRs) belong to the G protein-coupled receptor (GPCR)^[7] superfamily and produce downstream effects through second messengers and hence fall under the class of metabotropic receptors. On the other hand, the nicotinic acetylcholine receptors are ligand-gated ion channels and therefore are classified as ionotropic receptors.

Over the last few decades, five subtypes of muscarinic receptors have been identified, cloned and characterized, M_1 - M_5 .^[8, 9] These five subtypes are further classified on the basis of the G proteins with which they interact. The odd numbered subtypes, i.e., M_1 , M_3 , and M_5 couple preferentially to G-proteins of the G_{q/11} (pertussis toxin-insensitive) family.

In contrast, the even numbered subtypes of muscarinic receptors (M_2 and M_4) interact with the G_i/G_o (pertussis toxin-sensitive) family of G proteins.

Following ACh binding to the receptor, the $G_{q/11}$ coupled muscarinic receptors (M₁, M₃ & M₅) activate phospholipase C, which leads to the phosphatidylinositol trisphosphate cascade. This cascade is initiated by the enzymatic cleavage of phosphatidyl -4,5-bisphosphate (PIP₂) into two components, inositol 1,4,5-trisphosphate (IP₃) and 1,2-diacylglycerol (DAG). The formation of IP₃ leads to the release of Ca²⁺ from intracellular pools, (i.e., endoplasmic reticulum) while DAG is responsible for activation of protein kinase C.

The $G_{i/o}$ coupled muscarinic receptors (M₂ and M₄), on the other hand, inhibit adenylyl cyclase activity. These receptors also activate G protein-gated potassium channels, which leads to hyperpolarization of the membrane in excitable cells. In neurons, both M₂ and M₄ muscarinic receptors are located on axon terminals and are responsible for negative feedback of neurotransmission (by inhibiting neuronal excitability).

The various subtypes of muscarinic receptors are distributed throughout the body. However, it is their distribution in the brain that has lead to the increased investigation of their underlying role and identification as a potential targets in various neurological disorders.^[10]

The M_1 muscarinic receptors are found in the cerebral cortex and hippocampus regions of brain and therefore have been studied for their putative role in memory and cognitive function. The M_2 muscarinic receptor subtype is also found in abundance in regions of brain associated with cognitive and memory functions. M_3 muscarinic receptors also are expressed widely in the central nervous system including the diencephalon. The M_4 muscarinic receptors are highly expressed in the striatum and found at lower concentrations in cerebral cortex, hippocampus and midbrain.^[11, 12] Muscarinic M_5 receptors are expressed on the dopamine containing neurons of the ventral tegmental area (VTA).^[32]

1.1 Orthosteric and Allosteric Binding Sites

ACh interacts with mAChRs at a highly conserved region known as the orthosteric binding site. The orthosteric binding site is conserved throughout the five muscarinic subtypes for interaction with the endogenous ligand (ACh) and other small molecules. Most of the drugs targeting the muscarinic receptor, agonists as well as antagonists, mimic acetylcholine and thus interact with the orthosteric sites. An interaction with a highly conserved orthosteric site is a likely reason for the lack of highly selective muscarinic ligands.

Extensive mutation and cloning studies have revealed that the amino acids forming the binding site for orthosteric ligands are located within the exofacial portions of transmembrane (TM) helices, primarily TM domains III, V, VI, and VII.^[13] However, numerous studies suggest that muscarinic acetylcholine receptors also possess what are known as "allosteric" sites that are recognized by allosteric ligands or modulators. By definition, these allosteric binding sites are sites distinct from those involved in the binding of ACh. This distinction allows the allosteric and orthosteric ligands to bind to their target muscarinic receptor simultaneously. The binding of allosteric modulators

of binding of the orthosteric ligand. The allosteric ligand may exhibit positive, negative or neutral cooperativity towards the interaction of orthosteric ligands with the muscarinic receptor. Therefore, allosteric ligands can increase or reduce the affinity of the endogenous ligand for the orthosteric site. All five mAChR subtypes have been shown to exhibit allosteric modulation. The nature of modulation varies among the subtypes and depends on the choice of orthosteric and allosteric ligands and their concentrations.

The binding sites for allosteric muscarinic ligands are thought to be located near the orthosteric site but at an extracellular level, involving amino acid residues located in the extracellular loops and the outer portions of the transmembrane helices.^[13-16] The extracellular regions of the mAChRs (M₁-M₅), including the three extracellular loops, are less conserved than the TM regions which contain the binding site for orthosteric ligands. Because the allosteric muscarinic binding sites are located on these extracellular loops, they offer amino acid sequence differences which can be exploited to develop receptor subtype-selective allosteric muscarinic ligands.

1.2 Biotopic Ligands

The concept of bitopic ligands has evolved whereby a single ligand targets both the orthosteric and allosteric binding sites. This approach offers a unique way to confer selectivity to an otherwise nonselective orthosteric ligand – by targeting both the orthosteric and allosteric sites simultaneously. Rather than co-administration of two separate drug compounds, both orthosteric and allosteric recognition properties can be incorporated in a single therapeutic agent, thereby bridging two topographically distinct

domains. This approach increases the possibility of hitting both the targets at once since the two-site directed pharmacophores are linked together.

Adopting this approach, numerous compounds have been synthesized and, with the help of site-directed mutagenesis, a better understanding about the interactions taking place at the receptor level has been established.^[17] From these studies, it was evident that the bivalent ligands CDD-0273 & CDD-0304 (**Figure 1**) were interacting with both the transmembrane regions (orthosteric site) as well as the second and third outer loop of the M_1 muscarinic receptor subtype.

Figure 1. Compounds CDD-0273 and CDD-0304, cornerstones of the bivalent ligand approach targeting the M_1 muscarinic receptors.



The selectivity demonstrated by these compounds towards M_1 muscarinic receptor subtype was completely absent when extracellular loops of the muscarinic receptor subtype M_1 were replaced by corresponding amino acid sequences from M_5 receptor subtype. Following these encouraging results, a series of small ligands (**Figure 2**) was designed and synthesized to target the allosteric sites on the muscarinic receptors. These compounds incorporated some components of the bitopic ligands CDD-0304, namely the ethylene glycol linker and the 1,2,5-thiadiazole moiety. The synthetic work was undertaken by Dr. Aditya Maheshwari and described in his dissertation entitled, "Development of selective muscarinic agonists for the treatment of neurological disorders."^[18]



Figure 2. Series of potential allosteric ligands.

Following synthesis, the compounds were tested for their ability to inhibit the interaction of carbachol, a muscarinic receptor agonist, with the M_1 and M_5 muscarinic

receptor subtypes. Of all the compounds tested, the fumarate salt of CDD-0361, compound 1, was found to inhibit the effects of carbachol on the M_5 muscarinic receptor in a statistically significant manner. Subsequent studies aimed at understanding the nature of inhibitions can be found in the following chapters.

From these studies, compound 1 was identified as the lead compound and its potential therapeutic utility as a M_5 muscarinic receptor antagonist was studied.

1.3 Drug Addiction and Current Therapies

Drug addiction or substance dependence is a disorder characterized by compulsive drug seeking behavior with loss of control on the amount of intake. Drug dependence is accompanied by acute negative reactions like irritability, dysphoria and anxiety when the drug of abuse is either withdrawn or not available. Drugs of abuse, in general, increase activity in the part of brain controlling the responses to natural reward. The key circuitry involved in the rewarding properties associated with abused drugs is the mesolimbic dopaminergic system. Dopaminergic neurons with cell bodies in the ventral tegmental area (VTA) of the midbrain project towards the limbic forebrain, chiefly to the nucleus accumbens (NA).^[1, 2]

Cocaine, one of the most commonly abused psychostimulants, is thought to act as an indirect agonist at the dopaminergic synapses. By blocking the presynaptic re-uptake of monoamines, cocaine leads to an increased concentration of dopamine in the synapse which in turn is responsible for the rewarding properties of the drug. The dopaminergic neurons, on which cocaine has been shown to act, are those whose cell bodies are in the ventral tegmental area (VTA) and project towards the nucleus accumbens (NA). Studies have demonstrated the ability of cocaine to increase the level of dopamine (DA) in the NA following administration.^[19] Also, activation (electrical stimulation) of dopaminergic neurons in the VTA was followed by an increased extracellular level of DA in the NA.^[20] Intuitively, development of dopamine receptor antagonists has been perceived as a suitable strategy to tackle the substance dependence problem. Much work has been focused on the development of D₁ and D₂ dopamine receptor subtype antagonists.

been associated with severe side effects including inhibition of feeding behavior, reduced locomotion and dysphoria. Studies focusing on dopaminergic antagonists have also led to the increased uptake of cocaine in animal models as a result of compensatory response to decreased reinforcing ability.

Another approach envisioned to counter the problem of cocaine dependence is the development of cocaine antagonists. In several studies, addiction to cocaine has been directly related to its ability to increase the synaptic levels of dopamine by blocking the dopamine transporter (DAT).^[21, 22] Chimeric transporter studies have suggested that cocaine binding pocket differs from that of dopamine on the DAT.^[23] This led to the concept that blocking access of cocaine to the binding pocket on the DAT would abolish the effects of cocaine without interacting with dopamine binding and reuptake by the transporter. Multiple investigations have led to better understanding of the transporter but these studies have not produced a therapeutic compound thus far.^[24-26]

Moreover, a substitute pharmacotherapy targeting the DAT, that would help reduce the dependence on cocaine, is also being studied. DAT inhibitors have shown to decrease cocaine intake in animal models bolstering the idea that development of cocaine agonist can lead to a decreased dependence and future abstinence from cocaine.^[27] However, due to the cocaine-like reinforcing effects produced by DAT inhibitors, there is high probability of development of drug dependence for these inhibitors.

 γ -Amino butyric acid (GABA) neurons, located in the NA project towards the VTA and are inhibitory in nature. They act at the VTA through the GABA_B class of receptors. Activation of these receptors leads to decreased levels of DA in the NA. GABA related compounds posses the ability to attenuate the reinforcing effects of drugs of abuse like heroin, cocaine, nicotine etc. Previous studies have documented the effectiveness of GABA_B agonist baclofen in reducing the intake of cocaine in rat models. GABA_B antagonists diminished the effectiveness of baclofen in reducing the cocaine intake thereby highlighting the role and importance of GABA_B agonists.^[28]

In order to increase the synaptic concentration of GABA in the VTA, inhibitors of GABA-transaminase (GABA-T), a mitochondrial bound enzyme, have been studied. GVG, an irreversible inhibitor of GABA-T has been shown to decreased cocaine-induced DA levels in the corpus striatum and NA.^[29]

Currently, two major approaches are being employed to deal with the problem of substance abuse and dependence. One approach involves long-term agonist based substitution therapy, wherein opiods such as methadone are administered to patients in a controlled manner while avoiding the symptoms of drug withdrawal. Buprenorphine, a partial μ -opioid agonist, has also been employed in the long-term substitution therapy approach. Being a weak partial agonist and even an antagonist under some conditions, buprenorphine possesses a relatively weak abuse and dependence property as compared to methadone. However, the agonist substitution approach does not correct the underlying problem of addiction and over time patients have the tendency to abuse these substances instead. Also, they are associated with impairment of psychomotor performance such as driving ability. A considerable degree of mortality is associated with the agonist based substitution therapy.

The second approach being employed for managing drug dependence disorder is the use of opiate receptor antagonists such as naltrexone. Naltrexone is a competitive antagonist of the μ -opioid receptor and binds to these receptors in the ventral tegmental area (VTA) of the brain thereby inhibiting the dopaminergic system innervating the nucleus accumbens (NA). Naltrexone has also been shown to influence the conditioning process associated with alcohol drinking.^[30] It has been used clinically in the management of drug overdose cases. However, naltrexone has been associated with precipitation of an acute, life-threatening withdrawal syndrome while in use for treatment of opioid overdose. Withdrawal symptoms are also a major side effect observed while naltrexone is being used to manage drug dependence.

Overall, the current approaches do not provide a clinical solution to manage the drug dependence disorder by themselves. There exists a need to improve upon properties of available drugs to reduce side effects or to target other pathways circumventing the present approaches. The muscarinic acetylcholine receptors located in the VTA have been investigated in the past decade with the view of identifying one such pathway.

1.4 Role of M₅ Muscarinic Receptor Subtype in Drug Addiction

Of the five muscarinic receptor subtypes, the M₅ receptor gene was the last gene to be isolated and characterized. Using cloned antibodies designed for M₅ muscarinic receptor, immunoprecipitation studies were conducted to determine the location of these receptors.^[31] Subsequent studies focusing on isolating the mRNA of muscarinic receptors from the brain revealed that only M₅ mRNA was found near the dopaminergic neurons of the substantia nigra (SN) and ventral tegmental area (VTA).^[32, 33] Killing these neurons results in the loss of the M₅ receptors as well, thereby confirming the idea that muscarinic M₅ receptors are expressed on the dopaminergic neurons in these areas of the brain.

The ventral tegmental area (VTA) receives a major part of its cholinergic input from the laterodorsal tegmental nucleus (LDT). The dopaminergic neurons originating from the VTA in turn increase extracellular dopamine levels in the nucleus accumbens (NA). In M₅ receptor knockout mice ($M_5^{-/-}$), the electrical stimulation of laterodorsal tegmental area did not result in any increase in the level of dopamine in the nucleus accumbens, a result observed in the wild-type mice.^[3]

In a morphine induced conditioned place preference experiment, M_5 receptor knockout mice ($M_5^{-/-}$) displayed a significant reduction in the time spent in the morphine chamber as compared to the wild type mice. This was accompanied by reduction of dopamine levels in the nucleus accumbens following morphine administration. Moreover, the M_5 receptor knockout mice ($M_5^{-/-}$) did not exhibit a difference in degree of analgesia induced by morphine. In fact, the level of tolerance developed in both strains of mice after a high dose of morphine remained unchanged. Overall, the M_5 receptor knockout mice ($M_5^{-/-}$) studies indicated that M_5 receptors are involved in establishing the drug rewarding properties in the mice and knocking out the receptor results in decreased dependence on the drug. The lack of the M_5 receptor does not affect the analgesic properties of morphine.^[34]

An M₅ muscarinic receptor antisense oligonucleotide study provided new insight into the role of these receptors in sensitization induced by heroin.^[35] The repeated intake of any drug of abuse results in the progressive enhancement of locomotor activity, a phenomenon known as sensitization. It is believed that sensitization acts as an incentive which in turn leads to the compulsive drug seeking behavior and relapse. One of the major markers for sensitization is the FosB protein expression. There is a marked increase in the number of FosB positive neurons in the NA and dentate gyrus (DG) of the hippocampus following heroin administration in rats. M₅ muscarinic receptor antisense oligonucleotide injection in the VTA resulted in decreased heroin induced sensitization in rats. It was accompanied by diminished FosB expression in regions like the NA and DG, further highlighting the role of muscarinic M₅ receptors in the process of sensitization and hence drug dependence.

Hence, there prevails strong evidence linking the muscarinic M_5 acetylcholine receptor to its positive effect on the mesolimbic dopaminergic system and therefore in the development of drug seeking behavior. M_5 muscarinic receptors represent a unique target to bring about changes in the neurotransmitter levels in the nucleus accumbens. Antagonists for the muscarinic M_5 receptors possess the potential to block the process of drug dependence by abolishing the drug reward mechanism without affecting the analgesic property of useful clinical drugs like morphine. With no muscarinic M_5 selective antagonist currently available, this hypothesis remains untested.

1.5 Muscarinic M₅ Receptor Selective Compounds

Till date, no muscarinic M_5 receptor selective allosteric antagonist has been reported in the literature. Apart from the encouraging selectivity of compound **1** for M_5 receptors over M_1 receptors, only a handful of compounds have demonstrated muscarinic M_5 receptor selectivity. The few compounds that have shown selectivity for M_5 receptors, irrespective of their agonist or antagonist actions, include amiodarone^[36], VU0238429^[37], VU0365114 and VU0400265^[38] (**Figure 3**). Individually, these compounds provide a design scaffold for attaining either muscarinic M_5 receptor subtype selectivity, allosteric modulation or the desired antagonistic property.



Figure 3. Muscarinic M₅ receptor subtype selective compounds.

Based on the structural features of compound 1 and other M_5 selective allosteric compounds, a novel series of potential allosteric ligands for muscarinic M_5 receptor was designed, synthesized and characterized.

1.6 Synthetic Chemistry

Based on the compounds exhibiting selectivity for muscarinic M_5 receptors, namely amiodarone, VU0238429, VU0365114 and VU0400265, a general scaffold was designed. As outlined below, the compound tested previously, compound **1** can be divided into four sectors potentially responsible for its observed activity (as an M_5 antagonist).



While numerous modifications are possible in each of the four sectors, the rationale for design was based on key features found in the other M_5 selective ligands in order to retain/increase the M_5 selectivity along with the ability to allosterically inhibit the receptor. As depicted in **Figure 4**, the various substitutions at sector 1 included methanol, N-methyl Imidazole, N-methyl Indole, N-methyl Isatin and 2-Thiazole. Sector 2 was selected to be a phenyl group carrying the two substituents ortho, meta or para to each other. Sector 3 was maintained as 2-hydroxy ethyl amine throughout the library. The hydroxy was attached to the phenyl ring (sector 2) all times forming an ether linkage. On the other hand, the amine was always a tertiary amine with three possible variations comprising the sector 4. The three tertiary amines chosen to add variations to sector 4 in the library were dimethyl, diethyl or N-morpholine.



Figure 4. Designed library with the various substitutions made at the four sectors identified in compound 1.

Chapter Two

Results and Discussion

2.1 Lead Identification

A series of compounds based on CDD-0304, carrying the ethylene glycol linker and the 1,2,5-thiadiazole moiety (Scheme 2), was tested for the ability to modulate activity at the M₁ and M₅ muscarinic receptor subtypes. Since the compounds within this series were designed for their putative allosteric properties, apart from testing these compounds for their intrinsic activity, their effects on muscarinic agonist activity also were characterized at M₁ and M₅ muscarinic receptor subtypes. Phosphatidylinositol (PI) turnover assay revealed potential allosteric activity of these novel compounds. Carbachol, a known muscarinic receptor agonist, was used as a positive control in the PI turnover assay.

Within the series of compounds tested for their allosteric properties, two compounds were found to display interesting activity, compound **1** and **CDD-0362**. Figure **5** summarizes the results from the PI turnover assay conducted on cell lines expressing either the M_1 or M_5 muscarinic receptor subtype. The experiment was designed to determine the nature of the interaction of compounds at the M_1 or M_5 muscarinic receptor subtype. In order to accomplish this, the compounds were tested for both their intrinsic activity (1 μ M & 100 μ M concentration) and for their ability to modify the response of carbachol at the muscarinic receptor. The EC₅₀ concentration of carbachol was employed in order to access the activity of the synthesized compounds. Carbachol by itself (CCh + Vehicle) served as the positive control and comparisons were made to this value for statistical analysis. The level of significance (α) was set to 0.05 and P values < 0.05 were deemed statistically significant.

Figure 5. Stimulation of phosphoinositide metabolism by carbachol, compound 1 and CDD-0362 at M₁ and M₅ muscarinic receptors expressed in A9 L cells. Data represent the mean (\pm s.e.m.) of three experiments each performed in triplicate.



* Significantly different from CCh + Vehicle values (P < 0.05);

As illustrated in **Figure 5**, carbachol, a nonselective muscarinic receptor agonist, significantly stimulated PI metabolism at muscarinic receptors. Of the various compounds studied, 2-(4-methoxy-1,2,5-thiadiazol-3-yloxy)-N,N-dimethylethanamine fumarate salt (compound 1) was inactive at M_1 and M_5 receptors by itself at the concentrations tested (1 μ M and 100 μ M). Compound 1 did not decrease carbachol activity when compared to the response produced by carbachol alone at the M_1 muscarinic receptors.

In contrast, at the M₅ muscarinic receptors, in the presence of compound 1 (1 μ M or 100 μ M), carbachol-stimulated PI turnover was significantly decreased (P < 0.05). Thus compound 1 was identified as a potential lead for the development of M₅ muscarinic receptor antagonists.

As observed with compound 1, the quaternary iodide salt, 2-(4-methoxy-1,2,5-thiadiazol-3-yloxy)-N,N,N-trimethylethanamine (CDD-0362) did not produce any response by itself at either the M_1 or the M_5 muscarinic receptor subtype. However, CDD-0362 was able to inhibit the carbachol response at both M_1 and M_5 receptors at the 100 μ M concentration (P < 0.05).

Although both these compounds, compound **1** and CDD-0362, seemed promising enough for further studies, CDD-0362 was not considered a lead compound due to the expected poor CNS penetration owing to the presence of a quaternary amine.

In order to determine the kind of antagonism taking place at the receptor level, a Schild regression analysis was conducted for the lead compound, compound 1, at both M₁ and M₅ muscarinic receptors. To accomplish this, dose response curves for carbachol at these muscarinic receptors were produced in the presence of increasing concentrations of compound **1**. **Figure 6** illustrates the average values obtained from the PI turnover assays conducted on these cell lines. Figure 6. Schild regression analysis for compound 1 at M_1 and M_5 muscarinic receptors. Full dose response curves for carbachol were generated in the presence of increasing concentrations of compound 1.



Compound 1 - M₁ muscarinic receptors





The study revealed that 1 μ M of compound **1** did not modify the does-response curve for carbachol at either receptor subtype. On the other hand, at the M₁ muscarinic receptors, higher concentrations of compound **1** (10, 100 or 300 μ M) behaved as an agonist in presence of lower concentrations of carbachol. However, at higher carbachol concentrations, compound **1** acted as an antagonist decreasing the response produced by carbachol.

At M_5 muscarinic receptors, effects produced by compound 1 were even more complex in nature. While the 1 μ M concentration of compound 1 did not cause a change in the carbachol response, 10 μ M and 300 μ M concentrations of the compound led to a non-parallel right side movement of the carbachol dose response curve. Compound 1 at 100 μ M acted as an agonist in the presence of lower concentrations of carbachol along with the right side deviation of the dose response curve. The higher doses inhibited the effects of carbachol in a complex manner suggesting possible allosteric inhibition of M_5 receptors. Moreover, the plot between Log (DR-1) and Log (antagonist concentration) did not yield a straight line as expected with a competitive antagonist.

Overall, definite conclusions regarding the kind of antagonism could not be deduced. Nonetheless, the study encouraged us to ponder over the clinical relevance of M_5 antagonists which resulted in efforts leading up to the design and synthesis of series of potential allosteric M_5 selective muscarinic receptor ligands.
2.2 Synthetic Chemistry

Based on the compounds exhibiting selectivity for the muscarinic M_5 receptor subtype namely, amiodarone, VU0238429, VU0365114 and VU0400265, a general scaffold was designed. As outlined previously, the lead compound **1** can be divided into four sectors potentially responsible for its observed activity (as a M_5 antagonist).



The various modifications detailed in **Figure 4** were centered on the hydroxy benzyl alcohol core and hence a retro-synthetic pathway was designed in order to synthesize the library.

As detailed in **Scheme 1**, the desired final compounds, disubstitued aminoethoxy benzyl 1H-heterocycles, were synthesized using nucleophilic substitution of the good leaving group present in compound **A** and by the nucleophilic nitrogen of compound **B** generated *in situ*. Compound **A** was obtained from compound **C** following the substitution of hydroxyl group by the nucleophilic leaving group moiety in the presence of a base. Compound **C** was in turn synthesized as a result of the reaction of compound **D** and compound **E**.

Scheme 1: Retrosynthetic analysis of the designed library of muscarinic M₅ receptor ligands.



Following the rationale of having a good leaving group on compound **A**, which can be easily substituted by nucleophilic nitrogen generated from heterocycles such as isatin, indole and imidazole, various halides were explored. Numerous attempts were made to convert the hydroxyl group of compound **C** to an iodo, bromo or chloro moiety in order to facilitate the planned SN_2 reaction. However, all efforts to yield such a product failed. Multiple routes to obtain compound **C** such as appel chlorination, thionyl chloride, sodium iodide, PPh₃/CCl₄, I₂/PPh₃/imidazole, Br₂/PPh₃/imidazole also were unsuccessful.

Another strategy involved conversion of hydroxyl to mesylate followed by substitution with a halogen. The first step, conversion of hydroxy to mesylate, was found to work on compound \mathbf{C} while the second step, displacement of mesylate by halide did

not yield the desired product. Considering the encouraging result from the mesylation reaction, all further products were derived using this strategy.

The first compounds synthesized in the designed target library were (4-(2morpholinoethoxy)phenyl)methanol (compound **2**) and 1-(4-(2morpholinoethoxy)benzyl)-1H-indole (compound **4**). **Scheme 2** outlines the synthetic route taken for these compounds.

Scheme 2: Synthesis of compound 2 and compound 4.



(a) K_2CO_3 , acetone, reflux, 24 h; (b) N,N-diisopropylethylamine, methanesulfonyl chloride, dichloromethane, rt, 10 h; (c) Sodium hydride (60%), Indole, DMF, rt, 16 h.

The synthesis commenced with commercially available starting materials: 4-hydroxy benzyl alcohol and 4-(2-chloroethyl)morpholine hydrochloride. The reaction mixture was

refluxed for 24 h in the presence of excess of potassium carbonate in acetone. Before adding to the reaction, the amine was converted from the hydrochloride salt to the free base using a saturated sodium carbonate solution. Since the free base was volatile, the concentration of organic solvent following extraction was conducted below 30 °C.^[39]

Compound **2** was treated with methanesulfonyl chloride to yield the corresponding mesylate^[40], 4-(2-morpholinoethoxy)benzyl methanesulfonate (compound **3**), which was used immediately without further characterization. In order to obtain compound **4**, the corresponding mesylate, compound **3**, was added to a pre-cooled solution of indole and sodium hydride.^[41] Subsequently, the free base compound was converted to the fumarate salt^[42], compound **4**. The yield for the reaction was 38 % (over two steps) and established the protocol for such kind of nucleophilic substitutions.

Scheme 3 outlines the synthetic route adopted synthesize to (4 - (2 -(dimethylamino)ethoxy)phenyl)methanol (compound 5) 2-(4-((1H-indol-1and yl)methyl)phenoxy)-N,N-dimethylethanamine (compound 7). Due to the change of tertiary amine from morpholine to N,N-dimethyl amine, a few modifications were incorporated in the reaction conditions used for synthesis of compound 2 and compound 4. The solvent system for mesylation reaction was changed from dichloromethane to tetrahydrofuran due to the increased polarity of the tertiary amine; N,N-dimethyl amine immiscible was in dichloromethane. The mesylate intermediate, 4-(2-(dimethylamino)ethoxy)benzyl methanesulfonate (compound 6), was converted to compound 7 using a modified procedure as well. The temperature of the reaction was lowered to 0 °C during the sodium hydride addition and time of reaction was brought down to 4 h.



(a) K_2CO_3 , acetone, reflux, 24 h; (b) N,N-diisopropylethylamine, methanesulfonyl chloride, tetrahydrofuran, rt, 2 h; (c) Sodium hydride (60%), Indole, DMF, rt, 4 h.

In order to examine structure activity relationships for the designed library of compounds, other heterocycles were incorporated in place of indole. One such modification involved replacement of the N-methyl indole with thiazole. This was accomplished employing the Hantzsch thiazole synthesis protocol.^[43] As shown in scheme **4**, commercially available 4-methoxybenzonitrile was treated with sodium hydrosulfide in the presence of magnesium chloride to yield 4-methoxybenzothioamide (compound **8**). Compound **8** was refluxed with bromoacetaldehyde diethyl acetal in ethanol to give the crude product, 2-(4-methoxyphenyl)thiazole (compound **9**). Treatment

with hydrobromic acid and acetic acid under refluxing conditions led to generation of free hydroxy group, 4-(thiazol-2-yl)phenol (compound **10**).



Scheme 4: Synthesis of compound 11 and compound 12.

(a) 70 % NaSH, MgCl₂.6H₂0, DMF, rt, 8 h; (b) Bromoacetaldehyde diethyl acetal, EtOH, reflux, 10 h; (c) HBr/AcOH, reflux, 24 h; (d) N-(2-chloroethyl)-dimethylamine, K₂CO₃, acetone, reflux, 24 h; (e) N-(2-chloroethyl)-diethylamine, K₂CO₃, acetone, reflux, 24 h.

In the presence of excess of potassium carbonate, the β-chloroamines (N-(2-chloroethyl)-dimethylamine or N-(2-chloroethyl)-diethylamine) underwent nucleophilic substitution by the phenoxide ion generated from compound **10**. The two compounds obtained through this route were 2-(4-(thiazol-2-yl)phenoxy)-N,N-dimethylethanamine (compound **11**) and 2-(4-(thiazol-2-yl)phenoxy)-N,N-diethylethanamine (compound **12**) respectively.

Scheme 5: Synthesis of compound 29.



(a) 4-(2-chloroethyl)morpholine, K₂CO₃, acetone, reflux, 24 h.

Similarly, 4-(2-(4-(thiazol-2-yl)phenoxy)ethyl)morpholine (compound **29**) was obtained through the reaction of compound **5** with 4-(2-chloroethyl)morpholine (free base) in the presence of excess of potassium carbonate (Scheme **5**).

In order to explore the role of different spatial arrangements of functional groups on the benzyl ring and their effects on activity, compounds with ortho-, meta- or paraorientations were synthesized. Scheme **6** outlines the route taken to synthesize 1-(2-(2morpholinoethoxy)benzyl)-1H-indole (compound **15**), the first compound with the two substituents ortho to each other on the ring. The synthesis commenced with the reaction of 2-hydroxy benzyl alcohol with the free amine, N-(2-chloroethyl)-morpholine, in the presence of excess of potassium carbonate. The resulting product, (2-(2-morpholinoethoxy)phenyl)methanol (compound **13**), upon purification was subjected to mesylation of the hydroxyl group in the presence of N,N-diisopropylethylamine. The crude mesylate product, 2-(2-morpholinoethoxy)benzyl methanesulfonate (compound **14**), was used for the next reaction without subsequent characterization. The final compound, compound **15**, was obtained in 15% yield under the conditions used previously for compound **7**.

Scheme 6: Synthesis of compound 15.



(a) K_2CO_3 , acetone, reflux, 24 h; (b) N,N-diisopropylethylamine, methanesulfonyl chloride, dichloromethane, rt, 2 h; (c) Sodium hydride (60%), Indole, DMF, rt, 4 h.

Similarly, the meta-substituted 1-(3-(2-morpholinoethoxy)benzyl)-1H-indole (compound **18**), was obtained as detailed in scheme **7**. Due to the low boiling point of the free base, N-(2-chloroethyl)-morpholine, and other tertiary amines used to build the library, a significant loss of free base was seen during evaporation of solvent following extraction. To avoid the loss of free base and increase the yields of potassium carbonate driven nucleophilic substitution reactions, hydrochloride salt of the tertiary amines was directly used for synthesis of (3-(2-morpholinoethoxy)phenyl)methanol (compound **16**).

The marked increase in yield to 80 %, as compared to 42 % noticed during the synthesis of compound **15**, can be attributed to this change in reaction conditions.



Scheme 7: Synthesis of compound 18.

(a) K_2CO_3 , acetone, reflux, 24 h; (b) Triethylamine, methanesulfonyl chloride, tetrahydrofuran, rt, 2 h; (c) Sodium hydride (60%), Indole, DMF, rt, 4 h.

The column purified product, compound **16**, was subjected to mesylation under modified conditions. Instead of running the reaction in dichloromethane in the presence of excess of N,N-diisopropylethylamine, the reaction utilized tetrahydrofuran with an excess of triethylamine as the base. This was done in order to achieve uniformity for reactions with other amines, namely diethyl ethyl amine and dimethyl ethyl amine, employed to build the compound library. Since these two amines are not soluble in dichloromethane, freshly distilled tetrahydrofuran provided a suitable solvent for all the three tertiary amines. Following mesylation, N-alkylation of indole was achieved using previously established chemistry.

For establishing the structure activity relationships for the series, the indole group was replaced by imidazole as the heterocycle on the phenyl ring. Along the same lines, the imidazole analog of compound **18**, 4-(2-(3-((1H-imidazol-1-yl)methyl)phenoxy)ethyl)morpholine (compound **19**) was synthesized employing a similar strategy. Scheme **8** details the synthetic route adopted for compound **19**. The starting material for the reaction, compound **16**, served as the common intermediate and was obtained using the procedure described previously.

Scheme 8: Synthesis of compound 19.



(a) Triethylamine, methanesulfonyl chloride, tetrahydrofuran, rt, 2 h; (b) Sodium hydride (60%), Imidazole, DMF, rt, 4 h.

The next analog in the series was designed to incorporate the indole ring attached to the benzyl carbon along with N,N-diethylethanamine attached to the hydroxyl group on position 3. Scheme 9 below depicts the route for the synthesis of the analog, 2-(3-((1H-indol-1-yl)methyl)phenoxy)-N,N-diethylethanamine (compound 22). Commercially available 3-hydroxy benzyl alcohol was treated with N,N-diethylethanamine hydrochloride in the presence of potassium carbonate to obtain the intermediate (3-(2-(diethylamino)ethoxy)phenyl)methanol (compound 20). Subsequent mesylation yielded the product, 3-(2-(diethylamino)ethoxy)benzyl methanesulfonate (compound 21) which was subjected to nucleophilic substitution by the indole nitrogen to furnish compound 22.

Scheme 9: Synthesis of compound 22.



(a) K_2CO_3 , acetone, reflux, 24 h; (b) Triethylamine, methanesulfonyl chloride, tetrahydrofuran, rt, 2 h; (c) Sodium hydride (60%), Indole, DMF, rt, 4 h.

Meanwhile, the free benzyl alcohols also were synthesized and prepared for further characterization. For this purpose, the two commercially available benzyl alcohols, 4-hydroxy and 3-hydroxy benzyl alcohol, were treated with the tertiary amines in the presence of potassium carbonate in acetone. As shown below in scheme **10**, (4-(2-(diethylamino)ethoxy)phenyl)methanol (compound **23**) and (3-(2-(dimethylamino)ethoxy)phenyl)methanol (compound **24**) were obtained using this procedure.

Scheme 10: Synthesis of compound 23 and compound 24.



(a) K₂CO₃, acetone, reflux, 24 h.

With meta- and para- substituted benzyl compounds in hand, the ortho-substituted analogs were synthesized next. As detailed in scheme **11**, the synthesis of one such ortho-substituted analog, 2-(2-((1H-indol-1-yl)methyl)phenoxy)-N,N-diethylethanamine (compound **27**), commenced with 2-hydroxy benzyl alcohol. Following previously established protocols, compound **27** was obtained in 15 % yield (over two steps).

Scheme 11: Synthesis of compound 27.



(a) K_2CO_3 , acetone, reflux, 24 h; (b) Triethylamine, methanesulfonyl chloride, tetrahydrofuran, rt, 2 h; (c) Sodium hydride (60%), Indole, DMF, rt, 4 h.

In order to establish structure activity relationships between pharmacophores, an analog of CDD-0377 was synthesized with imidazole as the heterocycle instead of indole. As outlines in scheme **12**, 2-(3-((1H-imidazol-1-yl)methyl)phenoxy)-N,N-diethylethanamine (compound **28**) was synthesized from the previously synthesized intermediate **11** in the presence of excess sodium hydride. Standard work-up protocol followed by column purification yielded compound **28**.

Scheme 12: Synthesis of compound 28.



(a) Sodium hydride (60%), Indole, DMF, rt, 4 h.

All compounds were analyzed based on their ¹H and ¹³C NMR spectra and successful elemental analysis confirmed the composition for each member within the series. Moreover, the compounds from this library were characterized using the PI turnover assay employing cell lines stably expressing the M₅ muscarinic receptors for their possible antagonistic property.

2.3 Biological Evaluation

The series of CDD compounds were tested for their activity at the M_5 muscarinic receptors. Activity for all the synthesized compounds was accessed at 1 μ M and 100 μ M concentration in the absence and presence of EC₈₀ concentration of acetylcholine (4 μ M). Acetylcholine (ACh), as the endogenous agonist, was used in these studies to provide a more suitable control for evaluating the ability of compounds to modulate agonist activity. The EC₈₀ concentration was determined after conducting three successive full dose response curves for acetylcholine at the M₅ muscarinic receptors and provided a suitable point to identify compounds that decrease agonist activity. The data, obtained as counts per minute (CPM) after the PI turnover assay, was analyzed employing the two-way analysis of variance (ANOVA). The bar graphs were plotted in a manner to distinguish the activity observed with varying concentrations of test ligand in either the absence or presence of ACh.

In almost all the cases, ACh was found to produce stimulation significantly different than that seen with the vehicle. The difference was considered statistically significant in cases where ANOVA yielded result P<0.05. The results where therefore analyzed to see whether the CDD compounds were able to inhibit the stimulation of ACh in a manner to make the P value greater than 0.05 (P>0.05). This observation was interpreted as the ability of the CDD compound to inhibit the interaction of ACh at the M₅ muscarinic receptor and hence its role as a potential antagonist.

The first compound tested in the series for its activity was (4-(2-morpholinoethoxy)phenyl)methanol, compound **2**. As shown in **Figure 7**, the compound, by itself, did not produce any change in the baseline stimulation obtained in response to the vehicle. Acetylcholine significantly stimulated PI metabolism (P<0.001), while compound **2** did not have any impact on the PI response of ACh (P>0.05).

Figure 7. Stimulation of M_5 muscarinic receptors by acetylcholine in the absence and presence of compound 2.



Impact of compound **2** on the ACh induced PI turnover evaluated at two different concentrations, 1 μ M and 100 μ M. Data represent the mean (± s.e.m.) of three experiments each performed in triplicate.

The next compound from the series, (4-(2-(dimethylamino)ethoxy)phenyl)methanol, compound **5** was also tested for its activity. As depicted in **Figure 8** below, the change of para-substituted tertiary amine from morpholinoethoxy (compound **2**) to dimethyl aminoethoxy (compound **5**) brought no change in the activity profile for the two benzyl alcohols at the M₅ muscarinic receptor. Acetylcholine significantly stimulated PI metabolism (P<0.001), while the compound **5** did not have any impact on the PI response of ACh (P>0.05). As observed previously with compound **2**, compound **5** had no intrinsic activity and the varying concentration of compound **5** (1 μ M and 100 μ M) produced no change in baseline activity. Figure 8. Stimulation of M_5 muscarinic receptors by acetylcholine in the absence and presence of compound 5.



Impact of compound 5 on the ACh induced PI turnover evaluated at two different concentrations, 1 μ M and 100 μ M. Data represent the mean (± s.e.m.) of three experiments each performed in triplicate.

Structurally, compound **2** and compound **5** were further modified with the substitution of free hydroxyl group by nitrogen of the heterocycle indole to yield 1-(4-(2-morpholinoethoxy)benzyl)-1H-indole (compound **4**) and 2-(4-((1H-indol-1-yl)methyl)phenoxy)-N,N-dimethylethanamine (compound **7**) respectively.

As shown in **Figure 9**, the introduction of an indole moiety did not change the activity profile. The results for compound **4** were not very different from that observed previously with its direct benzyl alcohol analog, compound **2**. The ACh response remained significantly different against the background in both the presence and absence

of compound 4 (P<0.01). CDD-0371 failed to elicit any response by itself at both the tested concentrations (1 μ M and 100 μ M).

Figure 9. Stimulation of M₅ muscarinic receptors by acetylcholine in the absence and presence of compound 4.



Impact of compound 4 on the ACh induced PI turnover evaluated at two different concentrations, 1 μ M and 100 μ M. Data represent the mean (\pm s.e.m.) of three experiments each performed in triplicate.

Substitution of hydroxyl group by indole heterocycle, compound 7, produced a substantial change in the activity when compared to compound 5.

Figure 10. Stimulation of M₅ muscarinic receptors by acetylcholine in the absence and presence of compound 7.



Impact of compound 7 on the ACh induced PI turnover evaluated at two different concentrations, 1 μ M and 100 μ M. Data represent the mean (\pm s.e.m.) of three experiments each performed in triplicate.

As depicted in Figure 10, compound 7 did not elicit a response by itself at either of the tested concentrations. In the absence of compound 7, the ACh response was not significantly different from that produced by the vehicle (P>0.05). However, the presence of the compound, compound 7 at 1 μ M and 100 μ M, caused an increase in ACh response producing a statistically significant difference against the background (P<0.05). These results indicated that compound 7 exhibited possible positive allosteric modulation of ACh activity.

The other free benzyl alcohols, (4-(2-(diethylamino)ethoxy)phenyl)methanol (compound **23**) and (3-(2-(dimethylamino)ethoxy)phenyl)methanol (compound **24**), provided very different results and proved critical in developing structure activity relationships.

As shown in **Figure 11**, compound **23**, with a diethyl aminoethoxy substitution at the para position of benzyl alcohol seemed to enhance the activity of ACh. However, the effect was not statistically significant (P>0.05). Since the current experiment was designed to study inhibition of ACh, by employing EC_{80} concentration of ACh, further studies will have to be conducted to verify the results.

Figure 11. Stimulation of M_5 muscarinic receptors by acetylcholine in the absence and presence of compound 23.



Impact of compound 23 on the ACh induced PI turnover evaluated at two different concentrations, 1 μ M and 100 μ M. Data represent the mean (± s.e.m.) of three experiments each performed in triplicate.

In contrast, (3-(2-(dimethylamino)ethoxy)phenyl)methanol (compound 24) decreased the activity of acetylcholine at M_5 muscarinic receptors. As seen in Figure 12, the ACh response in the absence of a CDD compound was significantly different from the vehicle (P<0.01). However, in the presence of compound 24 (1 µM and 100 µM), the ACh response was found to be not significantly different from baseline activity (P>0.05). The dose dependent decrease in the ACh response suggests that compound 24 exhibits M_5 muscarinic antagonist properties. This result highlighted the importance of the tertiary amine and its relation to the benzyl alcohol, meta- in the case of compound 24.





Impact of compound **24** on the ACh induced PI turnover evaluated at two different concentrations, 1 μ M and 100 μ M. Data represent the mean (± s.e.m.) of three experiments each performed in triplicate.

The positional isomers of CDD-0371, 1-(2-(2-morpholinoethoxy)benzyl)-1H-indole (compound **15**) and 1-(3-(2-morpholinoethoxy)benzyl)-1H-indole (compound **18**), carried the indole and morpholine moieties and were characterized to observe the affects of position on activity, if any.

Figure 13. Stimulation of M_5 muscarinic receptors by acetylcholine in the absence and presence of compound 15.



Impact of compound **15** on the ACh induced PI turnover evaluated at two different concentrations, 1 μ M and 100 μ M. Data represent the mean (± s.e.m.) of three experiments each performed in triplicate.

As depicted in **Figure 13**, compound **15**, with the 1,2-disubstituted phenyl ring, produced an activity profile which could not be categorized. While compound **15** by itself did not change the level of activity as compared to the background (vehicle), the ACh response remained significantly different from the corresponding controls. However, the degree of difference varied in the three cases. The response elicited by ACh in the

absence of compound **15** was higher than the vehicle control (P<0.01). In the presence of compound **15**, the ACh response also was higher than the control levels (P<0.001).

On the other hand, as shown in **Figure 14**, compound **18** produced a similar effect to its constitutional isomer, compound **4**, by neither displaying an effect by itself at either concentrations nor changing the activity of ACh in its presence.

Figure 14. Stimulation of M_5 muscarinic receptors by acetylcholine in the absence and presence of compound **18**.



Impact of compound **18** on the ACh induced PI turnover evaluated at two different concentrations, 1 μ M and 100 μ M. Data represent the mean (± s.e.m.) of three experiments each performed in triplicate.

Within the series, indole heterocycle was replaced by imidazole in order to correlate the structural change and observed activity. Therefore, 4-(2-(3-((1H-imidazol-1-yl)methyl)phenoxy)ethyl)morpholine (compound **19**), a direct imidazole analog of

compound 18, was synthesized and characterized for its activity at the M_5 muscarinic receptor.

Figure 15. Stimulation of M₅ muscarinic receptors by acetylcholine in the absence and presence of compound 19.



Impact of compound 19 on the ACh induced PI turnover evaluated at two different concentrations, 1 μ M and 100 μ M. Data represent the mean (\pm s.e.m.) of three experiments each performed in triplicate.

As shown in Figure 15, change of heterocycle from indole to imidazole did not produce any significant change in activity profile. Apart from having no intrinsic activity by itself, compound 19 did not produce any significant change in the ACh response at either of the tested concentrations (1 μ M and 100 μ M).

Furthermore, in order to determine the structure activity relationships between diethylamine and the heterocycle indole, two regioisomers, 2-(3-((1H-indol-1yl)methyl)phenoxy)-N,N-diethylethanamine (compound **22**) and 2-(2-((1H-indol-1yl)methyl)phenoxy)-N,N-diethylethanamine (compound **27**), were characterized.

As depicted in **Figure 16**, like other compounds in the designed library, the 1,3disubstituted compound **22** did not produce any change in baseline activity on its own. The ACh stimulation was significantly different from the vehicle in the absence of compound **22** (P<0.001). In the presence of compound **22** at both the 1 μ M and 100 μ M concentrations, the PI turnover response obtained from ACh remained significantly different from respective controls (P<0.001 for1 μ M CDD-0377; P<0.05 for 100 μ M of compound **22**).

Figure 16. Stimulation of M_5 muscarinic receptors by acetylcholine in the absence and presence of compound **22**.



Impact of compound **22** on the ACh induced PI turnover evaluated at two different concentrations, 1 μ M and 100 μ M. Data represent the mean (± s.e.m.) of three experiments each performed in triplicate.

As seen in **Figure 17**, the change in spacial arrangement from meta- to orthodisubstitution along the phenyl ring, regioisomer compound **27**, led to a significant effect on the Acetylcholine response. Compound **27** exhibited no change in the baseline activity by itself at either the 1 μ M or 100 μ M concentrations.

Figure 17. Stimulation of M_5 muscarinic receptors by acetylcholine in the absence and presence of compound 27.



Impact of compound **27** on the ACh induced PI turnover evaluated at two different concentrations, 1 μ M and 100 μ M. Data represent the mean (± s.e.m.) of three experiments each performed in triplicate.

Although the ACh response remained significantly higher than the corresponding controls in the presence of vehicle (P<0.01) and compound **27** (P<0.001) at 1 μ M concentration, the ACh activity was not significantly higher in the presence of 100 μ M compound **27** (P > 0.05). The observed antagonist activity emphasizes the role of spacial arrangement of the two substitutions on the phenyl ring.

Next, the imidazole analog of compound 27, 2-(3-((1H-imidazol-1-yl)methyl)phenoxy)-N,N-diethylethanamine (compound 28), was tested for its biological activity at the M₅ muscarinic receptor. As shown in Figure 18, compound 28 was similar to its indole analog (compound 27) in having no effect at the receptor level by itself when compared to the vehicle. However, compound 28 decreased the ACh response at the M₅ muscarinic receptor in a dose dependent manner. In the presence of compound 28, the ACh response was significantly reduced (P > 0.05 when comparing ACh responses to corresponding response from controls).

Figure 18. Stimulation of M_5 muscarinic receptors by acetylcholine in the absence and presence of compound 28.



Impact of compound **28** on the ACh induced PI turnover evaluated at two different concentrations, 1 μ M and 100 μ M. Data represent the mean (± s.e.m.) of three experiments each performed in triplicate.

The observations with compound **28** emphasized the role of the heterocycle imidazole in the series.

In order to further study the effect of heterocycles on the activity, thiazole analogs were studied. Compounds, 2-(4-(thiazol-2-yl)phenoxy)-N,N-dimethylethanamine (compound**11**) and <math>2-(4-(thiazol-2-yl)phenoxy)-N,N-diethylethanamine (compound**12**), were the two thiazole compounds characterized for their behavior at the M₅ muscarinic receptors.

As shown in **Figure 19**, the thiazole compound with dimethylamine substitution, compound **11**, did not evoke any response at the receptors by itself. Acetylcholine responses at the receptor remained significantly different from the background values both in the absence and presence of compound **11** (P<0.001). The results indicated that compound **11** did not modulate M_5 receptor activity.

Figure 19. Stimulation of M_5 muscarinic receptors by acetylcholine in the absence and presence of compound 11.



Impact of compound **11** on the ACh induced PI turnover evaluated at two different concentrations, 1 μ M and 100 μ M. Data represent the mean (± s.e.m.) of three experiments each performed in triplicate.

As seen in **Figure 20**, the diethylamine substituted thiazole analog, compound **12**, gave similar results in the PI turnover assay. Compound **12** remained inactive on its own and was not able to interfere with the acetylcholine stimulation of the M_5 muscarinic receptors.

Figure 20. Stimulation of M₅ muscarinic receptors by acetylcholine in the absence and presence of compound 12.



Compound 12

The ACh responses remained significantly different to the controls employed in the experiments with a P value less than 0.001 each time.

In summary, 13 compounds were synthesized and characterized for their activity at the M₅ muscarinic receptor subtype. Two-way analysis of variance (ANOVA) was employed to analyze the results from phosphatidyl inositol (PI) turnover assay. Some of the key structure activity relationships deduced from the study includes:

The tertiary amines substituted on the 4-ethoxyamine benzyl alcohol within the _ series were not critical in determining the interaction of the compound at the M_5 muscarinic receptor. The dimethylamine, diethylamine or morpholine substitution

Impact of compound 12 on the ACh induced PI turnover evaluated at two different concentrations, 1 μ M and 100 μ M. Data represent the mean (± s.e.m.) of three experiments each performed in triplicate.

did not elicit any change in response from control values in the absence or presence of ACh at either 1 μ M or100 μ M concentration.

- On the other hand, the regiochemisty of the benzyl alcohols is of paramount importance as far as the desired M₅ antagonist is concerned. While compound 5, (4-(2-(dimethylamino)ethoxy)phenyl)methanol, did not show any signs of interaction at the M₅ muscarinic receptor in the absence or presence of ACh, the meta-substituted regioisomer, compound 24, displayed a dose dependent inhibition of acetylcholine interaction at the M₅ muscarinic receptor. Compound 24 serves as one of the lead compounds identified from the study for the development of M₅ muscarinic receptor antagonist. However, due to the lack of data concerning its interaction at other muscarinic receptor subtypes, no conclusions can be drawn about its selectivity at this point of time. Also, binding studies and schild regression analysis would provide insights about the nature of interaction of compound 24 with the M₅ muscarinic receptor.
- Substitution of hydroxyl groups at the benzyl with the nitrogen atom of a heterocycle gave insight about the importance of the benzyl position. While the indole analog of compound **2**, compound **4**, did not produce a change in activity profile, the presence of indole along with 4-ethoxy dimethylamine on the benzyl ring (compound **7**) changed the activity profile from being inactive to showing positive cooperativity. Although not a desired feature, the enhancement in ACh response by compound **7** underscores the necessity to avoid 1,4-substitution on the phenyl ring in future studies focusing on developing M₅ muscarinic receptor antagonists.

- The positional isomers of compound 4, with either ortho- or meta- orientation of indole and ethoxy morpholine on the benzyl group, compound 15 and compound 18 respectively, did not produce any significant change in ACh stimulation of M₅ muscarinic receptors. These results highlighted the inability of morpholine to interact with the M₅ muscarinic receptor irrespective of the spacial arrangement of substituents on the phenyl ring.
- As reflected by results from compound **19**, even a change in the heterocycle from indole to imidazole did not yield the desired M₅ muscarinic receptor antagonist when N-substituted morpholine served as the tertiary amine in the compound.
- Diethylamine served as a suitable tertiary amine within the general scaffold in the presence of a heterocycle at the benzyl position. While compound **22**, with indole meta substituted to the ethoxy diethyl amine on the benzyl ring, was unable to interfere with the interaction of ACh to the M_5 muscarinic receptor, the regioisomer, compound **27** (with a 1,2-disubstitution), acted as an antagonist at 100 μ M concentration highlighting the importance of spacial arrangement of substituents.
- Finally, the change of the heterocycle in compound **22** from indole to imidazole (compound **28**) imparted antagonistic properties emphasizing the fact that diethylamine can be employed as a tertiary amine in future compounds. However, the heterocycle and orientation along the disubstituted phenyl ring could be key in bestowing antagonistic properties to the compound.
- Thiazole as a heterocycle instead of indole, as in case of compound **11** and compound **12**, did not block the activity of ACh in any manner.

Overall, the project involved characterization of previously synthesized novel compounds designed for their putative allosteric action on muscarinic receptors. This study led to the identification of the unique compound **1**, which displayed M₅ muscarinic receptor antagonist properties and was further studied to identify the nature of its interaction with receptors. Results from Schild regression analysis indicated that compound **1** was not a competitive antagonist at the M₅ muscarinic receptor.

Given the role of M_5 muscarinic receptors in the development of drug dependence, antagonists targeting these receptors present a unique approach to tackle the problem of drug addiction. Therefore, to develop a potent M_5 muscarinic receptor antagonist and to better understand the structure activity relationships, a library of novel compounds was designed and synthesized based on known M_5 muscarinic receptor selective ligands and compound **1**.

Following synthesis of a library of potential M_5 muscarinic receptor modulators, the novel compounds were tested for their ability to inhibit the stimulation of M_5 muscarinic receptors by acetylcholine at both 1 μ M and 100 μ M drug concentrations. The phosphatidyl inositol (PI) turnover assay was employed to access the activity of the synthesized compounds. Two-way analysis of variance (ANOVA) revealed promising results for few analogs including compound **24**, compound **27** and compound **28** as potential M_5 muscarinic receptor antagonists.

Further studies should provide insight about the selectivity of the compounds for M_5 muscarinic receptor subtype and increase our understanding about the structure activity relationships. These and other efforts could ultimately lead to the development of selective M_5 muscarinic receptor antagonists.

Chapter Three

Experimental Section

3.1 Synthetic Chemistry

All reactions were carried out in a chemical fume hood and under inert atmosphere (nitrogen). ¹H and ¹³C NMR spectra were obtained using the Varian Inova 600 MHz and 400 MHz spectrometer using standard deuterated solvents. The trimethylsilyl (TMS) peak was used as a reference for NMR spectra while using chloroform-d and DMSO-d6. Elemental analysis data was obtained through Atlantic Microlab, Inc., Atlanta, GA. Precoated silica gel IB-F flexible sheets from Baker-flex were used for thin-layer chromatography and product spots were observed either under UV light (254 nm) or were stained under iodine vapor. Column chromatography purifications were carried out using Silica gel 60 purchased from Fisher Scientific (200-400 mesh). THF was freshly distilled before each reaction by treating with sodium-benzophenone. All other anhydrous solvents and reagents were commercially obtained and dispensed under nitrogen atmosphere. Acetone was dried over molecular sieves before every reaction. Starting materials were obtained through various commercial sources including Sigma-Aldrich, Alfa Aesar or Fisher Scientific.
3.1.1 Experimental Procedure

General procedure 1 (GP1) for obtaining the β-chloroamines (free base)^[39]. The commercially available hydrochloride salts (20.0 mmol) were dissolved in water (10 mL) and the solution was basified (pH 8-9) using a saturated sodium carbonate solution. The basified solution was extracted using diethyl ether (3 x 15 mL). The combined organic extract was dried over MgSO₄ and filtered. The filtrate was evaporated to obtain the free base. Since the free base was volatile in nature, the water bath was maintained at or below 30°C during the evaporation. The residual oil was shown to contain diethyl ether by ¹H-NMR. This general procedure was applied to obtain free base amines including N-(2-chloroethyl)-dimethylamine, N-(2-chloroethyl)-diethylamine and N-(2-chloroethyl)-morpholine.

(4-(2-Morpholinoethoxy)phenyl)methanol (compound 2). To a solution of 4hydroxy benzyl alcohol (4.9 g, 40 mmol) in acetone (150 mL) was added K₂CO₃ (11 g, 80 mmol). The solution was refluxed for an hour. A solution of N-(2-chloroethyl)morpholine (free base from 50 mmol) in acetone (10 mL) was added drop wise to the above solution and the reaction was refluxed for 24 h. The solution was allowed to cool to room temperature and filtered. The filtrate was concentrated and a crude product was obtained (9.7 g). The residue was purified by column chromatography on silica gel (EtOAc-MeOH, 95:5) to afford compound **2** (4.5 g, 48%): ¹H NMR (CDCl₃, 600 MHz): δ 7.28 (d, 2H), 6.89 (d, 2H), 4.6 (s, 2H), 4.1 (t, 2H), 3.73 (t, 4H), 2.79 (t, 2H), 2.57 (br s, 4H). Anal: (C₁₃H₁₉NO₃.0.2H₂O) C, H, N. (4-(2-(Dimethylamino)ethoxy)phenyl)methanol (compound 5). To a solution of 4hydroxy benzyl alcohol (4.9 g, 40 mmol) in acetone (150 mL) was added K₂CO₃ (11 g, 80 mmol). The solution was refluxed for an hour. A solution of N-(2-chloroethyl)dimethylamine (free base from 50 mmol) in acetone (10 mL) was added drop wise to the above solution and reaction stirred at reflux temperature for 24 h. The solution was allowed to cool to room temperature and filtered. The filtrate was concentrated and a crude product was obtained (6.7 g). The residue was purified by column chromatography on silica gel (EtOAc-MeOH, 1:3) to afford compound **5** (4.4 g, 56%). ¹H NMR (CDCl₃, 400 MHz): δ 7.27 (d, J = 8.8 Hz, 2H), 6.87 (d, J = 8.4 Hz, 2H), 4.60 (s, 2H), 4.01 (t, J = 6 Hz, 2H), 2.72 (t, J = 6 Hz, 2H), 2.32 (s, 6H). Anal: (C₁₁H₁₇NO₂.0.22H₂O) C, H, N.

4-(2-Morpholinoethoxy)benzyl methanesulfonate (compound 3). Methanesulfonyl chloride (0.6 g, 5.5 mmol) was added dropwise over 5 min to a solution of (4-(2-morpholinoethoxy)phenyl)methanol (1.2 g, 5 mmol) and N,N-diisopropylethylamine (DIPEA) (1.3 g, 10 mmol) in 30 mL anhydrous DCM cooled to 0° C,. After addition, the mixture was stirred at 0° C for another 10 mins. The ice bath was removed and the reaction was allowed to warm to room temperature and stirred for 2 h. The reaction mixture was filtered through celite with dichloromethane and concentrated under reduced pressure to obtain the crude 4-(2-morpholinoethoxy)benzyl methanesulfonate as a yellow oil. The crude compound **3** (1.1 g) was used for the next reaction without further characterization.

1-(4-(2-Morpholinoethoxy)benzyl)-1H-indole fumarate (compound 4). Indole (0.9 g, 7.5 mmol) was dissolved in 30 mL of anhydrous DMF and cooled to -10°C. Sodium hydride (60% dispersion in oil, 0.2 g, 8.5 mmol) was added portion wise and the reaction maintained at -10°C for 45 min until the foam disappeared. To this solution, compound 3 (dissolved in 5 mL of DMF) was added dropwise and the reaction was slowly warmed to room temperature. After 16 h, the reaction was quenched with saturated NH₄Cl solution and ethyl acetate. The aqueous phase was extracted with ethyl acetate (3x) and the combined organic layer was dried over Na₂SO₄, filtered and concentrated. The crude product was purified using column chromatography on silica gel (EtOAc-DCM, 1:1) to give the desired product (0.4 g, 24%). In order to obtain the fumarate salt, the free base (1 mmol) was dissolved in 4 mL of isopropanol. Meanwhile, fumaric acid (1 mmol) was dissolved in 0.5 mL of methanol. This solution was further diluted using 5 mL of acetone. After stirring for 5 min, the fumaric acid solution was added to the free base solution in a single addition. The mixture was stirred at room temperature for 2 h which led to the formation of white slurry. An additional 10 mL of acetone was added to this mixture and stirred overnight. The next day, the white colored salt was filtered and dried under vacuum to yield the product (133 mgs, 36%). ¹H NMR (DMSO, 600 MHz): δ 7.52 (d, J = 7.8 Hz, 1H), 7.46 (d, J = 3 Hz, 1H), 7.44 (d, J = 7.8 Hz, 1H), 7.14 (d, J = 8.4 Hz, 2H), 7.07 (t, J = 7.2 Hz, 1H), 6.98 (t, J = 7.2 Hz, 1H), 6.85 (d, J = 9 Hz, 2H), 6.61 (s, 2H), 6.44 (d, J = 9 Hz, 1H), 5.31 (s, 2H), 4.01 (t, J = 6 Hz, 2H),3.55 (t, J = 4.8 Hz, 4H), 2.66 (t, J = 6 Hz, 2H), 2.48 (t, J = 1.8 Hz, 4H). ¹³C NMR (CDCl₃, 100 MHz): & 166.06, 157.68, 135.58, 135.58, 134.05, 130.26, 128.94, 128.50, 128.30,

121.04, 120.41, 118.99, 114.44, 110.19, 100.79, 66.03, 65.08, 56.91, 53.53, 48.58, 30.74. Anal: (C₂₁H₂₄N₂O₂.1.0C₄H₄O₄) C, H, N.

4-(2-(Dimethyamino)ethoxy)benzyl methanesulfonate (compound 6). Methanesulfonyl chloride (0.5 g, 4.1 mmol) was added dropwise over 5 min to a solution of (4-(2-(dimethylamino)ethoxy)phenyl)methanol (0.5 g, 2.7 mmol) and triethylamine (0.8 g, 8 mmol) in 25 mL anhydrous THF cooled to 0° C,. The mixture was stirred at 0° C for another 10 min. The ice bath was removed and the reaction was allowed to warm to room temperature and stirred for 2 h. The reaction mixture was filtered through celite with DCM and concentrated under reduced pressure to obtain the crude 4-(2-(dimethylamino)ethoxy)benzyl methanesulfonate as a yellow oil. The crude compound (0.4 g) was used for the next reaction without further characterization.

2-(4-((1H-Indol-1-yl)methyl)phenoxy)-N,N-dimethylethanamine (compound 7). To a solution of indole (0.4 g, 3 mmol) in 20 mL of anhydrous DMF at 0°C, sodium hydride (60% dispersion in oil, 0.2 g, 8.5 mmol) was added portion wise over 15 min. The reaction maintained at 0°C for 10 min after the addition was complete. The ice bath was removed and the reaction stirred at room temperature for an hour. The reaction was once again cooled to 0°C and the solution of compound **6** in 5 mL of DMF was added all at once and the reactants were slowly warmed to room temperature. After 4 h, the reaction was cooled to 0°C and quenched with saturated NH₄Cl solution and ethyl acetate. The aqueous phase was extracted with ethyl acetate (3x) and the combined organic layer washed with water followed by brine. The organic layer was dried over Na₂SO₄, filtered and concentrated. The crude product was purified using column chromatography on silica gel (EtOAc-MeOH, 1:2) to give compound **7** (0.14 g, 18%). ¹H NMR (CDCl₃, 400 MHz): δ 7.64 (d, J = 8 Hz, 1H), 7.29 (d, J = 2.8 Hz, 1H), 7.17 (t, J = 8 Hz, 1H), 7.12-7.09 (m, 2H), 7.04 (d, J = 6.8 Hz, 2H), 6.84 (d, J = 6.4 Hz, 2H), 6.53 (br s, 1H), 5.25 (s, 2H), 4.02 (t, J = 5.6 Hz, 2H), 2.71 (t, J = 5.6 Hz, 2H), 2.32 (s, 6H). ¹³C NMR (CDCl₃, 100 MHz): δ 158.48, 136.38, 129.78, 128.88, 128.57, 128.53, 128.34, 128.28, 121.77, 121.11, 119.63, 114.93, 109.89, 101.68, 66.08, 58.40, 49.77, 46.04. Anal: (C₁₉H₂₂N₂O.0.2H₂O) C, H, N.

4-Methoxybenzothioamide (compound 8). To a solution of 68% sodium hydrosulfide hydrate (2.9 g, 35 mmol) and magnesium chloride hexahydrate (3.8 g, 18.75 mmol) in 25 mL DMF was added 4-methoxybenzonitrile (2.5 g, 18.75 mmol) all at once and the mixture was stirred at room temperature for 8 hrs. The reaction mixture turned green and was then poured in water and the precipitate was filtered out. The crude product was resuspended in 1N HCl and stirred at room temperature for 30 min. The precipitate was filtered and washed with water to obtain 4-methoxybenzothioamide as a yellow solid (2.8 g, 90 %).

2-(4-Methoxyphenyl)thiazole (compound 9). Compound **8** (2.8 g, 16.9 mmol) and bromoacetaldehyde diethyl acetal (3.3 g, 16.9 mmol) were dissolved in 70 mL of ethanol. The mixture was refluxed for 10 h, cooled to room temperature and concentrated under reduced pressure. Water was added to the residue and the resulting aqueous layer was extracted with ethyl acetate (3x). The combined organic extracts were washed with water

and brine, and then dried over Na₂SO₄. Evaporation of the organic solvent under reduced pressure gave the crude compound. Column chromatography using silica gel (DCM-Hexane, 9:1) yielded the final compound, 2-(4-methoxyphenyl)thiazole as a yellow oil (2.3 g, 72%). ¹H NMR (CDCl₃, 600 MHz): δ 7.89 (d, J = 9 Hz, 2H), 7.80 (d, J = 3 Hz, 1H), 7.24 (d, J = 3 Hz, 1H), 6.94 (d, J = 9 Hz, 2H), 3.84 (s, 3H).

4-(Thiazol-2-yl)phenol (compound 10). Compound **9** (12 mmol) was dissolved in 48% HBr (42 mL) and acetic acid (12 mL), and the mixture was refluxed for 24 h. The reaction mixture, which turned black, was cooled to room temperature and concentrated. The residue was cooled to 0°C and saturated NaHCO₃ was added dropwise till the solution turned basic (pH=8). The resulting precipitates were washed with water to yield the final compound, 4-(thiazol-2-yl)phenol (2 g, 95 %), as an grey powder. ¹H NMR (DMSO, 400 MHz): δ 7.84 (d, J = 3.2 Hz, 1H), 7.78 (d, J = 4.4 Hz, 2H), 7.66 (d, J = 3.2 Hz, 1H), 6.87 (d, J = 4.4 Hz, 2H).

2-(4-(Thiazol-2-yl)phenoxy)-N,N-dimethylethanamine (compound 11). Compound 10 (0.23 g, 1.3 mmol) was dissolved in 20 mL of acetone and to this solution K_2CO_3 (0.36 g, 2.6 mmol) added. The mixture was stirred for 15 min at room temperature. A solution of N-(2-chloroethyl)-dimethylamine (3 mmol, obtained using general procedure 1 [GP1]) in acetone (5 mL) was added to the above mixture and stirred at room temperature for 10 mins. The reaction was then stirred under reflux conditions for 24 h. The solution was allowed to cool to room temperature and filtered. The filtrate was concentrated to obtain the crude product (0.35 g). Column chromatography using silica gel (EtOAc-MeOH, 1:1) gave the pure compound, 2-(4-(thiazol-2-yl)phenoxy)-N,N-dimethylethanamine (0.1 g, 30%). R_f = 0.32 (EtOAc-MeOH, 1:1). ¹H NMR (CDCl₃, 600 MHz): δ 7.89 (d, J = 8.8 Hz, 2H), 7.80 (d, J = 3.6 Hz, 1H), 7.25 (d, J = 3.6 Hz, 1H), 6.98 (d, J = 8.8 Hz, 2H), 4.11 (t, J = 5.6 Hz, 2H), 2.75 (d, J = 5.6 Hz, 2H), 2.35 (s, 6H). ¹³C NMR (CDCl₃, 100 MHz): δ 168.50, 160.55, 143.57, 128.18, 126.81, 118.07, 115.08, 66.33, 58.43, 46.15. Anal: (C₁₃H₁₆N₂OS) C, H, N.

2-(4-(Thiazol-2-yl)phenoxy)-N,N-diethylethanamine (compound 12). Compound **10** (0.22 g, 1.2 mmol) was dissolved in 20 mL of acetone and to this solution K₂CO₃ (0.35 g, 2.5 mmol) added. The mixture was stirred for 15 min at room temperature. A solution of N-(2-chloroethyl)-diethylamine (4 mmol, obtained using general procedure 1 **[GP1]**) in acetone (5 mL) was added to the above mixture and stirred at room temperature for 10 mins. The reaction was then stirred under reflux conditions for 24 h. The solution was allowed to cool to room temperature and filtered. The filtrate was concentrated to obtain the crude product (0.35 g). Column chromatography using silica gel (EtOAc-MeOH, 1:1) gave the pure compound, 2-(4-(thiazol-2-yl)phenoxy)-N,Ndiethylethanamine (0.27 g, 76%). R_f = 0.31 (EtOAc-MeOH, 1:1). ¹H NMR (CDCl₃, 600 MHz): δ 7.88 (d, J = 7.2 Hz, 2H), 7.80 (d, J = 7.8 Hz, 1H), 7.25 (d, J = 8.4 Hz, 1H), 6.96 (d, J = 7.2 Hz, 2H), 4.10 (t, J = 6 Hz, 2H), 2.90 (t, J = 6 Hz, 2H), 2.66 (q, J = 6 Hz, 4H), 1.08 (t, J = 6 Hz, 6H). ¹³C NMR (CDCl₃, 100 MHz): δ 168.53, 160.55, 143.56, 128.81, 126.72, 118.06, 115.03, 66.80, 51.74, 48.01, 11.95. Anal: (C₁₅H₂₀N₂OS) C, H, N. (2-(2-Morpholinoethoxy)phenyl)methanol (compound 13). To a solution of 2hydroxy benzyl alcohol (3.7 g, 30 mmol) in acetone (100 mL) was added K₂CO₃ (8.3 g, 60 mmol). The solution was refluxed for an hour. A solution of N-(2-chloroethyl)morpholine (free base from 45 mmol) in acetone (10 mL) was added drop wise to the above solution and the reaction was stirred at reflux temperature for 24 h. The solution was allowed to cool to room temperature and filtered. The filtrate was concentrated and a crude product obtained. The residue was purified by column chromatography on silica gel (EtOAc-DCM, 9:1) to yield the product (3 g, 42 %). ¹H NMR (CDCl₃, 400 MHz): δ 7.27-7.21 (m, 2H), 6.96-6.91 (m, 2H), 4.62 (s, 2H), 4.21 (t, J = 4.8 Hz, 2H), 3.71 (t, J = 4.4 Hz, 4H), 2.72 (t, J = 4.8 Hz, 2H), 2.50 (s, 4H). ¹³C NMR (CDCl₃, 100 MHz): δ 129.75, 129.19, 121.74, 113.67, 66.66, 64.85, 62.62, 57.99, 53.63. Anal: (C₁₃H₁₉N₁O₃.0.1 H₂O) C, H, N.

2-(2-Morpholinoethoxy)benzyl methanesulfonate (compound 14). To a solution of (2-(2-morpholinoethoxy)phenyl)methanol (0.6 g, 2.6 mmol) in 20 mL dichloromethane, N,N-diisopropylethylamine (DIPEA) (0.7 g, 5.3 mmol) was added and solution cooled to 0°C. To this solution, methanesulfonyl chloride (0.3 g, 2.9 mmol) was added dropwise under constant stirring. The mixture was allowed to warm to room temperature and stirred for 10 h. The reaction was quenched with water (20 mL) and extracted with ethyl acetate (3 x 30 mL). The organic extract was washed with saturated NH₄Cl solution, water and brine in that order. The extract was dried over MgSO₄ and concentrated to yield the crude product. The crude product was used for the subsequent reaction without further characterization.

1-(2-(2-Morpholinoethoxy)benzyl)-1H-indole (compound 15). Indole (0.2 g, 1.6 mmol) was dissolved in 8 mL of DMF and cooled to 0°C. Sodium hydride (60% dispersion in mineral oil) (0.2g, 4.8 mmol) was added portion wise to the above solution over 15 min. Once the addition was complete, the solution was further stirred at 0°C for 10 min. The reaction was allowed to warm to room temperature and stirred for 1 h. The solution was cooled to 0°C and compound 7 (in 5 mL DMF) was added rapidly. After the addition of mesylate, the reaction was stirred at room temperature for 4 h. Once the reaction was complete, it was cooled to 0°C and saturated NH₄Cl solution was added. Ethyl acetate (30 mL) also was added and the resulting mixture was stirred for 5 min. The aqueous phase was extracted with ethyl acetate (3x). The combined organic extract was washed with water (2x) and brine, dried over Na₂SO₄, filtered and concentrated to yield the crude product (0.8 g). The residue was purified by column chromatography on silica gel (EtOAc-DCM, 1:4) to yield compound 15 (150 mg, 15%). ¹H NMR (CDCl₃, 400 MHz): δ 7.63 (d, J = 8 Hz, 1H), 7.34 (d, J = 8 Hz, 1H), 7.25-7.20 (m, 1H), 7.18-7.15 (q, 2H), 7.12-7.07 (m, 1H), 6.87 (d, J = 8.4 Hz, 1H), 6.83-6.81 (m, 2H), 6.50 (d, J = 3.2 Hz, 1H), 5.32 (s, 2H), 4.15 (t, J = 5.6 Hz, 2H), 3.70 (t, J = 4.8 Hz, 4H), 2.80 (t, J = 5.6 Hz, 2H), 2.53 (t, J = 4.8 Hz, 4H). ¹³C NMR (CDCl₃, 100 MHz): δ 156.25, 136.52, 129.07, 128.80, 128.74, 128.67, 125.98, 121.64, 121.05, 121.03, 119.53, 111.32, 109.92, 101.41, 67.15, 66.16, 57.86, 54.25, 45.48. Anal: (C₂₁H₂₄N₂O₂.0.2 H₂O) C, H, N.

(3-(2-Morpholinoethoxy)phenyl)methanol (compound 16). To a solution of 3hydroxy benzyl alcohol (2.5 g, 20 mmol) in acetone (100 mL) was added K_2CO_3 (5.5 g, 40 mmol). The solution was stirred at room temperature for 10 mins followed by the addition of 4-(2-chloroethyl)morpholine hydrochloride (7.4 g, 35 mmol). The reaction mixture was stirred at room temperature for another 10 min. After refluxing for 12 h, the solution was allowed to cool to room temperature and filtered. The filtrate was concentrated and a crude product was obtained. The residue was purified by column chromatography on silica gel (Acetone-DCM, 1:2) to yield compound **16** (3.8 g, 80%). ¹H NMR (CDCl₃, 600 MHz): δ 7.21 (t, J = 8.4 Hz, 1H), 6.9 (br s, 2H), 6.76 (d, J = 8.4 Hz, 1H), 4.60 (s, 2H), 4.04 (t, J = 6 Hz, 2H), 3.67 (t, J = 4.8 Hz, 2H), 2.73 (t, J = 6 Hz, 2H), 2.52 (br s, 4H).

3-(2-Morpholinoethoxy)benzyl methanesulfonate (compound 17). To a solution of (3-(2-morpholinoethoxy)phenyl)methanol (0.5 g, 2.4 mmol) and triethylamine (0.8 g, 7.5 mmol) in 15 mL anhydrous THF cooled to 0°C, methanesulfonyl chloride (0.4 g, 3.6 mmol) was added dropwise over 5 min. The mixture was stirred at 0°C for another 10 min. The ice bath was removed and the reaction was allowed to warm to room temperature with stirring for 2 h. The reaction mixture was filtered through celite with THF and concentrated under reduced pressure to obtain the crude 3-(2-morpholinoethoxy)benzyl methanesulfonate as a yellow oil. The crude compound was used for the next reaction without further characterization.

1-(3-(2-Morpholinoethoxy)benzyl)-1H-indole (compound **18).** Indole (0.6 g, 5.0 mmol) was dissolved in 25 mL of DMF and cooled to 0°C. Sodium hydride (60% dispersion in mineral oil) (0.6 g, 15 mmol) was added portion wise to the above solution

over 15 min. Once the addition was complete, the solution was further stirred at 0°C for 10 min. The reaction was allowed to warm to room temperature and stirred for 1 h. The solution was cooled to 0°C and compound 9 (in 5 mL DMF) was added rapidly. After the addition of mesylate, the reaction was stirred at room temperature for 4 h. Once the reaction was complete, it was cooled to 0°C and saturated NH₄Cl solution was added. Ethyl acetate (30 mL) was also added and the resulting mixture stirred for 5 mins. The aqueous phase was extracted with ethyl acetate (3x). The combined organic extract was washed with water (2x) and brine, dried over Na₂SO₄, filtered and concentrated to yield the crude product. The residue was purified by column chromatography on silica gel (EtOAc-DCM, 2:1) to yield compound **18** (296 mg, 38%). $R_f = 0.5$. ¹H NMR (CDCl₃, 400 MHz): δ 7.64 (d, J = 7.6 Hz, 1H), 7.27 (d, J = 8 Hz, 1H), 7.20 (d, J = 7.6 Hz, 1H), 7.18-7.14 (m, 1H), 7.11 (d, J = 2.8 Hz, 1H), 7.09 (d, J = 7.2 Hz, 1H), 6.78 (dd, 1H), 6.71 (d, J = 7.6 Hz, 1H), 6.64 (br s, 1H), 6.54 (d, J = 3.6 Hz, 1H), 5.27 (s, 2H), 4.00 (t, J = 5.6 Hz, 1H), 5.27 (s, 2H), 4.00 (t, J = 5.6 Hz, 1H), 5.27 (s, 2H), 4.00 (t, J = 5.6 Hz, 1H), 5.27 (s, 2H), 5.27 (s, 2H)Hz, 2H), 3.70 (t, J = 4.8 Hz, 4H), 2.72 (t, J = 5.6 Hz, 2H) 2.51 (t, J = 5.6 Hz, 4H). 13 C NMR (CDCl₃, 100 MHz): δ 159.25, 139.38, 136.45, 129.98, 128.86, 128.44, 121.86, 121.14, 119.70, 119.45, 113.65, 113.36, 109.84, 101.89, 67.07, 65.79, 57.72, 54.24, 50.19. Anal: (C₂₁H₂₄N₂O₂. 0.15H₂O) C, H, N.

(3-(2-(Diethylamino)ethoxy)phenyl)methanol (compound 20). To a solution of 3hydroxy benzyl alcohol (2.5 g, 20 mmol) in acetone (100 mL) was added K_2CO_3 (5.5 g, 40 mmol). The solution was stirred at room temperature for 10 min followed by the addition of 2-chloro-N,N-diethylethanamine hydrochloride (6.9 g, 40 mmol). The reaction mixture was stirred at room temperature for another 10 min followed by reflux for 12 h. After refluxing, the solution was allowed to cool to room temperature and filtered. The filtrate was concentrated and a crude product was obtained. The residue was purified by column chromatography on silica gel (EtOAc-DCM, 2:1) to yield the product (2.3 g, 53%). ¹H NMR (CDCl₃, 600 MHz): δ 7.24 (t, J = 7.8 Hz, 1H), 6.91 (d, J = 6.6 Hz, 2H), 6.80 (dd, 1H), 4.64 (s, 2H), 4.03 (t, J = 6 Hz, 2H), 2.85 (t, J = 6 Hz, 2H), 2.62 (q, J = 7.2 Hz, 4H), 1.06 (t, J = 7.2 Hz, 6H).

3-(2-(Diethylamino)ethoxy)benzyl methanesulfonate 21). (compound Methanesulfonyl chloride (0.3 g, 2.8 mmol) was added dropwise over 5 min, to a solution of (3-(2-(diethylamino)ethoxy)phenyl)methanol (0.3 g, 1.4 mmol) and triethylamine (0.6 g, 5.6 mmol) in 10 mL anhydrous THF cooled to 0°C. The mixture was stirred at 0°C for another 10 min. The ice bath was removed and the reaction was allowed to warm to room temperature and stirred for 2 h. The reaction mixture was filtered through celite with THF and concentrated under reduced pressure to obtain crude 3-(2-(diethylamino)ethoxy)benzyl methanesulfonate as a yellow oil. The crude compound was used for the next reaction without further characterization.

2-(3-((1H-Indol-1-yl)methyl)phenoxy)-N,N-diethylethanamine (compound 22). Indole (0.4 g, 3.0 mmol) was dissolved in 14 mL of DMF and cooled to 0°C. Sodium hydride (60% dispersion in mineral oil) (0.4 g, 9 mmol) was added portion wise to the above solution over 15 mins. Once the addition was complete, the solution was further stirred at 0°C for 10 min. The reaction was allowed to warm to room temperature and stirred for 1 h. The solution was cooled to 0°C and compound **21** (in 5 mL DMF) was added rapidly. After the addition of mesylate, the reaction was stirred at room temperature for 4 h. Once the reaction was complete, it was cooled to 0°C and saturated NH₄Cl solution was added. Ethyl acetate (30 mL) also was added and the resulting mixture stirred for 5 min. The aqueous phase was extracted with ethyl acetate (3x). The combined organic extract was washed with water (2x) and brine, dried over Na₂SO₄, filtered and concentrated to yield the crude product. The residue was purified by column chromatography on silica gel (EtOAc-DCM, 4:1) to yield compound **22** (120 mg, 29%). R_f = 0.1. ¹H NMR (CDCl₃, 400 MHz): δ 7.64 (d, J = 8 Hz, 1 H), 7.27 (d, J = 8 Hz, 1H), 7.15 (q, J = 8 Hz, 2H), 7.11 (d, J = 2.8 Hz, 1H), 7.09 (d, J = 7.2 Hz, 1H), 6.77 (dd, 1H), 6.68 (d, J = 8 Hz, 2H), 6.54 (d, J = 7.2 Hz, 1H), 5.27 (s, 2H), 3.95 (t, J = 6.4 Hz, 2H), 2.89 (q, J = 7.2 Hz, 4H), 1.03 (t, J = 7.2 Hz, 6H). ¹³C NMR (CDCl₃, 100 MHz): δ 159.48, 139.34, 136.53, 130.01, 128.93, 128.51, 121.90, 121.18, 119.74, 119.32, 113.61, 113.48, 109.93, 101.91, 66.69, 51.91, 50.31, 48.05, 12.07. Anal: (C₂₁H₂₆N₂O.0.2 H₂O) C, H, N.

4-(2-(3-((1H-Imidazol-1-yl)methyl)phenoxy)ethyl)morpholine (compound 19). Imidazole (0.41 g, 6 mmol) and potassium iodide (1 g, 6 mmol) were dissolved in 20 mL DMF and the mixture was cooled to 0°C. To this solution, compound 17 (from 3 mmol of compound 16) dissolved in 5 mL of DMF was added. The mixture was stirred for 5 min followed by addition of NaH 60% dispersion in mineral oil (0.48 g, 12 mmol). The reaction was warmed to 75°C and stirred for 12 h. Once complete, the reaction was cooled to 0°C and quenched with water (30 mL). The reaction mixture was extracted with EtOAc (3x) and the combined organic extract washed with water, brine and dried over Na₂SO₄. The mixture was filtered and concentrated to yield the crude product. The residue was purified by column chromatography on silica gel (EtOAc-DCM, 1:1) to yield compound **19** (312 mg, 37%). $R_f = 0.03$ (EtOAc 100%). ¹H NMR (CDCl₃, 400 MHz): δ 7.54 (s, 1H), 7.27 (d, J = 8 Hz, 1H), 7.09 (s, 1H), 6.90 (br s, 1H), 6.85 (dd, 1H), 6.75 (d, J = 8 Hz, 1H), 6.67 (br s, 1H), 5.08 (s, 2H), 4.06 (t, J = 5.6 Hz, 2H), 3.73 (t, J = 4.4 Hz, 4H), 2.78 (t, J = 5.6 Hz, 2H), 2.56 (t, J = 4.4 Hz, 4H). ¹³C NMR (CDCl₃, 100 MHz): δ 159.35, 137.89, 137.59, 130.19, 129.94, 119.81, 119.46, 114.18, 113.78, 67.04, 65.91, 57.71, 54.23, 50.82. Anal: (C₁₆H₂₁N₃O₂.0.32C₄H₈O₂) C, H, N.

(4-(2-(diethylamino)ethoxy)phenyl)methanol (compound 23). To a solution of 4hydroxy benzyl alcohol (2.5 g, 20 mmol) in acetone (100 mL) was added K₂CO₃ (5.5 g, 40 mmol). The solution was stirred at room temperature for 10 mins followed by the addition of 2-chloro-N,N-diethylethanamine hydrochloride (6.0 g, 35 mmol). The reaction mixture was stirred at room temperature for another 10 min followed by reflux temperature for 12 h. After refluxing, the solution was allowed to cool to room temperature and filtered. The filtrate was concentrated and 8 grams of crude product was obtained. The residue was purified by column chromatography on silica gel (EtOAc-MeOH, 9:1) to yield compound **23** (4 g, 90%). R_f = 0.18 (EtOAc-MeOH, 9:1). ¹H NMR (CDCl₃, 400 MHz): δ 7.26 (d, J = 8.8 Hz, 2H), 6.85 (d, J = 8.8 Hz, 2H), 4.59 (s, 2H), 4.01 (t, J = 6.4 Hz, 2H), 2.85 (t, J = 6.4 Hz, 2H), 2.62 (q, J = 7.2 Hz, 4H), 1.06 (t, J = 7.2 Hz, 6H). ¹³C NMR (CDCl₃, 100 MHz): δ 158.51, 133.50, 128.71, 114.66, 66.57, 64.98, 51.79, 47.88, 11.82. Anal: (C₁₃H₂₁NO₂.0.2 H₂O) C, H, N. (3-(2-(Dimethylamino)ethoxy)phenyl)methanol (compound 24). To a solution of 3-hydroxy benzyl alcohol (3.1 g, 25 mmol) in acetone (100 mL) was added K₂CO₃ (6.9 g, 50 mmol). The solution was stirred at room temperature for 10 min followed by the addition of 2-chloro-N,N-dimethylethanamine hydrochloride (7.2 g, 50 mmol). The reaction mixture was stirred at room temperature for another 10 min followed by reflux temperature for 12 h. After refluxing, the solution was allowed to cool to room temperature and filtered. The filtrate was concentrated and 5 grams of crude product obtained. The residue was purified by column chromatography on silica gel to yield compound **24** (EtOAc-MeOH, 9:1) (3.1 g, 63%). R_f = 0.08 (EtOAc-MeOH, 9:1). ¹H NMR (CDCl₃, 400 MHz): δ 7.23 (t, J = 8 Hz, 1H), 6.91 (d, J = 6.4 Hz, 2H), 6.80 (dd, 1H), 4.63 (s, 2H), 4.02 (t, J = 5.6 Hz, 2H), 2.70 (t, J = 5.6 Hz, 2H), 2.30 (s, 6H). ¹³C NMR (CDCl₃, 100 MHz): δ 159.10, 143.20, 129.62, 119.31, 113.93, 112.85, 65.88, 65.08, 58.33, 45.95. Anal: (C₁₁H₁₇NO₂.0.25 H₂O) C, H, N.

(2-(2-(Diethylamino)ethoxy)phenyl)methanol (compound 25). To a solution of 2hydroxy benzyl alcohol (1.6 g, 13 mmol) in acetone (70 mL) was added K₂CO₃ (5.5 g, 40 mmol). The solution was stirred at room temperature for 10 min followed by the addition of 2-chloro-N,N-diethylethanamine hydrochloride (4.5 g, 26 mmol). The reaction mixture was stirred at room temperature for another 10 min followed by reflux for 12 h. After refluxing, the solution was allowed to cool to room temperature and filtered. The filtrate was concentrated and 4.4 g of the crude product **25** was obtained. The residue was purified by column chromatography on silica gel (EtOAc-DCM, 3:1) to yield the product (1.8 g, 62%). ¹H NMR (CDCl₃, 400 MHz): δ 7.26-7.21 (m, 2H), 6.95-6.92 (m, 2H), 4.60 (s, 2H), 4.14 (t, J = 5.2 Hz, 2H), 2.78 (t, J = 5.2 Hz, 2H), 2.57 (q, J = 7.2 Hz, 4H), 1.03 (t, J = 7.2 Hz, 6H).

2-(2-(Diethylamino)ethoxy)benzyl methanesulfonate (compound 26). To a solution of (2-(2-(diethylamino)ethoxy)phenyl) and triethylamine (0.9 g, 8.4 mmol) in 15 mL anhydrous THF cooled to 0°C, methanesulfonyl chloride (0.5 g, 4.2 mmol) was added dropwise over 5 min. The mixture was stirred at 0°C for another 10 min. The ice bath was removed and the reaction was allowed to warm to room temperature and stirred for 2 h. The reaction mixture was filtered through celite with THF and concentrated under reduced pressure to obtain the crude 2-(2-(diethylamino)ethoxy)benzyl methanesulfonate as a yellow oil. The crude compound was used for the next reaction without further characterization.

2-(2-((1H-Indol-1-yl)methyl)phenoxy)-N,N-diethylethanamine (compound 27). To a solution of indole (0.7 g, 5.6 mmol) in 30 mL of DMF cooled to 0°C, sodium hydride (60% dispersion in mineral oil) (0.7 g, 17 mmol) was added portion wise over 15 mins. Once the addition was complete, the solution was stirred at 0°C for 10 min. The reaction was allowed to warm to room temperature and stirred for 1 h. The solution was cooled to 0°C and compound 26 (in 5 mL DMF) was added rapidly. After the addition of mesylate, the reaction was stirred at room temperature for 4 h. Once the reaction was complete, it was cooled to 0°C and saturated NH₄Cl solution was added. Ethyl acetate (30 mL) was also added and the resulting mixture stirred for 5 min. The aqueous phase was extracted with ethyl acetate (3x). The combined organic extract was washed with water (2x) and brine, dried over Na₂SO₄, filtered and concentrated to yield the crude product (1.1 g). The residue was purified by column chromatography on silica gel (EtOAc, 100%) to yield compound **27** (150 mg, 15%). ¹H NMR (CDCl₃, 400 MHz): δ 7.64 (d, J = 8 Hz, 1H), 7.33 (d, J = 8 Hz, 1H), 7.21 (d, J = 7.6 Hz, 1H), 7.18-7.16 (m, 2H), 7.11-7.07 (m, 1H), 6.88 (d, J = 8 Hz, 1H), 6.78 (t, J = 7.6 Hz, 1H), 6.69 (d, J = 6.4 Hz, 1H), 6.52 (d, J = 3.2 Hz, 1H), 5.33 (s, 2H), 4.10 (t, J = 6.4 Hz, 2H), 2.90 (t, J = 6.4 Hz, 2H), 2.64 (q, J = 7.2 Hz, 4H), 1.07 (t, J = 7.2 Hz, 6H). ¹³C NMR (CDCl₃, 100 MHz): δ 156.22, 136.56, 128.85, 127.82, 128.71, 128.17, 126.10, 121.63, 121.01, 120.81, 119.49, 111.19, 109.99, 101.44, 67.06, 52.01, 48.10, 45.37, 12.22. Anal: (C₂₁H₂₆N₂O.0.1H₂O) C, H, N.

2-(3-((1H-imidazol-1-yl)methyl)phenoxy)-N,N-diethylethanamine (compound 28). Imidazole (0.24 g, 3.5 mmol) was dissolved in 20 mL DMF and the mixture was cooled to 0°C. To this solution NaH 60% dispersion in mineral oil (0.48 g, 12 mmol) was added with continuous stirring. After 5 mins, compound 21 (from 1.8 mmol of compound 20) dissolved in 5 mL of DMF was added. The reaction was warmed to 90°C and stirred for 12 h. Once complete, the reaction was cooled to 0°C and quenched with water (30 mL). The reaction mixture was extracted with EtOAc (3x) and the combined organic extract washed with water, brine and dried over Na₂SO₄. The mixture was filtered and concentrated to yield the crude product. The residue was purified by column chromatography on silica gel (EtOAc-Acetone, 9:1) to yield compound 28 (44 mg, 9%). R_f = 0.12 (EtOAc 100%). ¹H NMR (CDCl₃, 400 MHz): δ 7.54 (s, 1H), 7.24 (d, J = 7.6 Hz, 1H), 7.08 (s, 1H), 6.90 (s, 1H), 6.84 (dd, J = Hz, 1H), 6.73 (d, J = 7.6 Hz, 1H), 5.07 (s, 2H), 3.99 (t, J = 6 Hz, 2H), 2.85 (t, J = 6 Hz, 2H), 2.62 (q, J = 100).

7.2 Hz, 2H), 1.06 (t, J = 7.2 Hz, 6H). ¹³C NMR (CDCl₃, 100 MHz): δ 159.57, 137.83, 137.64, 130.19, 129.99, 119.66, 119.49, 114.20, 113.88, 66.80, 51.86, 50.92, 48.03, 12.03. Anal: (C₁₆H₂₃N₃O. 0.25C₄H₈O₂) C, H, N.

4-(2-(4-(Thiazol-2-yl)phenoxy)ethyl)morpholine (compound 29). Compound 10 (0.25 g, 1.4 mmol) was dissolved in 20 mL of acetone and to this solution K₂CO₃ (0.41 g, 3.0 mmol) added. The mixture was stirred for 15 min at room temperature. A solution of 4-(2-chloroethyl)morpholine (4 mmol, obtained using general procedure 1 [GP1]) in acetone (5 mL) was added to the above mixture and stirred at room temperature for 10 mins. The reaction was then stirred under reflux conditions for 24 h. The solution was allowed to cool to room temperature and filtered. The filtrate was concentrated to obtain the crude product (0.66 g). Column chromatography using silica gel (EtOAc-DCM, 3:2) gave the pure compound, 4-(2-(4-(thiazol-2-yl)phenoxy)ethyl)morpholine (0.2 g, 49%). R_f= 0.4 (EtOAc-DCM, 3:2). ¹H NMR (CDCl₃, 400 MHz): δ 7.91-7.88 (m, 2H), 7.81 (d, J = 3.2 Hz, 1H), 7.26 (d, J = 3.2 Hz, 1H), 6.98-6.94 (m, 2H), 4.16 (t, J = 5.6 Hz, 2H), 3.75 (t, J = 4.4 Hz, 4H), 2.83 (t, J = 5.6 Hz, 2H), 2.60 (t, J = 4.4 Hz, 4H). ¹³C NMR (CDCl₃, 100 MHz): δ 168.37, 160.34, 143.57, 128.19, 126.90, 118.13, 115.06, 67.07, 66.08, 57.72, 54.28. Anal: (C₁₅H₁₈N₂O₂S) C, H, N.

3.2 Biological activity

3.2.1 Phosphatidyl Inositol Turnover Assay:

The procedure for determining IP₃ levels following the stimulation of muscarinic receptors has been described previously.^[4] In brief, A9 L cell lines stably expressing

human muscarinic receptors (M_1 or M_5 receptors through plasmids obtained from Missouri S&T cDNA Resource Center) were seeded in 96-well tissue culture plates (100 μ l with approximately 30,000 cells).

The plates were incubated for 24 hrs at 37° C in an incubator conditioned with 5% CO₂. On the following day, prior to radiolabeling the cells, the plates were rinsed twice with 200 μ l PBS. Then, 100 μ l of inositol free (IF) DMEM supplemented with D-glucose (25 mM), L-glutamine (4 mM), BSA (0.6%) and [³H]-inositol (10 μ Ci/ml) was dispensed into each well. The radiolabeled cells were incubated overnight at 37° C with 5% CO₂. The following day test ligand dilutions were prepared in HBSS buffer supplemented with LiCl (10 mM) and HEPES (20 mM). Receptor activation was initiated by addition of 100 μ l of the appropriate concentration of test ligands (with the dilution buffer serving to measure basal [control] levels) to each well in triplicate sets. The cells were incubated subsequently at 37° C (5% CO₂) for 1 h. Receptor stimulation was terminated by rapid removal of all media. Then, 100 μ l of ice-cold formic acid (50 mM) was added to each well and incubated at room temperature for 20 min.

Meanwhile, to a 96 well, white, solid plate, 80 μ l of YSi-SPA beads (1 mg/80 μ l water) was added. Following incubation, the plate containing cells was tapped on all sides to obtain a homogeneous cell lysate. Once properly mixed, 20 μ l of cell extract in formic acid was added to corresponding wells containing SPA beads (in the white plate).

In order to get a blank reading corresponding to formic acid, $20 \ \mu$ l of formic acid also was assessed for activity. The 96 well (white) plates were sealed using a TopSeal A and contents were mixed via shaking for 1 h at 4° C at 200 rpm using an orbital shaker. After

standing at 4°C for 2 h or overnight, radioactivity in counts per minute (CPM) was determined using the Topcount NXT system. Activity was presented either as the percentage activation above basal levels (CPM-CNTRL/CNTRL-BLANK*100) or counts per minute. Carbachol (or acetylcholine) served as a positive control for muscarinic receptor activation in each assay.

3.2.2 Allosteric Inhibition: Experimental Design

For compounds designed and synthesized as possible allosteric modulators of M_5 receptors, the experimental setup was modified to evaluate the interaction of compounds in the presence and absence of agonist (carbachol or acetylcholine).

In order to access the interaction between the two ligands and receptor, a fixed concentration of the agonist (carbachol or acetylcholine) was selected. The EC₈₀ value for the agonist was determined by generating three full dose response curves for the agonist. Instead of a full dose-response curve, the synthesized compounds were tested for activity at two fixed concentrations, 1 μ M and 100 μ M. The compounds were also incubated with the radiolabeled cells in the presence of EC₈₀ concentration of carbachol (or acetylcholine) at the same two concentrations. HEPES buffer and DMSO served at control for baseline activity of the vehicle.

Once a compound of interest was identified, a Schild-type analysis was conducted to determine the nature of inhibition (competitive or non-competitive).^[44] Full dose response curves of agonists (carbachol or acetylcholine) were performed in the presence of fixed concentrations of antagonist. The following analysis using '*dose ratio*', the EC₅₀ value of agonist in the presence of antagonist divided by the EC₅₀ value of agonist

without the antagonist, was conducted in order to access the nature of inhibition taking place.

Analysis of variance (ANOVA) was used to determine if the means among two or more groups were equal or significantly different, assuming that the sampled populations are normally distributed. The P value was set at 0.05 to determine if the difference between sample means was statistically significant. Bonferroni post-tests to compare replicate means by row were selected for analyzing the data.

Since the stimulation of M_5 muscarinic receptors by ACh produced PI turnover responses significantly greater than those observed with the vehicle, the P values in all such comparisons for ACh (by itself) were found to be P<0.05. Moreover, in experiments where the presence of a CDD compound (1 μ M or 100 μ M) was able to inhibit the stimulation of M_5 muscarinic receptor observed with ACh, the PI turnover response ceased to be significantly different from the corresponding control and hence, in such cases, P values were found to be greater than 0.05 (P>0.05). CDD compounds able to produce such an inhibition in the activity of ACh were identified as promising M_5 muscarinic receptor antagonists.

Chapter Four

Future Work

In accordance with the supporting data for M_5 muscarinic receptor involvement in development of drug dependence, an antagonist at the receptor could serve as an ideal candidate for treatment of drug addiction. However, the antagonist needs to be subtype selective and target only the M_5 muscarinic receptor subtype to avoid side effects and complications associated with inhibition or interaction with other muscarinic subtypes.

Therefore, in order to determine the selectivity profile of the synthesized compounds, evaluations of agonist activity at the other four subtypes of muscarinic receptors, i.e., M₁-M₄, need to be conducted. Such studies would assess the selectivity of the lead compounds thereby bolstering the conclusions drawn previously from the structure activity relationships.

Apart from functional studies, the nature of binding of these synthesized compounds to the various receptor subtypes needs to be evaluated. An antagonist could block receptor activity by binding to the binding pocket for the agonist (ACh) at the receptor thereby inhibiting its effects. Alternatively, antagonists could act by stabilizing the inactive state through interaction with allosteric binding sites. Determining the mechanism of action of the compounds is crucial in determining how potential antagonists interact with M_5 muscarinic receptors. Also, in order to assess the nature of inhibition being exhibited by the lead compounds within the synthesized series, a Schild-type regression analysis should be performed.

The compounds found to be inhibiting the effects of ACh at the M_5 muscarinic receptors namely, compound 24, compound 27 and compound 28 can be further modified to extend the structure activity relationship analysis. The phenyl ring within these compounds presents numerous possibilities for substitution. One such modification, which is also present in the M_5 muscarinic receptor allosteric ligand amiodarone, involves the replacement of hydrogen atoms next to the ether linkage present in these compounds with iodine. Other modifications, including but not limited to changing the heterocycle, increasing the chain length between the oxygen and amine in the side chain, and modification of the tertiary amine etc. could reveal further information about the pharmacophore and ultimately lead to the development of potent M_5 muscarinic receptor antagonists.

Moreover, evaluation of compound **13** and compound **29** for their ability to modulate the action of acetylcholine at M₅ muscarinic receptor subtype needs to be undertaken.

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Chapter Five

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Appendix A

Novel Compounds



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Appendix B

Elemental analysis

Compound	Formula	% C	% H	% N	% C	% H	% N
		Cal.	Cal.	Cal.	Found	Found	Found
Compound 2	C ₁₃ H _{19.40} NO _{3.20}	65.80	8.07	5.90	64.68	8.00	5.76
	$[C_{13}H_{19}NO_3.$						
	0.2H ₂ O]						
Compound 5	C ₁₁ H _{17.44} NO _{2.22}	67.66	8.78	7.17	66.66	8.72	6.63
	$[C_{11}H_{17}NO_2.$						
	0.22H ₂ O]						
Compound 4	$C_{25}H_{28}N_2O_6$	66.36	6.24	6.19	66.18	6.15	6.28
	$[C_{21}H_{24}N_2O_2.$						
	$1.0 C_4 H_4 O_4$]						
Compound 11	$C_{13}H_{16}N_2OS$	62.87	6.49	11.48	62.72	6.44	11.31
Compound 12	$C_{15}H_{20}N_2OS$	65.18	7.29	10.14	65.00	7.38	9.88
Compound 7	$C_{19}H_{22.40}N_2O_{1.2}$	77.52	7.53	9.52	76.28	7.48	9.02
	$_{0}$ [C ₁₉ H ₂₂ N ₂ O.						
	0.2H ₂ O]						
Compound 15	$C_{21}H_{24.4}N_2O_{2.20}$	74.97	7.19	8.33	74.37	7.19	8.33
	$[C_{21}H_{24}N_2O_2.$						
	0.2 H ₂ O]						
Compound 18	$C_{21}H_{24.30}N_2O_{2.1}$	74.97	7.19	8.33	74.08	7.19	8.33
	5						
	$[C_{21}H_{24}N_2O_2.$						
	0.15H ₂ O]						
Compound 22	$C_{21}H_{26.4}N_2O_{1.20}$	78.23	8.13	8.69	77.73	7.98	8.60
	$[C_{21}H_{26}N_2O.$						
	0.2 H ₂ O]						
Compound 19	C _{17.28} H _{23.56} N ₃ O	66.88	7.37	14.62	65.4	7.61	13.68
	2.64						
	$[C_{16}H_{21}N_3O_2.$						
	$0.32C_4H_8O_2$]						
Compound 23	C ₁₃ H _{21.40} NO _{2.20}	69.92	9.48	6.27	68.66	9.48	6.11

	$[C_{13}H_{21}NO_2.$						
	$0.2 H_2O$]						
Compound 24	C ₁₁ H _{17.50} NO _{2.25}	67.66	8.78	7.17	65.88	8.72	6.88
	$[C_{11}H_{17}NO_2.$						
	0.25 H ₂ O]						
Compound 27	C ₂₁ H _{26.20} NO _{1.10}	78.23	8.13	8.69	77.52	8.09	8.40
	$[C_{21}H_{26}N_2O.$						
	0.1H ₂ O]						
Compound 28	C ₁₇ H ₂₅ N ₃ O _{1.50}	70.30	8.48	15.37	69.49	8.75	14.04
	$[C_{16}H_{23}N_{3}O.$						
	$0.25C_4H_8O_2$]						
Compound 13	$C_{13}H_{19.20}N_1O_{3.1}$	65.80	8.07	5.90	65.02	8.09	5.82
	0						
	$[C_{13}H_{19}N_1O_3.$						
	0.10 H ₂ O]						
Compound 29	$C_{15}H_{18}N_2O_2S$	62.04	6.25	9.65	62.21	6.25	9.63

Appendix C

NMR Data




































