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Development of a pharmacological screen for M{esc}b5{esc}s muscarinic antagonists

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A Dissertation

entitled

Development of a Pharmacological Screen for
M_5 Muscarinic Antagonists
by

Amanda C. Klein

Submitted to the Graduate Faculty as partial fulfillment of the
requirements for the Masters of Science Degree in Pharmaceutical Sciences

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An Abstract of

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Drug abuse and addiction is a major problem in the United States. The current treatment options only substitute the drug of abuse with a similar compound but this type of treatment does not address the underlying cellular behaviors. Studies have shown that M_5 muscarinic receptor locations and functions are involved in the reward pathway that is also involved in drug abuse and addiction. A selective M_5 muscarinic receptor antagonist could allow for better treatment options than currently available, however such a compound has yet to be identified. An introductory step in the form of a screening method to identify possible compounds as M_5 muscarinic receptor antagonists has been developed and testing of several compounds has ensued. The screening method has been able to characterize compounds as inhibitors of acetylcholine binding at M_5 muscarinic receptors or inactive at the M_5 receptor binding site.

For my family.

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Contents

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List of Abbreviations

ACh ..... Acetylcholine

ANOVA ..... Analysis of variance

CPM ..... Counts per Minute

DAG ..... Diacyl glycerol

DAT ..... Dopamine Reuptake Transporter

DMEM ..... Dulbecco's Modified Eagle Medium

DMSO ..... Dimethyl sulfoxide

EC_{80} ..... Effective Concentration of 80% of Agonist

GPCR ..... G protein coupled receptor

IP ..... Inositol phosphate

IP_2 ..... Inositol bisphosphate

IP_3 ..... Inositol 1,4,5-trisphosphate

mAChR ..... Muscarinic acetylcholine receptor

MAPK ..... Mitogen-activated protein (MAP) kinases

NAc ..... Nucleus accumbens

nAChR ..... Nicotinic acetylcholine receptor

PLC ..... Phospholipase C

PIP2 ..... phosphatidylinositol 4, 5-bisphosphate

PPN ..... Pedunculopontine Nucleus

LDT ..... Laterdorsal Tegmental Nucleus

SN ..... Substantia Nigra

VTA ..... Ventral Tegmental Area

Chapter 1

Introduction

1.1 Drug Abuse & Addiction

Drug abuse which may lead to drug addiction is an important, persistent problem for society. While the incidence of illicit drug use remains steady for cocaine and heroin, the use of prescription drugs, such as oxycodone (OxyContin®) and hydrocodone (Vicodin®), has increased, especially among young adults (National Survey on Drug Use and Health (U.S.) and United States. Substance Abuse and Mental Health Services Administration. Office of Applied Studies. 2003). Drug addiction manifests itself as compulsive drug use possibly leading to serious negative consequences such as failures in significant life roles, medical illness, or engagement in criminal activities in order to obtain drugs. Individuals involved with drug abuse value drugs over all other goals resulting in lives with a very narrow focus on obtaining and using drugs (Hyman, Malenka et al. 2006). Current treatment of drug addiction includes a regimen of compounds that mimic the activity of the abused

substance, but such approaches do not address the underlying molecular changes that have occurred because of prolonged drug abuse.

Addiction is associated with increased activity in neural pathways linked to reward mechanisms. Within the brain, the nucleus accumbens (NAc) is a part of a natural reward system that assigns events, behaviors, and actions to promote beneficial behavior for the good of the organism. Within the NAc, dopamine is released in response to natural rewards, such as food or sexual activities, and responses to addictive drugs have many commonalities that include hedonic (pleasure) responses, desire, and the rapid learning of predictive cues and efficient behavioral sequences for obtaining the reward. Natural rewards and addictive drugs differ in two ways; first being that the rewards from drugs tends to become overvalued with other rewards losing value, thus contributing to the compulsion and the narrowing of goals to those only related to drugs. Secondly, addictive drugs also serve no beneficial homeostatic or reproductive purpose to the individual but are often instead detrimental to the individual's health and functioning (Hyman, Malenka et al. 2006).

The dopamine reward system arises in the ventral tegmental area (VTA) which is located within the midbrain. The VTA contains dopaminergic neurons that project to the NAc, which is a major component of the ventral striatum, as well as the prefrontal cortex, amygdala, and hippocampus where dopamine is released. All addictive drugs cause an increase of synaptic dopamine within the

NAc through either direct (cocaine, amphetamine) or indirect (opioids, nicotine) actions (Hyman, Malenka et al. 2006). The direct or indirect actions that cause the release of dopamine from the VTA is specific to the type of drug and to where and what kind of receptors the drugs bind. For instance, opiates can bind to μ opiate receptors located on GABAergic interneurons that disinhibit the dopamine neurons that allows for the release of dopamine in the NAc (Hyman, Malenka et al. 2006). Opiates also activate other mechanisms that interact with the reward system than just the afore mentioned mechanism which makes it a complicated system but also allows for multiple types of therapeutics to be researched and used.

Cocaine acts within the NAc by blocking the dopamine reuptake transporter (DAT) by which dopamine and other monoamines are taken back into the synaptic terminal for repacking into vesicles or degradation. In the presence of cocaine dopamine accumulates within synapses allowing for longer activation of post-synaptic terminals (Hyman, Malenka et al. 2006). Amphetamines work in a similar manner as cocaine but instead of just blocking the DAT, they also release intracellular stores of dopamine within the neuron terminals allowing for an even larger amount of dopamine to be present within the pre- and post- synaptic neurons (Hyman, Malenka et al. 2006).

The dopaminergic neurons within the VTA are modulated by cholinergic neurons originating from the pedunculopontine nucleus (PPN) and laterdorsal

tegmental nucleus (LDT) (Yeomans, J.S. 1995, Role of Tegmental), as well as GABAergic interneurons and glutamate neurons from the amygdala (Hyman). The cholinergic projections to the VTA from the PPN and LDT can activate either nicotinic acetylcholine receptors (nAChR) or muscarinic acetylcholine receptors (mAChR). Both types of receptors contribute to brain-stimulation reward in rats, but nAChRs make small contributions to maintaining rewarding effects within the VTA while mAChRs contribute a larger rewarding effect, and therefore play a more prominent role in brain-stimulation reward (Yeomans, J., Baptista, M. 1996, Both nicotinic and muscarinic). The GABAergic interneurons also modulate VTA dopaminergic neuronal activity by tonic inhibition of dopaminergic neurons (Hyman, Malenka et al. 2006). Glutamate neurons from the amygdala synapse on and activate dopamine neurons .(Hyman, Malenka et al. 2006).

Overall this large circuit modulates dopamine release within the NAc by modulating dopaminergic neurons in the VTA. There are both excitatory and inhibitory neurons that synapse on the dopaminergic neurons to cause an action potential to release the dopamine within the NAc for normal rewards. Drugs of abuse work in both direct and indirect mechanisms by modulating neurons in both the VTA and NAc that are either excitatory or inhibitory with an overall effect of increased dopamine within the NAc. The overall increase of dopamine within the NAc thus contributes to the rewarding properties associated with

drugs of abuse; with increased use of such drugs the other properties of addition become manifest.

1.2 Acetylcholine Receptors

There are two major types of receptors that bind acetylcholine (ACh) as the endogenous ligand; the nicotinic acetylcholine receptors (nAChR) and muscarinic acetylcholine receptors (mAChR). While the two types of acetylcholine receptors both bind acetylcholine, the actions resulting from acetylcholine binding differs completely. The nAChRs are ionotropic receptors while mAChRs are G-protein coupled receptors (GPCR) that act through second messenger systems. Both classes have multiple subtypes of receptors, with five muscarinic receptors (M_1 through M_5) and three nicotinic receptors subtypes, N_M, N_N, central nervous system subtypes, with multiple subtypes in each major class resulting in five primary receptor subtypes (Goodman, Gilman et al. 2006). Both muscarinic and nicotinic receptors have locations throughout the brain as well as other locations throughout the body (Goodman, Gilman et al. 2006).

1.21 Muscarinic Receptors

Of the five subtypes of muscarinic receptors, all of which are GPCRs, a separation into two classes according to the subtype of GPCR that the receptor represents can be made. M_2 and M_4 receptors inhibit adenylate cyclase by activating the G_i family of G proteins, while M_1, M_3, and M_5 receptors stimulate

lipid metabolism by G_q-mediated activation of phospholipase C that has downstream effects of mobilizing intracellular calcium (Stahl and Ellis 2010).

While the different subtypes of muscarinic receptors are coupled to different G-proteins, these are not the only differences amongst them. The location of muscarinic subtypes also differs between receptors. The M_1 muscarinic receptor is located within the central nervous system predominately in the cerebral cortex, hippocampus and striatum, as well as in autonomic ganglia, gastric and salivary glands and enteric nerves (Goodman, Gilman et al. 2006). M_2 receptors are widely expressed in the central nervous system, the heart, smooth muscles and autonomic nerve terminals (Goodman, Gilman et al. 2006). Receptors of the M_3 subtype are the most widely expressed in the central nervous system more than any other muscarinic receptor but are also located in the heart and are abundant in smooth muscle and glandular tissues. Unlike the other muscarinic receptors, the M_4 and M_5 are located almost exclusively in the brain, with the M_4 located particularly in the forebrain and the M_5 receptor while expressed in low levels in the central nervous system, are predominately within dopaminergic neurons in the VTA and the substantia nigra (SN) (Goodman, Gilman et al. 2006).

Location of muscarinic receptors while useful by itself is not in itself sufficient enough to determine the overall function that the receptors portray. With molecular cloning and expression, characterizing specific subtypes of

muscarinic receptors with regard to their involvement in various signaling pathways and physiological roles has still been difficult because of a lack of small-molecule ligands that are highly subtype selective. Most of the precise physiological involvement of muscarinic receptors has been obtained through the evaluation of knock-out mice (Stahl and Ellis 2010). Activation of the M_1 receptor is thought to increase cognitive function in learning and memory, increase seizure activity, decrease dopamine release and locomotion, increase the depolarization of autonomic ganglia and increase secretions (Goodman, Gilman et al. 2006). Mice lacking the M_1 muscarinic receptor have a phenotype that has increased locomotor activity, increased extracellular dopamine levels in the striatum, loss of carbachol-mediated MAPK activation in CA 1 hippocampal pyramidal neurons, and selective impairment in nonmatching–to-sample working memory and consolidation, among other characteristics (Wess 2004). Mice deficient in the M_2 muscarinic receptor have a phenotype that includes characteristics such as the absence of oxotremorine-mediated tremor and reduced oxotremorine-mediated hypothermia, reduced muscarinic agonist-mediated analgesic responses, absence of muscarine-mediated desensitization of peripheral nociceptors, impaired performance in the passive avoidance test and enhanced ACh efflux in the hippocampus, along with lack of carbachol-mediated bradycardia in isolated heart atria (Wess 2004). With activation of the M_3 receptor, increases in the contraction of the smooth muscle, which is more predominant in some organs such as the bladder, is observed. The M_3 receptor

activation also results in an increase in secretion which is primarily seen in the salivary gland (Goodman, Gilman et al. 2006). Also in mice lacking the M_3 receptor, phenotypic traits such as increased pupil size and urinary bladder distension, pronounced impairments in carbachol-mediated contractions of different smooth muscle preparations, and reductions in body weight, mass of peripheral fat deposits, and food intake has been observed (Wess 2004). Phenotypic traits of mice deficient in the M_4 muscarinic receptor include increased locomotor activity under basal conditions and after administration of a D_1 dopamine receptor agonist, increased sensitivity to phencyclidine-mediated disruptions in prepulse inhibition, reduced autoinhibition of ^{3}H-ACh release in heart atria and urinary bladder, lack of muscarinic agonist-mediated analgesic responses, and increased basal ACh efflux in the hippocampus (Wess 2004). Mice lacking the M_5 receptor have observed phenotype characteristics that include a lack of ACh-mediated dilation of cerebral arteries and arterioles, lack of sustained increase in dopamine levels in the nucleus accumbens triggered by electrical stimulation of the LDT, reduced sensitivity to the rewarding effects of morphine and cocaine and reduced severity of drug withdrawal symptoms and slightly impaired pilocarpine-induced salivation and increased water intake after an extended period of food and water deprivation (Wess 2004).

1.22 Nicotinic Receptors

Nicotinic receptors are the other type of receptors that bind ACh and act as ligand-gated ion channels. Each receptor has a pentameric structure of homomeric α and β subunits, with eight α subunits and three β subunits found in humans having been cloned (Goodman, Gilman et al. 2006). Since there are numerous nAChR that exist because of diversity in subunits and the combinations of subunits that can be formed into pentameric receptors, only major subtypes of nAChR are mentioned. Of the subtypes of nicotinic receptors, the skeletal muscle nAChR, N_M, are located at skeletal neuromuscular junctions and peripheral neuronal nAChR, N_N, have locations within the autonomic ganglia and adrenal medulla (Goodman, Gilman et al. 2006). The other subtype of nAChR are central neuronal receptors and have the primary subunits of $(\alpha_4)_2(\beta_4)_3$ or $(\alpha_7)_5$ and are α-btox-insensitive or α-btox-sensitive respectively (Goodman, Gilman et al. 2006).

Nicotinic receptors containing either α_4 or β_2 subunits are the most common and are widely expressed throughout the brain with locations in the midbrain dopamine areas and the striatum (Zhou, Wilson et al. 2003). β2 nAChRs are expressed in a vast majority of dopamergic and GABAergic neurons in the SN and VTA and are also located on GABAergic afferents (Zhou, Wilson et al. 2003). α7 nAChRs are also commonly present in the midbrain dopamine areas and are expressed presynaptically on glutamatergic afferents, but the

α7receptors are minority type with low expression density (Zhou, Wilson et al. 2003).

1.23 M_5 Muscarinic Receptors

The study of muscarinic acetylcholine receptors has a long history beginning in the mid 1800's with Schmiedeberg and Koppe noting that:

> "muscarine does not destroy the contractile force of the heart muscle, but only oppresses it and prevents its natural manifestation from becoming apparent…. very low doses of atropine paralyse the vagal terminals…after the administration of a very small dose of atropine, muscarine is no longer able to arrest the heart" (Brown 1989).

Even though muscarinic receptors have had an extensive history, it was not until the latter 1980s that the existence of the M_5 muscarinic receptor is generally acknowledged. Through *in situ* hybridization and reverse-transcriptase PCR the location of the M_5 receptor has been found to be primarily localized to the substantia nigra, ventral tegmental area, hippocampus (CA1 and CA2 subpopulations), the outermost layer of the cerebral cortex, and the striatum in the brain, which are distinctly different than the locations of the other four muscarinic receptors within the brain (Raffa 2009).

The areas of the brain that the M_5 receptor is located within have captured the interest of many researchers especially since this is the only subtype of muscarinic receptors that is expressed in dopamine containing neurons of the VTA (Schmidt, Miller et al. 2009). Because the VTA plays a predominant role in the reward system of the brain with the M_5 receptors located on dopamine neurons located in the VTA, M_5 receptors could be a useful target for the development of new treatments for drug abuse and addiction. Important questions to be addressed include what kind of action would be useful in drug abuse at the M_5 receptor (agonist or antagonist), for which drugs of abuse would an M_5 modifying compound be useful, and what kind of other side effects could a M_5 receptor modulator produce. Another issue that also needs to be addressed is the role of nicotinic receptors which are also located within the SN and VTA in the context of the functions of each ACh receptors. Further exploration of the reward system circuitry would define the type of modulatory compound that would be useful at the M_5 receptor and further animal studies have uncovered at least some of the drugs of abuse that an M_5 modulatory compound would be useful for. A look at previous studies and review of the anatomical and functional locations in which the M_5 receptor is present could indicate possible side effects to be taken into account. Also studies in which both mAChRs and nAChRs are observed could demonstrate specific functions of each.

The type of M_5 muscarinic receptor modulator that is useful in drug abuse is dependent upon the circuitry and the overall response of the system. It is well known that the dopaminergic neurons located in the substantia nigra (SN) and VTA have a role in both stimulation induced rewards and drug induced rewards. It is believed that the neurons within those pathways are activated indirectly by non-dopaminergic axons syanapsing with the dopaminergic neurons, which include cholinergic neurons (Raffa 2009). Studies have shown that carbachol, a muscarinic agonist, increases dopamine release when added in the dorsal striatum or NAc and that ACh levels increase within the VTA during brain stimulation reward (Raffa 2009). Since M_5 receptor activation has an overall effect of increasing dopamine levels and administration of drugs of abuse also increase the overall dopamine levels, a selective M_5 muscarinic antagonist should prove useful against drug abuse and addiction.

Other studies of brain stimulation reward that examined both mAChRs and nAChRs within the VTA have concluded that nAChRs have only small contributions to maintaining rewarding effects on brain stimulation reward in rats, but mAChRs within the VTA play a more prominent role (Yeomans, J., Baptista, M. 1996). It also has been reported that nAChRs in the VTA activate dopamine neurons quickly and are needed for the stimulation and rewarding effects of nicotine in rats and that mAChRs within the VTA activate dopamine neurons slowly are responsible for the rewarding effects of hypothalamic

stimulation, however the mAChRs do not increase locomotion (Yeomans, Forster et al. 2001). Yeomans, et al. have also shown that both nicotine and muscarine increase firing rates of VTA and SN dopamine neurons by depolarizing currents, but the nicotinic currents peak in seconds and last for about a minute, whereas muscarinic currents peak in one to three minutes, last a few minutes, and do not desensitize. They also demonstrated that both muscarinic and nicotinic blockers inhibit the rewarding effects of hypothalamic or mesopontine stimulation, but muscarinic blockers are more effective in inhibiting the rewarding effects (Yeomans, Forster et al. 2001). Together these studies indicate that while both muscarinic M_5 receptors and nicotinic receptors are co-localized and the functions of each are linked, each type of receptor plays important roles alone.

Several important observations have been made in regard to drug abuse through the development of mice that lack the M_5 muscarinic receptor (referred to as M_5 knockout mice). Mice without M_5 receptors have attenuated reinforcing properties of morphine in conditioned place preference paradigms indicating that activation of the M_5 receptor has a role in the reinforcing properties of opioid agonists like morphine (Basile, Fedorova et al. 2002). The symptoms associated with opioid withdrawal such as teeth chattering, jumping, paw tremors and jumping also were dramatically decreased after naloxone administration in dependent mice that lacked M_5 receptors (Basile, Fedorova et al. 2002). However, Basile et al. did demonstrate that the analgesic effects of morphine

remained unaltered by the lack of M_5 receptors. The M_5 receptor is not only implicated in opioid abuse but also in cocaine as well. In a conditioned place preference paradigm, mice without the M_5 receptor have been shown to spend less time in the cocaine-paired compartment than control mice and in an elevated plus maze. Mice lacking M_5 receptors also exhibited less withdrawal-induced anxiety than control mice (Fink-Jensen, Fedorova et al. 2003). Studies have also shown decreased cocaine self-administration in naïve M_5 knockout mice (Thomsen, Woldbye et al. 2005).

A study performed by Schmidt et al. in 2009 on cocaine and amphetamine effects in M_5 knockout mice concluded that there is increased locomotor activity, sensitization and accumbal dopamine release from amphetamines in mice lacking M_5 receptors (Schmidt, Miller et al. 2009). They observed that cocaine induced comparable levels of hyper-locomotion, sensitization and evoked dopamine release in mice lacking M_5 receptors and mice with homozygous ($M_5^{+/+}$) M_5 receptor genes (Schmidt, Miller et al. 2009). The results that Schmidt et al. observed differed in many aspects of what others had previously reported in both cocaine and amphetamine studies. The authors concluded that possible reasons for these discrepancies include the line of mouse chosen for the studies differed between this study and previous studies and the back-crossing that was performed with the mice. They also stated that the M_5 receptor may be a valid treatment of abuse of amphetamines and its derivatives (Schmidt, Miller et al.

2009). This study shows an instance where controversies have arisen because of mixed results and/or results that differed from previous studies; and that only with further studies and experiments could any firm conclusions be drawn.

The majority of these studies suggest that the discovery of a selective M_5 antagonist would provide a useful treatment for opioid and cocaine addiction and dependence (Langmead, Watson et al. 2008) and may have potential use in the treatment of amphetamine abuse. Since the muscarinic receptors have high degree of homology between them, discovery of selective receptor-subtypes has proved a challenge with even with high throughput screening methods. A selective-M_5 antagonist however, would not have other adverse effects that are seen with other non-selective muscarinic antagonists because of the low abundance of the M_5 receptors throughout the rest of the body. Such compounds would be able to block the rewarding properties of drugs of abuse as well as the withdrawal symptoms of not only illegal drugs but also opioids and opioid-derivatives.

As the muscarinic receptors have high homology between subtypes, an introductory step to determine what types of compounds may be useful as M_5 muscarinic receptor antagonists is necessary. A screening method has been constructed to test both available non-selective muscarinic antagonists as well as newly synthesized compounds to determine if the compounds show any antagonistic properties for M_5 muscarinic receptors. Compounds that have

antagonistic properties at the M_5 receptor could then be used as lead compounds for further development. Also the chemical structure of these compounds could be studied to determine if there are any common features that lead to the M_5 antagonistic property without activating the other muscarinic receptors.

1.3 Developing Drug Addiction Therapeutics using M_5 Antagonists

When developing a therapeutic agent of any sort certain issues must be addressed. Drug addiction therapeutics have the usual issues that arise from developing any type of therapeutic but also other issues because of the drug abuse disease that a therapeutic is meant to treat. Issues that need to be addressed while developing a treatment for drug abuse and addiction include the properties of the compound, such as solubility, size, passage into the blood-brain-barrier, the selectivity of the compound, abuse potential of the compound itself, potential side effects, and inhibition of regular and normal functions of the body.

A M_5 antagonist would have to be a small molecule with a certain degree of solubility in order to cross the blood-brain-barrier. In addition, an M_5 antagonist should be selective for only the M_5 muscarinic receptor. A selective compound that only inhibited M_5 receptors would exhibit fewer side effects than a muscarinic antagonist that inhibited multiple receptor subtypes. For example

inhibition of M_3 receptors would result in xerostomia (dry mouth). Also the locations of the M_5 receptor within the body are limited almost exclusively to the brain which would further decrease side effects that could be anticipated from the inhibition of M_5 receptors within the body in general.

A major concern in the development of a treatment for drug abuse is the potential that the treatment itself may become addictive and abused itself. While the compound may never shows signs of this property until administered to humans, animal studies could test for the potential of the compound to become an abused and addicted substance. Self-administration studies using mice could provide important insights on the abuse potential of the drug while withdrawal symptoms could be measured in mice or rats after a period of prolonged compound administration to indict any addiction to the treatment compound. From studies previously reported using M_5 receptor deficient mice, a reasonable hypothesis would be that a selective M_5 receptor antagonist would not have abuse potential nor would it have an addiction profile since mice without the M_5 receptor have shown less preference for abused substances as well as decreased withdrawal symptoms.

A larger concern may be the inhibition of normal and regular rewards because a M_5 antagonist would inhibit dopaminergic neurons from releasing dopamine in the NA by cholinergic innervations at M_5 receptors. While this may be a valid issue, it is important to look at the other receptors, neurons, and

circuitry in the area before jumping to conclusions. An M_5 antagonist would only inhibit M_5 receptors on dopaminergic neurons in the VTA, but M_5 receptors are not the only type of receptor on these neurons. Dopamine would still be released upon activation of nicotinic receptors on the neuron cell bodies and other neurons releasing GABA, glutamate, and other neurotransmitters would still have modulating effects upon dopaminergic neurons in the VTA. While all the other components would not be affected by a selective M_5 antagonist in theory, studies would still need to be performed with such a compound before reaching any definite conclusions.

A selective M_5 antagonist for the treatment of drug abuse has both pros and cons like any other therapeutic treatment. Advantages of such a compound are that the M_5 receptor is expressed mostly in the target areas for drug abuse, the VTA and SN, which would minimize side effects from activation of M_5 receptors in other locations. Although M_5 receptors are located in other areas, side effects of a selective M_5 antagonist would be presumably decreased as compared to the other selective muscarinic receptor antagonists where the receptors are more diversely spread throughout the body. Also a selective M_5 antagonist would not completely inhibit dopaminergic neurons within the VTA, thereby not completely inhibiting the reward circuitry for natural rewards such as food or sex. Some disadvantages of a selective M_5 antagonist would be that there could be the possibility of side effects from inhibiting M_5 receptors in other

locations rather than the target area. It has been observed by Takeuchi et al. that M_5 knockout mice drank more than twice as much water as wild-type mice after a prolonged period of food and water deprivation (Takeuchi, Fulton et al. 2002). Another disadvantage could be the possibility of inhibiting normal rewarding properties at any level, which could be observed in animal models. The advantages and disadvantages of a selective M_5 antagonist cannot be completely understood and classified until such a compound is identified and tested in animal models. Either way the advantages of creating such a compound, even for research use is an important tool for researchers.

Chapter 2

Materials & Methods

2.1 Cells

A cell line of A9 L cells transfected with the muscarinic M5 receptor created by Ms. Maggie Xu in Dr. Messer's laboratory were used for all studies. The A9 L cell line is a mouse fibroblast cell line that expresses no muscarinic receptor mRNA nor do the cells bind muscarinic ligands (Brann, Buckley et al. 1987). The A9 L cells were transfected with a plasmid encoding M_5 muscarinic receptors by Ms. Maggie Xu. The A9 L cells that express M_5 muscarinic receptors therefore have no other muscarinic receptors present. In addition, the antibiotic, G-418, is added to each plate of cells sub-cultured to select cells that express M_5 receptors. Cells are frozen and stored in liquid nitrogen until needed for use. All experiments were done during cell passages 17 through 37.

2.2 Cell Culture

Cells are cultured in Dulbecco's Modification of Eagle's Medium (DMEM) with 4.5 g/L glucose, L-Glutamine & sodium pyruvate. To each bottle of DMEM, 57.5 ml of Fetal Bovine Serum (FBS), 11.5 ml L-Glutamine, 2.9 ml PS (penicillin-streptomycin, 10,000 units in strength) is added. DMEM and L-Glutamine are purchased from Cellgro via Fisher, the penicillin-streptomycin is from Fisher, and the FBS is purchased from Atlanta Biologicals. Appropriate amounts of cells (1:10 dilution) are transferred to a new plate for sub-culture when the original plate reaches 85% to 95% confluence of cells. Prior to use for sub-culturing,

DMEM media, 1x trypsin, and G-418 are warmed in a water bath. The plates are removed from the incubator, the old media is removed and discarded, and the plates are washed with 10 ml of PBS. PBS is removed and approximately 14-17 drops of trypsin are added around the plates to cover cells with a thin coating of trypsin. Plates are incubated at 37° in a 5% CO_2 incubator for 4 minutes. Immediately following this incubation, 10 ml of media is added to the plates and aspirated to lift cells from the plates. A 1:10 dilution of cells is then transferred to a new plate that is prepared during the incubation period. Preparation of new plates during the incubation period begins by taking a fresh plate and adding 10 ml of media along with 100 µl of G-418 antibiotic. Plates are labeled with the cell line, date, and passage number. Once the incubation period is complete the appropriate amount of cells are added. The plates are mixed

gently to disperse the cells evenly and then incubated at 37° C with 5% CO_2 until ready for assays.

2.3 PI Turnover Assay

The phosphatidylinositol (IP_3) turnover assay has usefulness in measuring the activation and inhibition of G_q-linked G protein coupled receptors (Brandish, Hill et al. 2003), such as that of the M_5 muscarinic receptor. The A9 L cell line expressing the M_5 muscarinic receptor is labeled with myo-inisitol, which is incorporated into cellular phosphatidylinositol 4,5-bisphosphate, that with agonist (carbachol or acetylcholine) binding to the M_5 receptor, is hydrolyzed by phospholipase C to inositol 1,4,5-trisphosphate (IP_3) and diacylglycerol (Brandish, Hill et al. 2003). Dephosphorylation of IP_3 is blocked in the presence of Li^+ which thus allows for the mass of soluble phosphates to become a quantitative readout of receptor activation by positively charged yttrium silicate beads that bind inositol phosphates but not inositol itself (Brandish, Hill et al. 2003).

Once the A9 L cells expressing the M_5 muscarinic receptors plate had reached confluence the cells were lifted from the plate using trypsin and counted with a hemocytometer. The cells were then transferred to a 96 well round bottom plate at a concentration of 30,000 to 40,000 cells/well. The cells are incubated for 24-48 hours before being washed twice with 200 µL PBS. After the wells have been washed, 100 µL of ^{3}H-myo-Insitol DMEM media is added to

each well then allowed to be incubated at 37°C overnight. The following day drugs, compounds, or controls are added to the plate to equal a 100 µL addition into each well. The plate is incubated at 37° C with 5% CO_2 for 1 hour. The contents of the plate are then dumped and 100 µL of 50 mM formic acid is added and the plate is allowed to rest at room temperature for 20 minutes. After the rest period, 20 µL from each well is added to another 96 well plate containing 80µL of 80mg/L wheatgerm agglutinin coated yttrium silicate spa beads. The plate containing the 100 µL mix of spa beads and compound/cells/formic acid was then covered with a clear plastic adhesive sticker and placed on a shaker for 1 hour in a 4°C environment. The shaker was then turned off, but the plate was allowed to rest overnight in the 4°C environment. The plate was analysized by Top Count for the counts per minute (CPM) of radiolabeled inositol phosphate production the following day.

2.4 Results & Statistics

All experiments, except tamoxifen & hyoscyamine experiment, were completed in triplicate (n=3) with each individual experiment being done in triplicate. The tamoxifen and hyoscyamine experiment was completed in duplicate with each individual experiment being done in triplicate. Raw data from the Top Count was analysized using Graph Pad Prism and Microsoft Excel.

All statistics were done using Graph Pad Prism's Two Way ANOVA analysis using Bonferroni post-hoc test and results were normalized using Graph

Pad Prism to determine the percentage inhibition of the compounds tested. Each figure in the results section contains the p value for each experiment.

2.5 Materials

During the cell culture procedure explanation, any and all purchase information for the materials is mentioned chronologically throughout the sub-culturing description. However, all the compounds used for the PI turnover assay were too numerous to mention in the respective method section so therefore purchase information about the materials used for the PI turnover will be described below.

A majority of the test compounds and some materials used were purchased from Sigma which includes: Hepes acid free solution, acetylcholine, hyoscyamine, thioridazine, himbacine, pirenzepine, and trihexyphenidyl. The yttrium silicate scintillation proximity assay (SPA) beads were purchased from Perkin Elmer and the compounds tamoxifen and carbachol were bought from Acros. Amiodarone was bought from MP Biomedicals.

Several of the compounds used in the PI turnover assay were newly synthesized by medicinal chemists in Dr. Messer's laboratory. The compound CDD-0361 was synthesized by Dr. Aditya Maheshwari, while P.S. Shantanu Rao synthesized the other compounds used which include CDD-0369, CDD-0370, and CDD-0371F.

Throughout the PI turnover assay other solutions that were prepared in house have been used. These include 50 mM formic acid, the working HEPES solution in which the compounds were dissolved in, the ^{3}H-IF-DMEM for radiolabeling cells, and PBS used to wash the cells.

The HEPES solution was prepared in 50 ml aliquots in which 41.4 ml of distilled water, 2 ml of 0.5 M LiCl (made in house), 4.6 ml of 10x Hanks Balanced Salt Solution (HBSS purchased from Sigma), and 2 ml of 1M HEPES free acid solution was combined and mixing gently. The working HEPES solution was stored at 4°C until needed for use.

The ^{3}H-IF-DMEM was prepared in 30 ml aliquots that contains 0.18 g of fetal bovine serum (BSA), 30 ml of Inositol Free Dulbecco's Modification of Eagle's Medium (IF-DMEM) purchased from Cellgro via Fisher, 30 µl of L-glutamine (purchased from Cellgro via Fisher) and 300 µl of ^{3}H-myo-inositol. The solution is stored at 4°C but is allowed to warm to room temperature before use.

Chapter 3

Objective

Previous studies have indicated that a selective M_5 antagonist would be useful in the treatment of drug abuse and dependence for opioids and opioid derivatives, such as morphine, hydrocodone, and condone, as well as cocaine. Unfortunately, no compounds are available that are selective for the M_5 muscarinic receptor. An important introductory step in the discovery of a selective M_5 muscarinic antagonist is to validate a system for assessing compounds for antagonist properties at the M_5 receptor. Known non-selective muscarinic antagonists along with newly synthesized compounds and other compounds of interest were tested for their ability to block acetylcholine activity at M_5 muscarinic receptors. Compounds that do indeed have antagonist properties then can be used as lead compounds for further chemical modification or be used in determining the structural properties that lead to the binding and blockade of M_5 receptor activity.

The present studies employed commercially available non-selective muscarinic antagonists, as well as newly synthesized compounds and other compounds of interest. Compound were tested for antagonistic properties at a low (1 μM) and intermediate (100 μM) concentrations, alone and in the presence of an EC_{80} concentration of acetylcholine (4 μM) in a M_5 muscarinic receptor expressing cell line.

Chapter 4

Results

4.1 Acetylcholine & Carbachol

Past studies have used the phosphoinositide turnover (PI) assay to assess inositol phosphate generation in A9 L cells expressing M_5 muscarinic receptors to measure compound properties in the presence of carbachol. While carbachol has a similar structure to acetylcholine as well as being an agonist at muscarinic receptors including M_5 receptors, it is not the endogenous ligand of the muscarinic receptors. The endogenous ligand to muscarinic receptors is acetylcholine so to ensure that the assay system would accurately reflect what previous studies have shown, full dose response curves of carbachol and acetylcholine were performed as shown on in Figure 4.1.Once the full dose response curve of acetylcholine was produced, the EC_{80} concentration of acetylcholine was determined using GraphPad Prism. The EC_{80} concentration of acetylcholine was determined to be approximately 4 µM and this concentration of acetylcholine was used in all subsequent studies.

Figure 4.1

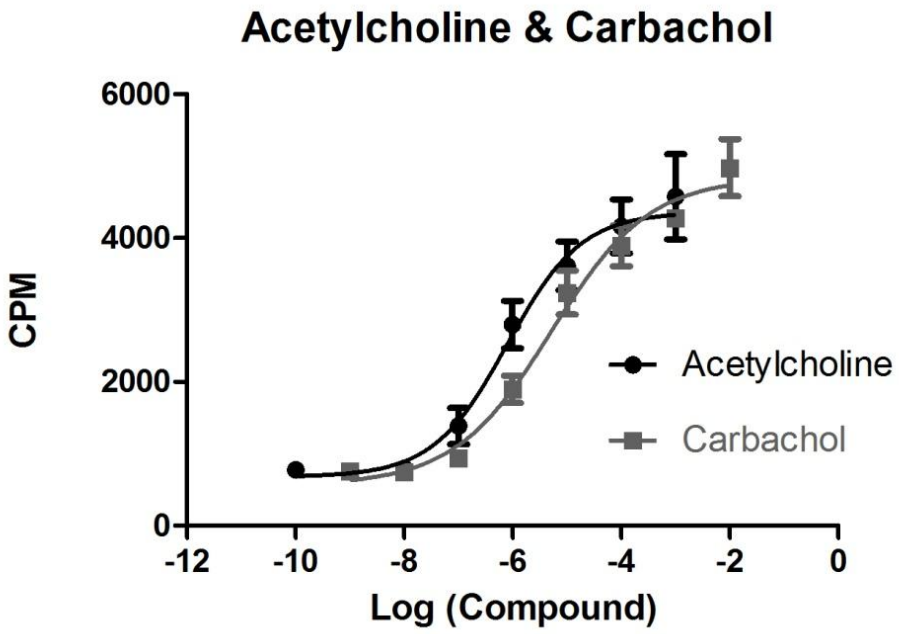

Figure 4.1: Acetylcholine is more potent than carbachol except upon reaching the saturation limit. All results are shown as the mean ± SEM

4.2 Hyoscyamine

Hyoscyamine is an active natural product that is the L-isomer of atropine. It is a non-selective muscarinic antagonist that has been used therapeutically for irritable bowel syndrome (Goodman, Gilman et al. 2006). Hyoscyamine was the first compound tested that had very potent antagonist effects in the presence of acetylcholine. By itself, hyoscyamine has no effects different than that of the control, HEPES which is represented as 0 hyoscyamine as shown by the CPM values of each. However, the concentrations of hyoscyamine which has been used for the remainder of compounds, 1 µM and 100 µM, both have complete inhibition in the presence of the EC_{80} value of acetylcholine. When the concentration was reduced to 1 nM hyoscyamine a marked reduction of activation of acetylcholine is seen. Consequently a dose dependent manner of acetylcholine blockage can be seen at the EC_{80} of acetylcholine in the presence of hyoscyamine. Overall there is a significant interaction between the presence and absence of acetylcholine at the different concentrations of hyoscyamine ($P<0.02$), with the varying concentrations of hyoscyamine having a significant effect ($P<0.01$) on the overall results, and the presence of acetylcholine has a significant effect ($P<0.05$) on the results.

Figure 4.2

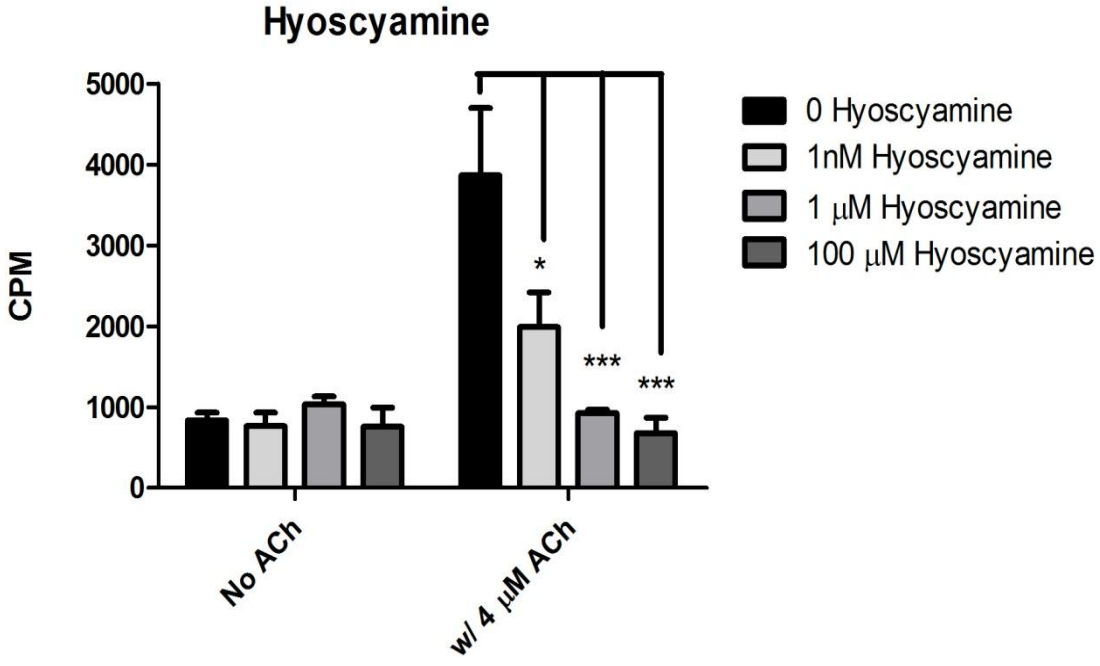

Figure 4.2: Hyoscyamine alone and in the presence of ACh at very low (1 nM), low (1 µM) and intermediate (100 µM) doses of hyoscyamine. All results are shown as the mean ± SEM. Results were analyzed by two way ANOVA; * indicates a significant statistical difference of $P<0.05$ between 4 µM ACh and 1 nM Hyoscyamine, ***indicates a very significant statistical difference of $P<0.001$ between 4 µM ACh and 1 µM hyoscyamine and 100 µM hyoscyamine.

4.3 Trihexyphenidyl

The compound trihexyphenidyl was also tested in the screening method for inositol phosphate production. Trihexyphenidyl is a tertiary amine (Goodman, Gilman et al. 2006) that acts as a M_1 selective muscarinic antagonist that may have possible binding at dopamine receptors. It is currently available as a therapeutic agent for the treatment of Parkinson's Disease. When the compound trihexyphenidyl was tested in the M_5 muscarinic receptor expressing cell line, very potent antagonistic effects were observed at both the low and intermediate concentrations of the compound in the presence of acetylcholine as shown in Figure 4.3. Trihexyphenidyl when alone at either concentration had no significant effect on inositol phosphate production in cells expressing the M_5 muscarinic receptor. At the two concentrations of trihexyphenidyl in the presence of acetylcholine, the inositol phosphate production was significantly decreased to the same level as that of control levels. . Overall there was a significant interaction between the presence and absence of acetylcholine at the different concentrations of trihexyphenidyl ($P<0.0002$), the varying concentrations of trihexyphenidyl also significantly ($P<0.0002$) decreased acetylcholine responses, and the presence of acetylcholine had a significant effect ($P<0.0003$) of inositol phosphate production within the cells. , and the presence of acetylcholine had a significant effect ($P<0.0003$) of inositol phosphate production within the cells.

Figure 4.3

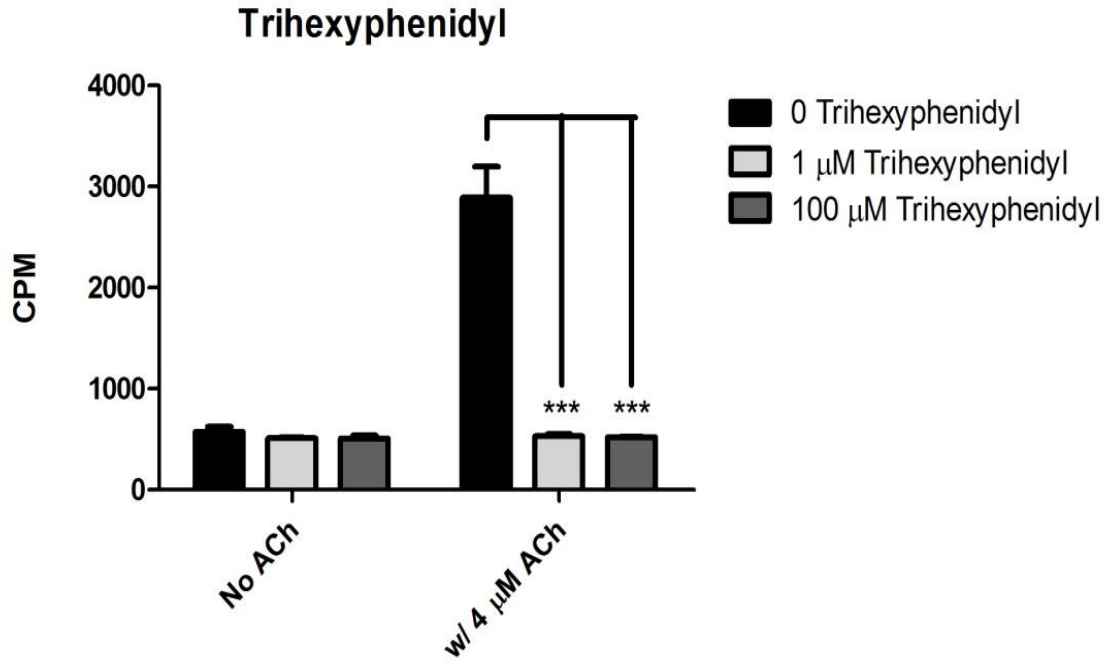

Figure 4.3: Trihexyphenidyl alone and in the presence of ACh at low (1 μM) and intermediate (100 μM) doses. All results are shown as the mean ± SEM. Results were analyzed by two way ANOVA; *** indicates an extremely significant statistical difference of $P<0.002$ between 4 μM ACh and 1 μM trihexyphenidyl and 100 μM trihexyphenidyl.

4.4 Pirenzepine

Pirenzepine is a partially selective muscarinic antagonist for M_1 muscarinic receptors but has similar affinities for the M_4 receptor also (Goodman, Gilman et al. 2006). It is a tricyclic drug that has inhibits gastric acid secretion at doses that have little effect on salivation and heart rate (Goodman, Gilman et al. 2006). Pirenzepine is used in some countries for peptic ulcer diseases because of its therapeutic profile (Goodman, Gilman et al. 2006). When pirenzepine was placed into the screening method a dramatic effect was observed in the presence of acetylcholine (Figure 4.4). At the low, 1 µM, concentration of pirenzepine in the presence of acetylcholine, there is a significant inhibition acetylcholine activated inositol phosphate production. Also the intermediate concentration of pirenzepine, 100 µM, in the presence of acetylcholine produced a significant decrease in M_5 receptor activation by acetylcholine that was significantly lower than that produced by the 1 µM concentration of pirenzepine in the presence of acetylcholine. Overall there was a significant interaction between the presence and absence of acetylcholine at the different concentrations of pirenzepine ($P<0.0003$), along with the varying concentrations of pirenzepine having a significant effect on the overall results ($P<0.0002$), and a significant effect of the presence of acetylcholine ($P<0.001$).

Figure 4.4

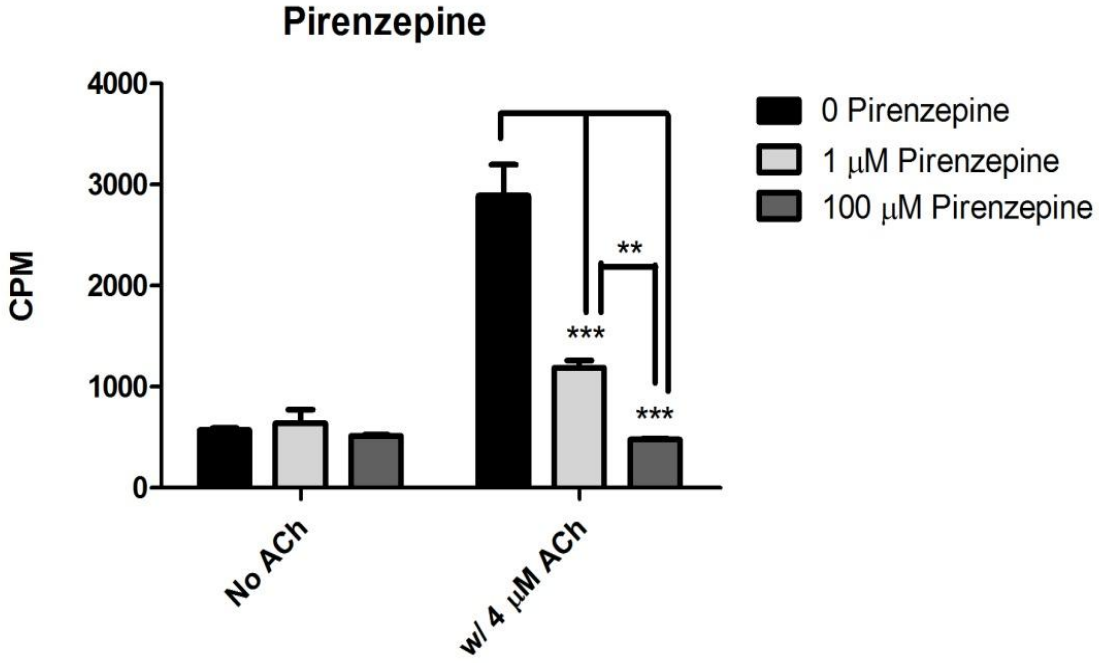

Figure 4.4: Pirenzepine alone and in the presence of ACh at low (1 μM) and intermediate (100 μM) doses. All results are shown as the mean ± SEM. Results were analyzed by two way ANOVA; ** indicates a significant statistical difference of $P<0.01$ between the 1 μM concentration of pirenzepine and 100 μM concentration of pirenzepine both in the presence of ACh, ***indicates an extremely significant statistical difference of $P<0.001$ between ACh and both the 1 μM and 100 μM concentrations of pirenzepine in the presence of ACh.

4.5 Himbacine

Himbacine is a complex piperidine alkaloid isolated from the bark of Australian magnolias with potent muscarinic receptor antagonist properties (Chackalamannil, Davies et al. 1999). It has more potent inhibitory properties at the M_2 and M_4 muscarinic receptors, which classifies himbacine as a selective muscarinic antagonist (Miller, Aagaard et al. 1992). When himbacine was tested in the PI turnover assay in the presence of acetylcholine (Figure 4.5), it produced a dose-dependent decrease in inositol phosphate production by acetylcholine. By itself, himbacine caused no increase in activation of the M_5 receptor above that of the control. At the lower concentration (1 μM) of himbacine, a modest decrease in inositol phosphate production was observed, however at the higher, 100 μM, concentration of himbacine, a significant decrease in acetylcholine activation of the M_5 receptor was seen. There also was a significant decrease in inositol phosphate production between both concentrations of himbacine in the presence of acetylcholine. Overall there was a significant interaction between the presence and absence of acetylcholine at the different concentrations of himbacine ($P<0.03$), with the varying concentrations of himbacine having a significant, effect on the overall results ($P<0.02$), and a significant effect of the presence of acetylcholine ($P<0.002$).

Figure 4.5

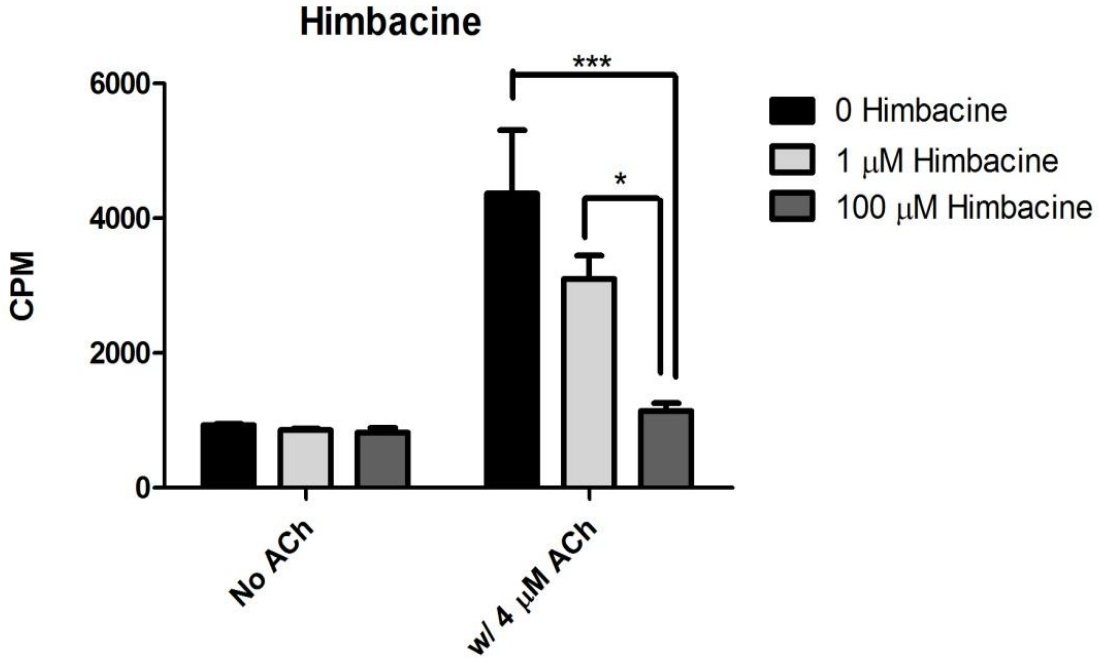

Figure 4.5: Himbacine alone and in the presence of ACh at low (1 μM) and intermediate (100 μM) doses. All results are shown as the mean ± SEM. Results were analyzed by two way ANOVA; * indicates a significant difference of $P<0.05$ between the two concentrations, 1 μM and 100 μM, of himbacine in the presence of ACh, *** indicates a significant difference of $P<0.001$ between ACh and the 100 μM concentration of himbacine in the presence of ACh.

4.6 Thioridazine

The compound thioridazine is a potent, non-selective dopamine antagonist that also has high affinity for muscarinic receptors. (Goodman, Gilman et al. 2006). When used clinically as an antipsychotic, thioridazine has a lower incidence of adverse extrapyramidial effects due to its muscarinic antagonist properties (Goodman, Gilman et al. 2006). At M_5 receptors within the screening method, thioridazine causes a significant decrease in acetylcholine activation at the M_5 receptor at both tested concentrations of thioridazine within the presence of acetylcholine (Figure 4.6). Interestingly, both concentrations of thioridazine produced very similar levels of inhibition, but not complete inhibition to the level of the control. Overall when thioridazine was tested in the screening method, a significant interaction between the presence and absence of acetylcholine at the different concentrations of thioridazine of was observed ($P<0.02$) with a significant effect observed with the presence of acetylcholine ($P<0.003$).

Figure 4.6

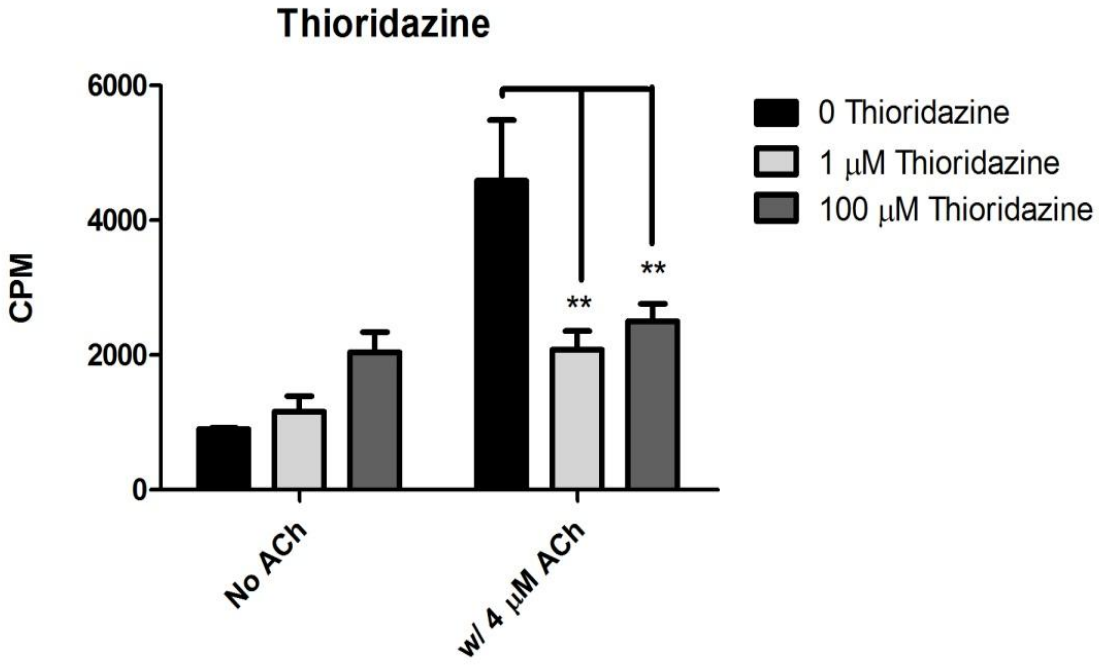

Figure 4.6: Thioridazine alone and in the presence of ACh at low (1 μM) and intermediate (100 μM) doses. All results are shown as the mean ± SEM. Results were analyzed by two way ANOVA; ** indicates a very significant statistical difference of $P<0.05$ between HEPES and ACh, # indicates a statistical difference of CPM of $P<0.01$ between ACh and either the 1 μM or 100 μM concentrations of thioridazine in the presence of ACh.

4.7 CDD-0361

Along with commercially available compounds, newly synthesized compounds were tested for antagonistic properties in the M_5 receptor expressing cells. The first newly synthesized compound that was utilized in this assay was CDD-0361, which is a novel compound synthesized by Dr. Aditya Maheshwari. The compound CDD-0361 had previously been tested in the presence of carbachol at M_1, M_3, and M_5 receptors but not for its ability to block the effects of acetylcholine. When CDD-0361 was tested with carbachol there was a modest inhibition of inositol phosphate production was observed with a slight preference towards M_5 receptors. In the current screening method, CDD-0361 produced a modest dose dependent inhibition of acetylcholine stimulated inositol phosphate production at the low and intermediate concentrations. Alone, CDD-0361 produced no change in M_5 receptor activity as compared to the control. Overall, in these experiments, acetylcholine had a significant effect on M5 receptor activity ($P<.0001$).

Figure 4.7

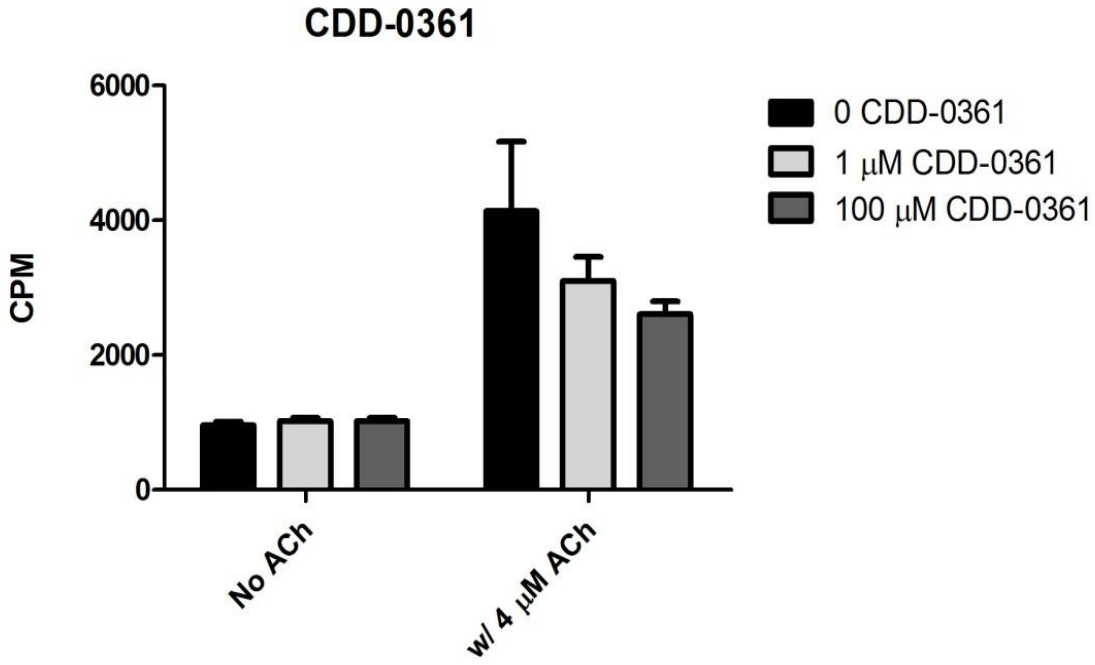

Figure 4.7: CDD-0361 alone and in the presence of ACh at low (1 μM) and intermediate (100 μM) doses. All results are shown as the mean ± SEM. Results were analyzed by two-way ANOVA.

4.8 CDD-0361 Full Dose Curves

The compound CDD-0361 is a newly synthesized compound, that unlike the other commercially available non-selective muscarinic antagonists utilized in the PI turnover assay, was synthesized in an attempt to create compounds that are M_5 receptor selective. Testing on CDD-0361 (shown in Figure 4.7) at low and intermediate concentrations in the presence of 4 μM (EC_{80} concentration) acetylcholine produced a modest dose dependent inhibition of inositol phosphate production. Further studies were conducted with CDD-0361 at the intermediate concentration and a high (2 mM) concentration. Acetylcholine dose response curves were generated in the presence and absence of CDD-0361 to further characterize the type of inhibition found at M_5 receptors. CDD-0361 inhibited activity at lower concentrations of acetylcholine (under approximately 100 μM) in a dose dependent manner at M_5 receptors.

Figure 4.8:

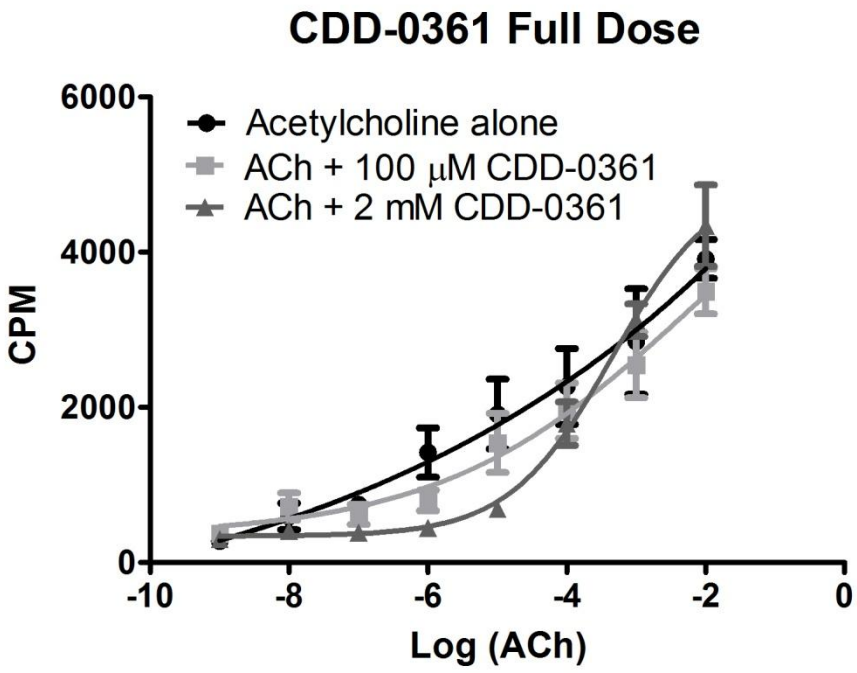

Figure 4.8: Dose response curves for acetylcholine alone and at differing concentrations (100 μM and 2 mM) CDD-0361. All results are shown as the mean ± SEM.

4.9 Amiodarone

Amiodarone is a widely used antiarrhythmic drug currently available as Cordaron® or Pacerone®. It is used for ventricular tachycardia arrhythmia or when other treatments are fibrillation resistant (Goodman, Gilman et al. 2006). Amiodarone acts in a noncompetitive manner blocking adrenergic receptors (Goodman, Gilman et al. 2006). Previous studies with amiodarone have found that amiodarone can enhance the maximal effect of acetylcholine but without increasing the potency of acetylcholine (Stahl and Ellis 2010); however the concentration of acetylcholine was much larger (1 mM) than those of this study. When amiodarone was utilized in the M_5 receptor expressing cells within the screening method, Figure 4.9, it caused only a modest decrease in acetylcholine-stimulated inositol phosphate production. Amiodarone did not increase M_5 receptor activity by itself. Overall, the presence of acetylcholine caused a significant increase in M_5 receptor activity ($P<0.0003$).

Figure 4.9:

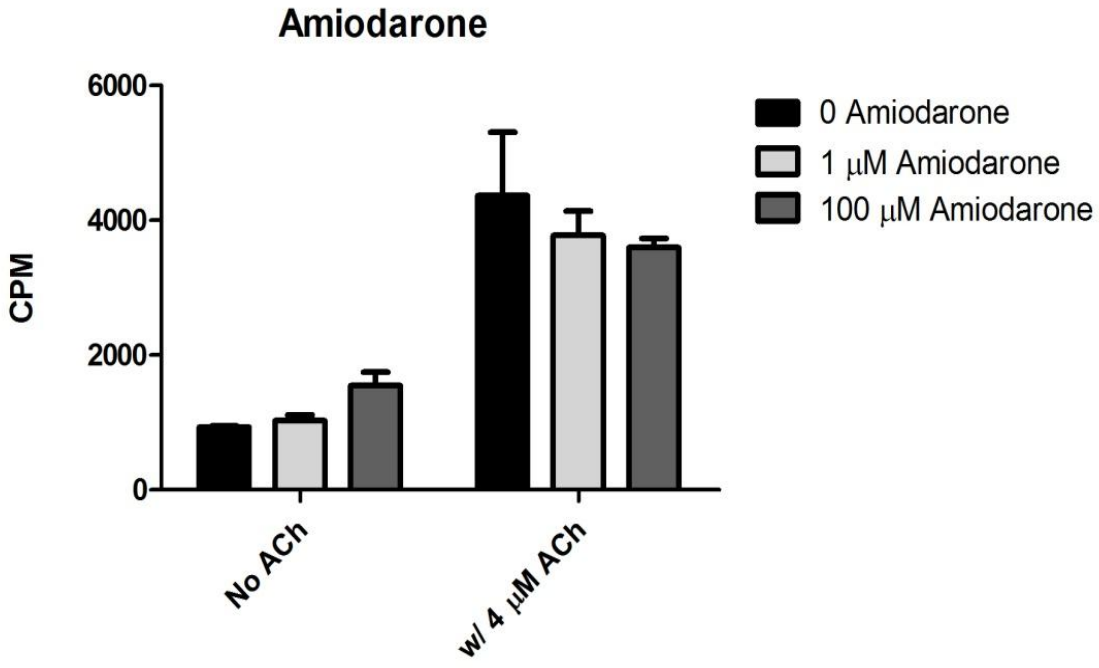

Figure 4.9: Amiodarone alone and in the presence of ACh at low (1 μM) and intermediate (100 μM) doses. All results are shown as the mean ± SEM. Results were analyzed by two way ANOVA.

4.10 Tamoxifen

The commercially available drug tamoxifen has been approved by the FDA as a treatment for breast cancer as well as a preventative agent for those who have a higher risk of developing breast cancer (Goodman, Gilman et al. 2006). Tamoxifen works by acting as a competitive antagonist at the estrogen receptor. It was originally developed in 1966 as an oral contraceptive but instead induces ovulation (Lazareno and Roberts 1989; Goodman, Gilman et al. 2006). Tamoxifen was tested in the M_5 receptor expressing cell line for antagonist activity because it has structural similarities to other compounds that were also tested that are of interest to our laboratory. When it was tested in the screening method complex characteristics were observed, Figure 4.10. At the low concentration of tamoxifen alone there was no difference in inositol phosphate production from the control, but at the intermediate, 100 μM concentration, tamoxifen significantly increased inositol phosphate production from that of the control ($P<0.01$). The 100 μM concentration of tamoxifen alone also significantly increased inositol phosphate production as compared to the 1 μM concentration of tamoxifen. When either concentration of tamoxifen is in the presence of acetylcholine, there is no significant difference between the inositol phosphate production between those and acetylcholine. Further, there is also a significant difference between the inositol phosphate production between the low and intermediate concentrations of tamoxifen in the presence of acetylcholine. The

two-way-ANOVA analysis revealed that there is a significant effect from the concentrations of tamoxifen on the inositol phosphate production ($P<0008$). Also the presence of acetylcholine causes a significant effect of on inositol phosphate production ($P<0.0002$). It should be noted that tamoxifen is not known to a muscarinic antagonist and was utilized in the screening method due to the compound's structural similarities to other compounds of interest to the laboratory. With the complex results observed, especially since there was an increase in inositol phosphate production of tamoxifen alone within the screening method, the statistical evidence given should not be taken as final. The statistics were given for a complete analysis of what the results reflect mathematically but tamoxifen's activity alone at the 100 μM concentration raises questions that need to be addressed.

Figure 4.10:

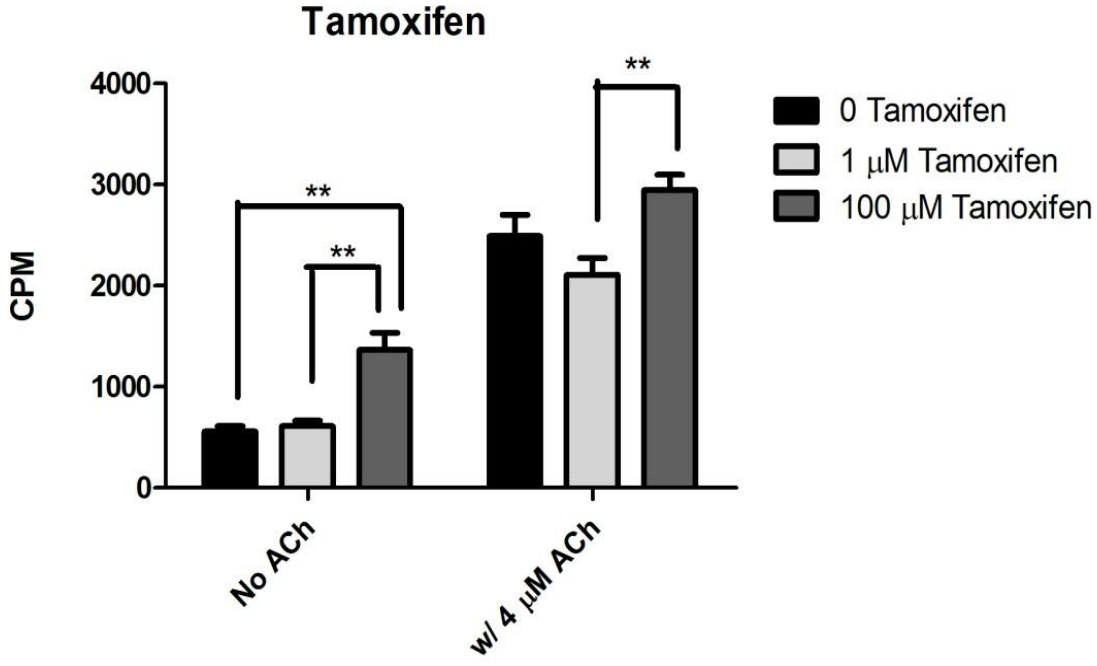

Figure 4.10: Tamoxifen alone and in the presence of ACh at low (1 μM) and intermediate (100 μM) doses. All results are shown as the mean ± SEM. Results were analyzed by two way ANOVA; ** indicates a very significant statistical difference of $P<0.01$ between columns connected by the bars.

4.11 Tamoxifen v Hyoscyamine

As mentioned previously, tamoxifen is not a muscarinic antagonist yet increased inositol phosphate production at the intermediate, 100 µM, concentration in the absence of acetylcholine. Further experiments were conducted to determine whether the increased inositol phosphate production was through M_5 receptors or was though another mechanism. Tamoxifen was tested in the presence of hyoscyamine which inhibits acetylcholine-stimulated inositol phosphate production. The hypothesis was that if tamoxifen's activity was mediated though M_5 receptors, then the presence of an M_5 receptor antagonist, such as hyoscyamine, should inhibit its activity. However when tamoxifen was tested in the presence of hyoscyamine, there was no change in the level of inositol phosphate production. This led to the conclusion that tamoxifen induced inositol phosphate production through a mechanism other than M_5 receptor activation.

Figure 4.11:

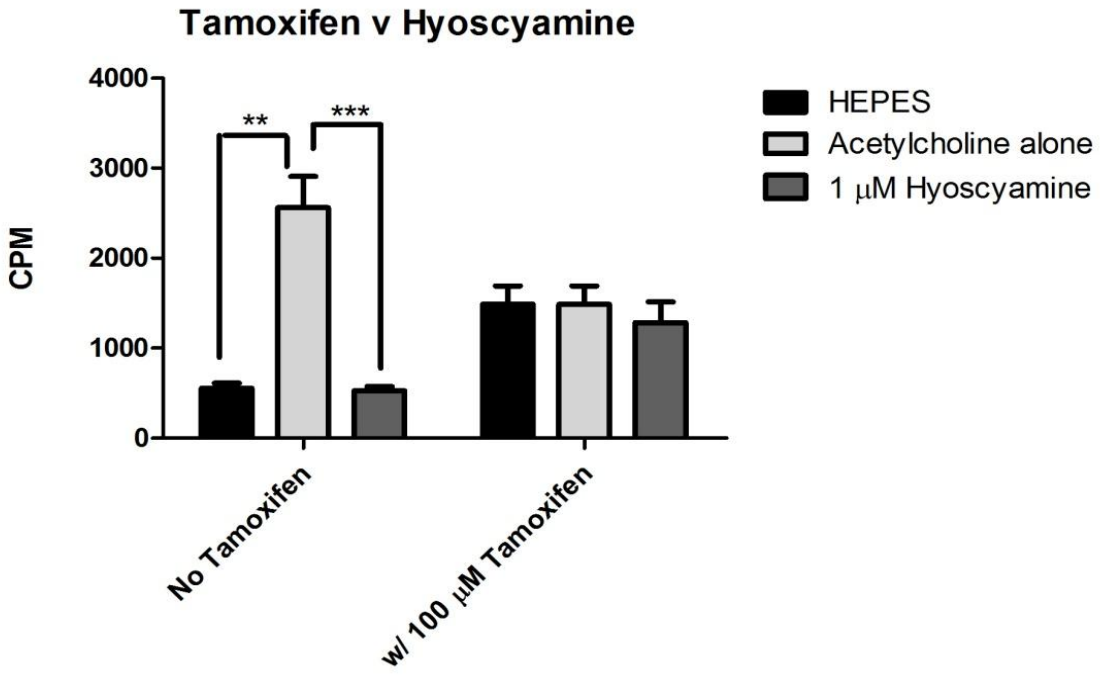

Figure 4.11: The control, HEPES, 4 μM ACh, and 1 μM hyoscyamine without the addition of tamoxifen and 100 μM tamoxifen alone, in the presence of ACh and in the presence of 1 μM hyoscyamine. All results are shown as the mean ± SEM. Results were analyzed by two way ANOVA; ** indicates a very significant statistical difference of $P<0.01$ between HEPES and ACh, ***indicates an extremely significant statistical difference of $P<0.001$ between ACh and 1 μM hyoscyamine.

4.12 CDD-0369

CDD-0369 was synthesized by P.S. Shantanu Rao. The compound has similar structural components as amiodarone as well as tamoxifen. When CDD-0369 was utilized in the screening method, it was observed that at both low and intermediate concentrations of CDD-0369 alone there was no activation of M_5 receptors when compared to the control HEPES. In the presence of acetylcholine, CDD-0369 at either low or intermediate concentrations did not inhibit inositol phosphate production. Overall, the two-way-ANOVA revealed that when CDD-0369 was tested in the screening method, a significant interaction between the presence and absence of acetylcholine at the different concentrations of CDD-0369 ($P<0.0001$).

Figure 4.12:

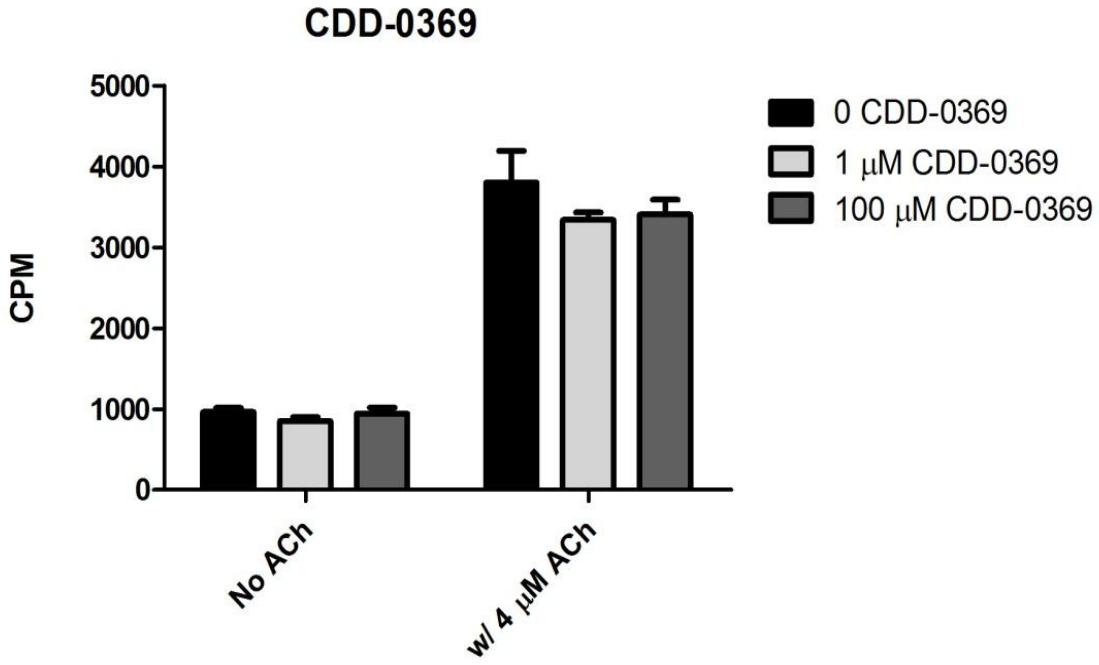

Figure 4.12: CDD-0369 alone and in the presence of ACh at low (1 μM) and intermediate (100 μM) doses. All results are shown as the mean ± SEM. Results were analyzed by two way ANOVA.

4.13 CDD-0370

The newly synthesized compound CDD-0370 was also utilized in the screening method. CDD-0370 also was synthesized by P.S. Shantanu Rao and shows structural similarities to amiodarone, tamoxifen, and CDD-0369. When tested CDD-0370 has similar characteristics to that of CDD-0369 in that at the low and intermediate concentrations of CDD-0370 when alone, has no inositol phosphate production from M_5 receptor activation. Also CDD-0370 at both concentrations in the presence of acetylcholine shows no inhibition of inositol phosphate production. Overall CDD-0370 when tested in the screening method, had a significant interaction between the presence and absence of acetylcholine at the different concentrations of CDD-0370 ($P<0.0003$).

Figure 4.13:

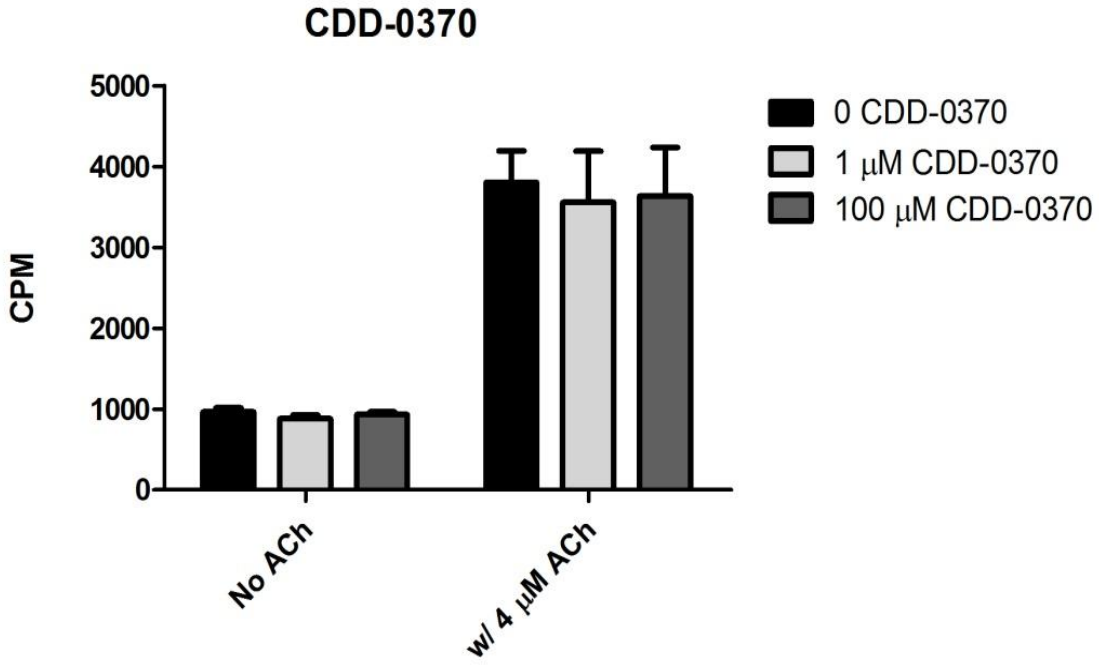

Figure 4.13: CDD-0370 alone and in the presence of ACh at low (1 μM) and intermediate (100 μM) doses. All results are shown as the mean ± SEM. Results were analyzed by two way ANOVA.

4.14 CDD-0371F

The last compound that was synthesized by P.S. Shantanu Rao is CDD-0371F. This compound also shares structural similarities to amiodarone, tamoxifen, CDD-0369 and CDD-0370. When CDD-0371F was utilized in the PI turnover assay through M_5 receptor expressing cells there was little effect observed on inositol phosphate production of CDD-0371F at either concentration tested on its own. CDD-0371F did not inhibit inositol phosphate production at either concentration in the presence of acetylcholine. Overall acetylcholine produced a significant increase in M5 receptor activity ($P<0.0002$).

Figure 4.14:

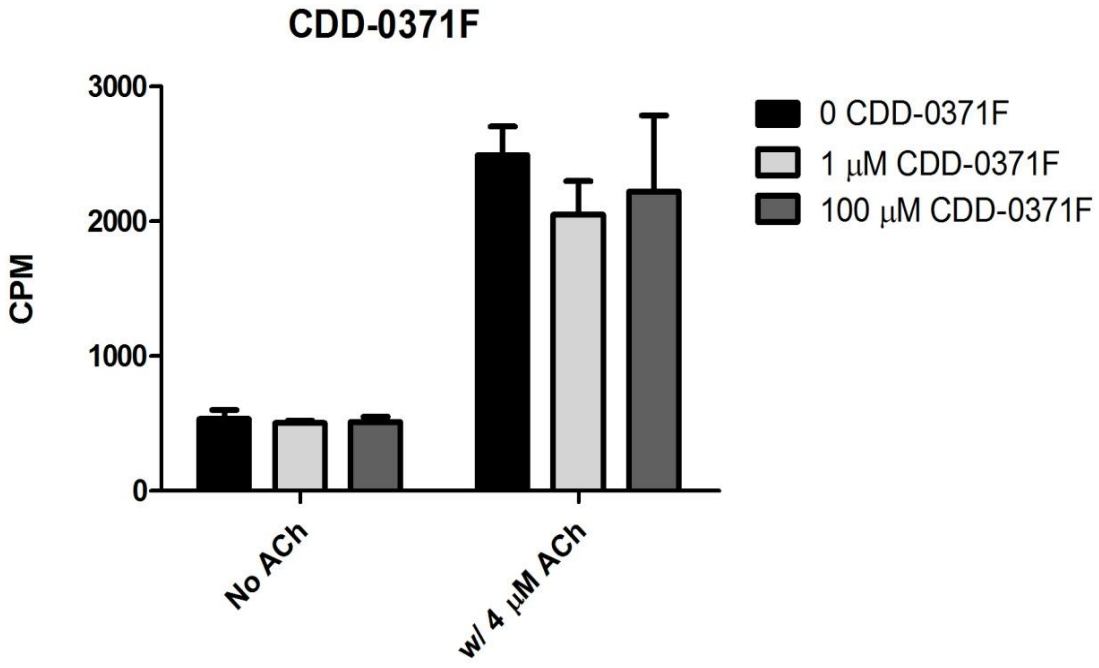

Figure 4.14: CDD-0371F alone and in the presence of ACh at low (1 μM) and intermediate (100 μM) doses. All results are shown as the mean ± SEM. Results were analyzed by two way ANOVA.

Chapter 5

Discussion

A M_5 muscarinic receptor antagonist would be useful in treating drug abuse and addiction for opioid, opioid derivates and cocaine as shown in previous studies in mice lacking M_5 muscarinic receptors. An antagonist of only the M_5 receptor would be of the greatest use because most side effects seen in muscarinic receptor antagonists arise from cross activation of other muscarinic receptors or subtypes in different locations than the target location. While the other muscarinic receptor subtypes have diverse and widespread functions throughout the body, the M_5 receptor has the greatest concentration of receptors, still at low levels, within the dopaminergic neurons in the VTA and substantia nigra. The M_5 receptor also has a low abundance of receptors throughout the rest of the body which would decrease side effects and cross activation of M_5 receptors at other locations.

Difficulties have arisen with synthesizing compounds that are selective for only one subtype of muscarinic receptor due to the high degree of homology between all the muscarinic receptor subtypes. This high homology has prevented successfully synthesizing an M_5 selective muscarinic receptor antagonist. An important introductory step in determining what types of compounds may be useful as M_5 muscarinic receptor antagonists is to categorize known muscarinic antagonists as active or inactive at M_5 receptors.

In order to test muscarinic antagonists, a screening method has been constructed to test compounds in a cell line expressing M_5 muscarinic receptors by measuring the concentration of soluble inositol phosphates which is a downstream effect of the G-protein, G_q, that is linked with the M_5 receptor. By measuring the change in the levels of inositol phosphates which is conveyed by counts per minute (CPM) compared to that of an EC_{80} concentration of acetylcholine, the compounds tested can be observed to have varying degrees of an antagonistic effect or no antagonistic effect.

The compounds are tested alone at low and intermediate concentrations, 1 μM and 100μM respectfully, for most compounds, in order to observe the activity of the compound at the M_5 receptor alone. This ensures that the compound either does not have any activity by itself or it has some activity alone. The compounds are also tested at the low and intermediate concentrations in the presence of the EC_{80} concentration, 4 μM, of acetylcholine to observe the activity

of the compound in a competitive manner with acetylcholine at the M_5 receptor. HEPES, which most of the compounds used in this study are dissolved in, is used as the negative control and allows for a comparison if a compound decreases the inositol phosphate production to levels below that of the basal level. However not all the compounds tested were soluble in HEPES, in those cases the compounds were dissolved into a HEPES solution containing various percent concentrations of DMSO. A control of DMSO in HEPES was then also done for those compounds in which the solutions also contained DMSO. The controls containing DMSO were tested at the highest concentration of DMSO that was added to the stock solutions of compounds.

The screening method was first tested to ensure proper accuracy by completing in triplicate full dose response curves of acetylcholine and carbachol. Carbachol was used in previous studies and binds at a M_5 receptor at a lower affinity than acetylcholine. After determining the EC_{80} concentration of acetylcholine, subsequent studies ensued to observe antagonistic effects of eleven compounds of either muscarinic antagonists, novel compounds, or compounds with structural similarities to other compounds of interest to the laboratory.

The compounds tested using the screening method reflect the functional activity of the compounds at M_5 receptors but not the binding of the compounds at the M_5 receptors. The screening method reflects the inhibition of inositol phosphate production via M_5 receptors, in which the buildup of inositol

phosphates ensues from biochemical inhibition by lithium chloride to cause degradation of inositol phosphates which naturally occurs. Activation of the M_5 receptors, which is linked to the G_q G-protein, initiates a downstream signaling cascade in which G_q proteins activate phospholipase C (PLC) which in turn cleaves phosphatidylinositol 4,5-bisphosphate (PIP_2) to diacyl glycerol (DAG) and inositol triphosphate (IP_3). IP_3 then binds to endoplasmic reticulum bound receptors which releases intracellular calcium. With the addition of lithium chloride IP_3 and other inositol phosphates that are negatively charged build up and bind to positively charged yttrium silicate beads which allow for scintillation counting and a measurement of activation of the M_5 receptor.

The compounds were also ranked in order of inhibition at the 1 μM concentration of the compound in the presence of acetylcholine with trihexyphenidyl, and hyoscyamine being the most potent inhibitors and CDD-0370 and amiodarone being the least. The full list of rankings for 1 μM concentrations of compound in the presence of acetylcholine amount of inhibition is as follows: Trihexyphenidyl (102%), hyoscyamine (101%), pirenzepine (73%), thioridazine (67%), himbacine (33%), CDD-0361 (26%), CDD-0371 (20%), tamoxifen (18%), CDD-0369 (13%), CDD-0370 (12%), and amiodarone (10%). When the lists of inhibition have been averaged together with the compounds that inhibit acetylcholine activation of inositol phosphate production

the most, the order of inhibition of the compounds are as follows: Trihexyphenidyl, hyoscyamine, pirenzepine, himbacine, thioridazine, CDD-0361, CDD-0371, CDD-0369, amiodarone, CDD-0370, and tamoxifen.

Of the eleven compounds utilized within the screening method a rank order of inhibition from greatest inhibition to least can be assigned by either concentration tested, 1 µM or 100 µM, of compound. The compounds have been ranked by calculating the amount of inhibition they have caused by normalizing the data. The EC_{80} concentration was set at zero percent inhibition and the controls for the compounds were set as 100 percent inhibition with the compounds then being calculated for their inhibitory actions accordingly. The ranking of the compounds level of inhibition at the 100 µM concentration of compound, of either novel compounds or muscarinic antagonists, has been calculated with pirenzepine and hyoscyamine both having more than 100% inhibition and CDD-0369, CDD-0370, and CDD-0371 having the least inhibition. The full list of rankings is as follows: Pirenzepine (104%), trihexyphenidyl (102%), hyoscyamine (102%), himbacine (95%), thioridazine (47%), CDD-0361 (42%), amiodarone (12%), CDD-0369 (12%), CDD-0371 (9%), CDD-0370 (9%), tamoxifen (-26%). Amiodarone and tamoxifen are not known to be muscarinic antagonists of any kind and as stated previously, are antiarrhiymic and breast cancer treatments respectively.

While the compounds' abilities to inhibit inositol phosphate production at different concentrations are important in a ranked list format, the figures of the compounds are no less important. The data presented here are the functional evaluation of the compounds and not the binding abilities of the compounds. From previous studies done with A9 L cells expressing M_5 receptors the binding affinities of the most of the muscarinic antagonists measured by the displacement of specific [^{3}H]-(R)-quinuclidinyl benzilate binding has been reported. The pK_i values (±SEM) for himbacine, pirenzepine, trihexyphenidyl and thioridazine are reported as 6.5 ± 0.05, 7.5 ± 0.12, 7.3 ± 0.06, and 7.4 ± 0.02 respectively.

Together the binding affinities and the functional assays represent a more accurate picture of the compounds than either can do apart. The compounds pirenzepine and trihexyphenidyl are both very potent inhibitors and have higher affinity for M_5 receptors some of the other compounds. While the evaluation of muscarinic antagonists at M_5 receptors is important, the evaluation of novel compounds is also very important. The novel compound CDD-0361, which had M_5 inhibition in mind when synthesized, portrays a complicated depiction of activation of M_5 receptors while acetylcholine is present. The other newly synthesized compounds CDD-0369, CDD-0370, and CDD-0371 are compounds that have not decrease inositol phosphate production as compared to the EC_{80} concentration inositol phosphate production levels.

Chapter 6

Conclusions

Overall a screening method was developed to test for M_5 muscarinic antagonists using an A9 L cell line transfected with the M_5 muscarinic receptor. The assay system utilized carbachol and acetylcholine to ensure accuracy by creating full dose response curves of each. After the full dose response curve of acetylcholine was preformed, an EC_{80} concentration was then calculated for acetylcholine and this concentration was used for the testing of consequent compounds. Muscarinic antagonists, newly synthesized compounds, and a few compounds of interest were then employed into the screening method with eleven compounds being tested overall.

Of the eleven compounds tested, three major categories can be described to classify the compounds upon their properties. The compounds hyoscyamine, trihexyphenidyl, and thioridazine can be classified as muscarinic receptor antagonists that have M_5 muscarinic activity, with hyoscyamine, trihexyphenidyl, and pirenzepine acting as very potent inhibitors. Thioridazine while dramatically inhibiting acetylcholine binding to M_5 receptors did not completely inhibit binding to basal level. Other compounds act in a

concentration dependent manner at M_5 receptors. These compounds include himbacine, and CDD-0361. A second major category of compounds are those that show no antagonist activity. These include the newly synthesized compounds CDD-0369, CDD-0370, and CDD-0371. The last major category would include compounds that are not known to be muscarinic antagonists but have interesting properties making them desirable to utilize in the screening method. The compounds that would be included into this category would be amiodarone and tamoxifen.

A few of the compounds displayed interesting characteristics that led to further experiments. The compound CDD-0361 is a newly synthesized compound that exhibited a dose dependent inhibitory effect when tested with acetylcholine. Full dose response curves using CDD-0361 were then completed using an intermediate and high concentration of the compound which resulted in a unique dose response curve. The curve at low concentrations of acetylcholine suppressed acetylcholine activity but at higher concentrations potentiated the response of acetylcholine.

Tamoxifen was another compound that exhibited interesting characteristics that warranted further investigating. The intermediate concentration of tamoxifen alone increased inositol phosphate production, but as the compound is known to act at estrogen receptors and not muscarinic receptors the cause for the increases of inositol phosphate production needed to be

addressed. Tamoxifen was placed in the presence of hyoscyamine, a potent antagonist at the M_5 receptor, to test whether the increased inositol phosphate production was caused by M_5 receptor activation or through another mechanism. The experiment resulted in concluding that M_5 receptor activation was not responsible for the increase of inositol phosphate production.

Future directions for this research and screening methods are diverse. This screening method could be used for future studies with any newly synthesized compounds, any unique compounds of interest, or to observe full dose response curves with any compound at the M_5 muscarinic receptor. Compounds that are developed or become of interest to the laboratory could be tested in the existing method for M_5 antagonist properties. The method could also be manipulated using other subtypes of muscarinic receptors, M_1 and M_3, to test compounds previously tested in the M_5 screening method or future compounds at whichever muscarinic receptor of interest, but testing at the three G_q G-protein linked muscarinic receptors is important for full understanding of the compounds. A screening method for antagonistic properties of compounds at M_2 and M_4 receptors also needs to be developed for a full analysis of the compounds tested in the M_5 screening method or future compounds.

These studies provided an introductory step in creating a selective M_5 muscarinic receptor antagonist. The compounds which have shown to be potent M_5 receptor antagonists could be further studied for structural similarities that

contribute to inhibition of acetylcholine. Also the compounds that are potent M_5 receptor antagonists should be tested in other muscarinic subtype systems to observe which subtypes the compounds are active at. If the compounds are active at all of the muscarinic receptor subtypes, then the usefulness at the M_5 receptor may diminish, but if the compounds are not active at other subtypes or only at higher concentrations then the compounds are potentially more useful.

Only with contributing sources of research and synthesis can a selective M_5 muscarinic receptor antagonist be produced. Only after the discovery of such a compound can other options for drug abuse and treatment become a feasible possibility. Further studies and research would still need to be completed but once a compound is produced then subsequent studies become much easier to perform. Animal studies to observe any concerns which the compound may have would need to be completed, as well as pharmacokinetic and pharmacodynamics studies to fully understand the actions of a selective M_5 antagonist on the body before possibly treating drug abuse and addiction in the human population.

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