The University of Toledo
The University of Toledo Digital Repository

Theses and Dissertations

2014

Pharmacological evaluation of a putative M5 antagonist at M1, M3 and M5 receptors

Teng Yan
University of Toledo

Follow this and additional works at: http://utdr.utoledo.edu/theses-dissertations

Recommended Citation
Yan, Teng, "Pharmacological evaluation of a putative M5 antagonist at M1, M3 and M5 receptors" (2014). *Theses and Dissertations*. 1722.
http://utdr.utoledo.edu/theses-dissertations/1722

This Thesis is brought to you for free and open access by The University of Toledo Digital Repository. It has been accepted for inclusion in Theses and Dissertations by an authorized administrator of The University of Toledo Digital Repository. For more information, please see the repository's About page.

A Thesis

entitled

Pharmacological Evaluation of a Putative M_5 Antagonist at
M_1, M_3 and M_5 Receptors

by

Teng Yan

Submitted to the Graduate Faculty as partial fulfillment of the requirements for the

Master of Science Degree in

Pharmaceutical Sciences

Dr. William S. Messer Jr., Committee Chair

Dr. Ming-Cheh Liu, Committee Member

Dr. Youssef Sari, Committee Member

Dr. Komuniecki, Dean
College of Graduate Studies

The University of Toledo

August 2014

Copyright 2014, Teng Yan

This document is copyrighted material. Under copyright law, no parts of this document may be reproduced without the expressed permission of the author.

An Abstract of

Pharmacological Evaluation of a Putative M_5 Antagonist at
M_1, M_3 and M_5 Muscarinic Receptors

by

Teng Yan

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

The University of Toledo

August 2014

Drug abuse and addiction is a major problem in the United States. Studies have shown that M_5 muscarinic receptors may be a target for the treatment of drug addiction because of their unique locations and functions in the brain reward system. Selective antagonists for M_5 muscarinic receptors might be useful in the treatment of drug abuse. GZ-002-05 was identified previously as a novel, M_5-selective muscarinic antagonist. Muscarinic receptor selectivity was characterized by measuring the effects of acetylcholine in the presence or absence of the compound using CHO cells expressing human M_1, M_3, or M_5 muscarinic receptors. A [^{3}H] arachidonic acid release assay measured receptor activity and helped delineate the nature of the interaction(s) between the compound and muscarinic receptor subtypes. The results suggest that GZ-002-05 may be very useful as a lead compound in the development of new therapeutic agents for the treatment of drug abuse.

For my love and family.

Acknowledgements

I would like to thank and give my appreciation to Dr. William S. Messer, my advisor, for the support and encouragement during the last two years. I have learned a lot from him not only in my research field, but also about life attitude. I am grateful to him for allowing me to be a part of his group and research plans.

I also would like to thank my thesis committee members Dr. Ming-Cheh Liu, Dr. Youssef Sari and my graduate faculty representative Dr. Caren Steinmiller for all their suggestions and valuable comments on my thesis and defense.

I am thankful to Dr. Ezdihar A. Hassoun, Dr. Fredrick Williams, and Dr. Ana Maria Oyarce for all the guidance and considerations that they have given me at UT and for the help when I needed it most.

I also am very grateful to Dr. Xingjian Jin and all the graduate students for all the help and guidance that they give me and without Dr.Jin I would have been lost. Dr. Jin taught me how to start the experiments and what approach to take when something unexpected happened.

Finally I would like to thank my family and friends for their support, love, and encouragement to follow my goals and dreams.

Table of Contents

Abstract iii

Acknowledgements v

Contents vi

List of Figures vii

List of Abbreviations viii

1 Introduction 1

2 Material & Methods 13

3 Result 17

4 Discussion 28

5 Conclusion and Future Study 32

References 33

A GZ 002-05 Chemical Structure 38

B K_B and pA_2 values 39

List of Figures

3.1 Acetylcholine and GZ 002-05 at M_1 receptors 19

3.1-2 GZ 002-05 at M_1 receptors baseline……………………………………….............20

3.2 Acetylcholine and GZ 002-05 at M_3 receptors..22

3.2-2 GZ 002-05 at M_3 receptors baseline 23

3.3 Acetylcholine and GZ 002-05 at M_5 receptors..25

3.3-2 GZ 002-05 at M_5 receptors baseline 26

List of Abbreviations

ACh ....Acetylcholine
AA…………………….Arachidonic Acid

CPM………………..Count Per Minute
CNS…………………Central Nervous System

DAG ....Diacyl Glycerol
DAT ....Dopamine Receptor
DMEM ....Dulbecco's Modified Eagle Medium
DMSO ....Dimethyl Sulfoxide

EC_{50}..........................Half maximal effective concentration

GPCRs......................G protein-coupled receptors

mAChR ....Muscarinic acetylcholine receptor

nAChR……………..Nicotinic acetylcholine receptor

PNS……………….. Peripheral Nervous System

LDT………………..Laterdorsal Tegmental Nucleus

SN…………………..Substantia Nigta

VTA………………..Ventral Tegmental Area

Chapter 1

Introduction

Acetylcholine Receptor

Acetylcholine is a major neurotransmitter that acts in both the central nervous system (CNS) and the peripheral nervous system (PNS). It was identified first in 1915 by Henry Hallet Dale. Based on pharmacological properties and the relative affinities of different molecules, acetylcholine receptors can be classified into two different families: nicotinic acetylcholine receptors (nAChR) and muscarinic acetylcholine receptors (mAChR). Nicotinic acetylcholine receptors also known as ionotropic acetylcholine receptors, function as ligand-gated ion channels, while muscarinic acetylcholine receptors are metabotropic receptors which act through second messenger systems. There are multiple subtypes of nicotinic acetylcholine receptors based on subunit composition. On the other hand, there are five subtypes of muscarinic acetylcholine receptors: M_1 through M_5. Both families of acetylcholine receptors are located throughout the brain as well as in other parts of the body (Goodman, Gilman, Brunton, Lazo, & Parker, 2006).

Muscarinic Acetylcholine Receptors

In recent decades, five different subtypes of muscarinic acetylcholine receptors have been identified and characterized. All of these five subtypes of muscarinic acetylcholine receptor are G-protein coupled receptors (GPCRs), but a separation of two different groups of muscarinic receptors can be made based on their interaction with G_i or G_q proteins. M_1, M_3 and M_5 receptors couple preferentially to the $G_{q/11}$ family of G proteins, which further activate phospholipase C (PLC) and participate in several different cellular signaling pathways (Alberts, 2002). In contrast, M_2 and M_4 receptor subtypes preferentially couple with the G_i family of G proteins, which decrease cAMP formation by inhibiting adenylate cyclase activity (Birnbaumer, 2007).

G_q coupled receptors (M_1, M_3 and M_5) activate phospholipase C (PLC,) which then cleaves phosphatidylinositol 4,5-bisphophate (PIP_2) into two components: diacylglycerol (DAG) and inositol 1,4,5-trisphosphate(IP_3). DAG remains bound to the membrane while IP_3 is released into the cytosol where it binds to IP_3 receptors, which further regulate calcium channels in the endoplasmic reticulum (ER) causing calcium concentration increases in the cytosol and a cascade of intracellular changes (Alberts, 2002).

G_i coupled muscarinic receptors (M_2 and M_4) inhibit the adenylyl cyclase pathway which decreases the production of cAMP from ATP. Both M_2 and M_4 muscarinic receptors activate a G protein-gated potassium channel, which is located in neurons and atrial myocytes.

Coupling with different families of G proteins is not the only difference among the muscarinic receptor subtypes. The presence of receptor subtypes located in different parts of the brain and throughout the body leads to unique physiological roles for eACh subtype of muscarinic receptors. Accordingly, muscarinic receptors may be suitable targets for the treatment of different neurological disorders. The M_1 receptor is most abundant in the cerebral cortex, hippocampus and neostriatum, and may have an important role in memory and cognitive function. M_2 muscarinic receptors are located throughout the brain and also have a role in cognitive and memory function (Comings et al., 2003). M_3 muscarinic receptors also are expressed throughout the brain but in a relatively low level. They do play important roles in peripheral tissues where they mediate smooth muscle contraction in the airways and in the urinary bladder, for example(Goodman et al., 2006). The M_4 muscarinic receptor is abundant in the neostriatum, and also in the cortex and hippocampus (Brann, Ellis, Jorgensen, Hill-Eubanks, & Jones, 1993), whereas the M_5 muscarinic receptor is predominately expressed in the substantia nigra (SN) and the ventral tegmental area (VTA), regions of brain containing dopamine neurons (Abrams et al., 2006).

Drug addiction and current therapies

Drug addiction is an important and severe social problem, because it is a chronic, sometimes relapsing neurological disease. Individuals with this disease not only have disorder in their own daily life but also have difficulty interacting with people around them. Drug addiction, also known as substance dependence, is a neurological disease in which patients compulsively use or seek a certain type of drug such as heroin or other

opiates and cocaine. Patients also suffer physiological changes in body function (termed withdrawal symptoms) when the drug is not present. Patients with drug addiction will value the drug over other activities that normally provide pleasure (such as food or sexual activity), and instead, will narrow their focus on drug seeking behavior.

A person experiences pleasure by natural rewards including food, water and sex – all of these are required for survival of the species. The experience of reward is achieved, at least in part, by the activation of the dopaminergic mesolimbic pathway. This key pathway of drug abuse and addiction originates in the ventral tegmental area (VTA) of the midbrain, and projects to the nucleus accumbens (NA), the amygdale,, and the hippocampus: the path way is widely believed to be the "reward" pathway of the brain and a key system involved in drug abuse and addiction (Berridge, 2007).

Cocaine is one of the most common psychoactive drugs that is made from the leaves of the coca plant (Aggrawal, Anil, 1995). It acts as a blocker of the dopamine transporter in the VTA synapse, leading to the accumulation of dopamine which will further activate the reward system in the midbrain. Studies show that cocaine can increase dopamine levels in the VTA area, a prominent source of dopaminergic neurons (Fiorino, Coury, Fibiger, & Phillips, 1993; Pettit & Justice, 1991). The increase of dopamine levels results in stimulation of the VTA projection to the NA to active the reward system. With continued use of cocaine, the body relies on the rewarding properties of cocaine instead of natural rewards (food, water and sex), and because of drug tolerance repeated, large doses of drug are needed for the patient to reACh the same level of the reward. That is how drug addiction develops.

The activation of the VTA is associated with dopamine accumulation in the NA area in the midbrain, as a result of signal transduction from the VTA to the NA. Cocaine elevates dopamine levels in the brain, so it is possible that dopamine receptor antagonists may be useful in the treatment of cocaine addiction. Drug development efforts have focused on antagonists for D_1 and D_2 dopamine receptors, with the goal of reducing of the effect of dopamine on the reward system. Unfortunately, dopamine receptor antagonists may have several side effects including Parkinsonism and increased levels of prolactin. Also, there are some animal behavior studies which report that: mice administered dopamine antagonists exhibit an increased uptake of cocaine, in order to compensate for the reduction in reinforcement by dopamine triggered by the dopamine antagonist.

Cocaine antagonists have been studied as another approACh to treat cocaine addiction. Cocaine addiction is directly related to the increased level of dopamine caused by cocaine binding to the dopamine transporter (DAT), which reduces dopamine reuptake (Giros, Jaber, Jones, Wightman, & Caron, 1996; Ritz, Lamb, Goldberg, & Kuhar, 1987). The cocaine binding site on the dopamine transporter (DAT) is distinct from the binding site of dopamine (Buck & Amara, 1994). This suggests that blocking the binding site for cocaine using a cocaine antagonist may not affect the binding site for dopamine, so that the binding and reuptake of dopamine by the dopamine transporter (DAT) would not be reduced. Although this area is a target for drug development, no therapeutic compound has been identified as yet through this approach (Deutsch, Shi, Gruszecka-Kowalik, & Schweri, 1996; Kopajtic et al., 2010; Singh, 2000).

However, the dopamine transporter (DAT) as a potential target for treatment of drug addiction has triggered the attention of many scientists. DAT inhibitors can decrease self-administration of cocaine in animal models suggesting that a cocaine agonist may also help reduce cocaine dependence and its future effect in the central nervous system. The successful replacement or substitution combined with the understanding of the neurobiological basis of cocaine dependence supports the idea that developing a substitute pharmacotherapy may be effective in reducing cocaine use in patients. (Howell & Wilcox, 2001). But the disadvantage is, such compounds will have a similar effect like cocaine (positive reinforcement) in the midbrain, and they have the potential of becoming abused themselves.

GABA (γ-Amino butyric acid) drugs have been studied as a potential treatment for addiction. An increasing number of experiments support the idea that GABA-related compounds may be able to reduce the acute reinforcement effects of cocaine, heroin, nicotine and alcohol in animal models. Some clinical studies also report that GABA compounds (such as baclofen) may have beneficial effects in the treatment of cocaine addiction and alcoholism: Baclofen, an agonist for the $GABA_B$ receptor reduces the intake of cocaine in a rat model (Brebner, Childress, & Roberts, 2002). The enzyme GABA-transaminase (GABA-T) converts GABA to succinate semi aldehyde and L-glutamate. An inhibitor of GABA-T could increase the level of GABA in the VTA area due to blockade of GABA-T preventing its use of GABA as a substrates. GVG, an irreversible inhibitor of GABA-T has been shown to decrease cocaine-induced DA levels in the corpus striatum and NA (Morgan & Dewey, 1998).

Two major approaches for treating substance addiction include substitution therapy over a pro-longed period and opiate antagonists. Although both of these two approAChes have significant effects for substance addiction, neither of them provides a clinical solution for the treatment of drug addiction at the present time. Methadone (one application of substitution therapy), a synthetic opioid, is given in a controlled manner to reduce symptoms associated with drug withdrawal and it is widely used all over the world in the drug addiction treatment medical centers. Buprenorphine (another example of substitution therapy), a semi-synthetic opioid that which targets the μ-opioid receptor is also widely used to reduce the symptoms of drug withdrawal. Both are used as a long term substitution therapy for opioid-dependent patients. But neither drug corrects the problems associated with opioid addiction, and with long term use (although in a controlled manner), the patient will become addicted to these substances instead. A μ-opioid receptor antagonist can inhibit indirectly the dopamine pathway in the VTA and NA area, and therefore may also be used as a treatment for addiction. Naltrexone, a μ-opioid receptor antagonist can be used for the treatment of alcohol dependence. Although the mechanism of action is not fully understood, it is likely due to modulation of the reward systems in the midbrain.(Matsuzawa, Suzuki, Misawa, & Nagase, 1999).

To sum up, addiction is a complex illness and none of the current approaches provide a solution for the underlying causes of drug addiction. New approaches need to be developed, inclueding new compounds that interact on different pathways than those described above. Another possibility is to try to decrease the side effects of the existing drugs. Over the last decade, the M_5 muscarinic receptor, which is located in the VTA, has

attracted attention. A selective antagonist for M_5 muscarinic receptors might be very valuable in the treatment of drug abuse.

M_5 receptor and role in drug addiction

The M_5 muscarinic receptor was the last subtype identified in the muscarinic receptor family. Two studies published during the late 1980s by Bonner et al.(Bonner, Young, Brann, & Buckley, 1988) and Liao et al. (Liao et al., 1989) were the first reports on the discovery of the new subtype muscarinic receptor. Later, studies also reported that the M_5 subtype receptor was the only subtype in muscarinic receptor family that located in the VTA of the midbrain (Basile et al., 2002; Vilaro, Palacios, & Mengod, 1990). The ventral tegmental area is the origin of the dopaminergic cell bodies of the mesolimbic dopamine system and it is wildly implicated in the reward system of the brain. This suggests that M_5 muscarinic receptor may play a very important role in the reward system, specifically in the substance abuse/addiction pathway.

Due to the lack of a selective M_5 receptor antagonist, M_5 muscarinic acetylcholine receptor mutant mice (M_5 $^{-/-}$) were used examine whether the M_5 receptor is a potential target for drug addiction. Past studies showed that dopamine release in the striatal area is modulated by the muscarinic receptor in this area. To determine which subtype is involved in this pathway-Zhang et al.(Zhang, Yamada, Gomeza, Basile, & Wess, 2002) used a non-subtype selective agonist compound to test [^{3}H] dopamine release in striatal slices prepared from WT and muscarinic knockout mice. They showed that the M_5 muscarinic receptor was the primary subtype responsible for stimulating dopamine

release. In 2000, Yeomans et al. showed that an infusion to the VTA of a M_5 antisense oligonucleotide, (which prevents M_5 receptor production,) to rats trained to bar-press for lateral hypothalamic stimulation resulted in a significant increase in bar pressing rates. This demonstrated that the M_5 muscarinic receptor is an important modulator of mesolimbic dopamine neurons and the brain stimulation reward system (Mochan et al., 1987; Yeomans et al., 2000).

Another study on cocaine addiction using M_5 KO mice was conducted by Fink-Jensen (Fink-Jensen et al., 2003). When they allowed the mice have free access to cocaine, M_5 KO mice exhibited significantly reduced cocaine self-administration as compared to the WT control mice. Also when given a free access between two chambers, one of which was associated with cocaine administration and the other one not associated with drug administration, the M_5 receptor deficient mice spent less time in the cocaine chamber than the WT mice did. Also studies using the light/dark transition test to evaluate cocaine withdrawal-induced anxiety revealed that the withdraw symptoms were less severe for M_5 KO mice than the WT mice. Further observations and studies were performed to evaluate additional factors that may influence the results in M_5 KO mice including (1) using a mouse model with a more pure genetic background to minimize the genetic differential and to compare various behaviors expressed with the wild type and genetic knockout mice, (2) allow a longer period of cocaine self-administration to evaluate additional conditions and responses in the mice, and (3) include both female and male mice to complete the experiment (Raffa, 2009).

Based on all the experiments and observations published by various researchers, there is strong evidence supporting a role for M_5 muscarinic receptors in modulating the mesolimbic dopamine system in the VTA and NA and therefor in the reward system of the brain in drug addiction pathways. Utilization of the M_5 receptor as a new and unique potential target for drug addiction provides another avenue for research opportunities to elucidate the mechanisms involved in addiction. A selective M_5 antagonist combined with other pharmaceutical therapies may produce a greater effect on substance abuse and addiction than current treatment options.

M_5 Receptor Selective Antagonist

There is little information about M_5 receptor selective antagonists as a therapy for drug abuse and addiction, because until now no M_5 receptor selective antagonist has been reported. This leave us with many pharmacological and pharmacokinetic issues to be addressed, including evaluating the properties of the compound itself (the size, solubility etc.) and other problems including whether the compound can cross the blood brain barrier, and the potential side effects of this compound.

As an ideal drug addiction therapy compound an M_5 selective antagonist should cross the blood brain barrier in order to target the VTA and NA in the brain. Also selectivity for M_5 receptor subtype is critical to limit the side effect profile associated with non-selective muscarinic antagonists which inhibit other subtypes of muscarinic receptors. Another question to be answered is whether an M_5 receptor antagonist compound is potentially addictive like methadone and buprenorphine. Due to the lack of selective M_5 compounds, no clinical data have been reported, but using an animal model

may provide a way to resolve some of these problems. A self-administration assay performed with mice could test the potential for abuse of the compound by administration over a pro-longed time period and would provide insight on the potential withdraw symptoms upon discontinuation of the treatment compound. The last question of potential interest is to evaluate whether the antagonist will block normal reward (i.e., food, water and sex) signals mediated by dopaminergic neurons in VTA and NA of the brain. This is a complex question because although the M_5 receptor is the only muscarinic receptor expressed in the VTA, there are also other types of neurotransmitter receptors in the area such as nicotinic and GABA receptors. Dopamine will still be released in this area through stimulation of other classes of receptors. In addition, except for dopamine, there are other classes of neurotransmitters that modulate neurons in the Na;(e.g., GABA, glutamate among others. These receptors and neurotransmitters would not be affected by a M_5 antagonist so interference with natural reward signal should be limited.

There are distinct advantages and disadvantages in suing selective M_5 antagonists for the treatment of substance abuse. Advantages of selective M_5 antagonists include their ability to target only M_5 receptors which are expressed mainly in the VTA, an area implicated in drug abuse and addiction. Although there are small amounts of M_5 muscarinic receptors expressed in other areas throughout the body, it is a very small proportion compared to the distribution of other subtypes of muscarinic receptors. M_5 antagonists would have minimal the side effects compared with nonselective muscarinic antagonists. The second advantages, as described above, is that M_5 selective compounds would not completely block the natural reward dopaminergic system in the brain, suggesting that the normal reward system signal should continue to be functional in

patients. Several studies have reported some of the potential disadvantages of these compounds in the knockout mice model. Takeuchi et al. reported that following prolonged periods of food and water deprivation, M_5 receptor deficient mice drank more than twice as much water as compared to the wild type mice once given access(Takeuchi et al., 2002). This finding suggests that selective M_5 antagonists may also affect other functions of body related to the distribution of the M_5 subtype muscarinic receptors. Also M_5 antagonists would not completely block the natural reward signal, thus an inhibition of natural reward may occur to lesser levels. In summary, there considerable interest in finding selective M_5 muscarinic receptor compounds so that such compounds can be tested for therapeutic efficacy in animal models and in humans.

Chapter 2

Material & Methods

Cells

A Chinese Hamster Ovary (CHO) cell line transfected with M_1, M_3 and M_5 receptors was obtained from Dr. John Ellis as a gift. CHO cells derived from the ovary of the Chinese hamster by T.T. Puck in 1957 (Tjio & Puck, 1958). It is widely used in many biological and medical research paradigms. Originally, there are no muscarinic receptors express in the CHO cells, so plasmids encoding M_1, M_3 and M_5 muscarinic receptors were transfected into the cells. The CHO M_1 cell line only expressed the M_1 subtype of muscarinic receptors, while the same is true for CHO M_3 and M_5 lines. Cells were stored in liquid nitrogen until used. An antibiotic G-418 was added to every plate that subculture to select cells with M_1, M_3 and M_5 receptors.

Cell Culture

The Dulbecco's Modification of Eagle's Medium (DMEM) with 4.5 g/L glucose, L-Glutamine & sodium pyruvate was used in CHO cell culture. For eACh 500ml of DMEM, 50 ml Fetal Bovine Serum (FBS), 10 ml Penicillin-streptomycin (10,000 units of penicillin and 10,000 µg of streptomycin) were added. The Dulbecco's Modification of

Eagle's Medium and the penicillin-streptomycin and the Fetal Bovine Serum were purchased from The Life Technologies.

Sub-culture process: DMEM media and 1x trypsin were warmed in a water bath (37º C). The plates were removed from the incubator to the hood, the old media was removed and discarded, and the plates were washed with 5 ml of PBS twice. PBS was removed with 2 ml of trypsin which was added to the plates to cover the cells with a thin coating of trypsin. Plates are then are put back into the incubator at 37º C with 5% CO_2 for around 5 minutes. After this incubation, 4 ml of media was added to the plates and mixed well to lift cells from the plates in to the media. A 1:6 dilution of cells was then transferred to a new plate which is prepared during the first incubation period. Preparation of new plates during the incubation period begins by taking a fresh plate and adding 5 ml of media along with 80 µl of G-418 antibiotic. Plates were all labeled with the cell line, its passage number and the date of sub-culture. Once the incubation period was complete, 1 ml of the cell dilution solution was added. The plates were mixed gently to disperse the cells well and then incubated at 37º C with 5% CO_2 until ready for assays.

Arachidonic Acid Release Assay

Measurement of [^{3}H] AA release was modified from the previous studies. (Stahl & Ellis, 2010). CHO cells were plated into a 96-well plate at a density of 30,000 cells/well in 0.2ml of DMEM media. After the cells were seeded in the 96-well plate, they were transfered to the incubator at 37° in 5% CO_2 until the cells were attached to the bottom of the plate. After approximately 3 hours incubation, the media was removed and

exchanged with 0.2 ml DMEM media with 0.2 μCi of [^{3}H] in each well. The cells then were incubated for another 18 to 22 hours before the assay was performed. Cells were rinsed twice using EM-BSA. Then the EM-BSA with experimental compounds was added to each well as planned (all the experimental compound stock solution were made with deionized water). Cells then were put back into the incubator for another 1 hour (37° and 5% CO_2). The amount of [^{3}H] AA released was count in the Eagle's basal media with 2 mg/ml fatty acid free bovine serum albumin (EM-BSA). The experiments were performed in EM-BSA with 20 μM HEPES buffer. EM-BSA media were collected after the last one hour incubation, and the [^{3}H] AA release was measured using a liquid scintillation counter or using the Top-Count machine.

Data analysis

Dose-response curves were fit to an empirical four parameter equation:

$$\mathbf{Y=Bottom + (Top-Bottom)/(1+10^{\wedge}((LogEC_{50}-X)))}$$

Where X is the log of concentration of the experimental compounds, Y is the amount of response. Top and Bottom have the same units as Y. EC50 is the concentration of compounds that produce 50% of the maximal effects.

Dissociation constants of the antagonist and the pA2 value were calculated by the equation:

$$\frac{\mathbf{EC_{50a}}}{\mathbf{EC_{50b}}} - \mathbf{1} = \frac{[\mathbf{B}]}{\mathbf{K_B}}$$

where EC_{50a} is the concentration of the agonist producing half the maximal response in the presence of the antagonist; EC_{50c} is the concentration of the agonist producing half the maximal response in the absence of the antagonist; [B] is theconcentration of the antagonist used; and K_B is the dissociation constant of the antagonist.Accordingly pA2 values could then be calculated as -log K_B(Jankovic, Milovanovic, & Jankovic, 1999).

Results and Statistics

All arachidonic acid release assay experiments were done in triplicate. Raw data from the Top Count and liquid scintillation were analyzed using Graph Pad Prism 5 and Microsoft Excel.

All statistical analyses were performed using Graph Pad Prism 5's Two Way ANOVA and the 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.

Materials

[^{3}H] Arachidonic acid was purchased from American Radiolabeled Chemicals, Inc. Most of the compounds and solutions used in the AA release assay were purchased from Sigma-Aldrich including albumin from bovine serum, acetylcholine, atropine, the liquid scintillation plastic vials, Hepes buffer solution and basal medium eagle solution. Other laboratory supplies such as cell culture plates, 96-well plate and pipette were purchased through Bio-Express. The putative M_5 receptor antagonist GZ-002-05 was obtained from Dr.Guangrong Zheng, University of Arkansas.

Chapter 3

Results

3.1 Acetylcholine and GZ-002-05 at M_1 receptors

Acetylcholine is one of the major neurotransmitters in the autonomic nervous system (ANS) and can stimulate both muscarinic and nicotinic receptors. Previous studies used [^{3}H] arachidonic acid (AA) release assay to measure muscarinic receptor activity in CHO cells expressing M_1 muscarinic receptors. To ensure the assay system would be able to reflect findings from previous studies correctly and accurately, full dose response curves of acetylcholine in M_1 muscarinic receptors were performed.

GZ-002-05, the compound obtain from Dr. Zheng Guangrong was identified previously as a putative M_5-selective muscarinic antagonist. It has the potential to be useful in treatment of drug abuse. To test the GZ-002-05's interaction properties at M_1

muscarinic receptors, an arachidonic acid release assay was performed in CHO cell that expressed M_1 muscarinic receptors. A 10 µM GZ-002-05 was used in the presence of various concentrations of acetylcholine to perform a dose response curve. At the concentration of 10µM, it did not change the maximal response for acetylcholine; however, it lowered the potency of Acetylcholine by change the EC_{50} significantly for acetylcholine from 1.8 µM to 14.3µM. Also the data suggest that GZ-002-05 would be able to lower the baseline activity of M_1 muscarinic receptors both in the presence and absence of acetylcholine. Overall, the data suggests that GZ-002-05 interacts as an inverse agonist and produces competitive interaction at M_1 receptors in the presence of acetylcholine. Figure 3.1 shows the full dose response curve of acetylcholine in the presence and absence of 10 µM GZ-002-05 in CHO cells expressed with M_1 muscarinic receptors. Once the curve was produced, the EC_{50} concentration and maximum response of acetylcholine was determined using GraphPad Prism. The EC_{50} concentration of acetylcholine without GZ-002-05 was determined to be approximately 1.8 µM and with the presence of 10 µM of GZ-002-05, the EC50 was 14.3 µM.

Figure 3.1

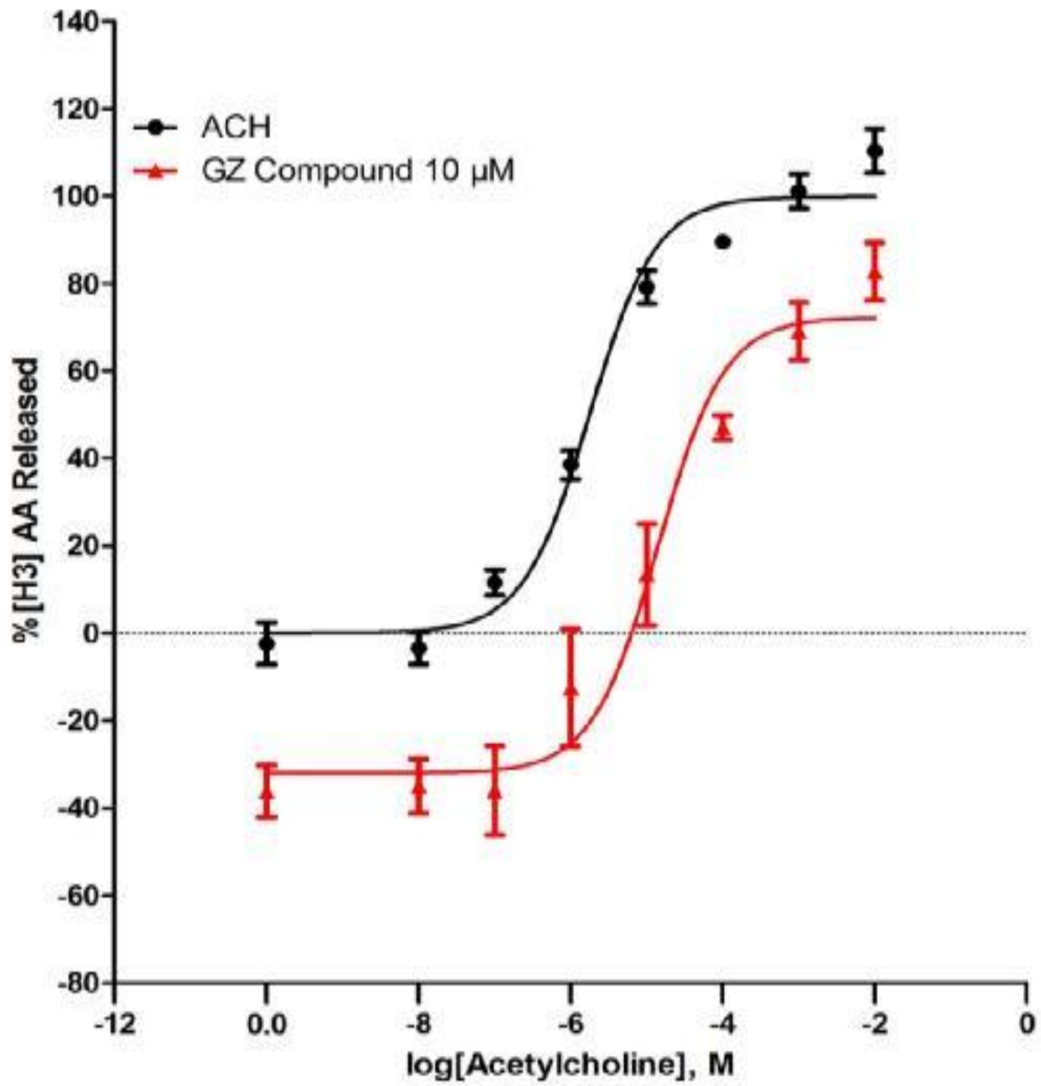


Figure 3.1: Acetylcholine alone and the in the presence of 10 μM GZ-002-05 at M_1 muscarinic receptors. Experiments were performed in triplicate. Data represent the mean (± S.E.M) of at least three experiments.

Figure 3.1-2

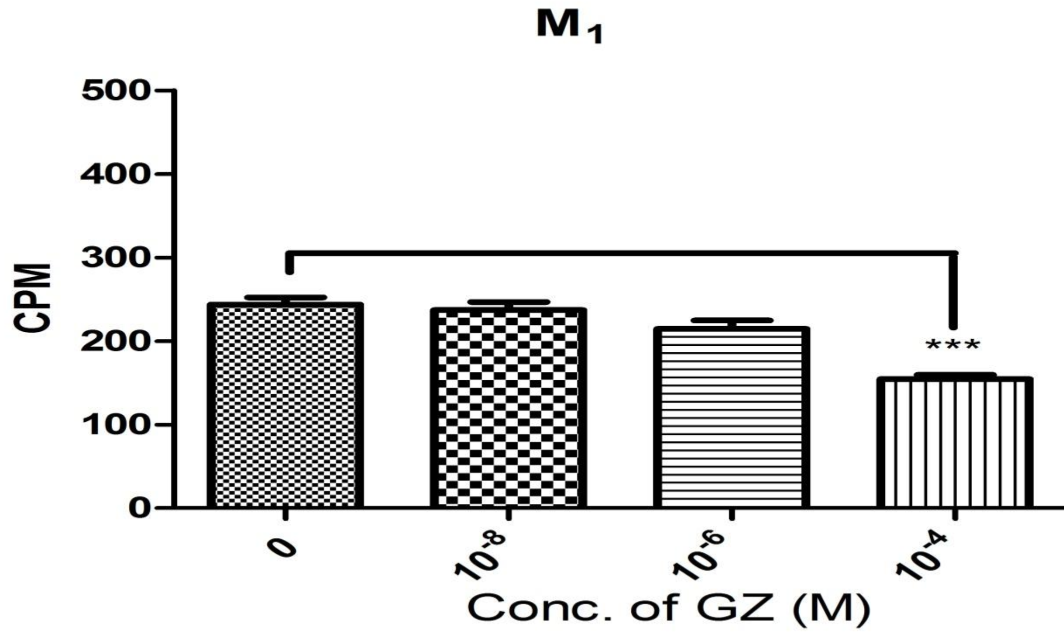


Figure 3.1-2: Various concentration of GZ-002-05 at M_1 muscarinic receptors. Experiments were performed in triplicate. All results are shown as the mean ±S.E.M. Results were analyzed by one way ANOVA; *** indicated a very significant statistical difference of $P < 0.001$ between reading media and GZ-002-05.

3.2 Acetylcholine & GZ-002-05 at M_3 receptors

Acetylcholine also acts as an agonist in M_3 muscarinic receptors, past studies have been done using the arachidonic acid release assay to test the interaction properties of acetylcholine in CHO cells that expressed M_3 muscarinic receptors . To make sure that the assay system would accurately reflect what previous studies have shown, a full dose response curve of acetylcholine at M_3 was performed using the arachidonic acid release assay. Also, in order to characterized the interaction of GZ-002-05 described above at M_3 muscarinic receptors, a full dose response curve of acetylcholine in the presence of 10 µM GZ-002-05 was performed with AA released assay which shown in Figure 3.2. The EC_{50} concentration and maximum response of both curves were determined using GraphPad after the curves were produced. The EC_{50} concentration of acetylcholine without GZ-002-05 was approximately 0.3 µM and in the presence of 10 µM of GZ-002-05, the EC_{50} concentration was 0.31 µM. Results suggest that, at 10 µM, GZ-002-05 neither shifted the EC_{50} value of acetylcholine nor depressed the maximum response of it. Interestingly, GZ-002-05 does depress the baseline activity at M_3 muscarinic receptors.

Figure 3.2

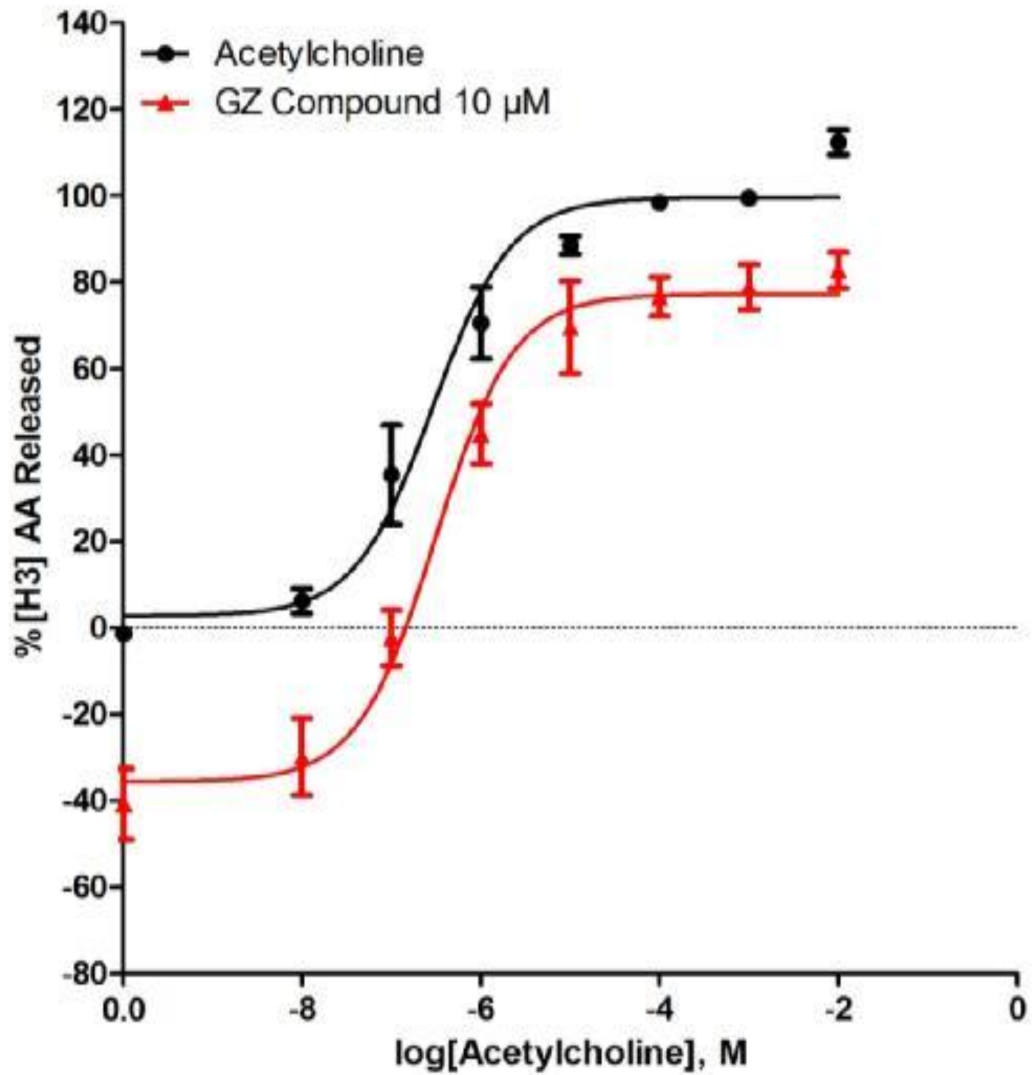


Figure 3.2: Acetylcholine alone and the in the presence of 10 μM GZ-002-05 at M_3 muscarinic receptors. Data represent the mean (± S.E.M) of at least three experiments, each performed in triplicate.

Figure 3.2-2

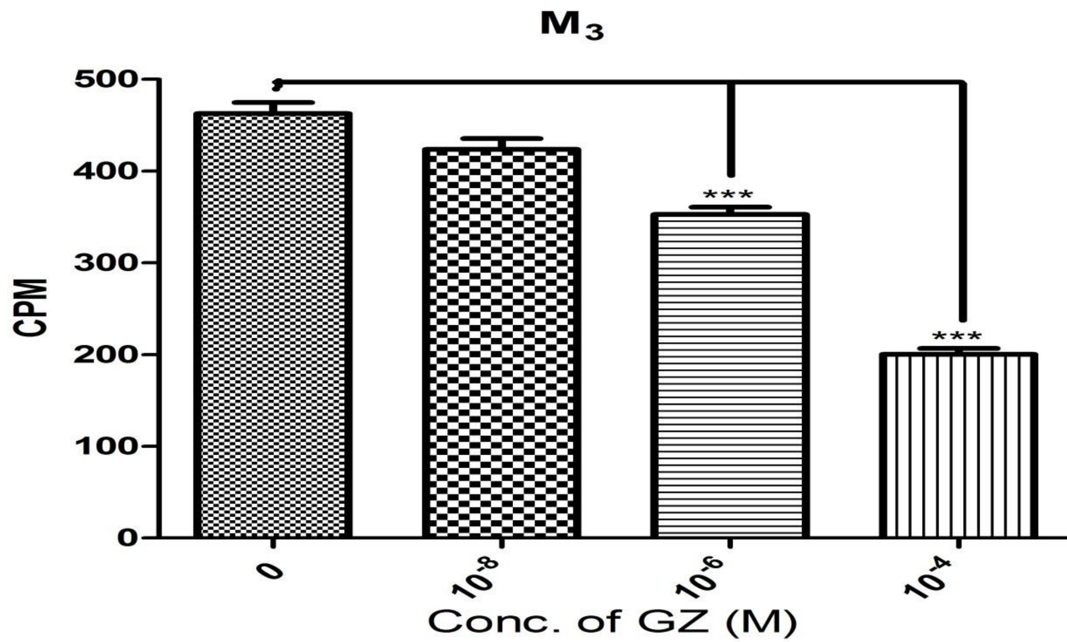


Figure 3.3-2: Various concentration of GZ-002-05 at M_3 muscarinic receptors. Experiments were performed in triplicate. All results are shown as the mean ± S.E.M. Results were analyzed by one way ANOVA; *** indicated a very significant statistical difference of $P < 0.001$ between reading media and GZ-002-05.

3.3 Acetylcholine & GZ-002-05 at M_5 receptors

Past studies have created an acetylcholine full dose-response curve by using [^{3}H] arachidonic acid (AA) release assay in CHO cells expressing M_5 muscarinic receptors which performed by Dr. Edward Stahl and Dr. John Ellis. To ensure the assay system would be able to reflect the results of previous studies correctly and accurately, full dose response curves of acetylcholine in M_5 muscarinic receptors were performed and shown in Figure 3.3. Once the curve was produced the EC_{50} concentration was determined using the GraphPad and found to be approximately 1.1 μM.

A 10 μM of GZ-002-05 was also used to characterized the selectivity of M_5 muscarinic receptors. By measuring the effects of acetylcholine in the presence of 10 μM of GZ-002-05 a full dose response curve was produced, as shown in Figure 3.3. At M_5 receptors 10 μM of the GZ-002-05 compound has an EC_{50} concentration of 0.51 μM which was determined using GraphPad and it inhibited the maximal response produced by acetylcholine (86 ±2.15%). Suggesting that the GZ-002-05 does not interact in a competitive manner at M_5 receptors and based on the data, the GZ-002-05 can also decrease the baseline activity of M_5 muscarinic receptors.

Figure 3.3

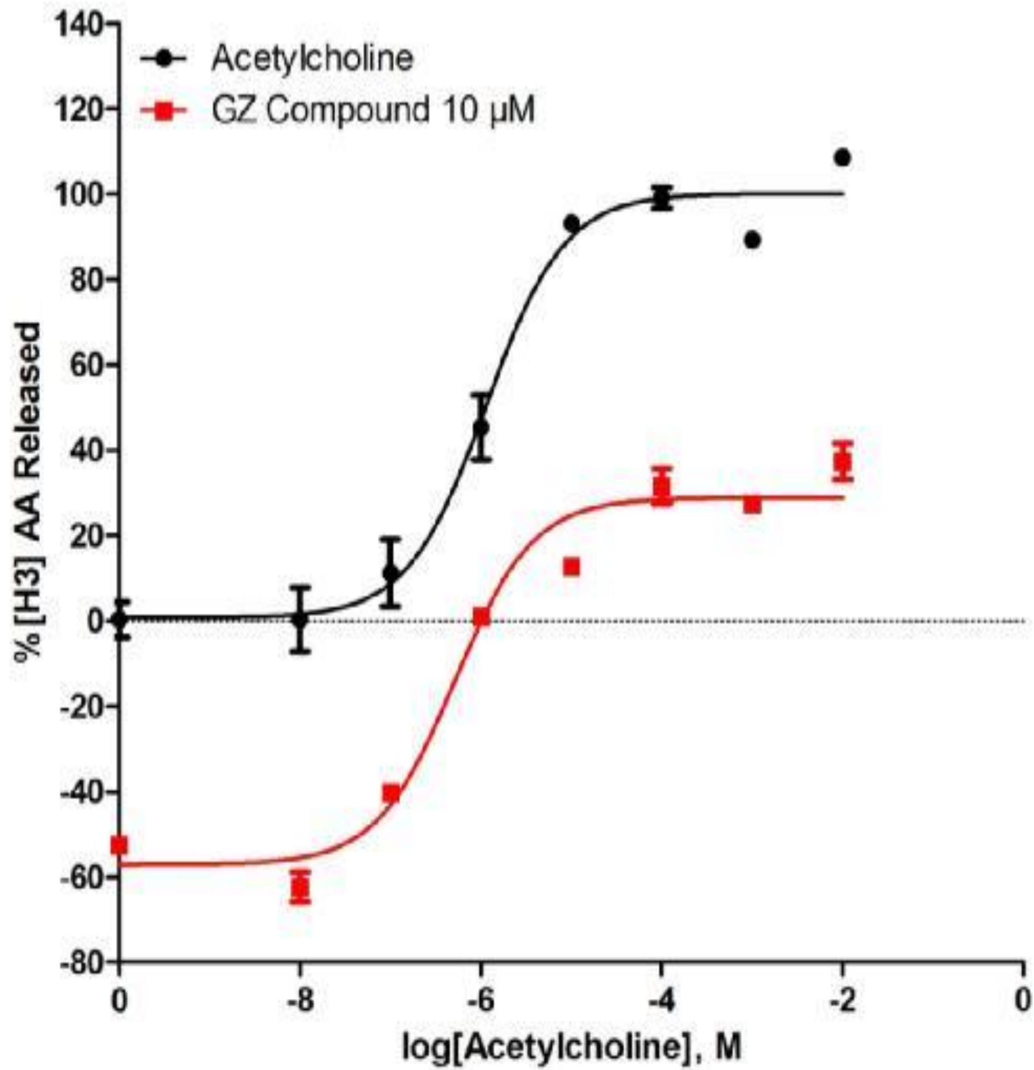


Figure 3.3: Stimulation of arachidonic acid release by acetylcholine alone and the in the presence of 10 μM GZ-002-05 at M_5 muscarinic receptors. Exp. Data represent the mean (± S.E.M) of at least three experiments, each performed in triplicate.

Figure 3.3-2

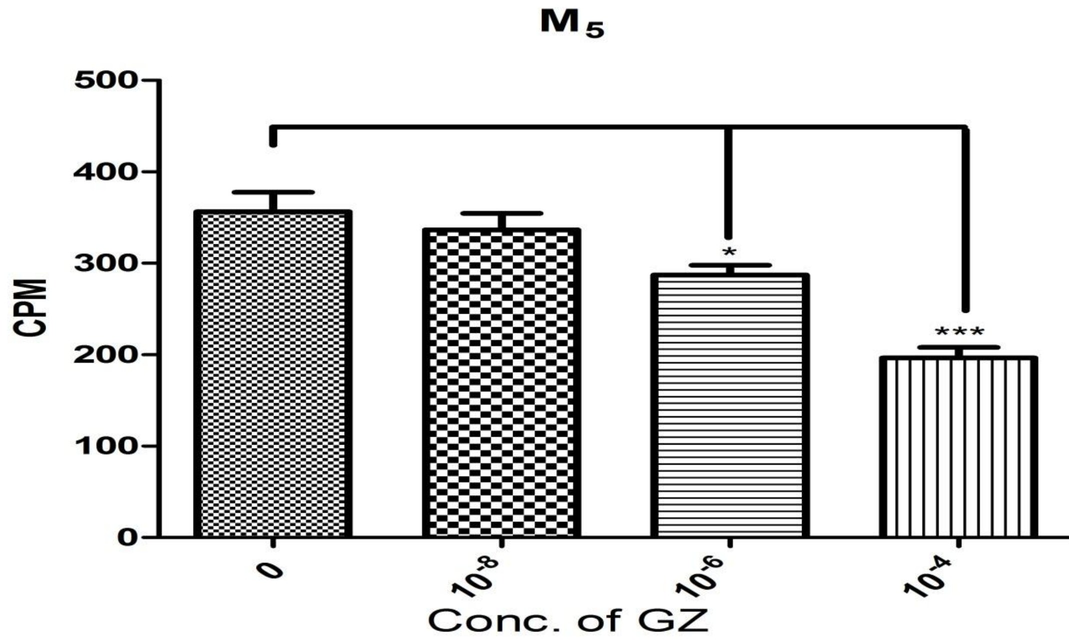


Figure 3.3-2: Various concentration of GZ-002-05 at M_1 muscarinic receptors. Experiments were performed in triplicate. All results are shown as the mean ±S.E.M. Results were analyzed by one way ANOVA; * indicated a very significant statistical difference of $P < 0.05$ between reading media and GZ-002-05. *** indicated a very significant statistical difference of $P < 0.001$ between reading media and GZ-002-05.

Table 1

Response parameters of acetylcholine with presence and absence of GZ-002-05

Experiments were performed in triplicate, and maximal response is expressed as a percentage of [3H] AA release stimulated by 1mM Ach. Data are the average of at least three experiments (mean ±S.E.M.).

		Log EC_{50}	**E_{max}**
CHO-M1			% *max*
	Acetylcholine	-5.774±0.047	100
	Ach + 10 µM GZ	-4.833±0.174	106.8±10.17
CHO-M3			
	Acetylcholine	-6.715±0.229	100
	Ach + 10 µM GZ	-6.528±0.091	113.4±10.16
CHO-M5			
	Acetylcholine	6.421±0.325	100
	Ach + 10 µM GZ	6.077±0.344	82.95±3.022

Chapter 4

Discussion

Previous studies in mice lacking M_5 muscarinic receptors suggested that a M_5 muscarinic receptor antagonist could be useful in the treatment of drug abuse and addiction (including opioid, and cocaine). A selective M_5 muscarinic receptor antagonist would be the optimal compound, because it would not produce many of the side effects resulting from the interaction with other muscarinic receptors in different locations through the body, especially M_1 and M_3 subtypes. While the other subtypes of muscarinic receptors have more diverse and widespread functions in the whole body, M_5 receptors have the most concentrated receptor levels in the dopaminergic neurons in the VTA which comprises an important part of the brain reward system. The low expression levels of M_5 muscarinic receptors in the other parts of body may limited the side effects from the blockade of M_5 receptors.

It has not easy to identify and synthesize a selective M_5 muscarinic receptor antagonist due to the high similarity among all the muscarinic receptors, especially M_1, M_3 and M_5 muscarinic receptors. These three receptor subtypes all belong to the Gq coupled receptor protein family, the high degree of homology results in all three subtypes

of the muscarinic receptors having a very similar binding sites which presents the difficulties in identifying a subtype selective antagonist. Testing the compound's interaction with different subtypes by measuring the effect of acetylcholine in the presence and absence of a various compounds can help us characterize the selectivity among the receptors subtypes of the compound.

To test the acetylcholine effects in M_1, M_3 and M_5 receptors an [3H] arachidonic acid release assay was performed in CHO cells expressing different subtypes of muscarinic receptors: M_1, M_3 and M_5. Measuring the concentration of downstream arachidonic acid is one way to identify muscarinic receptors linked to Gq coupled proteins. The Top-count machine was used to identify the release of arachidonic acid which presents as count per minute (CPM), the effects of different concentrations of acetylcholine with and without an antagonist were estimated. Full dose response curves were then produced using GraphPad and the EC50 value and maximal responses were calculated.

Acetylcholine alone was first tested at different concentrations at three of the muscarinic receptors subtypes: M_1, M_3 and M_5. A whole set of various concentrations, from 10^{-8} M to 10^{-2} M, were used to produce the full acetylcholine dose response curve for the different receptor subtypes. Then the curves were repeated with the presence of 10μM of GZ-002-05 at M_1 receptors, 10 and 30μM of GZ-002-05 at M_3 receptors and 1μM and 10μM at M_5 receptors. All of the concentrations were done in triplicate. The EC_{50} values and maximal response values were collected and used to analyze the GZ-002-05's selectivity.

Base on the results of the arachidonic acid release assays, the GZ-002-05 did not interact with the different muscarinic receptors in the way we supposed: interact as a selective competitive antagonist at M_5 muscarinic receptors. Interestingly, the results indicated that GZ-002-05 interact in a competitive manner at M_1 receptors, no change of acetylcholine effects at M3 receptors and more likely a non-competitive antagonist at M_5 receptors.

At M1 muscarinic receptors, a GZ-002-05 (10μM) did not depress the maximal response caused by acetylcholine; however, it did lower the potency of acetylcholine by increasing the EC50 value for acetylcholine significantly from 1.8μM to 14.3μM. This data indicated that GZ-002-05 produced a competitive interaction at M1 receptors but had no depression influence at concentration of 10μM.

In contrast, there is no significant change either EC_{50} values (0.29μM in acetylcholine group and 0.31μM in GZ-002-05 group) or maximal response at 10μM of GZ-002-05 at M_3 receptors. At a higher concentration (30μM) of GZ-002-05, there was still no change on either the EC_{50} values or the maximal response. This suggests that the compound did not influence acetylcholine activation of M_3 receptors.

At M_5 receptors, a lower concentration of GZ-002-05(1μM) was evaluated. At this concentration, there were no significant effects on the EC_{50} values nor the maximal response. However, at the higher concentration, 10μM, GZ-002-05 inhibited the maximal response elicited by acetylcholine (86 ±2.15%) with no significant effect on the EC_{50} values. That indicates that the compound does not depress the acetylcholine effects in a competitive manner.

It should also be pointed out, that the GZ-002-05 inhibited the baseline activity in the absence of acetylcholine at all three subtypes of muscarinic receptors. Although there is no estimate number of the level of inhibition, there is still a significant decrease in the baseline activity with the increased concentration of GZ-002-05, which suggests that GZ-002-05 may interact as inverse agonists at an unique site of muscarinic receptors.

Overall, all the data suggest GZ-002-05 has unique mechanisms of interaction with M_1, M_3 and M_5 muscarinic receptor subtypes. The results indicate that GZ-002-05 may be useful as a lead compound in the development of potential therapeutic agents for the treatment of drug abuse; however, further work is needed to understand how it acts at each muscarinic receptor subtype.

Chapter 5

Conclusions and Future Study

In summary, an arachidonic acid release assay was performed to characterize the pharmacological properties of GZ-002-05, a putative M_5 muscarinic antagonist, using CHO cells transfected with M_1, M_3 and M_5 receptor subtypes. The assay system examined the activity of acetylcholine in the absence and presence of GZ-002-05 at various concentrations. Comparisons of baseline activity, the EC_{50} values and maximal responses allowed a determination of the mechanism of interaction for GZ-002-05 at each muscarinic receptor subtype.

The results indicated that GZ-002-05 had a competitive interaction at M_1 receptors, minimal interaction with M_3 receptors and a non-competitive interaction at M_5 receptors. At all three muscarinic receptors, GZ-002-05 decreased baseline activity by itself, which suggests that it acts as an inverse agonist at all these three muscarinic receptors. Overall, this compound has very unique mechanism of acton among M_1, M_3 and M_5 muscarinic receptors, and could be useful as a lead compound in identifying and synthesizing of novel therapeutic agents for the treatment of drug abuse.

References

Abrams, P., Andersson, K. E., Buccafusco, J. J., Chapple, C., de Groat, W. C., Fryer, A. D., . . . Wein, A. J. (2006). Muscarinic receptors: their distribution and function in body systems, and the implications for treating overactive bladder. *Br J Pharmacol, 148*(5), 565-578. doi: 10.1038/sj.bjp.0706780

Alberts, B. (2002). *Molecular biology of the cell* (4th ed.). New York: Garland Science.

Basile, A. S., Fedorova, I., Zapata, A., Liu, X., Shippenberg, T., Duttaroy, A., . . . Wess, J. (2002). Deletion of the M_5 muscarinic acetylcholine receptor attenuates morphine reinforcement and withdrawal but not morphine analgesia. *Proc Natl Acad Sci U S A, 99*(17), 11452-11457. doi: 10.1073/pnas.162371899

Berridge, K. C. (2007). The debate over dopamine's role in reward: the case for incentive salience. *Psychopharmacology (Berl), 191*(3), 391-431. doi: 10.1007/s00213-006-0578-x

Birnbaumer, L. (2007). The discovery of signal transduction by G proteins: a personal account and an overview of the initial findings and contributions that led to our present understanding. *Biochim Biophys Acta, 1768*(4), 756-771. doi: 10.1016/j.bbamem.2006.09.027

Bonner, T. I., Young, A. C., Brann, M. R., & Buckley, N. J. (1988). Cloning and expression of the human and rat m5 muscarinic acetylcholine receptor genes. *Neuron, 1*(5), 403-410.

Brann, M. R., Ellis, J., Jorgensen, H., Hill-Eubanks, D., & Jones, S. V. (1993). Muscarinic acetylcholine receptor subtypes: localization and structure/function. *Prog Brain Res, 98*, 121-127.

Brebner, K., Childress, A. R., & Roberts, D. C. (2002). A potential role for GABA(B) agonists in the treatment of psychostimulant addiction. *Alcohol Alcohol, 37*(5), 478-484.

Buck, K. J., & Amara, S. G. (1994). Chimeric dopamine-norepinephrine transporters delineate structural domains influencing selectivity for catecholamines and 1-methyl-4-phenylpyridinium. *Proc Natl Acad Sci U S A, 91*(26), 12584-12588.

Comings, D. E., Wu, S., Rostamkhani, M., McGue, M., Lacono, W. G., Cheng, L. S., & MacMurray, J. P. (2003). Role of the cholinergic muscarinic 2 receptor (CHRM2) gene in cognition. *Mol Psychiatry, 8*(1), 10-11. doi: 10.1038/sj.mp.4001095

Deutsch, H. M., Shi, Q., Gruszecka-Kowalik, E., & Schweri, M. M. (1996). Synthesis and pharmacology of potential cocaine antagonists. 2. Structure-activity relationship studies of aromatic ring-substituted methylphenidate analogs. *J Med Chem, 39*(6), 1201-1209. doi: 10.1021/jm950697c

Fink-Jensen, A., Fedorova, I., Wortwein, G., Woldbye, D. P., Rasmussen, T., Thomsen, M., . . . Basile, A. (2003). Role for M5 muscarinic acetylcholine receptors in cocaine addiction. *J Neurosci Res, 74*(1), 91-96. doi: 10.1002/jnr.10728

Fiorino, D. F., Coury, A., Fibiger, H. C., & Phillips, A. G. (1993). Electrical stimulation of reward sites in the ventral tegmental area increases dopamine transmission in the nucleus accumbens of the rat. *Behav Brain Res, 55*(2), 131-141.

Giros, B., Jaber, M., Jones, S. R., Wightman, R. M., & Caron, M. G. (1996). Hyperlocomotion and indifference to cocaine and amphetamine in mice lacking the dopamine transporter. *Nature, 379*(6566), 606-612. doi: 10.1038/379606a0

Goodman, L. S., Gilman, A., Brunton, L. L., Lazo, J. S., & Parker, K. L. (2006). *Goodman & Gilman's the pharmacological basis of therapeutics* (11th ed.). New York: McGraw-Hill.

Howell, L. L., & Wilcox, K. M. (2001). The dopamine transporter and cocaine medication development: drug self-administration in nonhuman primates. *J Pharmacol Exp Ther, 298*(1), 1-6.

Jankovic, S. M., Milovanovic, D. R., & Jankovic, S. V. (1999). Schild's equation and the best estimate of pA2 value and dissociation constant of an antagonist. *Croat Med J, 40*(1), 67-70.

Kopajtic, T. A., Liu, Y., Surratt, C. K., Donovan, D. M., Newman, A. H., & Katz, J. L. (2010). Dopamine transporter-dependent and -independent striatal binding of the benztropine analog JHW 007, a cocaine antagonist with low abuse liability. *J Pharmacol Exp Ther, 335*(3), 703-714. doi: 10.1124/jpet.110.171629

Liao, C. F., Themmen, A. P., Joho, R., Barberis, C., Birnbaumer, M., & Birnbaumer, L. (1989). Molecular cloning and expression of a fifth muscarinic acetylcholine receptor. *J Biol Chem, 264*(13), 7328-7337.

Matsuzawa, S., Suzuki, T., Misawa, M., & Nagase, H. (1999). Roles of 5-HT3 and opioid receptors in the ethanol-induced place preference in rats exposed to conditioned fear stress. *Life Sci, 64*(21), PL241-249.

Mochan, W. L., del CastilloMussot, M., Barrera, R. G., P., A., Mexico, & Federal, D. (1987). Effect of plasma waves on the optical properties of metal-insulator superlattices. *Phys Rev B Condens Matter, 35*(3), 1088-1098.

Morgan, A. E., & Dewey, S. L. (1998). Effects of pharmacologic increases in brain GABA levels on cocaine-induced changes in extracellular dopamine. *Synapse, 28*(1), 60-65. doi: 10.1002/(SICI)1098-2396(199801)28:1<60::AID-SYN7>3.0.CO;2-A

Pettit, H. O., & Justice, J. B., Jr. (1991). Effect of dose on cocaine self-administration behavior and dopamine levels in the nucleus accumbens. *Brain Res, 539*(1), 94-102.

Raffa, R. B. (2009). The M5 muscarinic receptor as possible target for treatment of drug abuse. *J Clin Pharm Ther, 34*(6), 623-629. doi: 10.1111/j.1365-2710.2009.01059.x

Ritz, M. C., Lamb, R. J., Goldberg, S. R., & Kuhar, M. J. (1987). Cocaine receptors on dopamine transporters are related to self-administration of cocaine. *Science, 237*(4819), 1219-1223.

Singh, S. (2000). Chemistry, design, and structure-activity relationship of cocaine antagonists. *Chem Rev, 100*(3), 925-1024.

Stahl, E., & Ellis, J. (2010). Novel allosteric effects of amiodarone at the muscarinic M5 receptor. *J Pharmacol Exp Ther, 334*(1), 214-222. doi: 10.1124/jpet.109.165316

Takeuchi, J., Fulton, J., Jia, Z. P., Abramov-Newerly, W., Jamot, L., Sud, M., . . . Yeomans, J. (2002). Increased drinking in mutant mice with truncated M5 muscarinic receptor genes. *Pharmacol Biochem Behav, 72*(1-2), 117-123.

Tjio, J. H., & Puck, T. T. (1958). Genetics of somatic mammalian cells. II. Chromosomal constitution of cells in tissue culture. *J Exp Med, 108*(2), 259-268.

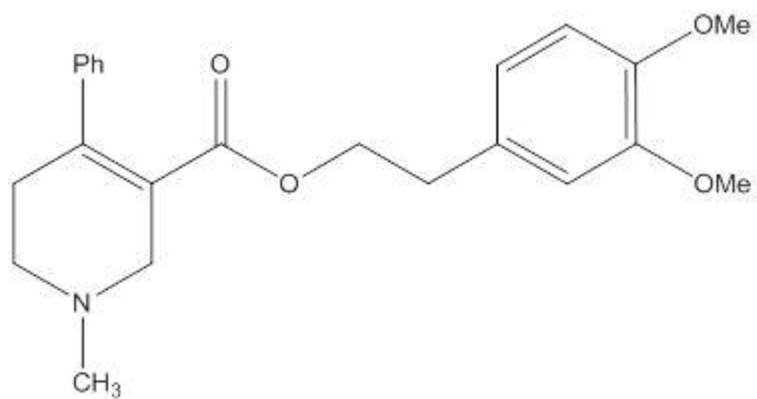
Vilaro, M. T., Palacios, J. M., & Mengod, G. (1990). Localization of m5 muscarinic receptor mRNA in rat brain examined by in situ hybridization histochemistry. *Neurosci Lett, 114*(2), 154-159.

Yeomans, J. S., Takeuchi, J., Baptista, M., Flynn, D. D., Lepik, K., Nobrega, J., . . . Ralph, M. R. (2000). Brain-stimulation reward thresholds raised by an antisense oligonucleotide for the M5 muscarinic receptor infused near dopamine cells. *J Neurosci, 20*(23), 8861-8867.

Zhang, W., Yamada, M., Gomeza, J., Basile, A. S., & Wess, J. (2002). Multiple muscarinic acetylcholine receptor subtypes modulate striatal dopamine release, as studied with M1-M5 muscarinic receptor knock-out mice. *J Neurosci, 22*(15), 6347-6352. doi: 20026644

Appendix A

GZ-002-05 Chemical Structure



Appendix B

K_B and pA_2 values

	【B】M	$EC_{50}a$ M	$EC_{50}b$ M	K_B	pA_2(-logKB)
M1	1.00E-05	1.50E-05	1.70E-06	1.28E-06	5.8934
M3	1.00E-05	2.96E-07	1.90E-07	1.79E-05	4.7465
M5	1.00E-05	8.375E-07	3.79E-07	8.27E-06	5.0827