

INVESTIGATING THE MECHANISM OF ACTION OF GUANOSINE BY THE G1
RECEPTOR

**INVESTIGATING THE MECHANISM OF ACTION OF GUANOSINE BY THE
G1 RECEPTOR**

By

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ABSTRACT

When released extracellularly, the purine nucleoside guanosine (Guo) can exert a wide range of physiological effects *in vitro* and *in vivo*. Guo can induce the release of neurotrophic factors such as nerve growth factor (NGF) and brain derived neurotrophic factor (BDNF) and can initiate the differentiation, growth and proliferation of neurons and glia. While structural and pharmacological evidence support the existence of a putative Guo binding site in the rat brain, there is a paucity of information on the mechanism through which Guo exerts these effects. Through bioinformatic research, our lab has identified an orphan G-protein coupled receptor as the first Guo receptor (termed G1R). The aim of this dissertation is to determine the mechanism of action of Guo using radioligand binding assays. It is hypothesized here that G1R is a distinct purinergic receptor for Guo. Using the calcium phosphate (CaP) co-precipitation (co-i.p.) method, *Drosophila Schneider 2* (S2) cells were stably and transiently transfected with G1R recombinant cDNA. A series of binding assays using tritiated Guo ([³H]-Guo) showed no difference in binding between CaP transfection groups and wild S2 controls that do not endogenously express G1R, suggesting that the [³H]-Guo may not have a high binding affinity for the G1R binding site. Preliminary experiments using the Lipofectamine® 3000 to transfect S2 cells showed higher G1R mRNA expression as well as increased binding affinity to Guo when compared to the CaP transfected groups. This suggests that the results in the CaP mediated groups may be due to low transfection efficiency. In conclusion, transfections using the CaP method resulted in too low of a transfection efficiency to see a difference in binding affinity between wild S2 and transfected S2 cells.

Findings from this work can be used to further examine the binding relationship of Guo to the G1R and optimize transfections using S2 cells and radioligand binding assays using purine based compounds.

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LIST OF ABBREVIATIONS

[3H]	Tritiated
[3H]-Guo	Tritiated Guanosine
7-TM	7-transmembrane
Akt	Protein Kinase B
ATP	Adenosine Tri-Phosphate
A₁R	Adenosine A₁ Receptor Subtype
A_{2A}R	Adenosine A_{2A} Receptor Subtype
A_{2B}R	Adenosine A_{2B} Receptor Subtype
A₃R	Adenosine A₃ Receptor Subtype
BDNF	Brain Derived Neurotrophic Factor
BK	Big Potassium
B_{MAX}	Maximal binding
BP	Base Pair
CaCl₂	Calcium Chloride
cAMP	Cyclic Adenosine Monophosphate
CaP	Calcium Phosphate
CdCl₂	Cadmium Chloride
cDNA	Complimentary DNA
CNS	Central Nervous System
CO₂	Carbon Dioxide

Co-i.p.	Co-Precipitation
CPMs	Counts per Minute
Ct	Cycle Threshold
CuSo₄	Copper Sulfate
DMEM	Dulbecco's modified Eagle's medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic Acid
DPMs	Disintegrations per Minute
DTT	Dithiothreitol
ECL	Electrogenerated Chemiluminescence
ECTO-PNPs	Purine Nucleoside Phosphorylases
EDTA	Ethylenediaminetetraacetic Acid
ERK1/2	Extracellular Signal-Regulated Kinase Proteins 1 and 2
EtoH	Ethanol
FBS	Fetal Bovine Serum
FGF-2	Fibroblast Growth Factor
G1R	G-Protein Coupled Receptor 1
GBPs	Guanine Based Purines
GDP	Guanosine 5' Diphosphate
GMP	Guanosine 5' Monophosphate
GPCR	G-Protein Coupled Receptor
G_{pp}[NH]_p	Guanylyl Imidiodiphosphate

GTP	Guanosine 5'Triphosphate
GUO	Guanosine
HCl	Hydrogen Chloride
His	Histidine
HRP	Horse Radish Peroxidase
I.C.V.	Intracerebroventricularly
I.P.	Intraperitoneal
kDa	Kilo Dalton
K_D	Dissociation Binding Constant
MAPK	Mitogen-Activated Protein Kinase
MgCl₂	Magnesium Chloride
mRNA	Messenger Ribonucleic Acid
NaCl	Sodium Chloride
Na₂HPO₄	Disodium Phosphate
NGN	Nerve Growth Factor
NMDA	<i>N</i>-methyl-D-aspartate
OGD	Oxygen/Glucose Deprivation
P₁	Purinergic Receptor Type 1
P₂	Purinergic Receptor Type 2
PBS	Phosphate-Buffered Saline
PCR	Polymerase Chain Reaction
PD	Parkinson's Disease

PEI	Polyethylenimine
PI3K	phosphatidylinositol-4,5 Biphosphate 3-kinase
PKA	Protein Kinase A
PKB/Akt	Protein Kinase B/Rac-Alpha Serine/Threonine-Protein Kinase
PMSF	Phenylmethane Sulfonyl Fluoride
pMT	Metallothionein Promoter
PSI	Proteasome Inhibitor
PTX	Pertussis Toxin
PVDF	Polyvinylidene Fluoride
qRT-PCR	Quantitative Reverse Transcription Polymerase Chain Reaction
RNA	Ribonucleic Acid
ROS	Reactive Oxygen Species
RT-PCR	Reverse Transcription Polymerase Chain Reaction
SCIs	Spinal Cord Injuries
S.D.	Standard Deviation
SDS	Sodium Dodecyl Sulfate
S2	Drosophila Schneider 2
TBS	Tris-Buffered Saline
TBT-T	Tris-Buffered Saline and Tween 20

TC6	Transfected C6 Cells
Tris	Trisaminomethane
TS2	Transfected S2 Cells
TUNEL	Terminal Deoxynucleotidyl Transferase (TdT) dUTP Nick-End Labeling
WC6	Wild Type C6 cells
WS2	Wild Type S2 cells

DECLARATION OF ACADEMIC ACHIEVEMENT

Stable transfections and some cell culture was performed by Ken Jiang until his departure in September 2015. Following this, finding new S2 cells, all transient transfections (both CaP and Lipofectamine® 3000) and tissue culture work were performed by me. All binding assays were performed by me with assistance from Ken (until his departure) and Ritesh Daya (until January 2015). All westerns were run by me with some assistance from Alan Zhu (undergraduate student) and some training from a former undergraduate student from my lab (Tina Wang). One round of PCR was run by Mahmood Aktar in the Tarnopolsky lab. The rest of the PCR was run by me in the Mishra lab with training from a Mishra lab student (Shreya Prashar). All samples for PCR, western blots and binding assays were collected by me.

SECTION 1: INTRODUCTION

1.1 Purines

Purine based compounds and their derivatives consist of a pyrimidine ring and an imidazole ring (Rosemeyer, 2004). Purines contain nitrogen in their ring structures and are aromatic organic compounds. Purine bases are best known for their role in forming nucleic acids which form deoxyribonucleic acid (DNA) and ribonucleic acid (RNA) (Rathbone et al., 1999). Additionally, they are mainly thought to have intracellular functions, with purine derivatives such as adenosine tri-phosphate (ATP) being important in cellular energy and guanine-based purine derivatives such as guanosine 5' triphosphate (GTP), guanosine 5' diphosphate (GDP) and guanosine 5' monophosphate (GMP) being involved in G-protein cell signalling (secondary messengers). However, over the past several decades evidence has supported an extracellular role for purines with receptors for adenosine and ATP being identified (Rathbone et al., 1999; Rosemeyer, 2004). Adenosine and ATP were shown to have trophic effect when released extracellularly and even act as neurotransmitters (Rathbone et al., 1999). Trophic substances induce changes in tissues or cells over a prolonged period of time and their effects are highly conserved across species both plant and animal based (Clark et al., 1993; Rathbone et al., 1999). In mammalian cell studies, extracellular purines have been shown to regulate gastrointestinal motility, cardiac muscle, renal function and respiratory function and reparative roles in the central nervous system (CNS) in the event of injury (Burnstock, 1991; Rathbone et al., 1999). With the identification of these extracellular purinergic effects, it is imperative to understand how they are being mediated.

1.2 Purinoceptors and Activation

There are two known purinergic receptor types that have been characterized in the nervous system (NS), P1 and P2 (Burnstock, 1976; Rathbone et al., 1999). The P1 classification identifies adenosine receptors and its subtypes (A_1 , A_{2A} , A_{2B} and A_3) and the P2 classification identifies ATP purinergic receptors and its subtypes (P_{2X1-7} , P_{2Y1-2} and P_{2u}). All P1 (adenosine) receptors are metabotropic while all P2 receptor subtypes are mixed, with some being metabotropic and others being ionotropic. While these receptors have a preference for adenosine and ATP, they have been known to be activated by other compounds (Rathbone et al., 1999; King & Burnstock, 2002). For example, it has been shown that adenosine receptors bind other purines such as inosine and ATP receptors are activated by uridine di- and triphosphates (Rathbone et al., 1999).

1.3 Chemical structure and synthesis of Guo

Guanosine ($C_{10}H_{13}N_5O_5$) is a guanine derivative, purine nucleoside that forms a glycosidic bond between its (9)nitrogen and the (1)carbon of its ribose ring (Figure 1) (Schmidt et al., 2007). Guanosine is released extracellularly after guanine nucleotides (such as GMP) are metabolized by 5'-ectonucleotidases (Figure 2) (Rathbone et al., 2008; Ciruela, 2013). Extracellular Guo can be further metabolized to guanine, by purine nucleoside phosphorylases (ecto-PNPs), which is then converted permanently to xanthine by guanine deaminases (Rathbone et al., 2008). Guanine derivatives, including Guo, are typically released from astrocytes *in vitro* and when stress conditions such as oxygen and glucose deprivation are induced (Rathbone et al., 2008).

Additionally, extracellular Guo has also been found to be released by neurons, other glial cells and oligodendrocytes under stress and basal conditions (Ciccarelli et al., 1999; Ciccarelli et al., 2000; Ciruela, 2013; Volpini et al., 2011). Guo's release from the immunocompetent astrocyte cells suggests its' involvement in neuroprotection and repair. Under basal conditions, extracellular Guo concentrations in the central nervous system (CNS) have been measured at 500nM and can increase significantly under stress conditions (Ciruela, 2013). For example, after focal stroke in rats Guo levels are increased and remain elevated for up to 7 days, supporting a possible role in repair (Uemura et al., 1991).

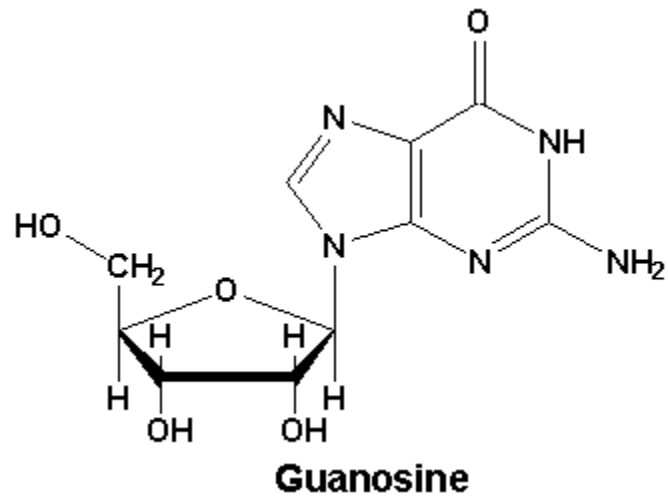


Figure 1. The chemical structure of Guanosine (Traversa et al., 2003)

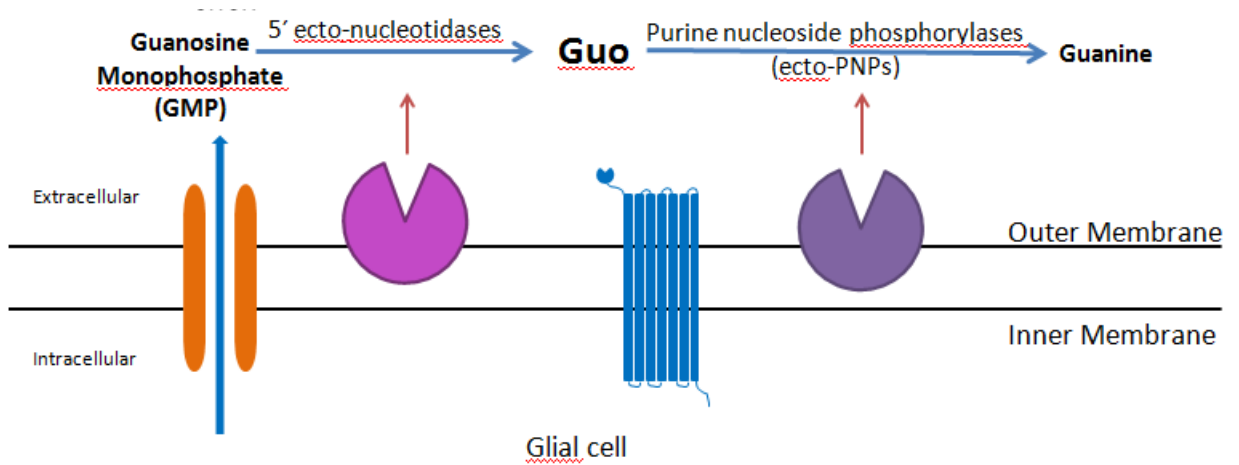


Figure 2. Extracellular synthesis and breakdown of Guo (schematic)

1.4 Guo and glutamate excitotoxicity

While adenosine and ATP are considered the major players of the purinergic system, extracellular Guo and other guanine based purines (GBPs) can also exert a myriad of effects on the CNS (Schmidt et al., 2007). Oxygen and glucose deprivation cause the creation of reactive oxygen species (ROS) which causes the release of neurotransmitters such as glutamate. The increased glutamate release can have an excitotoxic effect on cells causing an increased influx of sodium, potassium and calcium (Dal-Cim, et al., 2011; Dal-Cim et al. 2013). This can cause the creation of ROS and have other detrimental effects on the cell such as proteolysis and lipid peroxidation. Guanosine is one of many guanine derivatives that have been implicated in the involvement of glutamate transmission modulation. In a study by Dal-Cim et al. (2011), researchers examined the potential signalling pathways activated by released extracellular Guo in rat hippocampal slices exposed to oxygen and glucose deprivation (OGD) conditions. The study found that adding Guo (100 μ M) to slices subjected to 15 minutes of OGD followed by 2 hrs of re-oxygenation was neuroprotective (Dal-Cim et al., 2011). Further, Guo protected against OGD with the activation of PI3K pathway and increased glutamate uptake mediated by big potassium (BK) channels. Additionally, studies have shown that guanine derivatives can exert protective effects against seizures caused by quinolinic acid (N-methyl-D-aspartate (NMDA) receptor agonist; glutamatergic system stimulant) *in vivo* studies (Schmidt et al., 2000).

1.5 Trophic effects of Guanosine in disease and injury models

Guo has been found to release trophic substances, exert neuroprotective effects and stimulate the release of growth factors (nerve growth factor (NGF), brain derived neurotrophic factor (BDNF) and fibroblast growth factor-2 (FGF-2)) from nearby cells (Chang et al., 2008; Ciccarelli et al., 2000; Jiang et al., 2008; Rathbone et al., 1999; Rathbone et al., 2008). In a study by Su et al., (2009) Guo treatment increased the production and release of fibroblast growth factor -2 (FGF-2) in a proteasome inhibitor (PSI) induced model of Parkinson's Disease (PD). When administered intracerebroventricularly (i.c.v.). Guo has been shown to exert nociceptive and anxiolytic effects against pain models in mice (Schmidt et al., 2010).

Additionally, spinal cord injuries (SCIs) in rats that were treated with Guo resulted in remyelination (Jiang et al., 2003). They found that systematic administration of Guo could lead to remyelination and functional recovery in rats. Daily intraperitoneal (i.p.) injections of Guo solution (8mg) were administered to rats 34 days after spinal cords were surgically crushed (to a moderate severity) resulting in statistically significantly improvement in locomotor function compared to controls. Guo treatment increased remyelination as indicated by a fast blue (myelin stain) showing an increase in myelin in the Guo-treated animals compared to controls. Guo activated progenitor cells that were able to differentiate into oligodendroglia enabling remyelination of axons (Jiang et al., 2003).

The presence of extracellular Guo can also attenuate toxin/injury induced apoptosis. In a 2007 study, systemic i.p. administration of Guo (8mg/kg/day) was shown to reduce cellular apoptosis that can result from spinal cord injury in rats (Jiang et al., 2007). Guo treated animals had significantly fewer TUNEL-stained positive cells (indicative of apoptotic cell death) compared to controls (Jiang et al., 2007).

1.6 Putative Binding site for Guo

While there is strong evidence that supports Guo as a neuroprotective agent that is involved in the potential repair, differentiation and proliferation of different cell types (astrocytes, oligodendrocytes, progenitor cells), the mechanism through which Guo exerts its biological effects is still unclear (Cicarelli et al., 1999; Jiang et al. 2003). A study by Traversa et al. (2002) identified Guo high affinity binding sites in the rat brain. Traversa used whole rat brain samples and conducted binding assays. Results showed high affinity binding sites exist in the brain for Guo (Dissociation binding constant (K_D)= 95 nM and maximal binding (B_{MAX})=0.6 pmol/mg prot) (Traversa et al., 2002). While they were able to identify that these binding sites exist, they were unable to characterize them further. They speculated that the putative binding site for Guo is a G-protein coupled receptor (GPCR) due to the fact that tritiated Guo ($[^3H]$ -Guo) binding was significantly decreased by 50% when cells were pre-treated with pertussis toxin (PTX), an inhibitor of Gi/o coupled G-protein receptors (Traversa et al., 2002).

Additionally, Guo activates a number of intracellular signaling pathways including the elevation of cAMP (Su et al., 2013; Traversa et al, 2002; Traversa et al.,

2003; Volpini 2011), PI3kinase/Akt/PKB and ERK1/2, which are all characteristic of those activated by G-protein coupled receptors (Dal-Cim et al., 2013; Dal-Cim et al., 2011; DiIorio et al., 2004; Traversa 2002).

1.7 G-protein coupled receptors (GPCRs)

GPCRs, also known as guanine-nucleotide-binding protein-coupled membrane receptors or 7-transmembrane receptors (7-TM) are important for cellular signalling in eukaryotic cells (Palczewski & Orban, 2013). GPCRs are conserved in evolution and highly expressed in eukaryotic cells (Palczewski & Orban, 2013). They regulate a wide range of cellular processes through the activation of multiprotein complexes at the cellular membrane. More specifically, this complex contains heterotrimeric G proteins; an α -subunit bound to the GPCR and bound to it, a $\beta\gamma$ -subunit. G-proteins are able to hydrolyze GTP to GDP, rendering them active or inactive (respectively). Once activated the G-protein complex becomes unbound from the membrane bound receptor (Palczewski & Orban, 2013). These intermediary proteins (the $\alpha\beta\gamma$ complex) separate from the receptor. Following this, the α subunit dissociates from the $\beta\gamma$ complex. This allows them to interact with other intracellular effector proteins that can affect cellular processes (Palczewski & Orban, 2013).

1.8 G1R

The G1 receptor (G1R) is an orphan g-protein coupled receptor (Verleyen et al., 2014). It is found on chromosome 7q22 in humans and chromosome 6q16 in rats (O'Dowd et al., 1997). Northern blot analysis revealed G1R expression in the frontal

cortex, striatum, thalamus, caudate and putamen, using cDNA for G1R from human heart tissue. It is also expressed in cardiac myocytes and coronary arteries (O'Dowd et al, 1997). Its expression is highly conserved in mammals such as humans, rats and mice. It belongs to the class A rhodopsin-like GPCRs and has two known glycosylation sites in humans and rats (O'Dowd et al., 1997; Verleyen et al., 2014). Preliminary evidence indicates that the G1R is also expressed endogenously in astrocytes, C6 rat glioma cells, SH-SY5Y cells and N9 mouse microglial cells (unpublished collaborator's work). A study using HEK-293 cells over expressing G1R indicated that the receptor was coupled to Gi/o and activating this receptor would then cause inhibition of adenylyl cyclase and decreased cAMP and PKA (Adams et al., 2008). As there is no known endogenous ligand for this orphan receptor, its biological function remains unknown.

1.9 Drosophila Schneider 2 (S2) cells

Cell lines of *Drosophila melanogaster* were established by Kakpakov, Gvosdev, Platova and Polukarova (1969) and Echaliier & Ohanessian (1970) (Schneider, 1972). The S2 cell line is derived from the primary tissue culture of late stage *Drosophila melanogaster* embryos (20-24hours old). Cells are macrophage like in morphological appearance with cells presenting with a varying amount of different shapes and sizes with an average diameter of 25µm and an average length of 60 µm (Schneider, 1972). This cell type does not form a monolayer with more of the primarily round shaped cells aggregating into clusters that do not impede their overall health or grow. This semi-adherent cell line grows at an ideal temperature of 28°C.

SECTION 2: OBJECTIVES AND HYPOTHESES

The overall objective of this work is to determine the mechanism of action of extracellular Guo. Specifically, the focus of this work is to examine the binding of Guo to a specific receptor (G1R) that we believe is the putative binding site for Guo.

2.1 Objective 1: To determine the mechanism of action of Guo in an *in vitro* *Drosophila Schneider 2* (S2) model

It has previously been shown that there is a putative binding site for Guo in the rat brain (Traversa et al., 2002). Additionally, researchers have evidence that suggests that the putative binding site for Guo is a GPCR since studies aiming to characterize a putative binding site for Guo were able to significantly reduce specific binding through the use of GPCR ligand binding inhibitors such as guanosine triphosphate (GTP) and 5'-Guanylyl imidiodiphosphate ($G_{pp}[NH]_p$) and pertussis toxin (PTX) (lowers receptor affinity in G protein coupled pathways) (Traversa et al., 2002; Traversa et al., 2003). Research in our lab, along with research from collaborators, supports the evidence that the putative binding site for Guo is a GPCR and suggests that the G1R may be a putative binding site for Guo.

Our lab has preliminary evidence indicating that the G1R is capable of mediating the action of Guo as a signaling molecule. Our study (unpublished) showed that Guo inhibited apoptosis in G1R transfected S2 (TS2) cells when apoptosis was induced using 1 μ M actinomycin D (6hr. Incubation at 25°C) and failed to do so in wild type S2 (WS2) cells. This effect was also blocked by PTX, a known Gi/o protein pathway inhibitor found

in the literature (Traversa et al., 2003). This indicates that Guo induced inhibition of apoptosis is mediated by the G1R receptor in transfected S2 cells. Taken together, we believe that the G1R is the putative binding site for Guo.

2.1.1 Specific Aim #1: To determine if the physiological effects of Guo are mediated by G1 receptor binding in an in vitro S2 cell model

Our lab wanted to examine the Guo-G1R relationship in a simpler cell model. We found a cell line, Drosophila S2, that does not endogenously express the G1 receptor. To examine the ligand binding behaviour of Guo-G1R, we designed a system for recombinant DNA expression of G1R in the S2 cells. By co-transfecting the pMT-V5/His vector /G1R plasmid cDNA along with the pCoHygro vector into the insect cell line we will be able to investigate the relationship in a more isolated model. It is hypothesized here that the G1 receptor is the endogenous binding site for Guo. To investigate this, we will be running [3H]-Guo binding assays using the stably transfected S2 cells.

2.1.2 Specific Aim #2: To determine if CaP mediated stable transfections result in G1R positive protein expression compared to wild type cell (no G1R expression)

To ensure that binding affinity values are truly representative of the Guo-G1R relationship, and to confirm proper expression of the G1R protein, real time polymerase chain reaction (RT-PCR) and western blotting, probing for the G1R antibody, will be used. It is hypothesized that stable CaP mediated co-transfection of the pMT-V5/His vector /G1R plasmid cDNA and pCoHygro vector will result in positive G1R mRNA and protein expression in the TS2 cells.

2.2 Objective 2: To determine if the transient CaP transfection results in G1R protein expression

Another method of transfection used to express the G1R receptor in the S2 cells using CaP will be pursued. Transient transfection is a method by which the recombinant genetic material is expressed in the transfected cells but is not incorporated into the host cell's genome (Southern & Berg, 1982; Yang & Reth, 2012). This will allow us to investigate the G1R-Guo relationship in an environment that is not subjected to prolonged antibiotic exposure and ensure that findings are not the result of changes in cell behaviour or expression that can arise from the stable transfection method.

2.2.1 Aim #1: To determine if transiently expressing the G1R receptor in the S2 system will result in higher binding levels using [3H]-Guo compared to wild type cells

This study will investigate the Guo-G1R relationship in CaP transiently transfected S2 cells. It is hypothesized here that transiently transfected S2 cells will show higher binding than WS2 negative controls.

2.2.2 Aim#2: To determine if CaP mediated transient transfections result in G1R positive protein expression compared to wild type cell (no G1R expression)

To ensure that binding affinity values are truly representative of the Guo-G1R relationship, and to confirm proper expression of the G1R protein, RT-PCR and western blotting, probing for the G1R antibody, will be used. It is hypothesized that transient

transfection of the pMT-V5/His vector and G1R plasmid cDNA will result in positive G1R mRNA and protein expression in the TS2 cells.

2.3 Objective 3: To determine if the low binding results in CaP mediated stable and transiently transfected cells is due to low transfection efficiency or cell line

In this study, an alternative cell line and transfection method will be investigated. To ensure that G1R expression S2 cells line is not being hindered by post translational limitations in the S2 cell line itself, C6 rat glioma cells will be transiently transfected using the CaP method. The C6 rat glioma cells are part of a mammalian cell line that endogenously express the G1R (unpublished collaborator's works). Being that it endogenously expresses this membrane receptor, transfecting this cell line will allow us to quantify the endogenous expression levels of G1R in the WC6 cells and the over expressed levels in the TC6 cells and compare their [3H]-Guo binding values.

Secondly, S2 cells will be transiently transfected using the CaP and Lipofectamine® 3000 methods for comparison. The Lipofectamine® 3000 transfection method has a higher transfection efficiency compared to the CaP method (Santos et al., 2007; Chesnoy & Huang, 2000). By transfecting S2 cell in parallel using these different transfection methods we will be able to quantify the expression levels in both methods and determine which to utilize in future work. This will allow us to maximize the amount of G1R being expressed in the S2 model for our study.

2.3.1 Specific Aim: 1 To compare binding affinity of transfected cells to wild type cells in S2 and C6 glioma cells

C6 rat glioma cells will be transiently transfected with the G1R plasmid cDNA for over expression. C6 cells express endogenous levels of G1R that we will quantify using qRT-PCR and compare to the G1R expression levels that we are over expressing in C6 cells through CaP transient transfection. It is hypothesized here that wild type C6 cells will have high binding levels with the [3H]-Guo and transiently transfected C6 cells over expressing G1R binding will be even higher. The C6 binding levels (both wild and transiently transfected) will show higher expression levels than the S2 transfected cells.

2.3.2 Specific Aim :2 To compare the transfection efficiency of CaP transfection to Lipofectamine® 3000 transfection in S2 cells

Lipofectamine® 3000 is a form of lipid mediated transfection with higher transfection efficiency than CaP and Lipofectamine® 2000 (Chesnoy & Huang, 2000; Santos et al., 2007). It is hypothesized here that Lipofectamine® 3000 transiently transfected cells will have a higher G1R expression level when compared to the CaP transfected group.

SECTION 3: METHODOLOGY

3.1 Objective 1- To determine the mechanism of action of extracellular GUO

3.1.1 Specific Aim #1: To determine if the physiological effects of guanosine are mediated by G1 receptor binding in an in vitro S2 cell model

3.1.1.1 Recombinant cDNA Plasmid and Vectors

The G1R gene was cloned from rat striatum that was harvested in our lab. The RNA was extracted from the striatum using Trizol®. A G1R cDNA clone was then synthesized. cDNA synthesis was done using the qScript Flex cDNA kit (Quanta Biosciences; Cat. No. 95049-100) and transformed into One Shot® Top 10 Chemically Competent E. coli (Invitrogen; Cat. No. C404010) following manufacturer's protocol, and then extracted. The vectors used for stable transfection were pMT/V5-His A and pCo-Hygro selection vector from the Drosophila Expression System (DES® Kit; Invitrogen; Cat. No. K4120-01), provided by Dr. Roger Jacobs (Biology Department, McMaster University). pCoHygro was co-transfected into S2 cells with the G1R recombinant plasmid cDNA for stable transfection selection.

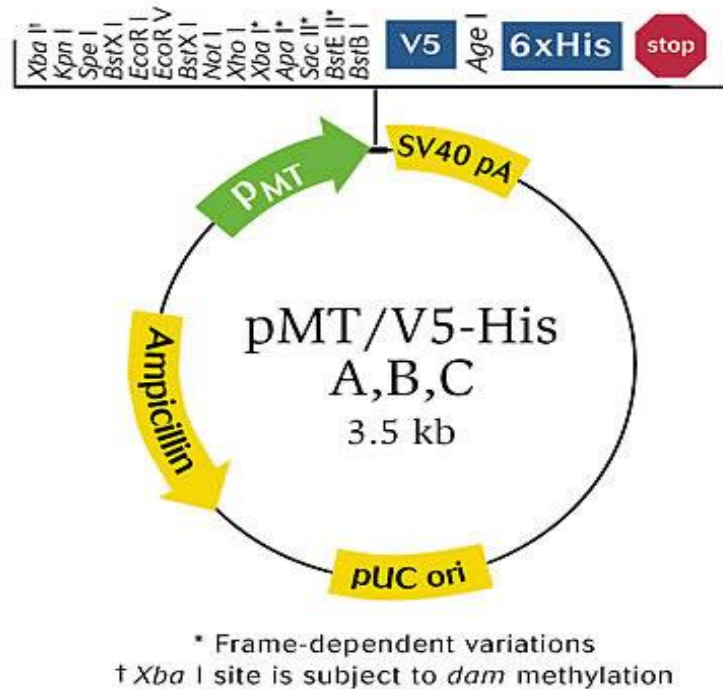


Figure 3: Inducible expression vector pMT/V5-His A map used for G1R transfection into the S2 cell line. Rat G1R cDNA was cloned in our lab and combined with an inducible expression vector pMT/V5-His-A for inducible expression of G1R.

(Figure retrieved from Invitrogen life technologies, https://tools.thermofisher.com/content/sfs/vectors/pmtv5his_map.pdf)

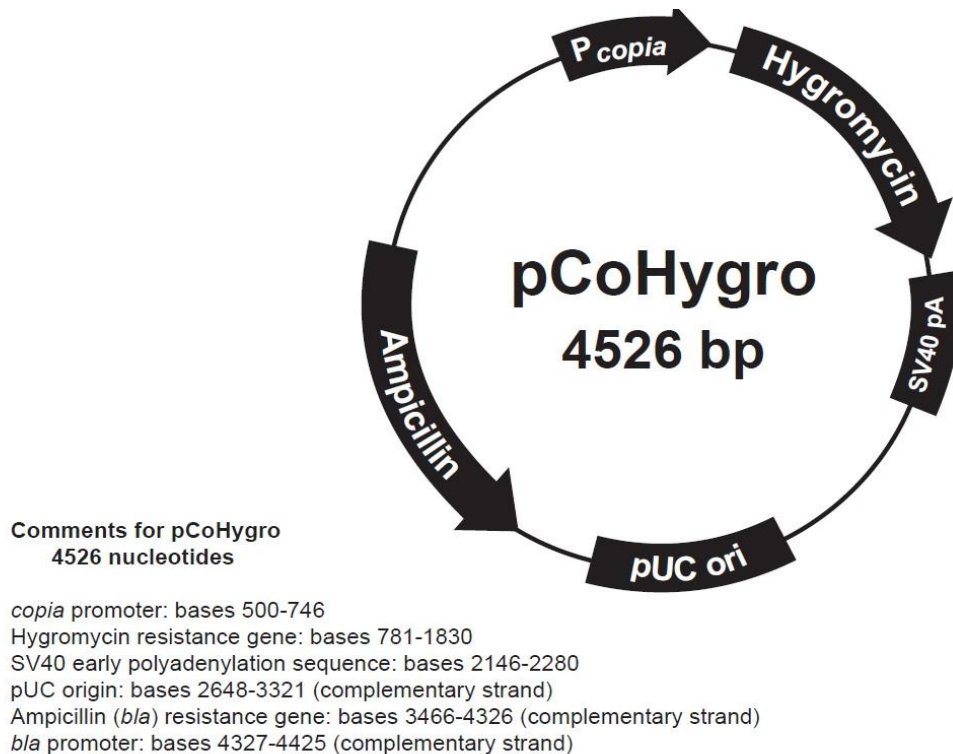


Figure 4. pCoHygro selection map used for stable transfection of the G1R into the S2 cell line. The pCoHygro vector possesses a Hygromycin resistance gene that is used for selection in transfected cells.

(Figure retrieved from Invitrogen life technologies, https://tools.thermofisher.com/content/sfs/manuals/des_man.pdf)

3.1.1.2 S2 cell tissue culture

The S2 cells are from an immortalized cell line and were obtained for stable transfection from Dr. Roger Jacob's laboratory (Biology Department, McMaster University). Cells were grown and maintained in serum free Schneider's Drosophila Medium (1X) (Gibco; Cat. No. 21720-024) at 28°C in 100mm² tissue culture dishes or 25cm² flasks. Cells were amplified, frozen and passaged following Drosophila Schneider 2 (S2) cells protocol (Invitrogen Life Technologies, Cat. No. R690-07). Cells were passaged every 3-4 days to a density of 2-4x10⁶/ml.

3.1.1.3 Cell Viability Assay

Cell viability was assessed using the Trypan Blue exclusion assay. The Trypan Blue exclusion assay is commonly used to determine cell viability in cell culture (Strober, 2015). Cells that are dead or dying have damaged cell membranes and will thus take on the dye. Conversely, healthy live cells with intact cellular membranes will exclude the dye (Strober, 2015). Cells were stained using 0.4% Trypan Blue stain (Gibco; Cat. No. 15250) in a 1:1 ratio and then loaded into a hemocytometer. Viability was measured by taking the average of the live cell counts and subtracting it from the total number of cells on the grid of the hemocytometer.

3.1.1.4 CaP Stable Transfections

The calcium phosphate transfection kit (Invitrogen life technologies, Cat. No. K278001) was used following manufacturer's protocol with slight modifications. Cells were thawed and passaged several times before seeding for transfection. Cells were

seeded at 1×10^6 cells/ml in 3mL in Drosophila Schneider 2 (S2) cells protocol (Invitrogen Life Technologies, Cat. No. R690-07) and grown for 16 hours at 28°C to a density of approximately 3×10^6 cells/ml. When cells reached the right density for transfection solution A (2M CaCl_2 , recombinant DNA $19\mu\text{g}$, $1\mu\text{g}$ pCoHygro and tissue culture water) to a total final volume of $300\mu\text{L}$ was prepared and added to Solution B $300\mu\text{L}$ of 2X HEPES-Buffered Saline (50mM HEPES, 1.5mM Na_2HPO_4 , 280mM NaCl, pH 7.1) drop wise with continuous mixing and while bubbling air through the solution. The combined solutions were incubated for 30-40 minutes and then added six drops (equalling $300\mu\text{L}$) to the S2 cells. S2 cells were homogenized in media in dish and then the CaCl_2 solution containing the recombinant G1R cDNA was added to the cells drop wise while shaking the dish. The solution was then homogenized and then incubated at 28°C . One to two hours later cells were homogenized to ensure no DNA had settled to the bottom of the dish. The cells were then incubated for 20 hours. After this incubation period the CaP solution was removed and cells were washed twice with complete medium. Fresh medium was added and cells were left to incubate at 28°C for 2 days. Selection began on day 5 with cells being centrifuged and re-suspended in complete medium containing 10% Fetal Bovine serum (FBS) (Sigma-Aldrich, F1051) and hygromycin-B (Invitrogen, 10687-010). Media was changed every 4 to 5 days for 3-4 weeks until resistant colonies appeared. Resistant colonies were then re-plated and maintained in selective medium (containing hygromycin-B). Cells were maintained until induction with CuSO_4 .

3.1.1.5 CuSO₄ Expression

CuSO₄ was used to activate the metallothionein promoter (pMT) in the inducible expression vector transfected into the S2 cells. A 500µM concentration of CuSO₄ was added to the dishes in a drop wise fashion (swirling after each drop to ensure proper mixing). Cells were induced with CuSO₄ a minimum of 24 hours before assaying.

3.1.1.6 Membrane Isolation Protocol

Cells were spun at 800xG for 10 minutes and collected and the media was discarded. Cells were washed with 50mL of Schneider's S2 medium, and then twice with phosphate buffered saline (PBS) to remove any residual CuSO₄. After washes, cells were lysed using a lysis buffer (5mM Tris and 5mM MgCl₂ adjusted to a final pH of 7.4). The lysate was then glass to glass homogenized (10 strokes) and then centrifuged for 30 minutes at 17200rpm. The pellet was then re-suspended in a binding assay buffer (50mM Tris and 1mM EDTA adjusted to a final pH of 7.4) and then re-centrifuged for 30 minutes at 17200rpm. The final membrane pellet was re-suspended by light homogenization in binding buffer immediately before use in binding assays. This protocol was adapted from Schetz et al. (2003) and slightly modified. Samples were then put on ice and optical density was tested using 540nm.

3.1.1.7 Binding Assays:

3.1.1.7.1 Saturation Isotherm Assays

To determine the binding affinity of Guo for the G1R, radioligand binding assays were used. The protocol used for the binding assays using tritiated Guo ([³H]-Guo) was adapted from Dr. Mishra's laboratory (Department of Psychiatry and Behavioural Neurosciences, McMaster University). The aim of a saturation isotherm assay is to determine the concentration at which the binding is maximized (B_{max}). Protein, assay buffer and tritiated ligand were added to Fisherbrand glass 12x75 glass tubes (Cat. No.14-961-26) to a total final volume of 200 μ L. 100 μ g of protein and 5nM of [³H]-Guo were combined in the tubes. The volume was brought up to 200 μ L using assay buffer (50mM Tris-HCl, 1mM EDTA mM , 0.1mM PMSF in EtoH, 5mMMgCl₂·6H₂O, 0.1mM DTT, 100 μ g/mL Bacitracin and 5 μ g/mL Soybean Trypsin-adjusted to a pH of 7.4 using HCl and filled to 500mL with dH₂O stored at 4°C). A dilution series of the tritiated ligand was done to determine saturation using a fixed amount of protein. Samples were run in triplicate. Samples were incubated at 25°C for 45 minutes and then filtered through a protein harvester (Brandel, USA) and washed 8 times using ice cold filtration buffer (50mM Tris-HCl and 1mM EDTA adjusted to a pH of 7.4 using HCl and filled to 2L with dH₂O stored at 4°C). After filtration, filter paper was removed from the harvester and sections corresponding to tubes were cut in the SD-24 Brandel dispenser. Filter paper was the placed in white scintillation tubes and filled with scintillation cocktail. Samples were left to incubate in liquid scintillation cocktail (Beckman Coulter, USA) at room temperature for 24 hours and then put into the Beckman scintillation counter for counting. Both transfected and wild type cells were used to determine B_{max} and non-specific binding for this experiment.

3.1.1.7.2 Binding Assay Counting

After a 24 hour incubation period the samples are loaded into the Beckman counter that generated counts per minute and disintegrations per minute that we can use to assess binding affinity.

3.1.1.7.3 Displacement Assays

Serial dilutions of cold Guo (unlabeled displacement agent) were prepared for displacement assays. Samples were prepared in triplicate. All glass tubes are prepared with the same amount of protein (receptor) and [3H]-Guo with varying amounts of cold Guo to determine binding affinity to the G1R. Assay buffer was used to bring the final concentration to 200 μ L. The binding assay was conducted in the same method as fully described in section 3.1.1.7.1.

3.1.1.8 Binding Assay Optimization for purinergic binding

3.1.1.8.1 Filter paper pre-treatment

After it was determined that the background binding was interfering with the readings, polyethylenimine (PEI) (Sigma Aldrich, Missouri, USA, Cat. No. 40,870-0) was used to coat the filter paper before filtration. This technique is commonly found in the literature (Schepmann et al., 2010; Traversa et al., 2002). It is considered effective because the PEI is a cationic polymer that neutralizes the negative charge of the glass fiber filter paper to reduce binding (Traversa et al., 2002). Whatman GF/B filter paper was pre-soaked for 30 to 60 minutes or overnight in 0.5% or 1% PEI (diluted in double

distilled water) at 4°C. Before filtering samples through the pre-treated filter paper, the filter paper was washed 4x using ice cold filter buffer.

3.1.1.8.2 Binding Assay using centrifugation filtration method

All steps for the binding assay followed the Mishra lab binding assay protocol (as described in section 3.1.1.7.1) up to the point of incubation in the water bath for 1 hour at 25°C. After incubation samples were removed and transferred to 1.5mL microcentrifuge tubes for centrifugation to separate the bound ligand to the receptor from the free floating ligand in the solution and 200µL of ice cold filtration buffer was added to the samples. Samples were then gently homogenized and spun in the microcentrifuge for 10 minutes at 6000 rpm. The supernatant was discarded and samples were rewashed with 200µL of cold filter buffer and re-spun for 10 minutes at 6000rpm. The supernatant was discarded a second time and the pellet was dissolved in liquid scintillation cocktail (Beckman Coulter, USA) at room temperature. Tubes were incubated for 24hrs and then put into the counter.

3.1.1.8.3 Binding Assay Counting

After a 24hour incubation period in scintillation cocktail, samples were loaded into the Hidex 300 SL automatic liquid scintillation counter (MCG, USA) to be counted. DMPs are generated using the MikroWin 300 SL software and analysed for binding affinity.

3.1.2 Aim #2 Specific Aim #2: To determine if calcium phosphate mediated transfections result in GIR positive protein expression compared to wild type cells (no GIR expression)

3.1.2.1 Protein Extraction

Protein was extracted using the I-PER[®] Insect Cell Protein Extraction Reagent (Thermo Scientific, Cat. No. 89802) following manufacturer's protocol with slight modifications. This reagent contains a non-ionic detergent that is commonly used for insect cell cytoplasmic protein extraction (Thermo Scientific, 89802). This procedure is recommended for insect cells that are grown in suspension (Thermo Scientific, 89802). Cells were centrifuged at 5000 x G for 10 minutes. 8 μ L of Lysozyme and 8 μ L DNase I was added to 1mL of I-PER. 100-150 μ L of lysis buffer was added to the cell pellet. Samples were incubated for 2 hours at -20°C. Samples were removed from the freezer re-centrifuged at 14.5xG for 10 minutes at -4°C. The pellet was discarded and the supernatant was kept and stored at -20°C. Protein concentrations were determined using the spectrophotometer at 540nm.

3.1.2.2 Western Blotting

15% sodium dodecyl sulfate (SDS) polyacrylamide gels (1.5mm thick) were used to separate 12-20 μ g of protein before polyvinylidene fluoride (PVDF) membrane transfer. Membranes were then blocked overnight with milk protein. After blocking the membranes were briefly rinsed with TBS-T and probed for 2 hours with the following primary antibodies: GIR (name not revealed for confidentiality) (Novatein Biosciences,

USA) 1:1000 and Anti-His (GenScript, USA; A00186-100) 1:1000. After washing with TBS-T blots were incubated for an hour with secondary antibodies: Goat anti-rabbit HRP (ab6721) 1:6000 (Abcam, USA) and Goat Anti-mouse (GenScript, USA; A00160). Membranes were then washed with TBS-T and treated with equal parts of high sensitivity electrogenerated chemiluminescence (ECL) detection reagents (Amersham, RPN2106) to cover the membrane. The final volume required is 0.125mL/cm² of membrane. After 1 minute the ECL solution was removed and the membrane was covered in Saranwrap and taken to the dark room for exposure. Membranes were exposed to autoradiography film (Amersham Hyperfilm™ ECL, Cat. No. 45001507) for varying amounts of time (1second-5minutes). The film was then developed using the X-ray film developer Konica Minolta model SRX-101A (Pathology and Molecular Medicine Center for gene therapeutics, McMaster University).

3.1.2.3 RNA Extraction

Cells were lysed using 1ml Trizol™ (Invitrogen, Burlington, ON) and stored at -20°C until RNA extraction occurred. Real-time reverse transcription-polymerase chain reaction (RT-PCR) S2 samples were prepared using the RNeasy™ Mini Kit from Qiagen (Qiagen, Mississauga, ON) following the manufacturer's protocol. The Trizol cell samples were thoroughly homogenized and then left to incubate at room temperature for 5 minutes. Chloroform was then added to each sample and the samples were then shaken by hand for 15 seconds. The aqueous phase was taken and combined with 70% ethanol to precipitate the RNA. This solution was then transferred to a RNeasy Mini column and spun at 8000 x G for 15 seconds at room temperature. Flow through was discarded. RTW

buffer was added to the column and re-spun at the same speed and time. Samples were then washed twice with RPE buffer before RNase-free water was added and then centrifuged for 1 minute at 8000 x G to elute. RNA concentration was determined using the Nanodrop 2000 spectrophotometer (NanoDrop Technologies Inc., USA) and ND-1000 V3.5.2 software at 260/280nm.

3.1.2.4 DNase treatment and cDNA synthesis

DNase treatment was performed using on-column DNase digestion following the RNeasy Mini Kit part 2 (Cat. No. 74104 and 74106) following manufacturer's protocol.

3.1.2.5 Real-time reverse transcription-polymerase chain reaction (RT-PCR)

G1R expression was determined by RT-PCR. The test was run to confirm the presence of the G1R mRNA in the stably transfected cells and the lack of mRNA expression in the wild S2 cells. Issues with western blotting and not being able to visualize a G1R protein band led us to question whether the cells were correctly expressing the receptors or not. This RT-PCR was run to confirm that there was in fact expressing in the transfected cells and none in the wild type cells.

3.1.2.6 Statistical Analysis

Data collected in this work was analysed using Graph Pad Prism 7 software. Unpaired t-tests assuming equal or unequal variances were used to assess the data. Significance was set at $p < 0.05$, using a two-tailed critical value.

3.2 Objective 2 To determine if the transient calcium phosphate transfection results in G1R protein expression

3.2.1 Aim #2 To determine if transient over expression the G1R receptor into the S2 system will result in more binding using [3H]-Guo compared to wild type cells

3.2.1.1 S2 Cell Tissue Culture

S2 cells were obtained from Dr. Andre Bedard's biology laboratory (Biology Department, McMaster University). The S2 cell line obtained is an immortalized cell line. All tissue culture procedures followed Drosophila Schneider 2 (S2) cells protocol from (Invitrogen life technologies, Cat. No. R690-07). Cells were cultured in SFX serum free insect media (Hyclone Laboratories, Cat. No. SH30278.02) at 28°C.

3.2.1.2 CaP Transient Transfections

The CaP transfection kit (Invitrogen life technologies; Cat. No. K278001) was used following manufacturer's protocol with slight modifications. Cells were thawed and passaged 4 times before seeding for transfection. Cells were seeded at 1×10^6 cells/ml in 6ml of SFX insect medium and grown for 16 hours at 28°C to a density of approximately 3×10^6 cells/ml. Solution A (72µl of 2M CaCl₂, 43ul of Recombinant DNA(40µg) and 485µl of tissue culture water) was added to Solution B- 600ul of 2X HEPES-Buffered Saline (50mM HEPES, 1.5mM Na₂HPO₄, 280mM NaCl, pH 7.1) drop wise with continuous mixing and while bubbling air through the solution. The now combined solutions are incubated for 30-40 minutes and then added drop wise to the S2 cells. S2 cells were homogenized in media in dish and then the calcium chloride solution

containing the recombinant G1R DNA was added to the cells drop wise while shaking the dish. The solution was then homogenized and then incubated at 28°C. One-2 hours later cells were homogenized to ensure no DNA had settled to the bottom of the dish. The cells were then incubated for 20-22 hours. After this incubation period cells were washed and re-suspended in complete SFX medium containing 10% FBS (no selection agent). following the Invitrogen protocol and re-suspended in media and left to grow for 1-4 days until inducing expression.

3.2.1.3 CuSO₄ induction

While troubleshooting the low expression of the G1R, we modified the concentration of the inducing agent. For this round of experiments 800µM was used to induce expression instead of 500 µM following the same protocol fully described in section 3.1.1.5.

3.2.1.4 Binding Assays

Due to concerns that the membrane isolation protocol was not isolating the protein correctly, the binding assays in this round of experiments were performed using whole cells. If the cells are correctly expressing the receptors on their plasma membranes, then cell lysis was deemed not be necessary. Binding assays were performed using whole cells (for both WS2 and TS2). Cells in both groups (transfected and wild type) were cultured to the same cellular density and prepared and run using the same protocol as outlined in section 3.1.1.8.2.

3.2.1.5 Binding Assay Counting

After a 24hour incubation period in scintillation cocktail, samples were analysed using the Hidex 300 SL scintillation counter.

3.2.2 Aim #2: To determine if calcium phosphate mediated transient transfections result in GIR positive protein expression compared to wild type cell (no GIR expression)

3.2.2.1 Protein Extraction

Protein was extracted using the I-PER[®] Insect Cell Protein Extraction Reagent from (Thermo Scientific; Cat. No. 89802) following manufacturer's protocol with slight modifications (as fully described in section 3.1.2.1).

3.2.2.2 Western Blotting

8% sodium dodecyl sulfate (SDS) polyacrylamide gels (1.5mm thick) were used to separate 12-20µg of protein before polyvinylidene fluoride (PVDF) membrane transfer. Membranes were then blocked overnight with milk protein. After blocking the membranes were briefly rinsed with TBS-T and probed for 2 hours with the following primary antibodies: 1:1000 and Anti-His (GenScript, USA; A00186-100) 1:1000. After washing with TBS-T blots were incubated for an hour with secondary antibodies: Goat anti-rabbit HRP 1:6000 (Abcam; ab6721) and Goat Anti-mouse (GenScript; Cat. No.A00160). Membranes were then washed with TBS-T and treated with equal parts of high sensitivity ECL detection reagents (Amersham; Cat. No. RPN2106) to cover the

membrane. The final volume required is 0.125mL/cm² of membrane. After 1 minute the ECL solution was removed and the membrane was covered in Saranwrap and taken to the dark room for exposure. Membranes were exposed to autoradiography film (Amersham Hyperfilm ECL™, Cat. No. 45001507) for varying amounts of time (1second-5minutes). The film was then developed using the X-ray film developer Konica Minolta model SRX-101A (Pathology and Molecular Medicine Center for gene therapeutics, McMaster University).

3.2.2.3 RNA Extraction

S2 cells were treated with Trizol[®] (Invitrogen; Cat. No.15596026) and homogenized using a 21G 1 ½ needle (pipette in and out 10 times). Chloroform was added to each sample and shaken for 15 seconds by hand. RNA was precipitated using isopropyl alcohol, washed with 75% ethanol and then solubilized into nuclease free water. The optical densities of the samples were measured using the Nanodrop 2000 spectrophotometer and stored at -80°C for DNase treatment and cDNA synthesis.

3.2.2.4 DNase treatment and cDNA synthesis

DNase treatment to removed genomic DNA was done using DNase treatment reagents (Qiagen) for 5-15µg/mL of RNA sample. Samples were run for 40 minutes at 37°C and 10 minutes at 65°C (EDTA added in between to quench the reaction). Reverse transcription was performed using 2.2µL of DNase treated RNA and reverse transcriptase enzyme Omniscript (Qiagen) reagents run for 60 minutes at 37°C and then 5 minutes at 93°C.

3.2.2.5 RT-PCR

Both forward and reverse primers were manufactured by Mobix and used for PCR and DNA sequencing (see Table 1). This method allows for an assessment of the transfection efficiency of the calcium phosphate transfection method. All reactions were performed in triplicate using ROX and SYBRGREEN (Qiagen) and the StartaGene® Mx3000P Multiplex quantitative PCR system (Agilent Technologies, USA). The thermal profile used to detect the primers was: 40 cycles of 94°C 30s, 57°C for 30s, and 72°C for 1 minute.

3.2.2.6 Agarose Gel and DNA Sequencing

Amplified DNA bands were separated on a 1.5% agarose gel stained with ethidium bromide and run at 200V for 20 minutes at room temperature. Bands were cut out and treated using the Freeze 'N Squeeze DNA Gel Extraction Spin Columns kit (BIO-RAD) and samples were sent to Mobix for DNA sequencing. DNA sequencing was then analyzed to confirm correct amplification of desired product. The sequence was then run through a BLAST software to confirm the correct expression of the G1R sequence.

3.2.2.7 Statistical Analysis

Data collected in this work was analysed using Graph Pad Prism 7 software. Unpaired t-tests assuming equal or unequal variances were used to assess the data. Significance was set at $p < 0.05$, using a two-tailed critical value.

Objective 3 To determine if the low binding results in calcium phosphate mediated stable and transiently transfected cells is due to low transfection efficiency or cell line

3.3.1 Aim #1 To compare binding affinity of transfected cells to wild type cells in two different cell lines

3.3.1.1 S2 cell culture

Cells were obtained from Dr. Andre Bedard's laboratory in the Biology Department of McMaster University. Cells were maintained as fully described in section 3.2.2.1.

3.3.1.2 C6 cell culture

Rat C6 glioma cells were selected to ensure proper insertion and expression of the GIR. Rat C6 glioma cells were maintained in Dulbecco's modified Eagle's Medium (DMEM, Invitrogen) with penicillin/streptomycin, 10% fetal bovine serum and fungizone (2.5 µg/mL, Invitrogen; Cat. No. 15240096) at 37°C under 5% CO₂ and air.

3.3.1.3 CaP Transient Transfections of C6 and S2

The CaP transfection kit (Invitrogen life technologies, Cat. No. K278001) was used following manufacturer's protocol with slight modifications (described fully in section 3.2.1.2). After this incubation period cells were washed and re-suspended in complete SFX medium containing 10% FBS and left to grow for 1-4 days until inducing expression.

3.3.1.4 CuSO₄ induction

C6 cells were induced using 800 μ M and S2 cells were induced using 800 μ M and 1200 μ M to determine if increasing the induction agent concentration will increase expression. Cells were induced using the method fully described in section 3.1.1.5.

3.3.1.5 Binding Assay using C6 Glioma and S2 cells

The C6 cell binding assay was performed using whole transiently transfected and wild C6 cells. Cells in both groups (transfected and wild) were cultured to the same cellular density and prepared as per binding assay protocol (minus the membrane protein isolation protocol) (as described fully in section 3.1.1.8.2). This method was repeated for S2 cells for comparison.

3.3.2 Aim #2: To compare the transfection efficiency of calcium phosphate mediated co-ip to Lipofectamine® 3000

3.3.2.1 Lipofectamine 3000 transfections in S2 cells

GIR recombinant DNA plasmid was transfected into S2 cells using Lipofectamine 3000[®] (Invitrogen, USA). Modifications were made to the manufacturer's protocol due to the fact that Lipofectamine 3000 is designed and has only been tested by the manufacturer on mammalian cells. This method of transfection was used instead of the recommended for the insect cell line, Cellfectin, due to the fact that it was kindly provided by Margaret Fahnestock's lab for a preliminary experiment. Since Lipofectamine 3000[®] is used for adherent cells modifications to the protocol had to be made to account for insect cells grown in suspension. 1500 μ L of solution A (48 μ g of

diluted plasmid DNA, SFX serum free media and P3000®) and 1500µL solution B (Lipofectamine 3000® and SFX serum free media) were combined and added drop wise to the 100mm² dish containing S2 cells grown in suspension at a density of 4x10⁶/mL at a total volume of 6mL. Cells were then incubated in the solution for two different incubation periods (12 hours and 24 hours) to ensure the cells would survive the exposure to the Lipofectamine 3000® reagents. After 12 and 24 hours the cells were washed and re-suspended in complete SFX medium containing 10% FBS. Cells were then left to grow for 24 hours before inducing expression.

3.3.2.2 CuSO₄ induction

S2 cells were induced using 800µM and 1200µM CuSO₄ to determine if increasing the induction agent concentration will increase expression. Cells were induced using the method fully described in section 3.1.1.5.

3.3.2.3 RNA extraction

S2 cells were treated with Trizol® (Invitrogen, Burlington, Ontario) and followed the same extraction protocol as fully described in section 3.2.2.3.

3.3.2.4 qRT-PCR

Quantitative RT-PCR was done following the protocol outlined in section 3.2.2.5.

3.3.2.5 Protein Extraction for S2 and C6 cells

0.1% SDS was used to extract protein for the S2 cells grown in suspension and C6 glioma cells. This reagent is an anionic detergent used for nuclear/sub-cellular protein extraction.

This method is recommended if you are trying to obtain a signal for a protein that has low expression. 100 μ L of 0.1% SDS was added to samples. Samples were then vortexed and centrifuged for 10 minutes at 17200rpm. Supernatant was transferred to a new tube and its concentration was tested.

3.3.2.6 Western Blotting

8% sodium dodecyl sulfate (SDS) polyacrylamide gels (1.5mm thick) were used to separate 30-50 μ g of protein before polyvinylidene fluoride (PVDF) membrane. Membranes were then blocked overnight with protein. After blocking the membranes were briefly rinsed with TBS-T and probed for 2 hours with the following primary antibodies: Anti-His (GenScript; A00186-100) 1:1000 and Anti-V5-FITC Ab (Novex; 46-0308) 1:500. After washing with TBS-T blots were incubated for an hour with secondary antibodies: Goat Anti-mouse (GenScript; A00160). Membranes were then treated and exposed as previously describe in section 3.1.2.2.

3.3.2.7 Statistical Analysis

Data collected in this work was analysed using Graph Pad Prism 7 software. Unpaired t-tests assuming equal or unequal variances were used to assess the data. Significance was set at $p < 0.05$, using a two-tailed critical value.

Gene	Primer (5'-3')	Product Size (Base Pairs)
G1R	Forward TGACAATGGGCAGAGCTGTA	75Bps
G1R	Reverse TGAAGGGAATCAGGAATGAGA	75Bps

Table 1. G1R forward and reverse primers with their corresponding product size.

Primers amplified a 75bp product that matched chromatograph sequencing results.

Sequence was then blasted and correct gene (G1R) was matched.

Representative Sequences of G1R	Length
Genomic	7081bps
Transcript	4587bps
Polypeptide	469aa

Table 2. Sequences for G1 receptor and corresponding lengths.

SECTION 4: RESULTS

4.1 Objective 1

4.1.1 [³H]-Guo shows high binding to both TS2 and WS2 cells in Saturation Isotherm Assays

TS2 cells show higher binding to [3H]-Guo when compared to WS2 cells (Figure 5). Following 500µM CuSO₄ induction, saturation isotherm binding assays were run to determine the amount at which G1 receptors in the transfected samples became saturated with [3H]-Guo. The Bmax and dissociation constant (Kd) for the transfected cells were 8025 fmol/g and 7.98nm, which were deemed too high to be representative of the binding relationship of Guo and the G1R alone. Additionally, WS2 cells showed similar high binding with a Bmax of 7291 fmol/g and Kd of 7.78, which could not be explained. No significant difference was found between groups.

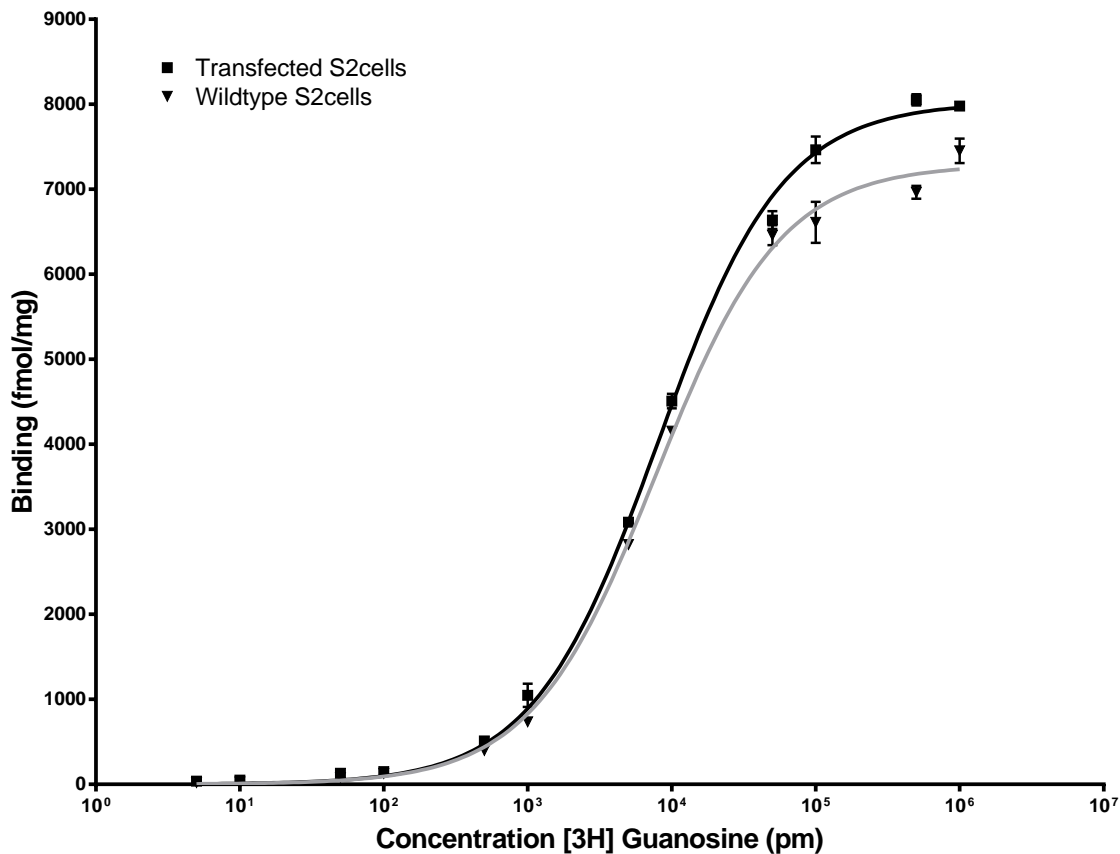


Figure 5: Transfected S2 cells have higher binding to [3H]-Guo compared to wild S2 cells. Following stable transfection and CuSO₄ induction, a saturation isotherm binding assay using wild type and stably transfected S2 cells was performed. Error bars represent S.D. The Bmax for transfected cells was 8025 fmol/mg and the Kd value was 7.98nM. The Bmax for wild type S2 cells was 7291 fmol/mg and the Kd value was 7.78nM. There was no significant difference found between the two sample sets. Samples were run in triplicate. Throughout this paper hot guo refers to radiolabeled guo and cold guo refers to unlabeled guo.

4.1.2 Pre-treating filter paper with PEI does not reduce high background binding

[3H]-Guo shows high affinity for the fiber glass filter paper when blank (no protein) binding assays were run (Figure 6). Soaking the filter paper in 0.5% PEI had no effect on [3H]-Guo filter paper binding. Different filter paper types (GF/B and GF/C), concentrations of PEI (1% and 0.5%) and increasing the number of washes with filter buffer, all failed to reduce [3H]-Guo binding to the filter paper and showed similar results. [3H]-Guo binding to the filter paper resulted in an average of 3647.28 DPMs and was being displaced with cold Guo resulting in an average of 1578.77 DPMs. High background binding was the cause of the inconsistent data found in previous saturation isotherm assays and could potentially explain the high binding that was found in the WS2 samples. While PEI appeared to reduce background binding in the literature, it failed to do so here. These findings suggest that the labeled guanosine is highly attracted to the filter paper. No statistical analysis was deemed necessary for the assessment of this data. It was simply to confirm that high background binding was taken place.

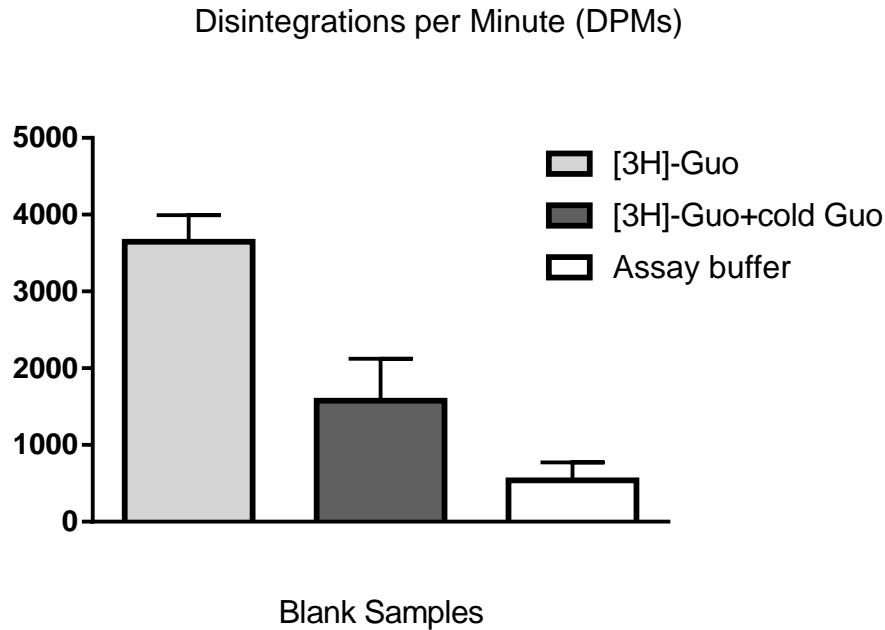


Figure 6. [3H]-Guo is highly attracted to the fibreglass filter paper in blank tests.

Following inconsistent findings in the saturation isotherm experiments, blank tests were run using no protein to assess the levels of background binding. High binding was found between [3H]-Guo and the filter paper. Error bars represent SD. Samples were run in triplicate. The filter paper (GF/C; Brandel, USA) was soaked overnight in 0.5% PEI polymer at 4°C. GF/B filter paper test showed similar results. 2nM of [3H]-guanosine was used along with 100uM of cold guanosine for displacement. No statistical analysis was performed. Samples were run in triplicates.

4.1.3 *[3H]-Guo binding showed no difference between WS2 and stably transfected S2 cells in repeated binding assays using centrifugation*

[3H]-Guo showed significantly higher binding in WS2 cells compared to TS2 cells in a binding assay using centrifugation (Figure 7; * $p < 0.05$). After 500 μ M CuSO₄ induction to express G1R and a 24 hour incubation, TS2 isolated membrane protein was used in a binding assay using centrifugation. WS2 isolated membrane protein was used as a negative control. Once optimized, the binding assay protocol used in these experiments was modified from the filtration method using a harvester and filter paper, to separation via centrifugation. 180 μ g of protein was used for all samples with 2nM [3H]-Guo. 100 μ M of cold Guo was used for displacement. Binding was measured using DMPs recorded by the Whatmann Scintillation counter. This experiment was repeated twice using the same parameters and no significant difference in binding was found between TS2 and WS2 groups (Figure 8; with A) $p = 0.31$; B) $p = 0.06$).

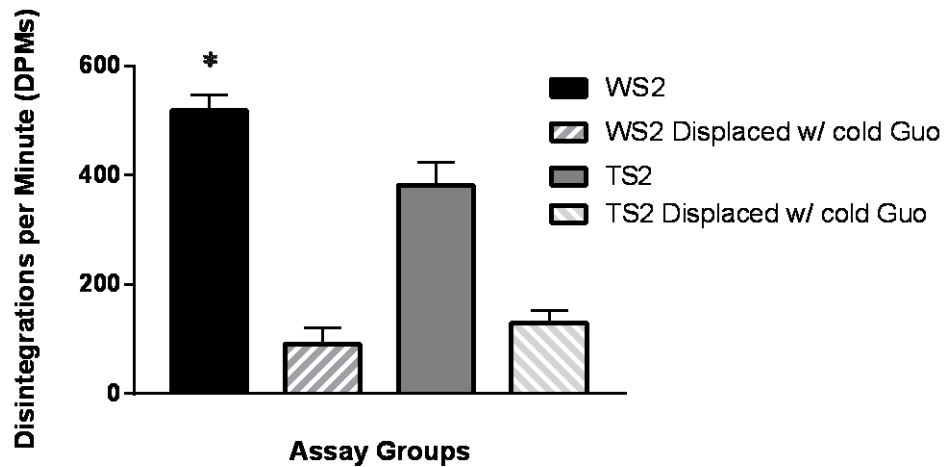
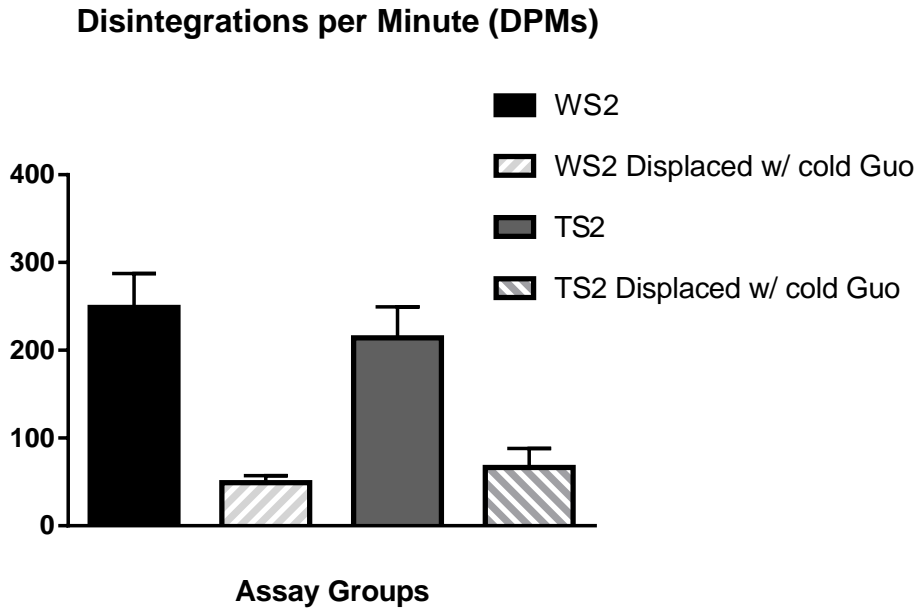


Figure 7. Centrifugation Binding Assay using [3H]-Guo and Wild type and G1R Transfected S2 cell membranes. This binding assay was performed on April 24th 2015. 100ug of protein was used from both wild and transfected samples along with 2nM [3H]-Guo and 100uM of cold Guo for displacement. Error bars represent S.D. Samples were run in triplicate. Significance was found between wild and transfected groups with wild type samples showing higher binding affinity for [3H]-guanosine (Student's t-test, *p<0.05).

A)



B)

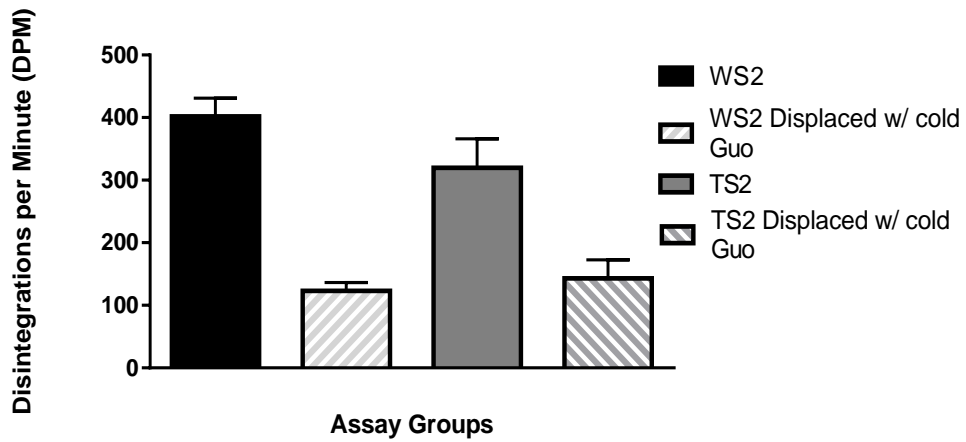


Figure 8. [3H]-Guo shows no significant difference in binding with WS2 and TS2 cells. Following a 500 μ M CuSO₄ induction and membrane protein isolation, A) no significant difference was found in DPM levels in WS2 and TS2 groups (Student's t-test, p=0.31) and B) similar results were found when this experiment was repeated. 180ug of protein in both wild and transfected samples as well as 2nM [3H]-guanosine and displacement using 100uM of cold Guo (Student's t-test, p=0.06). Error bars represent S.D. and samples were run in triplicate.

4.1.4 *Preliminary RT-PCR test confirms G1R expression in stably transfected S2 cells*

Stably transfected S2 cells expression G1R mRNA while WS2 cells do not. To determine S2 cells were successfully transfected G1R, RT-PCR was run for TS2 cells and WS2 cells as a negative control. Amplification plot values (Appendix 1; Figure 16) indicate WS2 cells have no G1R expression with no Cq values being generated. Additionally, results showed TS2 cells were successfully transfected with the G1R recombinant cDNA with Cq values of 25.84 and 25.64 and rat brain positive control samples showing Cq values of 21.62 and 22.59.

4.1.5 *No expression of G1R found in western blot when probed for G1R or His-tag on vector*

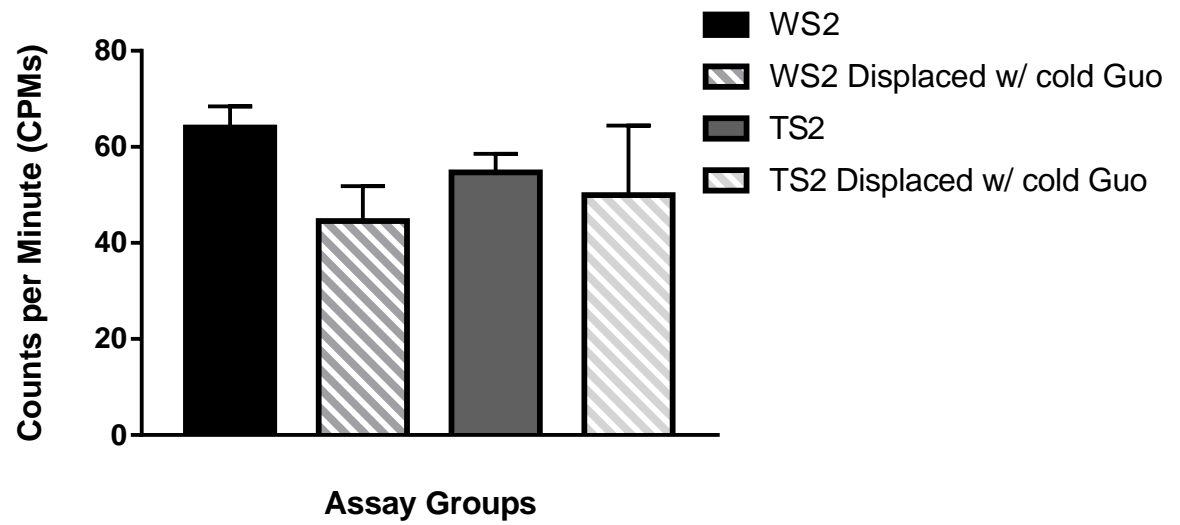
Previous western blots performed in our lab using older stably transfected S2 samples generated bands corresponding to the dimeric size of the G1R receptor. While common for GPCRs to form dimers and other order oligomers, these bands were not found in more recent samples. While RT-PCR confirmed mRNA expression western blots failed to show bands in the appropriate kilo Dalton (kDa) range (56kDa) when using G1R and His-tag antibodies (Appendix 1; Figure 17).

4.2 Objective 2

4.2.1 *No significant difference in binding affinity to [3H]-Guo between transient TS2 and WS2 cells*

No difference was found in binding assay results between stably transfected S2 and WS2 cells. In this study, cells were transiently transfected and assays performed in a short time frame, (within 5 days post transfection) to ensure that expression of the transfected G1R plasmid would not be lost. Transiently transfected cells were grown to the same density as WS2 cells for comparison. Whole cells were used in place of membrane protein to confirm that the protein of interest was not being discarded in the membrane isolation process. Cells were induced using either 500-800 μ M CuSO₄ for 24 hours before assaying. Cells were grown to a density of 5x10⁶ cells/ml and 20 μ L of the whole cell samples were used with 2nM [3H]-Guo and 100 μ M of cold Guo for displacement. No significant difference was found between groups (Figure 9; A) p=0.86, B) p=0.83).

A)



B)

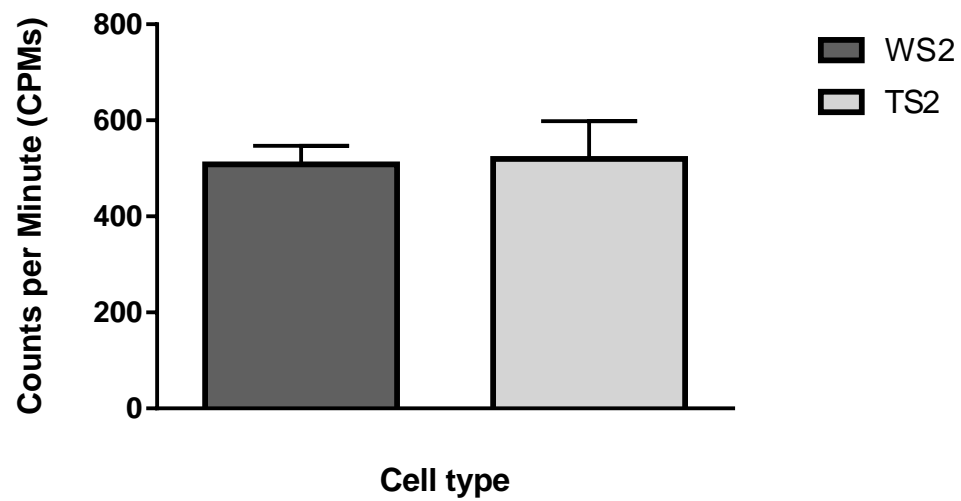


Figure 9. Transient transfected S2 cells do not show a difference in binding compared to WS2 in Centrifugation Binding Assay using [3H]-Guo. Following transient transfection and 500 μ M CuSO₄ induction, A) whole cells were used for the [3H]-Guo binding assay. TS2 and WS2 cells were grown to the same density (5 \times 10⁶ cells/ml). Twenty microliters of whole cell samples were used along with 2nM [3H]-guanosine and 100 μ M of cold Guo for displacement. No significant difference was found between WS2 and TS2 binding with [3H]-Guo (Student's t-test, p=0.086). B) The same parameters were used in this experiment only no cold Guo was used for displacement. No significant difference was found between groups (Student's t-test, p=0.83). This experiment was repeated several times with similar results. Samples were tested in triplicate.

4.2.2 Preliminary RT-PCR test confirms G1R expression in Transiently Transfected S2 cells

Transiently transfected S2 cells show expression of G1R mRNA and wild type cells do not. To determine the successful transient transfection of the G1R, RT-PCR was done using both WS2 and TS2 cells. Amplification values (Appendix 1; Figures 18 &19) showed WS2 cells generated no Cq value and that TS2 cells generated Cq values of 25.84 and 25.64 with rat brain control samples showing Cq values of 21.62 and 22.59, confirming that WS2 cells do not express the G1R and that the transiently transfected S2 cell do.

4.2.3 No expression of G1R found in western blot when probed for His-tag and V5-tags

While RT-PCR confirmed mRNA expression western blots failed to generate bands in the appropriate kilo Dalton (kDa) range (56kDa) when using the G1R and His-tag and V5 tag antibodies (No corresponding figure).

4.3 Objective 3

4.3.1 *C6 experiments show no significant difference between [3H]-Guo binding in TC6 and WC6 cells*

C6 rat glioma cells are a mammalian cell line that endogenously expresses G1R. In this study, C6 cells were transiently transfected with recombinant G1R plasmid cDNA to over express the G1R protein and then induced using 800 μ M CuSO₄ to compare transfected G1R levels to endogenous (baseline) expression levels. Twenty microliters of whole cell samples were used from both groups with 2nM [3H]-Guo and 100uM of cold Guo for displacement. Transiently transfected C6 (TC6) cells showed no difference in [³H]-Guo binding when compared to wild C6 (WC6) cells (Figure 10, p=0.76). From looking at the data, results were deemed too variable within groups and need to be repeated.

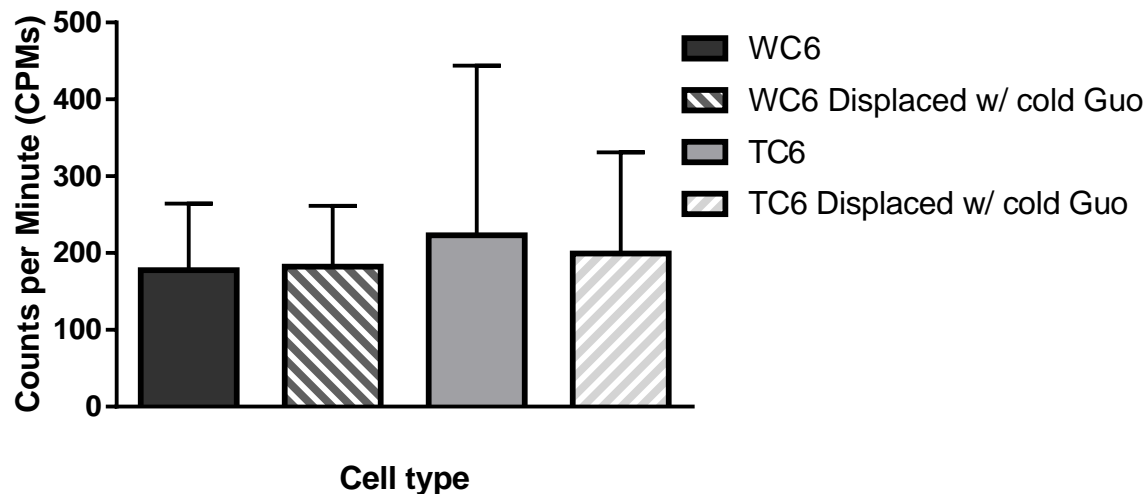


Figure 10. No significant difference was found in [3H]-Guo binding between transiently transfected C6 and WC6 cell. Transiently transfected C6 (TC6) and wild C6 (WC6) cells were cultured to the same density. Twenty microliters of whole cell samples were used from both groups with 2nM [3H]-Guo and 100uM of cold Guo for displacement. No significance was found between the wild and transfected group binding affinities. Error bars represent S.D. (Student's t-test, $p=0.76$). Samples were run in triplicate.

4.3.2 *WC6 have significantly higher copy numbers of G1R when compared to TC6 cells*

qRT-PCR results showed high G1R expression in WC6 cells when compared to TC6 cells. WC6 cells had an average of 284.1 per 77.4ng of cDNA compared to 40.11 for TC6 (Figure 11,**p<0.05). Cells were induced with 800 μ M CuSO₄ and incubated for 24 hours before assay. This suggests that the induction failed and was potentially detrimental to the transfected cells. Cells appeared stressed with irregular morphology. Again, this work is preliminary and needs to be optimized and repeated to confirm the findings presented here.

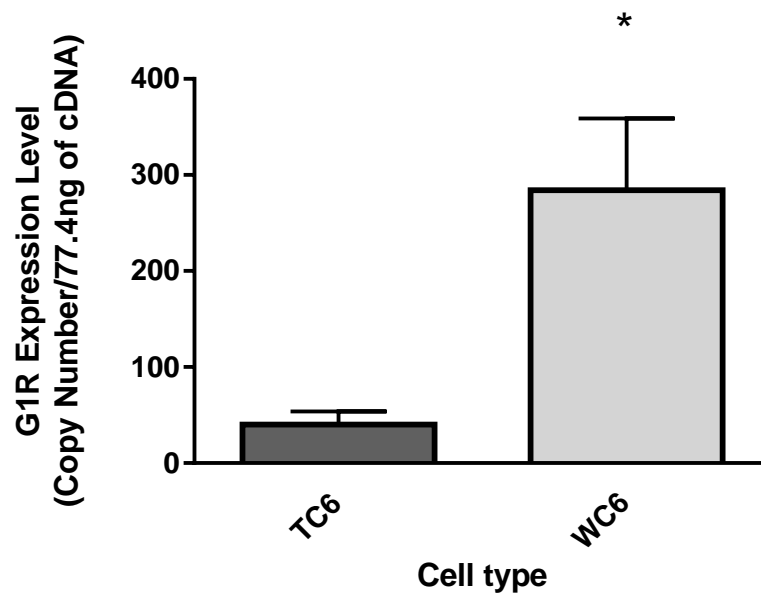
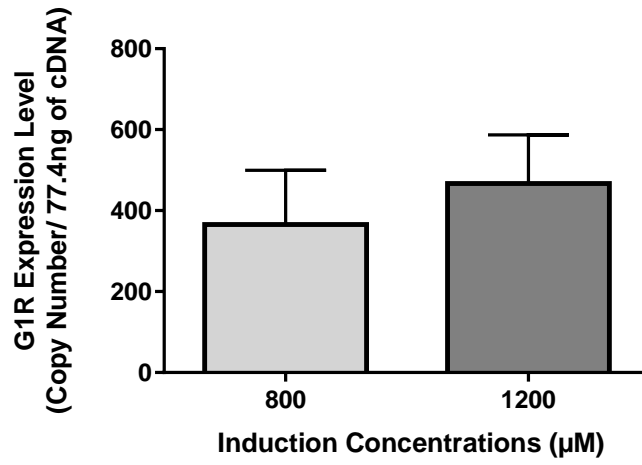


Figure11. Significantly higher copy numbers in WC6 cells that endogenously express G1R compared to transiently transfected C6 cells. Following transient transfection of C6 cells and a 24 hour incubation with 800 μ M CuSO₄ induction copy numbers in TC6 cells were significantly reduced compared to endogenous levels in the WC6 group. (Student's t-test; **p<0.05) Error bars represent S.D. Samples were run in triplicate.

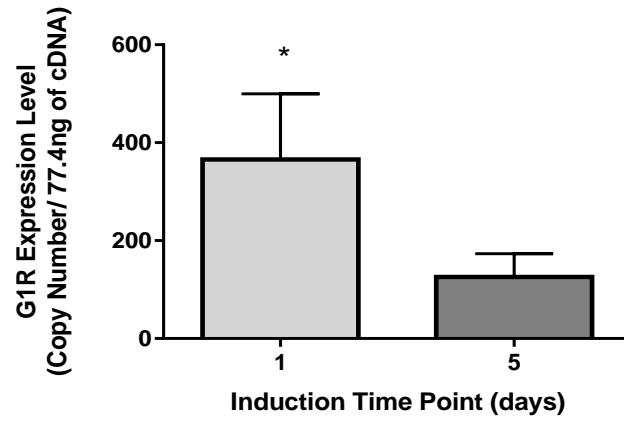
4.3.3 800 μ M and 1200 μ M CuSO₄ induction comparisons reveal no significant difference in G1R expression

Following transient transfection in S2 cells, two different concentrations of CuSO₄ were tested to compare differences in G1R expression levels. Results show no significant difference in G1R expression measured by copy numbers/77.4ng of cDNA (Figure 12A, $p=0.38$). Additionally, cells were induced and assayed at two different time points in both groups. Cells were assayed on day 1 post-induction and then again day 5 for comparison. Day 1 post-induction cells expressed significantly higher G1R copy numbers compared to the day 5 group in both the 800 μ M and 1200 μ M treated samples (Figure 12B, $*p<0.05$; Figure 12C, $*p<0.05$).

A)



B)



C)

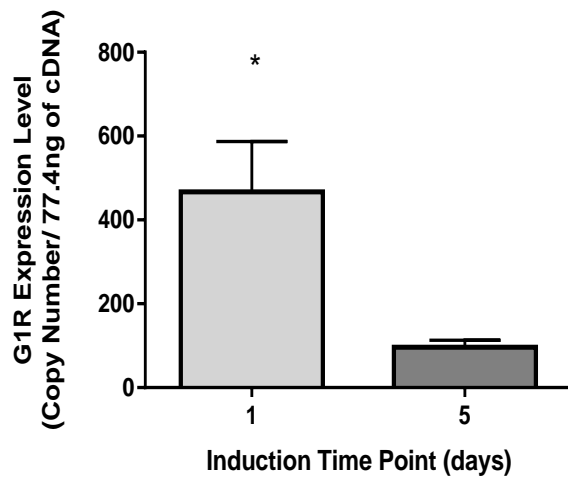


Figure 12. No significant difference found between 800 μ M and 1200 μ M CuSO₄ induction G1R expression levels. Following CaP transient transfection, A) cells induced with CuSO₄ showed no significant difference in G1R expression between 12 and 24 hour incubation groups (Student's t-test, $p=0.39$) B) The 800 μ M CuSO₄ induction group has a significantly higher G1R expression on day 1 when compared to day 5 post induction (Student's t-test, $*p<0.05$) C) The 1200 μ M CuSO₄ induction group has a higher G1R expression on day 1 when compared to day 5 post induction (Student's t-test, $*p<0.05$). Error bars represent S.D. Samples were run in triplicates.

4.3.4 S2 Cells survive 12 and 24 hour incubations with Lipofectamine® 3000 reagent during transfection

S2 cells survived both 12 and 24 hour incubations with the Lipofectamine® 3000. A cell viability assay was used to determine cell survival. Additionally, different incubation periods resulted in no significant difference in mRNA G1R expression (Figure 13, $p=0.35$). However, the 24 hour incubation period produced slightly higher copy number values with an average of 2049.3 copy numbers per 77.4ng of cDNA compared to 1860.3 in the 12 hour incubation group.

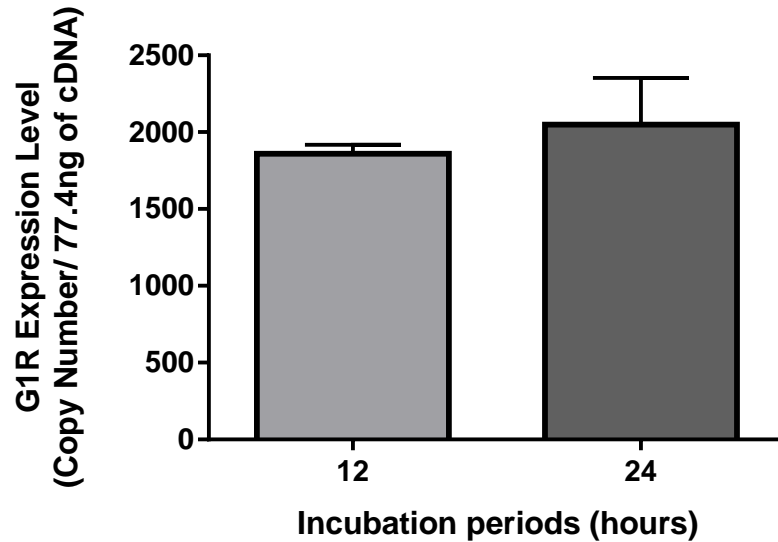
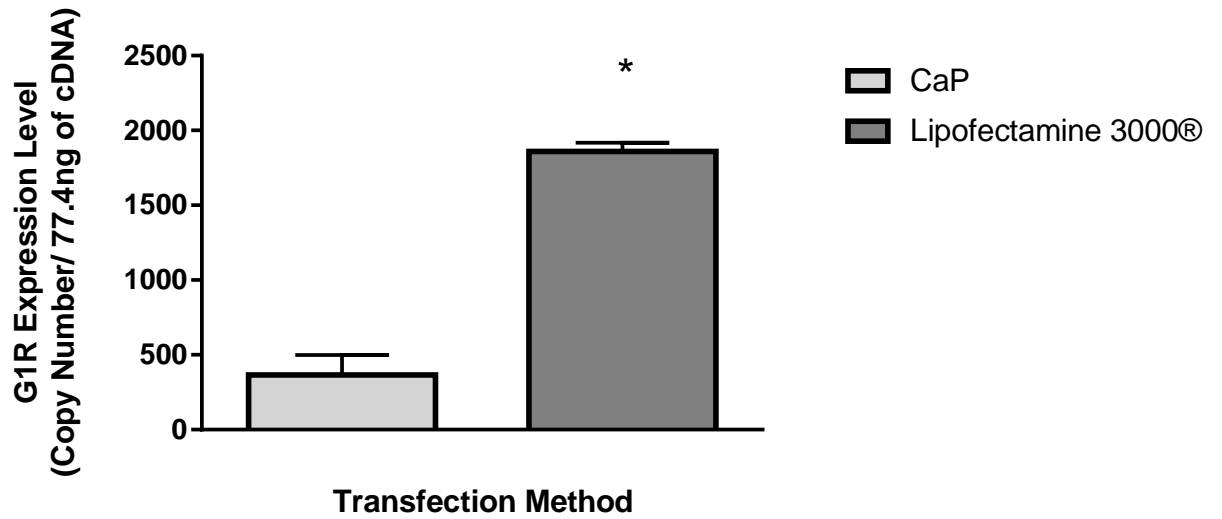


Figure 13. No significant difference was found between 12 and 24 hour incubations with Lipofectamine® 3000 in transfections using S2 cells. Following transfection using Lipofectamine® 3000, cells required a 24 hour incubation. Two time points were run (12 and 24) to ensure Lipofectamine® 3000 reagent would not be too toxic for cells. Both groups survived transfection and were induced for 24 hours with 800 μ M CuSO₄. Error bars represent S.D. No significance was found (Student's t-test; $p= 0.35$) samples were run in triplicate. This experiment was repeated an additional time with similar results.

4.3.5 Lipofectamine® 3000 transfected S2 cells have significantly higher G1R expression when compared to CaP transfected S2 cells

Cells were split into two groups for transfection. One group was transiently transfected using CaP and the other was transiently transfected using Lipofectamine® 3000. Following transfection expression was induced in both groups using 800 μ M CuSO₄ for 24 hours before assaying for qRT-PCR. Lipofectamine transfected cells had significantly higher G1R expression when compared to CaP transfected cells (Figure 14 A&B; Student's t-test, *p<0.05). WS2 cells were kept as a negative control and showed negligible copy numbers.

A)



B)

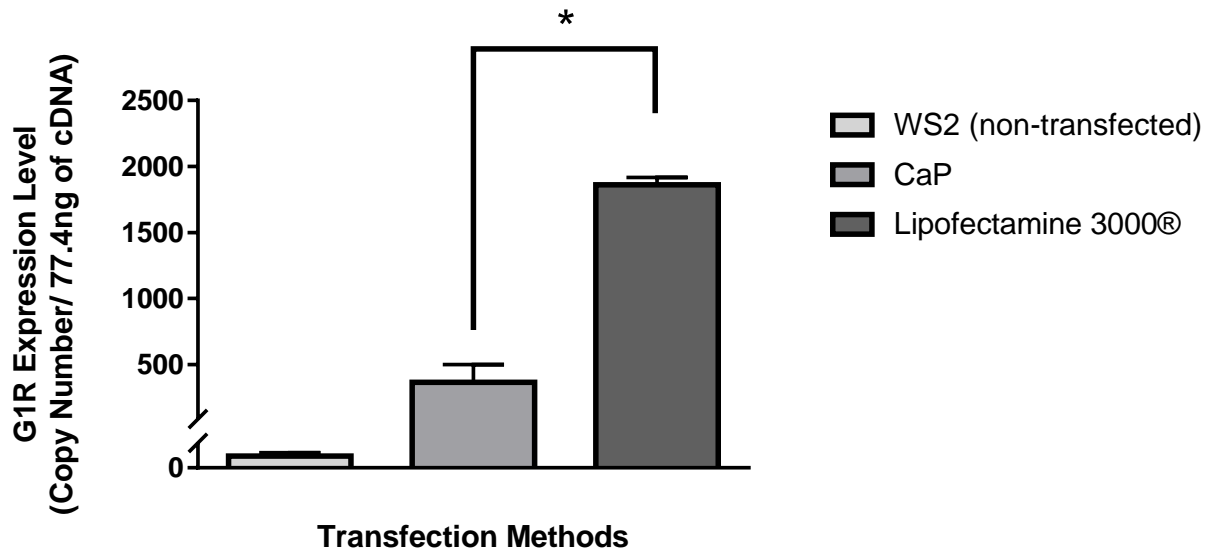


Figure14. Lipofectamine® 3000 transfected S2 cells have significantly higher G1R expression when compared to CaP transfected cells and WS2 cells (negative control).

Following 800 μ M CuSO₄ induction, A) cells transfected with Lipofectamine® 3000 had higher G1R expression compared to CaP transfected cells (Student's t-test,*p<0.05). Error bars represent S.D. Samples were run in triplicate. B) WS2 negative control

comparison to CaP and Lipofectamine® 3000 transfection groups. Error bars represent S.D. Samples were run in triplicate.

4.3.6 No significant difference was found in binding between CaP and Lipofectamine® 3000 transfected cells in a preliminary binding assay using [3H]-Guo

Following 800µM CuSO₄, CaP and Lipofectamine® 3000 transfected cells were run in a preliminary binding assay. This binding assay was performed on a smaller scale with no displacement (unlabeled Guo) groups to see if the highly expressed G1R Lipofectamine® 3000 transfected cells would show higher binding. Whole cell samples were used. Cells were grown to the same density and 20µL of whole cell samples were taken for the assay. 2nM [3H]-Guo was used for this assay. While Lipofectamine®3000 transfected cells showed slightly higher CPM counts, significance was not reached (Figure 15; Student's t-test, p=0.43).

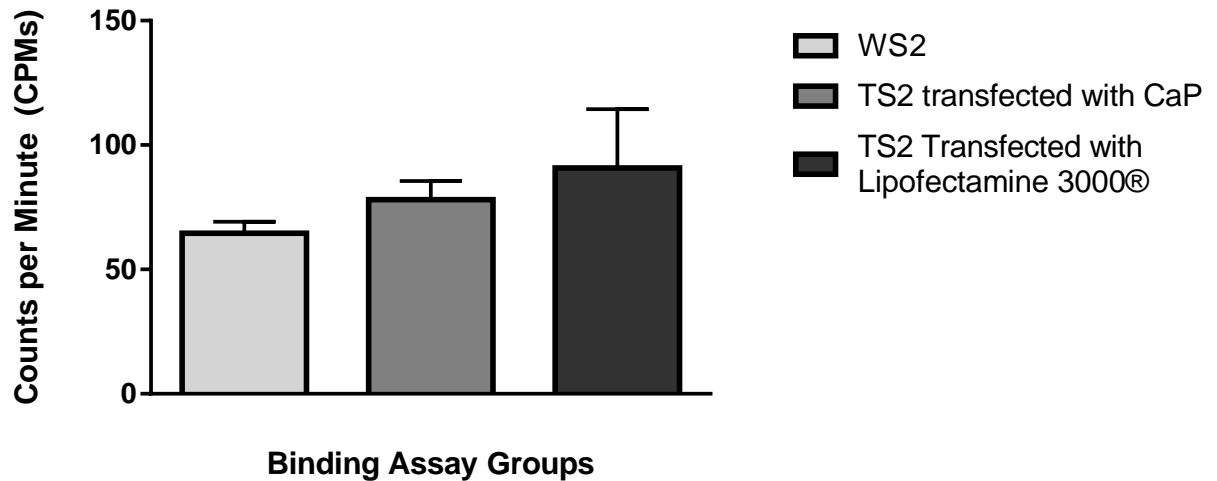


Figure 15. No significant difference in binding was found between Lipofectamine® 3000 and CaP transfected cells. Following 800 μ M CuSO₄ induction, Lipofectamine® 3000 and CaP transfected cells were used for a preliminary binding experiment. 20 μ L of whole cell samples (grown to the same density) were used for this experiment. 2nM [3H]-Guo was used as well. No significant difference was found between groups (Student's t-test, p=0.43). Error bars represent S.D. Samples were run in triplicate. This preliminary whole cell binding experiment needs to be optimized and repeated.

5 Section Discussion

5.1 Objective 1

5.1.1 *[3H]-Guo shows high binding to both TS2 and WS2 cells in Saturation Isotherm Assays*

In this study, we used [3H]-Guo in a saturation isotherm experiment to support our claim that G1R is the endogenous receptor for Guo (Figure 5). Stably transfected S2 and WS2 cells were compared to assess binding affinity of [3H]-Guo to G1R. S2 cells were chosen because they do not endogenously express G1R, as so confirmed by DNA sequencing. This allowed for the examination of the Guo-G1R relationship in a more simplistic system and isolated model. Results indicated high background binding with the WS2 cell group having comparable binding results to the TS2 group. Additionally, we observed high binding in the absence of insect and mammalian tissue (blank tests) (Figure 6). It was determined that tritium labeled Guo was highly attracted to the fiber glass filter paper. Background binding is a common issue found in ligand binding assays and a common solution is filter paper pre-treatment with PEI (Roche et al., 1985; Traversa et al, 2002).

After attempts to reduce the background binding, by soaking filter paper in PEI, were unsuccessful, the separation of the (bound) protein-[3H]-Guo complex and the free (unbound) [3H]-Guo was modified to the centrifugation method (Traversa et al, 2002). It is possible that [3H]-Guo had too high an affinity for the filter paper to be reduced by PEI pre-treatment (Pollard et al., 2010; Schiedel et al., 2007). PEI solution concentrations used were similar to those found in the literature (0.5% & 1%; Traversa et al., 2002).

Perhaps, though not confirmed by literature, higher concentrations may have rectified this issue. Additionally, contamination could be a potential consideration for such results. Using unfiltered buffers may have resulted in a high microbial presence in solution, bringing with them other high affinity binding sites for [3H]-Guo (Schiedel et al., 2007). Due to the vast nature of troubleshooting options, as well as time restraints, the paper filtration method was abandoned for centrifugation and experiments were repeated.

5.1.2 [3H]-Guo binding showed no difference between WS2 and stably transfected S2 cells in a series of binding assays using centrifugation

[3H]-Guo binding assays play a critical role in assessing the putative receptor for Guo. Once the centrifugation method was optimized, the saturation isotherms were repeated following 500 μ M CuSO₄ induction (as in previous experiments). Results from the first assay revealed significantly higher binding in WS2 cells when compared to TS2 cells (Figure 7). However, these results were never replicated when repeated (Figure 8 A&B). Repeated experiments indicated no significant difference in [3H]-Guo binding between TS2 and WS2 groups. Further, RT-PCR results indicated that the TS2 cells expressed G1R mRNA while WS2 cells do not (Appendix; Figure 16). Conversely, protein expression was absent in TS2 cells probed using G1R and His-tag antibodies (Appendix; Figure 17). This suggests the G1R expression may have been lost over time or expression may have been too low (PCR results were not quantitative) to generate a protein band for the stably transfected cells. Another potential reason for these findings could be that while stably transfected cells were expressing G1R mRNA, G1R protein was not making it to the cellular membrane. It was determined that since PCR samples

and western blot samples were taken at different time points, they may in fact be representing two different expression efficiencies in the stably transfected cells. With this in mind, it is reasonable to suggest that the G1R mRNA expression confirmed via PCR had since been lost upon sampling for western blots. While this is an inducible expression system, gene silencing can still occur (Moritz et al., 2015).

Losing gene expression in stably transfected cells can occur for several reasons (Moritz et al., 2015). One such reason is promoter methylation. Promoter methylation is a common issue that can result in loss of gene expression or loss of productivity (Moritz et al., 2015). Multi-site methylation accounted for productivity loss in stably transfected Chinese hamster ovary cells (CHO) (Moritz et al., 2015). Other methods of G1R silencing (RNA-mediated) were also possible and difficult to rule out. Through RNA interference (RNAi) for example, gene expression can be silenced at, and after transcription (Ulvila, 2006; Sanders & Bowman, 2012). The G1R gene could have been silenced while cells still maintained antibiotic resistance (to Hygromycin B), thus allowing cell survival in selective media with little to no G1R expression. An antibiotic is commonly used to select for cells expressing the gene of interest (Yang & Reth, 2012). The gene of interest, along with an antibiotic resistant gene, are co-transfected into cells so that cells not expressing the transfected gene will die following antibiotic treatment (Yang & Reth, 2012). However, cells growing in selective media can bring along a new set of potential issues.

Cells were maintained in a selective media containing Hygromycin B at a concentration of 300µg/ml. Another potential reason for loss of expression in stably transfected cells could be that the concentration of the antibiotic was too low to maintain

selection pressure on transfected cells (Southern & Berg, 1982; Yang & Reth, 2012). Modifying the concentration of the selection agent could have potentially increased efficiency. Long term exposure to antibiotics can have detrimental effects on S2 cells, producing unstable and unforeseen changes in cell behaviour and slow cell growth (Yang & Reth, 2012). With these reasons in mind, it was decided to repeat experiments with a transiently transfected cell population.

5.2 Objective 2

5.2.1 No significant difference in binding affinity to [3H]-Guo between transient TS2 and WS2 cells

To rule out issues that arise from stable transfection, experiments were repeated in transiently transfected S2 cells. Experiments were repeated using CaP mediated transient transfection to ensure the differences between groups in our initial (stable) transfections were not due to loss of expression or low expression in the stably transfected cells. Transiently transfected cells express the gene of interest but do not incorporate it into their genome. It does not get replicated and expression is lost quickly (Kingston et al., 2003). In this study, cells were transiently transfected using CaP method and binding assays were performed following 24 hour induction using 500 or 800 μ M CuSO₄. Results showed no difference in binding between TS2 and WS2 groups (Figure 9 A&B). Transfected cells were being grown in selection free media and not stably transfected. Results were similar to the first round of experiments with RT-PCR confirming G1R expression (Figures 18& 19) and western blotting failing to produce bands corresponding

to the sizes of either the monomeric or dimeric forms of G1R. Further, V5 and His-tag antibodies failed to produce bands when probed. Further, blots probing for His showed high non-specific binding. *Drosophila* cells contain many histidine proteins naturally and this could account for the high non-specific binding in western blots (Invitrogen, 2012).

Concerns of low expression and cell line selection became relevant after the optimized centrifugation binding assays from transiently transfected cells showed no difference between the stably and transiently transfected S2 cell groups. With recombinant mammalian receptor cDNA being transfected into an insect cell line we were unsure that the S2 cell line was capable of handling G1R protein expression. Post-translational modification issues may be inhibiting the G1R protein from being properly folded, glycosylated and inserted into the cellular membrane (Parodi, 2000; Zhang et al., 2003). This concern led us to design another set of experiments using a mammalian cell line to determine if the *Drosophila* S2 insect cell line was hindering recombinant protein expression (Girard et al., 2001).

5.3 Objective 3

5.3.1 C6 experiments show no significant difference between [³H]-Guo binding in TC6 and WC6 cells and qRT-PCR results confirm failed CuSO₄ induction

Concerns that the S2 cell line was not properly equipped to handle the expression of G1R protein led to experiments using a mammalian cell line that endogenously expressed G1R. C6 rat glioma cells that positively express G1R, confirmed by RT-PCR, were used (unpublished collaborator's works). Cell were transiently transfected using the

CaP method and induced with 800 μ M CuSO₄. Cells were incubated in 800 μ M CuSO₄ for 24 hours and checked repeatedly. At 24 hours cells appeared damaged, lacking characteristics of healthy C6 glioma cells. Cells were not adhering to the plate, appeared different in morphology and appeared smaller. While cells were still viable, it was evident that the 800 μ M CuSO₄ induction left them damaged. Cell samples were still taken for binding and qRT-PCR assays. Binding assay results showed high variability and, qRT-PCR results confirmed induction was unsuccessful (Figures 10&11 respectively). It is possible that the 800 μ M CuSO₄ induction was too high a concentration for the C6 cells. Alternatively, other induction agents such as cadmium chloride (CdCl₂) have been used in the literature to induce expression in place of CuSO₄ and could potentially be tried (Johansen et al., 1989; Santos et al., 2007).

5.3.2 Lipofectamine® 3000 transfected cells show higher GIR expression when compared to CaP transfected cells

Optimizing and repeating C6 experiments was unfeasible in the time allowed. With inconclusive C6 cell line results, and due to cost and time restraints, focus was shifted to the transfection method. After controlling for issues with stable transfection, binding assays, immunoblotting, induction concentration and cell line resulted in no difference in binding between groups, low transfection efficiency became a concern. In this study, we compared the transfection efficiency of CaP to Lipofectamine® 3000 in the Drosophila S2 cell line. qRT-PCR results indicate Lipofectamine® 3000 transfected groups significantly expressed higher GIR mRNA copy numbers than CaP transfected groups (Figure 14 A&B). This suggests that low transfection efficiency was the cause for

previous results. The CaP transfection method has an efficiency rate of ~30% (Yang & Reth, 2012).

Lipofectamine® 3000 is a lipid-mediated transfection method and yields a higher transfection efficiency (~70%) and lower cytotoxicity when compared to other transfection methods based on company comparisons (Andronikou et al., 2014; Santos et al., 2007; Chesnoy & Huang, 2000). It is only recommended for mammalian cells (as indicated on the manufacturer's website), however can be used successfully in insect cells as demonstrated here. Modifications to the manufacturer's protocol had to be made to account for differences between mammalian and insect cell lines. For instance, concerns that Lipofectamine® 3000 would be too toxic for the insect cells led us to transfect cells using two different time points (12 and 24 hours). Results showed that cells survived both incubation periods and further, the 24 hour incubation group had slightly higher G1R expression (though not significantly) (Figure 13). A second consideration was that S2 cells are semi-adherent while mammalian cells are adherent. It is recommended in the protocol that the cells be seeded to $0.25-1 \times 10^6$ cells/ml which is too low for S2 cells to grow. Cells were therefore seeded at 2×10^6 cells/ml for these experiments which did not appear to negatively affect expression.

S2 cells were transfected using CaP method parallel to the Lipofectamine® 3000 transfected cells for comparison. Two additional parameters of the CaP method were assessed at this time, induction concentrations (800 μ M and 1200 μ M) and time points following CuSO₄ induction (24hours post induction and 5 days post transduction). Results showed that while 1200 μ M induced cells had higher G1R mRNA expression,

statistical significance was not reached (Figure 12 A), suggesting that induction concentrations and incubation periods in the CaP method have the potential to be further optimized to increase expression in an inducible expression system. Additionally, both the 800 μ M and 1200 μ M groups had higher G1R expression 24 hours after induction compared to day 5 post induction (Figure 12 B & C). This information confirms that transient G1R expression is highest in the first few days post induction and declines quickly as cells grow. A more thorough time course should be run in future transfections on days post transfection to determine maximum expression of our protein.

Lastly, a preliminary binding assay was run to compare binding between CaP transfected and Lipofectamine® 3000 transfected S2 cells. Binding levels showed no significant difference between groups and further, were comparable to WS2 binding levels (low) (Figure 15). This may be due to the fact that the membrane isolation protocol was abandoned for whole cell assays. The reasoning for this was that if the receptors are not making it into the membrane, the protein of interest may be getting discarded during the membrane isolation protocol. As a result, we are working with less protein and potentially less protein of interest if some of the highly expressed G1R concentrations are representative of a subcellular population of G1R protein as well. These experiments need to be optimized and repeated. Additionally, binding assays using the membrane isolation protocol need to be run.

There is an incredible amount of variability in expression within and between transfection methods that result in the successful introduction of recombinant cDNA into cell lines. While both stable and transient CaP transfections were successful (produced

positive G1R mRNA expression), it was not enough to see a difference in [3H]-Guo binding in TS2 cells and non-specific binding in WS2 cells. These results explain the previous findings of positive mRNA expression, absent protein expression and comparable levels of [3H]-Guo binding between WS2 and TS2 cells.

Section 6 Conclusions, Implications & Future Directions

6.1 Conclusions

Guo is a purine nucleoside that possesses neuroprotective and neurorestorative properties (Rathbone et al, 1999). Guo can aid in repair of damaged cellular tissue and promote proliferation and differentiation of different cells in vivo and in vitro (Rathbone et al., 2008). Uncovering the mechanism of action of Guo is imperative to our understanding of extracellular Guo as a signaling molecule and the role of extracellular GBPs in the nervous system. The findings presented here have revealed that while saturation isotherm and displacement assays are acceptable methods to determine and quantify binding parameters between Guo and G1R, modifications needed to be made to ensure background binding and displacement would not occur. Also, while insect and mammalian cell lines are ideal systems to host and express recombinant mammalian receptor cDNA, additional factors need to be taken into account to ensure proper gene of interest expression and cell survival. Lastly, selecting the ideal transfection method to maximize efficiency and expression is of the utmost importance when designing experiments. We have shown that the Lipofectamine® 3000 transfected cells produce higher G1R expression compared to CaP transfected cells and that low G1R expression

using the CaP transfection method was the reason for our low binding and absent protein expression results.

6.2 Implications of this work

Findings from this work will allow us to develop more efficient models for the examination of the G1R-Guo relationship to confirm that G1R is the endogenous binding site for Guo. This work has significant implications for the development and design of therapeutic pharmacological agents geared towards enhancing the neuroprotective effects of Guo. Identifying the binding site for Guo will provide imperative information necessary for the understanding of how Guo works as a signaling molecule and provide more information on the extracellular role of the guanine based purinergic system in the CNS. Also, this information will help us to understand the nature and function of this orphan receptor (G1R). Specifically, findings from this work can be used to optimize transfection efficiency in an S2 model and increase expression, optimize radioligand binding assays using purine based compounds and further examine the relationship of Guo to the G1R.

6.3 Future Directions

There are several factors that should be considered in the continuation of this project. Several ideas for more efficient study design when examining the Guo-G1R relationship are presented here.

6.3.1 Transfection Methods and Optimization

Choosing the best transfection method and protocol for the cell line and gene of interest is the most important decision that must be made when optimizing transfection efficiency. The CaP method has been a common method used in insect cell lines and S2 cells (semi-adherent cell line) in particular. While low transfection efficiency using the CaP method was determined to be the reason for negative protein expression and non-significant binding results, techniques to increase the CaP transfection efficiency should be investigated. Cell starvation and adding DMSO to the boost efficiency are common techniques used to optimize CaP efficiency (Yang & Reth, 2012).

Also, if stable transfections were to be repeated, prolonged induction incubation periods should be considered. For our experiments using stably transfected cells, cells were assayed following 24-48hours incubations with CuSO_4 (induction agent). There are findings in the literature that suggest waiting until the fifth day post transfection will increase the expression of the gene of interest (Santos et al., 2007).

With modifications to its manufacturer's protocol, Lipofectamine® 3000 transfection method allowed for successful use in an insect cell line. However, it is recommended that Cellfectin® be used for optimal efficiency in insect cell lines based on manufacturer's suggestions. It is unknown whether Cellfectin will produce higher copy numbers than those seen here using Lipofectamine 3000. However considering the Cellfectin reagent is optimized for use in insect cells, it should be investigated.

Lastly, alternative induction concentrations and methods should be investigated. While a small group of different concentrations were tested here to elucidate ideal

conditions to maximize expression, a wider range of concentrations should be tested to determine an optimal concentration. 500 μ M was used here because it was manufacturer recommended, while 800 μ M and 1200 μ M were comparable concentrations to those found in the literature (Yen et al., 1988; Wang et al., 1993; Tota et al., 1995; Santos et al., 2007; Yang & Reth, 2012). Additionally, it should be mentioned that cadmium chloride (CdCl₂) and sodium butyrate (NaBu) has also been shown to successfully induce the metallothionein promoter in S2 cells (Johansen et al., 1989; Santos et al., 2007). A more thorough study investigating different induction agents and incubation times should be performed to maximize G1R expression for CaP mediated transfection in S2 cells.

6.3.2 Green Fluorescent Protein (GFP) tagged vector alternative

With difficulties in visualizing bands for the protein of interest, probing for tags on our vectors became a primary focus. In this study we probed for the His-tag and V5-tags with little to no success. At the time, it was difficult to determine if the issues were to do transfection optimization or immunoblot optimization. It was also difficult to ascertain the exact transfection efficiency that was being produced. With low transfection efficiency now known to be the central issue, options for faster and more time efficient estimations of gene expression should be sought out. GFP or fluorescence tagged vectors could be used in future transfections with G1R cloned cDNA to ensure successful transfection efficiency by allowing us to visualize the protein of interest within the cell (Jiang & Chen, 2006; Santos et al., 2007). Expression could be detected using confocal microscopy or epifluorescence (Santos et al., 2007; Yang&Reth, 2012).

6.3.3 Surface Plasmon Resonance

Binding assays provide a wealth of information about the relationship between two ligands. They determine, if these two ligands interact, the Bmax, the Kd and compare binding affinity of a series of other compounds to a receptor of interest (Pollard, 2010). However, with high background binding being an issue, and the centrifugation method producing lower CPM and DPM values, searching for an alternative method to evaluate the G1R and Guo binding is a priority. Multi-parametric surface plasmon resonance (SPR) is an alternative method to the more classically known binding assay that can be used to assess molecular interactions, structure and function (Guo, 2012; Hesselink & Findlay, 2013). This biosensor technology uses refractive index changes to measure binding between two ligands (Guo, 2012; Hesselink & Findlay, 2013). This technique is a fast and efficient way to quantify the G1R-Guo relationship. In brief, the ligand (G1R) is fixed to a metal turf and the analyte (Guo) flows over top of the turf. The system measures the change in refractive index (Guo, 2012). If the analyte binds to the ligand there will be an increase in refractive index on the turf (Guo, 2012). This technology offers a better assessment of biomolecular interactions without the issues we face with binding assays.

6.3.4 Different Cell lines

The S2 insect cell line has been used extensively in the literature to express a wide variety of recombinant mammalian proteins with a myriad of posttranslational modifications (Johansen et al. 1995, Tota et al, 1995; Aldecoa et al., 2000; Santos et al.,

2007). While posttranslational modification issues can be protein/receptor (G1R) specific, it is rare and uncommon. Regardless, C6 rat glioma cells were transiently transfected to confirm that the insect cell line was not inhibiting the expression of the mammalian G1 membrane receptor. Findings for these C6 experiments were inconclusive with qRT-PCR results suggesting that induction using 800 μ M CuSO₄ had failed. Irrespective of this fact, these experiments should be optimized and repeated. Findings from these experiments can provide invaluable information regarding G1R expression in different cell lines and if a mammalian cell line expression system would better express the G1R receptor compared to an insect cell line.

APPENDIX 1: SUPPLEMENTAL DATA

Objective 1

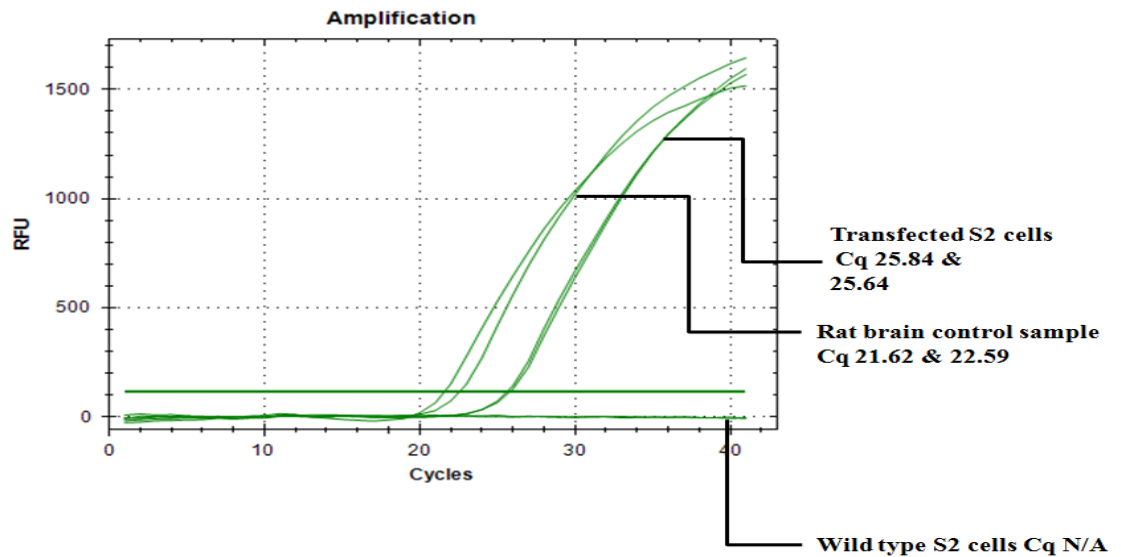


Figure 16. Transfected S2 cells express G1R mRNA. Following stable transfection of S2 cells using the CaP co-i.p. transfection method, G1R mRNA was expressed. Error bars represent the S.D. This test was done once to simply confirm both expression of G1R in transfected cells, and the lack of expression in wild S2 cells. Rat brain samples were used as a positive control. No statistical tests were used to analyze this data. Samples were run in duplicates.

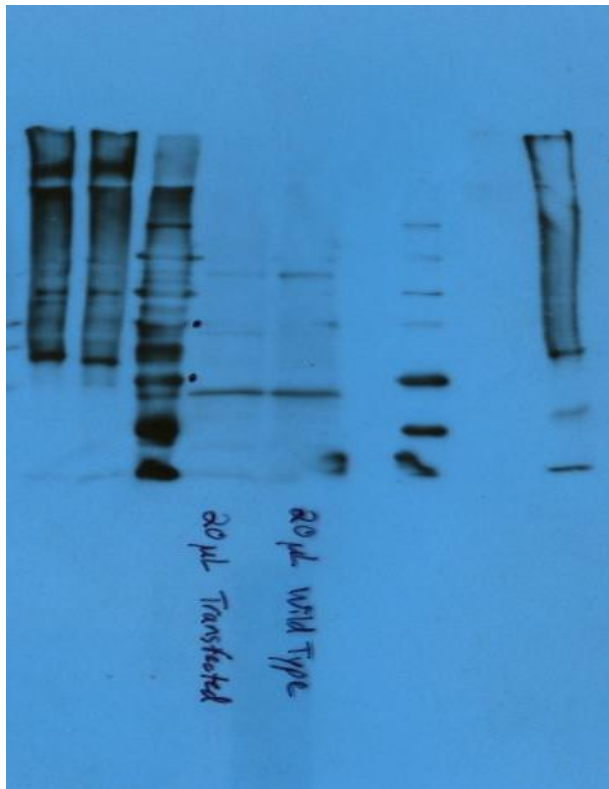


Figure17. Stable transfected cell samples revealed no bands when probed for His-Tag in Western blot. Protein of interest is approximately 56kDa.

Objective 2

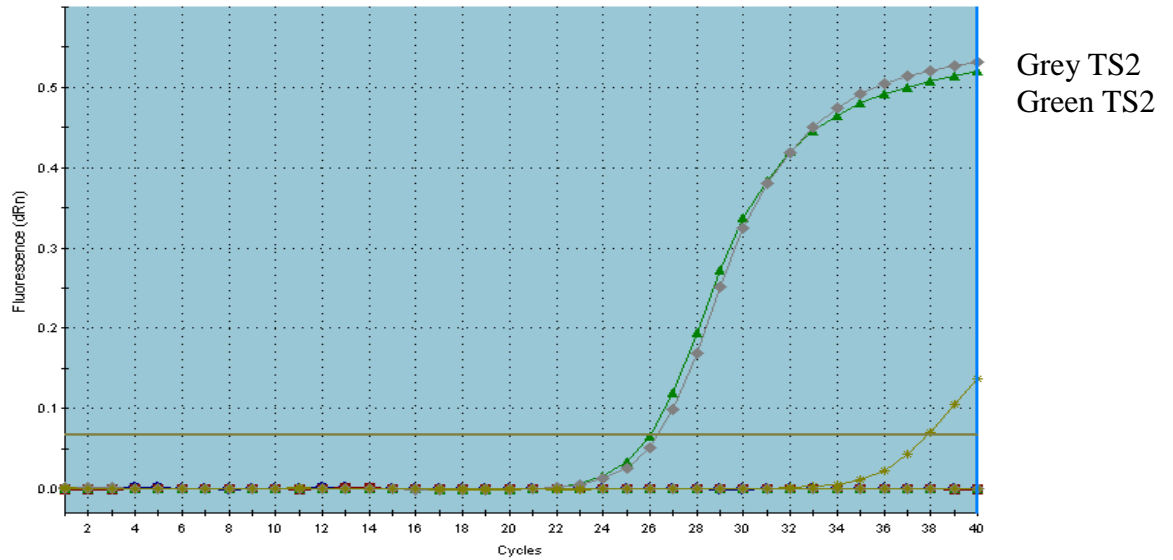


Figure18. Transfected S2 cells express G1R. Twenty-four hours after copper sulfate induction cells were assayed to confirm expression of G1R. Results showed no Ct values for WS2 cells or C6 cells (control) and Ct values from 26-26.38 for TS2 cells.

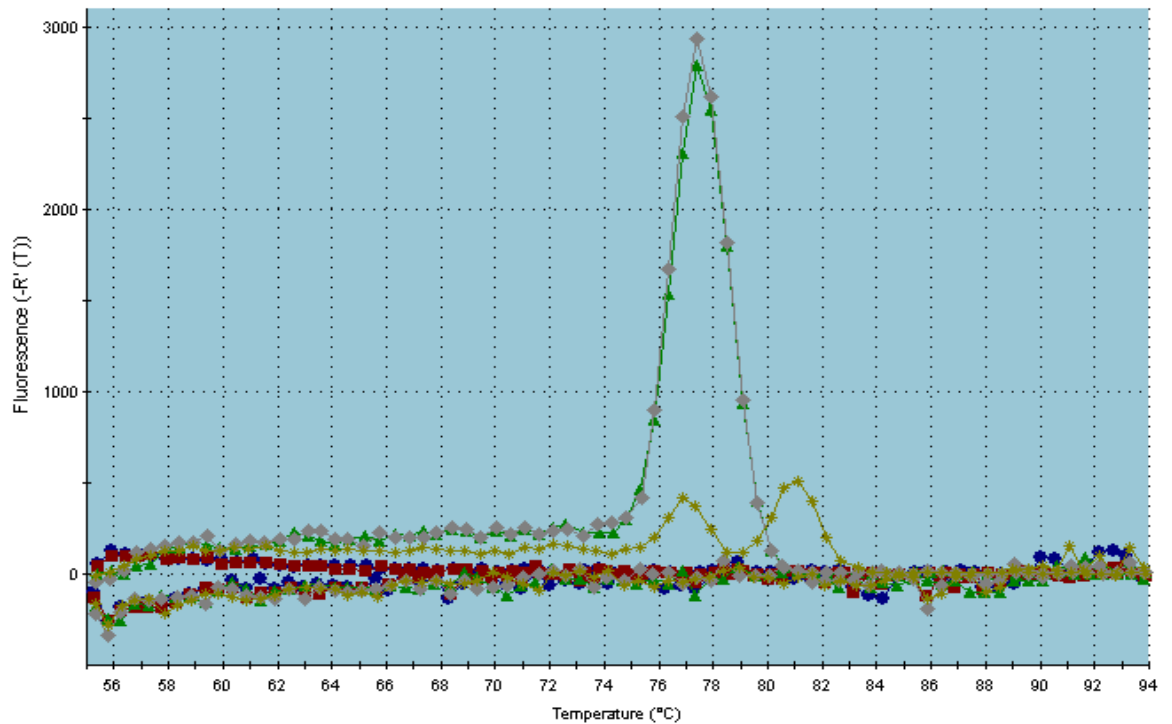


Figure 19. RT-PCR Dissociation Curve for transiently transfected S2 cell samples compared to WS2 and C6 cells. During the final temperature increase in the qRT-PCR run, the fluorescence is measured. This fluorescence is known as a dissociation curve. We use this curve to determine the specificity of our PCR reaction to ensure that it is our product that is being amplified. Twenty-four hours after copper sulfate induction cells were assayed to confirm expression of GIR. Results showed no Ct values for WS2 cells or C6 cells (control) and Ct values from 26-26.38 for TS2 cells.

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