Expression of Soluble Guanylate Cyclase in Rat and Human Hepatocytes

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Soluble Guanylate Cyclase (sGC) is a key enzyme in the nitric oxide (NO) signaling pathway. sGC binds NO to produce cyclic guanosine-3′,5′-monophosphate (cGMP). The NO-sGC-cGMP pathway is directly involved in a number of physiological functions including smooth muscle vasodilation. Deficiencies in this system, such as reduced NO tone, can result in cardiovascular dysfunction. Stimulators of sGC have been developed to synergize with and enhance NO signaling, creating potential therapies for a number of diseases. Currently, it is known that sGC is expressed in tissues such as the brain, kidney, lung, and liver. However, as an intracellular enzyme, sGC expression in specific cell types within these tissues remains to be explored.

This study aims to uncover the expression of sGC in rat and human hepatocyte cells, the major cell type in the liver responsible for metabolizing and detoxifying the body. To begin, the expression of sGC subunits α1 and β1 was assessed by immunohistochemistry (IHC) staining on a paraffin fixed rat liver slice. The results uncovered the localization of sGC in stellate, endothelial and hepatocytes surrounding the vasculature in a rat liver. To confirm positive expression of sGC in hepatocytes, branched DNA (bDNA) probes for sGC were used to confirm expression of sGC in isolated, cryopreserved rat and human hepatocyte cells. Finally, the functionality of the sGC enzyme was shown in vitro by stimulating both rat and human hepatocytes in the presence of an sGC stimulator with and without the NO-donor (DETA). Target engagement by the sGC stimulator was determined through the quantitation of cGMP.
Dedication

This thesis is dedicated to my loving and supportive family. To my wonderful parents whose love, support, and encouragement throughout my entire life has enabled my greatest successes. Mom and dad, you have always encouraged me to follow my passions and dreams, and I wouldn’t be where I am today without you. In addition, this work is dedicated to my loving fiancé. I can’t thank you enough for your patience and support throughout all of my schooling and especially throughout the writing of this work.
Acknowledgments

This research was conducted in the laboratory of Ironwood Pharmaceuticals and would not have been possible without the support of all of my colleagues. A very special thanks goes to my research directors, Dr. Peter Germano and Dr. Maria Ribadeneira, for all their encouragement, knowledge, and dedication. In addition, I would like to thank my research advisor Dr. Denkin, who has been instrumental in guiding this work.
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Chapter I

Introduction

As drug discovery progresses, researchers are identifying new protein targets of interest. Some proteins are expressed throughout multiple tissue tissues and may have an impact on several different diseases. Soluble guanylate cyclase is currently a protein target of interest for treating a variety of cardiovascular diseases. However, the protein has been identified in several tissues and cell types throughout the body including the liver, lungs, kidneys, and brain.

Soluble Guanylate Cyclase (sGC)

Soluble guanylate cyclase is a heterodimeric protein composed of two homologous α and β subunits. The most prevalent and commonly studied isoform is the α1β1 protein, however the α2 and β2 subunits have also been identified (Derbyshire 2012). Currently, it is the only known receptor for nitric oxide (NO), a signaling agent produced by nitric oxide synthase (NOS) (Denninger et al., 1998). Figure 1 depicts the sGC signaling pathway in smooth muscle cells. NO binds to the sGC heme which activates the enzyme causing a cascade of downstream events. Upon activation or stimulation, sGC catalyzes the conversion of guanosine 5’-triphosphate (GTP) to cyclic guanosine 3’,5’-monophosphate (cGMP).
This consequent rise in cGMP is what allows sGC to transmit an NO signal to the downstream elements of the signaling cascade such as cGMP-dependent protein kinase, cGMP-gated cation channels, and cGMP-regulated phosphodiesterase (Denninger et al., 1998).
Since the discovery of the NO/cGMP pathway in the 1980s, much has been done to characterize and better understand the mechanism of the pathway’s signal transduction. The most well understood mechanisms of this pathway include smooth muscle relaxation and blood pressure regulation (Warner et al., 1994); platelet aggregation and disaggregation (Buechler et al., 1994); and neurotransmission (Warner et al., 1994).

Vasodilation caused by sGC activation is the result of increasing cGMP in vascular smooth muscle. Although the precise mechanism by which cGMP reduces vascular tone is unknown, literature suggests that it is associated with changes in membrane potential. It has been shown that a PKG-dependent activation of calcium-sensitive potassium channels leads to the hyperpolarization of pulmonary artery smooth muscle cell membrane potential and inhibition of calcium influx (Archer et al., 1994). As
expected, vasodilation of the smooth muscle leads to a decrease in blood pressure clinically.

In addition to its presence on smooth muscle cells, sGC is also present in platelets. When activated by NO, sGC becomes involved in the inhibitory action of NO on platelet function. NO interferes with the initial activation of platelets preventing both adhesion and aggregation (Wang et al., 2013). With this, our knowledge of sGC activation has contributed greatly to the understanding of platelet function and provided several therapeutic possibilities.

sGC Stimulators and Activators

To capitalize on its vasodilatory effects, sGC has become a desired therapeutic target. Two classes of drugs are known to increase sGC activity: sGC stimulators and activators. Stimulators function synergistically with NO to stabilize the enzyme’s nitrosyl-heme complex which sensitizes sGC to low levels of bioavailable NO. In addition, stimulators can also directly increase sGC activity in the absence of NO given the heme group is present. In contrast, sGC activators function by taking the place of the NO-heme complex. To do so, activators either bind the unoccupied heme-binding pocket, or replace the weakly bound oxidized heme (Das Gupta et al., 2014). The development of sGC modulators is seen as beneficial for conditions needing induced vasodilation in conditions with diminished NO bioavailability and/or where there is tolerance to organic nitrates. Bayer Pharmaceuticals has been a front runner in the development of sGC stimulators and activators. Cinaciguat, their sGC activator, did not show very compelling
results in the clinic as the drug trial was terminated early due to several cases of hypotension (Erdmann et al., 2021). However, their drug, Adempas (Riociguat), is the first sGC stimulator approved by the FDA for the treatment of pulmonary hypertension. The success of Riociguat provides great promise for the sGC pathway and has led several researchers to continuously investigate the potential therapeutic possibilities of sGC as a target.

sGC in the Liver

The functionality of the sGC signaling cascade is clinically apparent in its effects on vasodilation. As research continues, sGC has been discovered in several other tissues and cell types. Currently, sGC has been identified in the brain, lung, kidney and liver. As the second largest organ in the body, the liver performs many essential functions related to digestion, metabolism, immunity, and the storage of nutrients. The liver is a vital organ which keeps other organs alive by producing energy and nutrients. In addition, the liver has the incredibly capacity to regenerate dead or damaged tissue.

The liver is composed of several cell types including hepatocytes (parenchymal cells), vascular endothelial cells (LSECs), Kupffer cells (macrophages), bile duct cells, and Stellate cells (HSCs). Figure 2 demonstrates the anatomy of cell types in a normal liver. Hepatocytes are the most predominant cell type in the liver accounting for 70-85% of the total liver mass. Hepatocytes are multifunctional and play a role in protein synthesis, protein storage, the transformation of carbohydrates, the synthesis of cholesterol, bile salts and phospholipids, the detoxification, modification and excretion of
exogenous and endogenous substances, and the initiation of formation and secretion of bile. Kupffer cells are monocytes that line the liver sinusoids near endothelial cells. They remain close to hepatocytes via the space of Disse. Kupffer cells are able to take up and destroy foreign material such as bacteria. The main function of LSECs is still highly debated; however it is believed these cells play a role in liver regeneration (Kmiec 2001). Finally, HSC are known as perisinusoidal cells located between the sinusoids and the hepatocytes in the space of Disse. HSCs are of major interest in the study of liver fibrosis as they are the major cell type involved in the formation of scar tissue in response to liver damage (Iwakiri et al., 2014).

![Figure 2. Anatomy of liver cell types. The liver comprises of mainly of hepatocytes with Kupffer, HSCs, and LSECs dispersed throughout the vasculature. From “Cellular Distribution and Function of Soluble Guanylyl Cyclase in Rat Kidney and Liver” by Theilig et al, 2001, J. Am. Soc Nephrol.](image)

As a multifunctional organ that is involved in several diseases, the liver is gaining interest as a therapeutic target. With increasing prevalence and no treatment options to date, liver fibrosis/cirrhosis is currently one of the most widely studied diseases. Liver fibrosis results from the accumulation of extracellular matrix proteins, including collagen,
which occurs in most chronic liver diseases. Advanced liver fibrosis results in cirrhosis, liver failure, and portal hypertension and requires a liver transplant. The most common causes of liver fibrosis are HCV infection (now treatable), alcohol abuse, and nonalcoholic steatohepatitis (NASH) (Batallar and Brenner, 2005). With much advancement of therapies for HCV, many researchers have turned their focus on NASH.

NASH is linked to the increased consumption of fructose, trans fats, and an overall energy imbalance. NASH is believed to begin as nonalcoholic fatty liver disease (NAFLD) in which the liver accumulates excessive fat and becomes steatotic. These patients progress to NASH after developing steatosis due to oxidative stress that leads to chronic inflammation, ROS formation, and an influx of inflammatory components. Once the patient has developed inflammation, the disease may further progress and lead to the formation of fibrosis which may further worsen to cirrhosis (cancer of the liver) (Cohen et al., 2011).

Potential Role of sGC in Treating Liver Disease

Although not much is currently known about sGC function in the liver, researchers have begun to investigate the potential therapeutic benefits sGC may provide as a target in treating liver diseases. As previously mentioned, the progression of NASH occurs via three main insults; steatosis, inflammation, and fibrosis. Although the direct role of sGC in NASH is unknown, there is rationale for each individual insult.

The initial insult leading to NASH is steatosis. Steatosis is the infiltration and accumulation of fat in liver cells. Recently, the sGC pathway has been studied in obesity
models. The sGC stimulator BAY 41-8543 was synthesized by Bayer Pharmaceuticals, a leading researcher in the field of sGC. Recently, BAY 41-8543 was studied in an obesity model in which mice were fed a high fat diet. According to Hoffman, sGC stimulation by BAY 41-8543 protected mice from diet induced obesity and resulted in a 37% reduction in body mass, with a 15% reduction in relative fat mass compared to vehicle high-fat diet mice. In addition, it was shown that adipocyte size and hepatic lipid content were significantly decreased (Hoffman et al., 2015). The results from this recent study help uncover the potential therapeutic benefits sGC stimulation may have on hepatic steatosis and provide rationale for sGC as a target in the “first-hit” of NASH. Hepatocytes are known to be the main depository for lipid/fat accumulation in the liver (Kmieć, 2001), and fat accumulation in the liver leads to the first “hit” in NASH that leads to steatosis. If sGC is expressed on hepatocyte, a stimulator has the potential to prevent fat accumulation in liver cells as eluted to by Hoffman et al’s studies.

The second major insult in NASH is inflammation. There is significant literature surrounding the benefits of sGC stimulation on inflammatory responses throughout the body. It is known the NO production by the vascular endothelium maintains anti-inflammatory influence on the blood vessel wall (Ahluwalia et al., 2004). As a NO receptor, sGC is expressed in endothelial cells, however its function is unknown. Using endothelial nitric oxide synthase (eNOS) knockout mice, researchers investigated the role of sGC in the regulation of adhesion molecule expression and leukocyte recruitment by treating the mice with the sGC stimulator BAY41-2272. Upon treatment, the eNOS-/- mice showed reduced leukocyte rolling and adhesion, suggesting the role of sGC in anti-inflammatory response (Ahluwalia et al., 2004).
In addition to reduced leukocyte rolling and adhesion in eNOS-/- mice, another study demonstrated the effect of an sGC stimulator on circulating tumor necrosis factor (TNF-α). TNF-α is a cytokine involved in systemic inflammation. Using eNOS -/- mice, Ott et al., showed that treatment with the sGC stimulator riociguat significantly decreased the circulating plasma levels of TNF-α compared to the control diseased mice (Ott et al., 2012). Taken together, there is much rationale to believe sGC stimulators have an effect of inflammation. Although not much is known about the effect of sGC on inflammation in the liver, there is significant literature supporting its anti-inflammatory effect throughout other tissues in the body. It is quite plausible; sGC’s anti-inflammatory properties extend to tissues beyond what has been currently studied.

In the liver, it is suggested the Kupffer cells play a role in inflammation. Previously it has been shown that rat hepatocytes have inducible NO synthesis in response to co-cultured Kupffer cells exposed to lipopolysaccharide (LPS) (Billiar et al., 1989). It was further shown that inflammatory cytokines from Kupffer cells and LPS maximally stimulates NO synthesis in rat hepatocytes (Curran at al., 1990) and human hepatocytes (Nussler et al., 1995). With the ability to increase NO synthesis in hepatocytes as a response to inflammatory stimuli, it is plausible that hepatocytes do in fact play a role in the anti-inflammatory response of the liver. Since NO is a target for sGC, it is possible that sGC is a downstream target in response to inflammation in the liver. If sGC is expressed on hepatocytes, these cells may provide a target for the treatment of liver injury and/or disease, specifically the inflammation component of NASH.
Finally, the last major insult in NASH is the progression to fibrosis. Once steatosis and inflammation occur in the liver, this resulting cascade causes a progression in NASH which leads to fibrosis (Oh et al., 2008). Liver fibrosis is the excessive accumulation of the extracellular matrix proteins including collagen. Activated hepatic stellate cells are major collagen-producing cells that become activated by fibrogenic cytokines such as TGF-β1, angiotensin II, and leptin (Bataller and Brenner, 2005). Some work has been done to demonstrate the effect of sGC stimulation on fibrosis. To date it is known that sGC is expressed in HSCs, the key player in the progression of fibrosis (Thelig et al., 2001) and LSECs (Xie et al., 2012). To demonstrate direct antifibrotic effects of sGC stimulation on HSCs, the pig serum and bile duct ligation models of fibrosis were used. Both models show that once fibrosis is induced in rats either by injection of pig serum or ligation of the bile duct, upon treatment with sGC stimulator BAY 41-2272 there is a dose responsive reduction in the pro-fibrotic marker α-SMA (Nowatzky, 2012). Since α-SMA is upregulated in HSCs as fibrosis progresses, this data demonstrates that a sGC stimulator can target HSCs and impact fibrosis.

In addition to using a sGC stimulator, a sGC activator BAY 60-2770 also showed an effect on the pig serum and CCL4 models of fibrosis in rat. Chronic dosing of CCL4 in rats and mice leads to increased collagen deposition resulting in liver fibrosis. When rats in both models were treated with BAY-60-2770, the major component of collagen, hydroxyproline, significantly decreased (Knorr et al. 2008). The effects of both the sGC stimulator and activator on liver fibrosis demonstrate the potential role sGC may play in the treatment of liver fibrosis and/or other liver diseases.
Previously, it was mentioned that sGC is known to be expressed on HSC’s, however it remains to be explored whether sGC is expressed on the most abundant cell type, hepatocytes. The investigation of sGC expression in the liver is a fairly new area research, and researchers simply haven’t begun to investigate its expression in hepatocytes. The focus initially has been to investigate the expression of sGC in “bad actor” cells of the liver such as HSCs. Since HSCs are known to directly cause fibrosis, it is likely researchers began their investigation with cell types directly linked to disease. As we begin to learn more about liver disease at the cellular level, it is becoming apparent that hepatocytes may in fact play a greater role than originally thought. Since the liver is comprised mainly of hepatocytes with a smaller number of HSCs, Kupffer cells and LSECs, it is plausible that if sGC is in fact expressed on hepatocytes there may be several beneficial effects of targeting sGC in the liver.
Chapter II

Materials and Methods

Several techniques were used to determine the expression of sGC on human and rat hepatocytes. The techniques include IHC staining of a naïve liver slice to determine the localization of sGC in various cell types in the liver, and bDNA analysis of isolated rat and human hepatocytes for mRNA expression of sGC, in addition to the identification of sGC on hepatocytes, the functionality of the sGC protein was determined in rat and human hepatocytes by treatment with a sGC stimulator and a NO donor to observe target engagement by cGMP quantification using ELISA.

Immunohistochemistry

Immunohistochemistry (IHC) staining enables the detection of specific proteins within tissue samples and cell types. Using this technique, the specific location of a protein within a tissue can be identified. Antibodies specific for sGCα1 and sGCβ1 were used to detect the expression of both sGC subunits in naïve Sprague Dawley rat liver. In this experiment, the detection of both sGC subunits will be explored in all cell types of the liver with specific focus on hepatocyte cells, which are easily discernable by microscopy.
Paraffin Fixed Liver Slice

Male Sprague Dawley naïve rat liver was isolated, perfused with PBS, and preserved in formalin. The sample was shipped to Mass Histology where it was paraffin fixed and several slides were prepared per the company protocol. The slides were subsequently shipped back for IHC staining.

Immunohistochemistry Staining for sGCα1 and sGCβ1

Immunohistochemistry staining was performed on the tissue slices using antibodies specific for sGC α1 and sGC β1 (http://www.abcam.com/). Four slides were prepared and stained for the following: H and E, sGC α1, sGC β1, and null antibody for background determination. To begin, the slides were deparaffinized and rehydrated. To do so, the slides were treated with xylene three times for 5 min each. Subsequently the slides were treated with 100% ethanol two times for 10 min, then with 95% ethanol twice for five minutes each. Finally, the slides were rinsed with water twice for five minutes each. Next, to unmask the antigen the slides were boiled in 10mM sodium citrate (pH 6) and maintained at sub-boiling temperature for 10 minutes. The slides were then cooled for 30 minutes on bench top. Once the slides were cooled, they were washed in water three times for five minute intervals. After removing excess water from the slides, a barrier was created around the liver slice using a PAP pen, and peroxidase activity was quenched using 3% hydrogen peroxide for ten minutes. The slides were then rinsed in water.
sGCα1 and sGCβ1 Antibody Staining

To stain for antibody detection, the slides were washed in PBS containing 0.1% Tween 20 pH7.6 (PBS-T) for five minutes. They were then blocked in PBS-T with 5% normal goat serum for 1 hour at room temperature. Three slides were stained with 5µg/mL anti-sGCα1 (Abcam), 10µg/mL anti-sGCβ1 (Abcam), and blank antibody diluent (Abcam) at 4°C overnight. The slides were then washed in PBS-T three times for five minute. Biotin goat anti-rabbit antibody was then added and slides were incubated at room temperature for one hour. The slides were then washed and incubated with vactastain for 30 minutes are room temperature. Next, the slides were washed three times in PBS-T for five minutes and visualized using DAB. Once developed, the slides were immersed in water and then counterstained in hematoxylin for five minutes. They were then rinsed two times in Blue in Scott’s water.

Mounting the Slides

Finally, the slides were dehydrated and mounted with Permount. To dehydrate, the slides were rinsed twice in 95% ethanol, twice in 100% ethanol, and twice in xylene. The slides were then visualized under microscopy for sGC expression.
Expression of sGC

The branched DNA assay (bDNA), is a technique used to detect and quantitate RNA within tissue or cell types. The bDNA assay was used to quantify sGCα1 and sGCβ1 in isolated primary rat hepatocytes. The sGC-specific rat probes were previously purchased by Ironwood Pharmaceuticals. To detect sGC in human hepatocytes, qPCR was used. The two techniques of sGC expression employed for rat and human hepatocytes were previously established techniques in my research lab. For cost effectiveness, different techniques were applied to both species.

Hepatocyte Resuspension

Male rat and pooled human hepatocytes were purchased from IVAL (In Vitro ADMET Laboratories). Male rat hepatocytes were from a single donor (Lot RSM110). Pooled human hepatocytes were a pooled lot of 10 donors (Lot H1050). Hepatocytes were thawed and resuspended in UCRM per the manufacturers protocol. The cells were counted manually using trypan blue.

bDNA

Rat hepatocyte cells were lysed using QuantiGene sample processing kit per manufacturer’s instruction (Affymatrix, Fremont, CA). To measure gene expression in the cell lysates, QuantiGene 2.0 Plex Assay (Ponomics/Affymetrics, Santa Clara, CA) was used per the user manual. Analytes were measured using Luminex MAGPIX™
Median fluorescence intensity (MFI) were generated for each gene target and normalized to house-keeping gene Polr2a.

qPCR

RNA from human hepatocytes and HEK293 cells was extracted using the Qiagen Tissue Lyser II. Cells were homogenized in Trizol for 2 minutes. Subsequently, 200µL of chloroform was added to each sample. The samples were then transferred to Quiagen mini-columns following the protocol associated with the Qiagen RNeasy Mini Kit. Total RNA was measured using Nanodrop 2000. The High Capacity cDNA Kit from Life Sciences was used to prepare cDNA and samples were run on RT-PCR. To amplify and quantitate the mRNA from both cell lines, the Taqman Gene Expression Assay was used.

Functional sGC Testing

Although sGC proteins can be identified using techniques such as IHC and bDNA described above, such techniques are unable to determine functionality of identified proteins. The presence of a protein’s mRNA does not confirm the its functional activity. To determine if the sGC expressed within hepatocyte cells is functional, isolated primary rat and human hepatocyte cells were incubated with an sGC stimulator in the presence of an NO donor (DETA) and analyzed by ELISA for the presence of cGMP. An increase in cGMP by sGC in response to stimulation will confirm the functionality of the protein.
Hepatocyte Incubation with sGC Stimulator

Cells were plated into 96-well v-bottom plates at 220,000 cells per well into HBSS with 0.5mM IBMX. Cells were incubated at 37°C for 15 minutes. 10μM sGC stimulator with 100μM DETA was added and incubated for 20 minutes. Cells were lysed with 10% acetic acid in water. Cells were then spun for 15 minutes at 3200 RPM and the supernatant was removed and analyzed for cGMP by ELISA kit from Enzo (cat# ADI-900-014).
Chapter III.

Results

The purpose of this study was to evaluate the potential expression of sGC on hepatocytes. To do this, IHC staining was used to determine the expression of both α and β sGC in a paraffin fixed rat liver slice. Since hepatocytes are the major parenchymal cell type comprising approximately 80% of the total number of cells in the liver, and are easily distinguished from other non-parenchymal cell types via microscopy, the expression of sGC in hepatocytes is easily discerned. To confirm sGC protein expression, isolated hepatocytes from both human and rat were evaluated by RT-PCR. Finally, to validate the functionality of sGC in hepatocytes, an sGC stimulator was incubated with hepatocyte cells to confirm target engagement via cGMP readout.

IHC Staining for sGC in Rat Liver Slice

To evaluate the expression of sGC α1 and β1 in a rat liver slice, paraffin fixed liver slices from freshly isolate male sprague dawley rats were obtained. The slices were prepared according to protocol and stained using sGC α1 and β1 antibodies obtained from Abcam. Figure 3 shows the results of IHC staining in a normal male rat liver slice.
H and E staining of the rat liver slice highlighting the nuclei and cytoplasmic regions within the liver (Figure 3a). The localization of sGC α1 (Figure 3c.) confirms the literatures previous finding that sGC is expressed in rat stellates (Thelig et al., 2001). In addition, it appears that sGC α1 may also be expressed in hepatocytes, mainly those cells surrounding the vasculature. The presence of sGC β1 (figure 3d) is detectable in stellate cells, and likely in hepatocytes surrounding the vasculature, however the specificity of the sGC β1 antibody is poor making it difficult to confirm. The negative control (Figure 3b) confirms that the antibody specificity observed in figures 2a and 2b are real. These results suggest that sGC may be expressed on hepatocytes, however the lack of specificity by the antibodies for sGC α1 and β1 warrant alternative methods of sGC detection.
Figure 3. IHC staining of male SD rat liver slice. (a.) H and E staining shows the localization of nuclei (blue) and cytoplasmic regions (pink). (b.) Shows a liver slice untreated with sGC antibodies demonstrating negligible non-specific antibody staining. (c.) shows the expression of sGC α1 in stellate cells as well as some hepatocytes surrounding the vasculature. (d.) the expression of sGC β1 in stellate cells and potentially in some hepatocytes surrounding the vasculature.
Confirmation of sGCα1β1 Protein Expression

The results of the IHC staining suggest sGC α1 and β1 may be expressed in rat hepatocyte cells. However, since IHC is not quantifiable and the data suggests the expression of the sGC subunits may be low, RT-PCR was used to confirm the presence of sGC subunits in commercially available isolated rat and human hepatocyte cells.

To determine the expression of sGC α1 (gene name Gucy1a3) and β1 (gene name Gucy1b3) in rat hepatocytes, hepatocytes from a pool of 10 donors was analyzed by branched DNA (bDNA). The bDNA technique was chosen to analyze the rat hepatocyte cells, because the sGC α1 and β1 probe genes in rat have been well characterized. The expression data was normalized to a standard housekeeping gene, Polr2a. The relative gene expression data shows that Gucy1a3 and Gucy1b3 are detectable in rat hepatocytes, suggesting the presence of both sGC α1 and β1 in rat hepatocytes. It also appears that the relative gene expression of Gucy1a3 is approximately 2-fold greater than the expression of Gucy1b3 in rat hepatocytes (Figure 4).
Figure 4. Relative expression of Gucy1a3 and Gucy1b3 in isolated rat hepatocyte cells. Gucy1a3 (sGCα1) shows greater expression relative to Gucy1b3 (sGCβ1) in rat hepatocytes.

The presence of sGC α1 and β1 mRNA in rat hepatocytes confirms the IHC staining observed previously (Figure 3). However, the question remains as to whether sGC α1 and β1 mRNA is detectable in human hepatocyte cells. A pool of hepatocytes from 5 human donors was analyzed to determine the presence Gucy1a3 and Gucy1b3. Since the bDNA probes for Gucy1a3 and Gucy1b3 are not as well characterized in human, RT-PCR was used to determine the gene expression. HEK-293 cells were used as a control in this study, as the presence of both sGC subunits have been previously well characterized (Hasan et al., 2014). Although the expression of both Gucy1a3 and Gucy1b3 were low comparable to the HEK-293 cells (Table 1), mRNA for both sGC subunits was detected in human hepatocyte cells (Figure 5).
Table 1.

Relative Expression of Gucy1a3 and Gucy1b3 in Human Hepatocytes and HEK-293 Cells

<table>
<thead>
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<th>Relative Expression</th>
<th>Gucy1a3</th>
<th>Gucy1b3</th>
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<tr>
<td>Human Hepatocytes</td>
<td>0.0012</td>
<td>0.0005</td>
</tr>
<tr>
<td>HEK-293 Cells</td>
<td>0.0161</td>
<td>0.0079</td>
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</table>

The control cell line, HEK293 cells are known express high levels of sGCα1 and sGCβ1. In comparison, human hepatocytes appear to express low levels of sGCα1 and sGCβ1.

Figure 5. Relative Expression of Gucy1a3 and Gucy1b3 in Human Hepatocytes and HEK-293 Cells. Main peaks represent sGCα1 (Gucy1a3) and sGCβ1 (Gucy1b3) in HEK293 cells which are known to have high levels of both sGC subunits. sGCα1 and sGCβ1 are also expressed in hepatocytes (minor peaks). Expression is hepatocytes is less relative to HEK293 cells.
Functional Activity of sGC

The mRNA expression results indicate that both human and rat hepatocytes contain mRNA encoding sGC α1 and β1. However, the presence of mRNA does not confirm that the sGC protein is functional. To assess the functionality of sGC in vitro, rat and human hepatocytes were incubated with a proprietary sGC stimulator (X) and the output of cGMP (biomarker for sGC target engagement) was measured by ELISA. Rat and human hepatocytes were incubated alone, with a NO-donor (30 μM and 100 μM DETA), with 10 μM sGC stimulator X, and with a NO-donor (30 μM and 100 μM DETA) + 10 μM sGC stimulator X (Table 2).

Table 2.

Incubation Conditions for Rat and Human Hepatocytes

<table>
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<th>Incubation Conditions</th>
<th>DETA</th>
<th>sGC Stimulator X</th>
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<tr>
<td>1</td>
<td>0μM</td>
<td>0μM</td>
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<tr>
<td>2</td>
<td>30μM</td>
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<td>3</td>
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</tr>
<tr>
<td>6</td>
<td>100μM</td>
<td>10μM</td>
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Rat and human primary hepatocytes were incubated using the six conditions described above. Conditions 2-6 were compared relatively to the vehicle (condition 1).
Both rat and human hepatocytes showed a dose-proportional increase in cGMP upon stimulation of sGC with DETA alone and in combination with an sGC stimulator (Figure 6 and Figure 7). The data in rat and human hepatocytes showed synergy between treatment with sGC stimulator and 30μM DETA as there is a 2-fold increase in cGMP from treatment with the sGC stimulator alone. Treating the cells from both species with sGC stimulator and 100μM DETA did not show a significant increase in cGMP from the cells treated with sGC stimulator and 30μM DETA. This suggests that NO has reached saturation by treatment with 30μM DETA.
Figure 6. cGMP Production by rat hepatocytes. Vehicle levels of cGMP were undetectable. There is an apparent dose response in cells treated with 30 μM and 100μM DETA, respectively. Additionally, there is an increase in cGMP when comparing sGC stimulator alone to the combination of the sGC stimulator and 30μM DETA. There is no difference in cGMP levels when cells are treated with sGC stimulator and either 30 μM or 100 μM DETA suggesting the system is saturated.
Figure 7. cGMP Production by Human Hepatocytes. Vehicle cells show undetectable levels of cGMP. There is an apparent dose response when cells are treated with 30 μM and 100μM DETA, respectively. Additionally, there is an increase in cGMP when comparing sGC stimulator alone to the combination of the sGC stimulator and 30μM DETA. There is no difference in cGMP levels when cells are treated with sGC stimulator and either 30 μM or 100 μM DETA suggesting the system is saturated.
A direct comparison of cGMP output by human and rat hepatocytes under the conditions specified in Table 2 is shown in Figure 8. These results suggest that both rat and human hepatocytes contain functional sGC. In addition, it appears that the rat and human hepatocytes have a similar response (cGMP concentration) to DETA and sGC stimulator alone. However, when treated with 10μM sGC stimulator and 30μM DETA, human hepatocytes produce 2-3-fold greater cGMP.

Figure 8. Comparison of cGMP Production by Rat and Human Hepatocytes. Similar response profile in treatment groups when comparing rat and human hepatocytes.
Results Summary

The expression of functional sGC was successfully identified in rat and human hepatocytes. Immunohistochemistry staining provided the initial evidence that sGC may be expressed in rat hepatocytes, however conclusive results proved difficult due to the presence of all liver cell types in the liver slice.

Confirmation of sGC expression in hepatocytes was determined through positive mRNA detection in isolated human and rat hepatocytes. At Ironwood Pharmaceuticals where this work was completed, a bDNA panel for sGC expression in rat had been previously made. Since the kit was available, it was more cost-effective to use the existing bDNA assay for sGC expression in Rat. Both sGCα1 and sGCβ1 were detected in isolated rat hepatocytes.

Although the bDNA assay is more high-throughput, Ironwood did not have an existing bDNA panel for human samples. Since generating the bDNA probes specific for sGC are costly, qPCR was used to determine the expression of sGC in human hepatocytes. As seen in rat, isolated primary human hepatocytes showed expression of both sGCα1 and sGCβ1.

To confirm functional activity of sGC, individually isolated rat and human hepatocytes were incubated with a proprietary sGC stimulator. An increase in cGMP in the treated cells compared to the vehicle treated samples, suggests that sGC is functional and can be stimulated in vitro. Collectively, these results strongly support that sGC is present and functional in rat and human hepatocytes. To date, little is reported about sGC and its functionality in hepatocytes. Thus, these data contribute greatly to the sGC
literature and provide a foundation for new potential applications for sGC targeting in liver disease.
Chapter IV.

Discussion

Understanding the structure, function, and implications of sGC is a growing area of research among many drug companies to date. The broad disposition of sGC throughout many cell types in the body along and its demonstrated role in the regulation of fat accumulation (Hoffman et al. 2015), inflammation (Ahluwalia, et al. 2004), vasodilation, platelet aggregation, and fibrosis (Nowatzky 2012), make it an attractive therapeutic target for many diseases. Understanding the localization of sGC in additional cell types broadens the spectrum of potential uses of sGC stimulation as a therapy.

Through this work, it has been shown that sGC is expressed in rat liver through IHC staining. The IHC confirmed the expression of sGC in stellate cells as well as potential sGC expression on hepatocyte cells. Because IHC is not quantitative and its utility highly depends on the specificity of the antibodies to your target that are available, these results were not conclusive. However, the results of this experiment display the first reported finding of sGC expression in hepatocytes.

Although the results of the IHC staining do not definitely conclude that sGC is expressed on hepatocytes, it insinuates that the expression is low and may be isolated in specific populations of hepatocytes within the liver. Figure 3c shows that positive staining of sGCα1 in hepatocytes may be isolated to populations surrounding the vasculature. Since sGC is stimulation causes vasodilation, such localization is plausible. In addition, the literature has reported that certain proteins such as transporters within the...
liver have zone-dependent localization. For example, OATP transporters in human have been reported to be expressed greater in zone 2 of the liver than zones 1 and 3. Therefore, zonal dependent expression of other proteins such as sGC is plausible. Further IHC studies on liver slices from different zones of a rat liver would be necessary to confirm these results. Zonal-specific liver slices are very difficult to obtain, and would require a skilled histologist to verify and obtain.

Since the results of the IHC staining for both sGC subunits in rat was inconclusive, performing the same study in human liver slices would not be useful. In addition, human liver slices are more costly than rat liver slices, therefore the expense and high likelihood of inconclusive data made the same study in human unfeasible. Instead, a more conclusive and cost-effective assay to confirm that both sGC α1 and β1 are expressed on rat and human hepatocytes was to look for mRNA expression of both proteins in isolated cells. Rat and human hepatocytes are commercially available, readily obtained, and are relatively inexpensive. In addition, looking at isolated hepatocytes ensured that the read on sGC protein expression is in fact in the cell type of interest.

Since IHC staining leaves the possibility that hepatocytes could be misidentified for other cell types or result in non-specific staining, it was difficult to conclusively determine sGC expression on hepatocytes. To enable cell type-specific sGC expression, isolated rat and human hepatocytes were used to determine sGCα1 and β1 expression by mRNA. Using bDNA for rat hepatocytes and RT-PCR for human hepatocytes, it was determined that both sGC subunits were present in the hepatocytes of both species, provided further confidence in the IHC data that sGC is expressed on hepatocytes. Although it does appear the mRNA for sGC is expressed on hepatocytes of both species,
the relative expression was low. This supports the hypothesis the sGC expression may be zonal on hepatocytes rather than a ubiquitous expression across all hepatocytes in the liver.

The detection of sGC mRNA on rat and human hepatocytes provides confidence that the sGC subunits are in fact present in the cells. However, the expression of the protein doesn’t confirm its functionality. To determine if the sGC detected on the hepatocytes was functional, the cells were incubated with an sGC stimulator with and without DETA-NO to determine if the target could be engaged. Since stimulation of sGC causes an increase in cGMP, a functional read on cGMP was determined. Both rat and human hepatocytes showed a cGMP response to the presence of an sGC stimulator as well as a further increase in cGMP in the presence of the stimulator and DETA-NO over vehicle. This study suggests that the sGC present in both rat and human hepatocytes is functional.

Although it is known that sGC is expressed in the liver, particularly in stellate cells, sGC expression on hepatocytes and its implications are a limited area of research. As the most abundant cell type in the liver, hepatocytes have several responsibilities including metabolism, detoxification, protein synthesis and storage, and biliary elimination. Since hepatocytes are important regulators in the liver, many liver diseases result in damage and/or fat accumulation within these cells.

Wang et al. previously demonstrated that NO provides cytoprotection against inflammation and tissue damage (Wang et al., 2002). Currently, the best known mechanism for NO-mediated signal transduction is through the sGC pathway via an increase in cGMP. In addition, it has been shown that NO-mediated hepatoprotection
depends partly on cGMP (Wang et al., 2002). NO works synergistically with sGC to stimulate the production of cGMP. Although the literature suggests that sGC is a plausible pathway through which NO-mediated hepatoprotection occurs, research surrounding the expression of sGC on hepatocyte cells has yet to be reported.

In addition to hepatoprotection against inflammation, sGC stimulation has also been shown to decrease fat accumulation in the liver. Hoffman et al. showed that the commercially available sGC stimulator, BAY 41-8543, protects against diet induced obesity (DIO) in mice. Upon treatment with BAY 41-853, the DIO mice showed improved metabolism, decreased liver steatosis, reduced insulin levels, and decrease adipocyte size (Hoffman et al., 2015). Fat accumulation and steatosis is the first insult in the progression of many liver diseases. For example, in the disease Nonalcoholic steatohepatitis (NASH), fat accumulation in the liver is believed to be the first insult in the progression of the disease. Steatosis triggers a cascade of liver insults including inflammation and fibrosis. As the disease progresses, eventually the liver becomes cirrhotic and the patient must receive a liver transplant to survive.

The stimulation of sGC on hepatocytes may prevent or reverse steatosis preventing the progression of liver diseases. It is plausible that sGC stimulation in hepatocytes may reverse hepatotoxicity or prevent the progression of liver disease. This work confirms the presence and functionality of sGC on hepatocyte cells, and warrants additional research to understand the potential impact sGC stimulation may have on liver diseases. These results may provide an additional therapeutic target as researchers continue to find treatments for devastating liver diseases.
Study Limitations

The above-mentioned experiments each have their intrinsic limitations. To begin, successful IHC staining depends largely on the quality of the antibodies used. In addition, optimization of the concentrations of antibodies used is paramount for quality data. It can also be difficult to distinguish different cell types in the liver. Because you’re determining expression of a protein based on staining observed under magnification, there may be subjective results based on the “viewer.”

To limit the subjective bias of IHC, mRNA expression provided a more quantitative result for expression in both rat and human hepatocytes specifically. Since the results are compared to a control (house-keeping gene), it is easier to confirm a positive or negative expression result. Although you cannot determine an absolute quantitative amount of mRNA with this technique, you can determine whether the expression is low, moderate, or high. One major consideration in analyzing individual rat and human hepatocytes is the cell purity. Although isolated hepatocytes have high purity, there is always the possibility of contamination by other cell types. It is possible that the hepatocytes were contaminated with other cell types, such as stellate cells, that can give a false-positive result for sGC expression. In addition to the concern of cell purity, mRNA expression does not confirm functional sGC. It is possible that the gene is expressed independent of the protein.

Although this study demonstrates that sGC is functional in hepatocyte cells via cGMP production, it does not show that sGC stimulation can positively impact the cells. Further studies are needed to explore the benefits of sGC stimulation in hepatocytes, including its impact of metabolism, fat accumulation, steatosis, and glucose regulation.
Understanding the benefits and implications of sGC stimulation on hepatocytes is critical in determining the viability of the target in liver disease. If this mechanism proves beneficial, my research sets the groundwork for a potentially exciting new area of interest for researchers exploring alternative benefits of sGC.

Future Directions

As described above, the presence and functionality of sGC in hepatocytes is novel and has yet to be well characterized in the literature. In addition to the novelty of this data in the literature, it may provide great therapeutic strategy for Ironwood and other researchers in the field of sGC. Since the expression of sGC on hepatocytes was previously unknown, the results of these studies have the potential to expand the literature surrounding sGC and offer new insight into additional roles the enzyme may play within the body. Specifically, in the liver, fat accumulation and inflammation are common insults that lead to liver injury and fibrosis. Since studies have shown that fat infiltrates hepatocytes and causes steatosis (Cohen et al., 2011) and that hepatocytes respond to inflammatory signals released from Kupffer cells (Curran et al., 1990), sGC expression on hepatocytes may provide an additional target cell type for the treatment of liver injury and disease.

Previously, it has been shown that sGC is present on stellate cells, the “bad actors” in liver fibrosis. Activation of stellate cells causes an infiltration of collagen within the liver leading to fibrosis (Knorr et al., 2008). Through in vivo models of liver fibrosis, it has been shown that treatment with an sGC stimulator causes decreased
collagen deposition and reversal of fibrosis (Xie et al., 2012). Since sGC has been shown to reverse and prevent fibrosis through stellate cells, the expression of sGC on hepatocytes may have additional benefits for treating and preventing liver disease.

The presence of sGC on hepatocytes not only expands potential therapeutic areas of interest, but also opens the door for a targeted chemical strategy. Because hepatocytes contain several uptake and efflux transporters, we would have the potential to minimize the systemic effects of our drugs by taking on a hepatocyte-targeted chemical strategy. Continuing to build the literature surrounding sGC will undoubtedly aid the understanding of sGC’s downstream signaling cascade and the implications it may have when disrupted in certain diseases, specifically in this case, the liver.
V. References


