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TRANSCRIPTIONAL REGULATION OF ALDO-KETO REDUCTASE 1D1

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BY

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ABSTRACT

Bile acids are physiological detergents and surfactants with recently identified roles as signaling hormones. Maintenance of physiologically normal bile acids levels is fundamentally important as a deregulation within the bile acid synthesis pathway has the potential to result in an unbalanced bile acid pool. This may result in any number of pathological down stream effects and can exacerbate various diseases and disorders. Chenodeoxycholic acid (CDCA) and cholic acid (CA) are two major primary bile acids in humans with CDCA being more hydrophobic and toxic than CA. Aldo-keto reductase 1D1 (AKR1D1) and 12-alpha-hydroxylase (CYP8B1) are the two key enzymes responsible for the synthesis of CDCA and CA, respectively.

It remains largely unknown how AKR1D1 and CYP8B1 are regulated to maintain homeostatic CDCA and CA concentrations under physiological conditions. Likewise, little is known regarding the regulation of their synthesis under pathological conditions, or the mechanisms by which this regulation occurs. To date, much focus has been on CYP8B1 expression as the key regulator of bile acid synthesis, and also as the determining factor for the CDCA to CA ratio within the bile acid pool. We hypothesize that due to the increased toxicity associated with elevated CDCA concentrations, combined the knowledge that CDCA is a potent ligand for various signaling pathways, the liver is consistently altering AKR1D1 expression, and not CYP8B1, in an effort to maintain physiologically normal liver function.

Further knowledge pertaining to the regulation of individual primary bile acids may shed light on novel mechanisms by which various disorders and diseases, which are attributed to bile acid dysregulation, can be treated or prevented. For example, it

has been established that the CDCA composition of the bile acid pool is lower in patients with diabetes; however, the mechanism by which this occurs is unclear. Understanding the role of bile acid synthesis in diabetic models may identify a mechanism by which CDCA production is being regulated. Hepatocellular carcinoma (HCC) is the most prevalent type of liver cancer. It has been identified that high concentrations of CDCA are toxic to the hepatocytes and can have carcinogenic effects; therefore a better elucidation of the regulation of CDCA production may prove important for prevention or progression of HCC. Intrahepatic cholestasis of pregnancy (ICP) is a disorder that occurs within the third trimester of pregnancy as a result of elevated bile acid levels. Although AKR1D1 expression is known to increase during pregnancy, the role of AKR1D1 in pregnancy has not yet been established.

Experiments were carried out *in-vitro* in a human liver carcinoma cell line and *in-vivo* in mice as well as on human liver tissue and tissue sections. Our data revealed that endogenous AKR1D1, and not CYP8B1, is transcriptionally and translationally regulated by primary bile acids, CDCA and CA, by a negative feedback or positive feed-forward mechanism, respectively. Likewise, with the use of a human AKR1D1 promoter reporter, the transcriptional regulation of AKR1D1 expression was investigated, and confirmed endogenous results. Additionally, results indicated that in conditions of metabolic disorder, (i.e., diabetes) or in liver cancer, human hepatic AKR1D1 expression is significantly decreased as compared to normal human tissue. Furthermore, a substantial link was established between AKR1D1 expression, estrogen and conditions of ICP in *in-vitro* and *in-vivo* experiments.

With knowledge of the cooperative capabilities of primary bile acids on the expression of AKR1D1, we next investigated potential signaling pathways involved in AKR1D1 regulation. *In-vitro* and *in-vivo* activation of critical bile acid (Farnesoid X Receptior, FXR), lipid (peroxisome proliferator activated-receptor, PPAR) and cholesterol (Liver X Receptor, LXR) signaling pathways were examined for implications on AKR1D1 expression. Our results demonstrated that while FXR was not involved in the regulation of AKR1D1, PPAR activation decreased AKR1D1 expression and LXR activation resulted in an increase in AKR1D1 expression. Furthermore, a substantial link was established between estrogen signaling and AKR1D1 expression *in-vitro* as well as in pregnant mice.

In conclusion, CDCA and CA are key regulators of AKR1D1, but not CYP8B1, expression. Such coordinated down-regulation and up-regulation of AKR1D1 by primary bile acids represents a mechanism by which the liver maintains homeostatic CDCA and CA levels under physiological condition. The data also indicates that LXR and PPAR signaling pathways are involved in regulating AKR1D1 with the possible identification of response elements in the promoter region. Moreover, the data demonstrates that changes to the bile acid composition of diabetic patients are due to a down regulation of AKR1D1 resulting in a decreased production of CDCA. Our data also demonstrates that in circumstances of HCC, the liver may alter bile acid synthesis through the down regulation of AKR1D1 expression, in a negative feedback manner to decrease production of CDCA. Finally, the role of AKR1D1 with respect to pregnancy was identified, substantiating a link between estrogen and AKR1D1 expression. In summary, our results confirm our hypothesis

that the liver alters AKR1D1 expression in an effort to minimize toxicity associated with CDCA production and maintain homeostatic CDCA to CA levels.

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DEDICATION

To my Maman and Baba, everything is always for you.

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CHAPTER 1

INTRODUCTION

Cholesterol and Bile Acid:

Cholesterol is an amphipathic, waxy, fat-like substance that is synthesized endogenously by our livers. It is a fundamental structural component of cell membranes as well as a precursor for many intracellular biochemical pathways. Cholesterol is found in all animals, and thus, it is in many of the foods that we ingest. The liver plays a primary role in not only the digestive and detoxification process but also the synthesis of cholesterol and movement of excess cholesterol from the bloodstream as required.

Bile acids are the end product of cholesterol catabolism. Bile acids are produced in the liver and secreted from the hepatocyte into the bile by the membrane-associated transporter, bile salt export pump (BSEP), and stored in the gallbladder (Russell, 2003). Upon ingestion of a meal, the gallbladder empties the bile into the intestinal tract where the bile acids aid in the emulsification and absorption of dietary fats and lipids. Approximately 95% of bile acids are reabsorbed through the upper intestines, circulated back to the liver via the portal vein, and then sent back to the liver (Russell, 2003). This transport of conserved bile acid from the intestine to the liver is known as enterohepatic circulation, and BSEP is the rate-limiting step. The composition of the re-circulating bile acid not only dictate the rate of enterohepatic circulation, but also regulate the activation and repression of various signaling pathways that range from the regulation of bile acid synthesis to various metabolic pathways in the liver, gastrointestinal tract and beyond.

The conversion of cholesterol to bile acids is the primary pathway by which cholesterol is removed from the liver (Russell, 2003). There are two types of primary bile acids synthesized from cholesterol, cholic acid (CA) synthesized from sterol 12-alpha hydroxylase (CYP8B1) and chenodeoxycholic acid (CDCA) synthesized from aldo-keto reductase 1D1 (AKR1D1) (Russell, 2003). These two bile acids differ greatly with respect to their individual roles in cholesterol solubilization and absorption. CA is more hydrophilic due to the hydroxylation of the bile acid steroid nucleus in the C-12α position and is therefore more efficient in the absorption and excretion of cholesterol in the intestine (Chiang, 2009). CDCA is highly hydrophobic and therefore inhibits cholesterol absorption and excretion (Chiang, 2009). In addition to these processes, secondary bile acids are generated by modifications that are carried out by intestinal bacteria. Primary and secondary bile acids can also be further conjugated with either glycine or taurine, and this enhances their water solubility. This results in 13 total primary and secondary, conjugated and unconjugated bile acids.

At physiological concentrations bile acids are not harmful to the body, they are essential due to their detergent-like properties, however bile acid accumulation can be toxic to hepatocytes. The hydrophobicity of the bile acid pool contributes to the toxicity and thus it is essential to regulate the concentration of individual bile acids and the size of the total bile acid pool. The ratio of CA to CDCA concentrations in the bile acid pool affects how much cholesterol is absorbed from the food we eat and how much is excreted. At physiological concentrations, a 1:1 ratio of CA to CDCA is maintained. A sustained balance of hydrophilic to hydrophobic bile acid is essential to support normal, physiologically healthy liver function. The circumstances that can

arise due to a deregulation of the CA to CDCA ratio are not restricted to the bile acid synthesis pathway. Due to the signaling characteristic of circulating bile acids, and their role as physiological ligands in the activation and suppression of numerous pathways, many pathological consequences can occur as a result of a deregulation in the CA to CDCA ratio. Such consequences might include the exacerbation of diabetic symptoms, hepatocellular damage or death, or liver tissue inflammation that result in cirrhosis, cholestasis, and ultimately, the progression of liver cancer (Perez, 2009). The elucidation of the underlying mechanisms by which this bile acid ratio affects the progression or exacerbation of such disorders will lead to novel approaches for the maintenance of the CA to CDCA ratio of bile acid composition. Moreover, once the mechanistic pathway, through which AKR1D1 and CYP8B1 regulate the production of CDCA and CA, respectively, is understood, novel pathways that control the ratio of CA to CDCA production will be exposed. These pathways can then be manipulated to maintain physiological concentrations in conditions of metabolic disease and distress and as a precursor for disease prevention.

Nuclear Receptors and Bile Acid*:*

It is important to note that bile acid signaling is not restricted to the liver. Specifically, through enterohepatic circulation, bile acids are circulated from the intestines through portal blood back to the liver, and serve as endogenous ligands responsible for the activation of numerous transcription factors and signaling pathways (Houten, Watanabe, & Auwerx, 2006). The Farnesoid X Receptor (FXR) is known as the bile acid nuclear receptor as bile acids, more potently CDCA, serve as the physiological ligand and activator (Lefebvre, Cariou, Lien, Kuipers, & Staels,

2009). FXR activation ultimately serves a protective role in the maintenance of low bile acid concentrations to support homeostasis by triggering transcription of target genes involved in bile acid metabolism (Eloranta & Kullak-Ublick, 2008). When there is an over-production of bile acids in the liver, the bile acids bind to and activate FXR (Hageman, Herrema, Groen, & Kuipers, 2010). FXR will then activate the transcription of the small heterodimer partner (SHP), which in turn binds to and inhibits the transactivation of the Liver Homolog-1 receptor (LHR-1). This results in a negative feedback regulation of cholesterol 7-alpha hydroxylase (CYP7A1) expression, the rate limiting step in bile acid synthesis (Chiang, 2009). A down-regulation of CYP7A1 results in a decrease in the production of bile acids and this allows circulating concentrations to stabilize to physiological levels (Lefebvre et al., 2009). In the intestines, when FXR is activated due to access bile acids, fibroblast growth factor-19 (FGF19) expression on the enterocytes is up-regulated and a signal is sent from the intestines to CYP7A1 in the liver to decrease bile acid synthesis for the regulation of bile acid concentrations (Chiang, 2009). As a ligand of FXR, CDCA also serves to control the efflux of bile acid from the liver through the activation of BSEP in an effort to maintain physiologically normal liver function in the absence of high bile acid levels (Y. Chen et al., 2013).

When cholesterol levels are high, the liver x receptor (LXR) is activated (Zhao & Dahlman-Wright, 2010). Oxysterol, an oxidized metabolite of cholesterol, acts as an endogenous ligand for LXR. LXR is activated in response to an accumulation of cholesterol, and this leads to the induction of genes responsible for the maintenance of cellular cholesterol levels in both the liver and intestines (Faulds, Zhao, & Dahlman-

Wright, 2010). Activation of LXR also results in an increase of CYP7A1 expression in an effort to produce sufficient bile acid which aids in the emulsification and absorption of the excess cholesterol (Ory, 2004). Therefore, activation of LXR signaling has implications on overall cholesterol homeostasis via regulation of bile acid synthesis.

Peroxisome proliferator-activated receptors (PPAR's) are a group of transcription factors that play a role in the regulation of lipid metabolism (Ory, 2004; Pineda Torra, 2002). PPAR's function as a group of three receptor isoforms, PPARα, PPARβ, and PPARϒ (Ory, 2004). Specifically, PPARα is responsible for the regulation of fatty acid oxidation and lipid and glucose metabolism (Ory, 2004). PPARβ is responsible for increased insulin sensitivity, and increased energy expenditure while PPARϒ plays a part in the management of insulin resistance (Ory, 2004). Taken together, the activation of PPAR has implications for bile acid synthesis and bile composition, as well as disorders such as hyperlipidemia and diabetes (Yoshikawa et al., 2003).

These nuclear receptors, and many more, serve as examples of how bile acid concentrations directly and indirectly affect the overall synthesis of various pathways, and paint the picture of a very intricate system taking place to ensure homeostasis. Further understanding of the complex role and regulation of these nuclear receptors with respect to bile acid synthesis and AKR1D1 and CYP8B1 expression under physiological bile acid conditions will further uncover their role and response in diseased conditions.

Disease and Bile Acid:

The liver plays a role in both metabolism and detoxification. Any deviation from normal physiological function may result in any number of pathological outcomes. This holds true for bile acid synthesis as well. A deregulation of bile acid synthesis, bile acid composition and bile acid pool size may result in the exacerbation of metabolic disorders, gallstone formation, cholestasis, cirrhosis, and even cancer (Y. Chen et al., 2013; Eloranta & Kullak-Ublick, 2008; Hofmann, 2014; Jansen, 2007; Martinez-Augustin, 2008). Type-2 Diabetes (T2D) is a very common metabolic disorder characterized by hyperglycemia, insulin resistance and insulin deficiency. It has been shown that under physiologically normal conditions insulin inhibits CYP7A1 expression, while glucagon increases it, and this regulates bile acid synthesis (Li, Chanda, Zhang, Choi, & Chiang, 2010). Elevated cholesterol levels can exacerbate T2D symptoms as well (Li et al., 2012; Prawitt, Caron, & Staels, 2011); however the mechanism is poorly understood. As a result of a deregulation of glucose homeostasis associated with T2D, elevated glucagon and glucose levels due to fasting fail to decline or be suppressed after a meal has been ingested (Nguyen & Bouscarel, 2008). This may be a mechanism by which the liver produces more bile acid in order to regulate glucose and lipid homeostasis after ingestion of a meal. Therefore, bile acid synthesis increases due to insulin deficiency and resistance, and indicates that metabolic disorders such as diabetes affect the rate of bile acid synthesis (Prawitt et al., 2011). Studies have shown that the bile acid pool size and composition are significantly altered in patients with uncontrolled T2D (Li et al., 2012). More significantly, it has been established that in patients that present with uncontrolled T2D, concentrations of the individual bile acid CDCA were significantly lower in the

bile acid pool with no indication of change in CA concentrations (Brufau et al., 2010; Prawitt et al., 2011). Therefore, because AKR1D1 is responsible for the synthesis of CDCA, gaining a better understanding as to the role of AKR1D1 in T2D models can identify a mechanism by which CDCA synthesis may be manipulated to regulate metabolic distress.

Hepatocellular carcinoma (HCC) is the most prevalent type of liver cancer and a deregulation of bile acid synthesis and homeostasis has been linked to HCC formation (Y. Chen et al., 2013). This is due to the fact that, because of the potent surfactant capabilities of bile acids, an accumulation of bile acids at high concentrations is potentially toxic and can subject the liver to carcinogenic outcomes (Halilbasic, Claudel, & Trauner, 2013). It has been established that high concentrations of the more hydrophobic bile acids, such as CDCA, is toxic to the hepatocytes and can effect overall liver function (Lefebvre et al., 2009). This toxicity may result in liver inflammation and hepatocyte DNA damage that may promote the occurrence of carcinogenesis (Martinez-Augustin, 2008). Bile acid accumulation may also lead to cholestasis, and this has been linked to HCC progression (Y. Chen et al., 2013). Regulation of the production and accumulation of highly toxic bile acids, such as CDCA, may be a mechanism by which the occurrence or progression of HCC can be controlled. In order to effectively control the production and accumulation of CDCA through bile acid synthesis, AKR1D1 expression must be tightly regulated. Understanding the pathogenesis that results from the accumulation of bile acids, such as the highly hydrophobic and toxic CDCA, via AKR1D1, may provide insight into a

novel therapy for prevention or treatment of diseases such as HCC and diabetes, among others.

Another disorder that is linked to uncontrolled bile acid concentrations is intrahepatic cholestasis of pregnancy (ICP). ICP is a condition that can be seen in certain pregnant women in their third trimester of pregnancy (Glantz, Marschall, & Mattsson, 2004). Pregnant women with ICP symptomatically present jaundice-like conditions in the presence or absence of pruritus (Glantz et al., 2004). ICP is characteristically associated with increased serum bile acid concentrations in combination with a dysregulation of individual bile acids in the CDCA to CA ratio (Egan et al., 2012). In physiologically normal conditions, including normal healthy pregnancies, the ratio of CDCA to CA concentrations is essentially equal; however, in conditions of ICP, women present with serum bile acid concentrations that contain 4 times more CA in conjunction with a decrease in CDCA production (Egan et al., 2012; Laatikainen, Lehtonen, & Hesso, 1978). Diagnosis is most commonly made through the measurement of serum bile acid levels and if ICP is left untreated, there will be stresses onto the mother, and fetus as well (Glantz et al., 2004). Thus, uncontrolled bile acid levels can result in adverse and possibly fatal consequences to the fetus (Glantz et al., 2004). While the mechanism by which ICP occurs is still not definitively understood, it has been documented that increased estrogen levels and changes in expression of BSEP may contribute to ICP (Song et al., 2014). Uncovering the transcriptional changes in expression of the genes that are responsible for the production of primary bile acids, such as AKR1D1, in the presence of estrogen may provide a potentially important link between pregnancy and cholestasis.

CYP8B1:

Sterol 12α-hydroxylase, CYP8B1, is cytochrome P450 enzyme responsible for the synthesis of the more hydrophilic bile acid, cholic acid (CA) (Ishida, Gotoh, Yamashita, Yoshida, & Noshiro, 1999). To date, there has been much attention given to CYP8B1 linking its expression to the regulation of the CDCA to CA ratio within the bile acid pool (M. Zhang & Chiang, 2001). The foundations of these claims have been the targets of controversy, as the mechanism behind CYP8B1's control of the bile acid ratio remains unclear. There have been further reports which suggested a decreased trend in CYP8B1 expression with either CDCA or CA treatment of hepatocytes (Liu et al., 2014) while others have claimed that hydrophobic bile acids, and not hydrophilic bile acids, regulate the expression of CYP8B1 (M. Zhang & Chiang, 2001). Changes in the expression of CYP8B1 have been documented in cases of diabetes with claims that CYP8B1 expression is increased in the absence of insulin (Ishida et al., 1999).

AKR1D1:

Aldo-keto reductase 1D1, AKR1D1, is a 5β-reductase enzyme responsible for the synthesis of the hydrophobic primary bile acid, chenodeoxycholic acid, CDCA. The 5β-reductase, NADPH-dependent, activity of AKR1D1 incorporates a 90° bend in the structure of the enzyme and this is believed to add necessary solubility characteristics to subsequently produced bile acids (M. Chen & Penning, 2014).

AKR1D1 is involved in the metabolism of several steroid hormones and also in the regulation of several hormone dependent processes. In fact, AKR1D1

expression has been linked to regulating time of parturition (Sheehan, Rice, Moses, & Brennecke, 2005). It has also been documented that, during pregnancy, increased expression of AKR1D1 serves to suppress contractions and thereby prevents pre-term birth (Pařízek, Koucký, & Dušková, 2014).

There are many mutations associated with AKR1D1 expression and an inborn mutation of AKR1D1 can manifest itself into several liver-related abnormalities (Chaudhry et al., 2013; Drury, Mindnich, & Penning, 2010). For example, there is the potential for cholestasis as a result of alteration in the bile acid synthesis pathway and while treatment with primary bile acids tends to alleviate the condition, children born with an AKR1D1 deficiency that go untreated do not survive (Drury et al., 2010).

AKR1D1 is the only enzyme in humans that is capable of catalyzing a 5β-reduction in bile acids and steroids (M. Chen & Penning, 2014). AKR1D1 expression is also necessary for bile acid synthesis. While CYP7A1 is the rate-determining enzyme for total bile acid synthesis, little attention has been given to the enzymes that are responsible for the production of the individual primary bile acids. Following CYP7A1, AKR1D1 or CYP8B1 are next synthesized for the production of either CDCA or CA, respectively. While CYP8B1 has been identified as the enzyme that dictates the production of CDCA or CA and therefore influences the bile acid pool ratio, AKR1D1 expression should not go unnoticed. This is due to the fact that AKR1D1 is not only responsible for the production of the primary bile acid CDCA, but also for the synthesis of CA. After conversion of CYP7A1 to CYP8B1, another subsequent synthesis of CYP8B1 to AKR1D1 results in a 5β-reduction that is

necessary for CA synthesis. It is for this reason that AKR1D1 should be considered a more potent player in bile acid synthesis.

Conclusion:

Due to CDCA's highly toxic hydrophobic nature, controlling its production via AKR1D1 regulation is fundamentally important. Understanding the regulation of AKR1D1 in bile acid synthesis will ultimately unveil a novel approach to regulate CDCA production and the overall composition of the bile acid pool. The investigation into AKR1D1's regulation can serve to provide drug therapies for such metabolic disorders as hyperlipidemia and diabetes, as well as treatment for disorders such as ICP, cardiovascular disease and liver cancer by regulating the production of the hydrophobic bile acid, CDCA.

CHAPTER 2

COORDINATED REGULATION OF ALDO-KETO REDUCTASE 1D1 BY CHENODEOXYCHOLIC ACID AND CHOLIC ACID

1. INTRODUCTION

Recently, it has been discovered that the role of bile acids is not only to serve as biological detergents, emulsifiers and the major route for cholesterol elimination, but to also to serve as intricate signaling hormones responsible for the regulation of numerous metabolic and regulatory processes such as cholesterol, lipid, energy, and glucose metabolism (Perez, 2009). Accordingly, impaired bile acid synthesis and signaling can lead to the occurrence, or exacerbation, of metabolic disorders such as obesity, diabetes, cholestasis, liver injury or cancer (Hofmann, 2014; Li & Chiang, 2014; Perez, 2009).

One trend that is observed for many bile acid related cardiovascular and metabolic disorders is an imbalance in the bile acid pool size and composition (Chiang, 2009). Deregulation of CDCA and CA levels is reported to be associated with various disease conditions including intrahepatic cholestasis, diabetes and hepatocellular carcinoma (HCC). AKR1D1 and CYP8B1 are the two key enzymes for CDCA and CA synthesis, respectively. Due to their detergent-like properties, excessive bile acid accumulation can be toxic and therefore the size of the pool is tightly regulated (Chiang, 2009). On the other hand, bile acid can either be hydrophobic and more toxic in nature, such as CDCA or hydrophilic such as CA (Russell, 2003). The composition of individual bile acids, or the ratio of CDCA to CA in the bile acid pool will greatly affect various physiological processes, such as the

rates of absorption and excretion of cholesterol and lipids(Russell, 2003). CDCA more commonly inhibits cholesterol absorption while CA promotes cholesterol absorption (Russell, 2003). For this reason, deregulation of the ratio or alteration of the bile acid pool composition may also lead to pathological consequences and influence disease conditions (Lefebvre et al., 2009). Currently, it remains largely unknown how AKR1D1 and CYP8B1 are regulated to maintain homeostatic CDCA and CA levels. Furthermore, little is understood with regard to the underlying mechanisms that lead to bile acid pool alterations, which, in turn, give rise to physiological homeostasis or pathological circumstances.

Bile acids circulate between the liver and intestines via the tightly synchronized enterohepatic circulation. Bile acids also function as signaling molecules, and not only control their own synthesis and regulation but also the gene expression of important nuclear receptors that are involved in various signaling pathways (Handschin & Meyer, 2005; Houten et al., 2006; Zwicker & Agellon, 2013). Many nuclear receptors and signaling pathways are also involved in the regulation of bile acid production through the rate limiting step of bile acid synthesis, cholesterol 7a-hyroxylase, CYP7A1 (Russell, 2003). The Farnesoid X Receptor (FXR) is also known as the bile acid receptor due to the fact that bile acids, mainly CDCA, serve as endogenous ligand (Chiang, 2009; Eloranta & Kullak-Ublick, 2008; Hageman et al., 2010; Russell, 2003). As bile acid levels increase FXR signaling results in not only a decrease of CYP7A1 expression and an increase in expression of essential transporter proteins necessary for bile acid movement (Chiang, 2009; Eloranta & Kullak-Ublick, 2008; Lefebvre et al., 2009).

Liver x receptor (LXR) is involved in cholesterol and lipid homeostasis (Ory, 2004; Zhao & Dahlman-Wright, 2010). Oxysterols activate LXR in response to an accumulation of cholesterol, and this results in an increase of bile acid synthesis via CYP7A in order to produce sufficient bile acid to aid in the emulsification and absorption of excess cholesterol (Faulds et al., 2010; Ory, 2004; Zhao & Dahlman-Wright, 2010). Peroxisome proliferator-activated receptors (PPAR's) are another group of transcription factors that play a role in the regulation of lipid metabolism (Ory, 2004; Pineda Torra, 2002). PPAR agonists include fatty acids and fibrates which have implications on bile acid synthesis and bile composition, and also for disorders such as hyperlipidemia and diabetes (Mansour, 2014; Ory, 2004; Yoshikawa et al., 2003). Research to understand how the activation of LXR or PPAR has implications on bile acid synthesis and/or bile acid related disorders may reveal novel mechanisms through which to regulate individual bile acid production.

A deregulation in the CDCA to CA ratio can result in toxicity and the exacerbation of diabetic symptoms, hepatocyte damage, death, or inflammation leading to cirrhosis, and ultimately, the progression of liver cancer (Hageman et al., 2010; Li et al., 2012; Nguyen & Bouscarel, 2008; Perez, 2009). While it is known that bile acids regulate their own total synthesis by repression of CYP7A1 expression, little research has been done to investigate the regulation of individual bile acids (Russell, 2003). It is understood that AKR1D1 is responsible for the production of CDCA, and CYP8B1 is responsible for the production of CA, however regulation of their individual synthesis remains unclear. Furthermore, little is known with regard to their involvement in the regulation and maintenance of the CDCA to CA bile acid ratio.

Due to CDCA's highly toxic hydrophobic nature, physiological control of the production of CDCA, via AKR1D1 regulation, may logically be more fundamentally important for bile acid homeostasis and healthy liver function. It is the critical requirement of the liver to maintain homeostatic bile acid concentrations through the control of bile acid concentrations, therefore we hypothesize that regulation of AKR1D1, and not CYP8B1 is essential for healthy liver function.

At present, the regulation of AKR1D1 expression is not clearly understood. Thus, research to elucidate the mechanism by which AKR1D1 regulates the production of bile acid can uncover potentially innovative approaches for the modulation of the ratio of CDCA to CA in order to maintain physiologically normal concentrations. Such coordinated regulation of bile acid will also be beneficial for the optimization of expression of bile acid associated target genes. Moreover, advancement in our knowledge on the overall regulation of bile acid synthesis, which leads to a balanced pool of hydrophobic and hydrophilic bile acids, may reveal novel mechanistic signaling pathways through which bile acid production and pool composition may be managed. In the present study, we examine the expression of AKR1D1 and CYP8B1 under physiologically normal bile acid concentrations and assess their regulation under activation of key cholesterol and lipid metabolism regulating pathways.

2. MATERIALS AND METHODS

2.1 Chemicals and Reagents:

Chenodeoxycholic acid (CDCA) and cholic acid (CA) were purchased from

Sigma-Aldrich. PPAR agonist GW7647 and LXR agonist GW3965 were obtained from Tocris Biosciences. Cell culture reagents Dulbecco's modified Eagle's medium (DMEM), and fetal bovine serum (FBS) as well as Taqman master-mix and RT-PCR probes were purchased from Life Technologies. RNA Bee for RNA isolation, dimethyl sulfoxide (DMSO), and propanediol were obtained from Fisher Scientific. Primary and secondary antibodies for western blotting were purchased from SantaCruz Biotechnologies. All western blotting gels, buffers, and markers were purchased through BioRad Laboratories. Complementary DNA synthesis kit was purchased through Promega. Protease inhibitor, Halt, and BSA protein quantification reagent were purchased from ThermoScientific.

2.2 Treatment of HepG2 Cells

HepG2 cells were seeded in 12-well plates at a cell density of 4.0×10^5 in 1.0 mL DMEM supplemented with 10% (v/v) FBS, 1% (v/v) penicillin/streptomycin and 1% (v/v) NEAA and cultured in a 5% CO_2 incubator overnight at 37°C before treatment. After overnight incubation, cells were treated with 25uM CDCA or 25uM CA prepared in DMSO or a DMSO control treatment for 30 hours in 1 ml DMEM supplemented with 1% FBS, 1% antibiotics and 1% NEAA. All treatments were at 0.1% of total volume medium. HepG2 cells were also seeded as described above and treated with LXR synthetic agonist GW3965 (1uM) or PPAR synthetic agonist GW7647 (25uM) prepared in DMSO or a DMSO negative control 30 hours in 1mL DMEM containing 1% FBS, 1% penicillin/streptomycin and 1% NEAA for a total concentration of 0.1%.

2.3 HepG2 RNA Isolation and Quantitative Real-Time Polymerase Chain Reaction (RT-PCR) Analysis

Thirty hours after treatment of HepG2 cells, cells were washed twice with 1X PBS and homogenized by pipetting with 0.25mL RNA-Bee reagent. Homogenates were then transferred to 1.5mL microcentrifuge tube. 100uL chloroform was added to each tube and shaken vigorously for 30 seconds. The samples were then incubated on ice for 5 minutes and then centrifuged at 12,000 G's for 15 minutes. The supernatant was then transferred to a clean microcentrifuge tube and 0.5 mL propanediol was added. The samples were then incubated at room temperature for 5 minutes after which they were spun at 12,000 G's for 10 minutes. The supernatant was discarded and the remaining pellet was washed with 70% ethanol in DEPC treated water and spun at 7,500 G's for 5 minutes. The supernatant was then discarded and the pellet was air dried for 10 minutes before being reconstituted in 30 uL DEPC water. RNA was quantified by nano-drop and normalized to 2ug for reverse transcription into complementary DNA (cDNA) (Promega). Applied Biosystems 7500 System Sequence Detection Software was used for Taqman RT-PCR data analysis. Using relative quantification (RQ), transcript levels of AKR1D1, CYP8B1 and CYP7A1 were normalized against glyceraldehyde-3-phosphate dehydrogenase (GAPDH).

2.4 HepG2 Western Blotting

Cells were washed in 1X PBS and homogenized in radioimmunoprecipitation assay (RIPA) buffer with 1% (v/v) protease inhibitors Halt and 1% (v/v) phenylmethanesulfonylfluoride (PMSF). The total cell lysate was homogenized by

vigorous pipetting and collected into a 1.5 mL micro-centrifuge tube to be spun at 10,000 RPM at 4°C for 10 minutes. The supernatant was collected into a clean micro-centrifuge tube and quantified using a standard BSA assay. After quantification, samples were normalized to 10ug of HepG2 protein lysate. A 1:1 dilution of protein to laemilli sample buffer containing 1% (v/v) beta-mercaptoethanol was incubated at 95°C for 5 minutes, spun, and loaded into a 4-20% gradient SDS-PAGE gel for 1 hour at 120V. Proteins were transferred from the gel using a semi-dry transfer apparatus onto methanol-wetted PVDF membranes at 20V for 30mins. For antibody detection, membranes were blocked for 3 hours in 5% (w/v) skim milk dissolved into a 1X tris-buffered saline solution containing 0.05% Tween20 (1X TBST). Membranes were then incubated overnight at 4°C in a 1:500 dilution of primary antibody in 10mL of 5% skim milk dissolved into a 1X TBST. Membranes were probed for AKR1D1, CYP8B1, CYP7A1 (SantaCruz) or against GAPDH (SigmaAldrich). After overnight incubation membranes were washed 3 times in 1X TBST and incubated with a corresponding 1:2000 HRP-conjugated secondary antibody for 2 hours. Chemiluminescent signals produced by HRP enzymes were detected using enhanced chemiluminescent substrates (Biorad Clarity Western ECL Substrate) and imaged under a Carestream Gel Logic 2200 Pro camera for 8 minutes. Expression of protein was quantified and normalized against GAPDH expression. Where possible the same blot was stripped and re-probed with different antibodies.

2.5 Plasmid Construct

Human AKR1D1 promoter reporter was prepared by cloning a -5.0kb fragment

upstream of the transcription start site of human AKR1D1 into a PGL4.10-Luc vector (Promega). A PCR amplification was preformed using Promega GoTaq Long PCR system. The DNA fragment was PCR amplified using human genomic DNA as a template as well as primers with the following sequences with the Promega GoTaq Long PCR reaction assembly. The thermal cycling parameters were set to a 2 minute initial denaturation at 94°C followed by 35 cycles of a 30 second denaturation at 94°C and 5 minutes of annealing and extension at 65°C. The PCR was concluded with a final extension at 72°C for 10 minutes. The 5kb hAKR1D1 PCR fragment was digested at the NheI and XhoI (New England BioLabs) sites over night at 37 °C and ligated into the PGL 4.10 luciferase reporter vector overnight at 16°C. The plasmid was then transformed using DHα5 High Efficiency competent cells (Invitrogen) onto ampicillin treated agar plates at 37°C for 16-18 hours. Following incubation, colonies were picked and grown in an ampicillin/LB broth while shaking for 16-18 hours. DNA from cultures was purified using a Promega Mini-prep kit. Reporter construct was sequence-verified before use in the experiments. Dr. Matthew Stoner provided expression plasmids for FXRα2. Dr. Bingfang Yan at the University of Rhode Island provided expression plasmids for LXRα, LXRβ, PPARα, PPARβ, and PPARγ.

2.6 Reporter Luciferase Assay

HepG2 cells were seeded in 24-well plates at a cell density of 2.5 x 10^5 in 0.5 mL DMEM supplemented with 10% (v/v) FBS, 1% (v/v) penicillin/streptomycin and 1% (v/v) NEAA and cultured in a 5% CO_2 incubator at 37°C overnight before treatment. After overnight incubation, cells were treated transiently transfected with GenJet Version II reagent. For all transfections, a standard concentration of 100ng/ul

hAKR1D1 promoter reporter was used. Additionally, 10ng/ul null-Renilla luciferase plasmid was used as an internal control. Nuclear receptor expression plasmids (FXRα2, PPARα, PPARβ, PPARγ, LXRα, LXRβ) were also transfected at 100ng/ul. Twenty-four hours after transfection, cell medium was replaced with corresponding agonist treatment. Treatment with 25uM CDCA, 50uM CDCA, 25uM CA, 1uM GW4064, 1uM GW3965, 20uM GW7647 prepared in DMSO or a DMSO control treatment for 30 hours in 1 ml DMEM supplemented with 1% FBS, 1% antibiotics and 1% NEAA. All treatments were at 0.1% of total volume medium. Following 30 hours treatment, cells were washed once with phosphate-buffered saline and lysed with 100ul of 1X passive lysis buffer (Promega) by rocking at room temperature for 15 minutes. Following cell lysis, 10ul of cell lysate was transferred into a white 96-well plate for measurement of luciferase activity. A Dual-Luciferase Reporter assay system was used to which measures the reporter vector firefly luciferase activity and subsequently the renilla luminesce of the null internal control. The luminescence signal of the reporter vector was normalized based on the null reading.

2.6 CDCA and CA Treatment of CD-1 Mice

Eighteen male and female CD-1 mice were bred in-house and randomly separated into 3 groups of 6 mice at 6-8 weeks of age. The mice had free access to food and water and were on a 12-hour dark/light cycle. The mice were treated with CDCA or CA (SigmaAldrich) dissolved in propanediol, as well as a propanediol negative control. Mice were injected in the intraperitoneal cavity (IP) with treatment chemicals at 5-mg/kg body weight every 12 hours for 3 days for a total of 6 injections. Twelve hours after the last injection livers were harvested and processed for mRNA

and protein determination. The Institutional Animal Care and Use Committee (IACUC) at the University of Rhode Island approved all animal studies.

2.7 LXR and PPAR Synthetic Agonists Treatment of C57BL/6 Mice

Eighteen C57BL/6 male and female mice were bred in house and randomly separated into 3 groups of 6 mice at 6-8 weeks of age. The mice were treated with GW3965 or GW7647 (Tocris) dissolved in propanediol as well as a propanediol negative control. The mice had free access to food and water and were on a 12-hour dark/light cycle. Mice were injected in the intraperitoneal cavity (IP) with treatment chemicals at 10-mg/kg body weight every 12 hours for 3 days for a total of 6 injections. Twelve hours after the last injection livers were harvested and processed for mRNA and protein determination.

2.8 Mouse Liver RNA Isolation and Quantitative Real-Time Polymerase Chain Reaction (RT-PCR) Analysis

100mg of mouse liver was homogenized in 1 mL RNA-Bee for RNA extraction. 300 uL chloroform was added to each tube, which was then shaken vigorously for 30 seconds. The samples were then incubated on ice for 5 minutes before being centrifuged at 12,000 G's for 15 minutes. The supernatant was then transferred to a clean microcentrifuge tube and 0.5 mL propanediol was added. Samples were incubated at room temperature for 5 minutes and then spun at 12,000 G's for 10 minutes. The supernatant was discarded and the remaining pellet was washed with 70% ethanol in DEPC treated water and spun at 7,500 G's for 5 minutes.

The supernatant was then discarded and the pellet was air dried for 10 minutes before being reconstituted in 30 uL DEPC water. RNA was quantified by nano-drop and normalized to 2ug for reverse transcription into complementary DNA (cDNA) (Promega). Applied Biosystems 7500 System Sequence Detection Software was used for Taqman RT-PCR data analysis. Applied Biosystems 7500 System Sequence Detection Software was used for Taqman RT-PCR data analysis. Using relative quantification (RQ), transcript levels of AKR1D1, CYP8B1 and CYP7A1 were normalized against glyceraldehyde-3-phosphate dehydrogenase (GAPDH).

2.9 Mouse Liver Tissue Western Blotting

100mg of liver tissue was homogenized in 1 mL of Sucrose-Tris buffer containing 1% Halt. The total homogenate was spun in a micro-ultra centrifuge at 100,000g's at 4C to separate the cytosolic fraction from the membrane fraction. Cytosolic fraction protein was collected and transferred to a clean 1.5 mL microcentrifuge tube. The protein was quantified using a standard BSA assay. After quantification, protein samples were normalized to 100ug of cytosolic protein. A 1:1 dilution of protein to laemilli sample buffer containing 1% (v/v) betamercaptoethanol was incubated at 95°C for 5 minutes, spun, and loaded into a 4-20% gradient SDS-PAGE gel for 1 hour at 120V. Proteins were transferred from the gel using a semi-dry transfer apparatus onto methanol-wetted PVDF membranes at 20V for 30mins. For antibody detection, membranes were blocked for 3 hours in 5% (w/v) skim milk dissolved into a 1X tris-buffered saline solution containing 0.05% Tween20 (1X TBST). Membranes were then incubated overnight in a 1:500 dilution of primary

antibody in 10mL of 5% skim milk dissolved into a 1X TBST. Membranes were probed for AKR1D1, CYP8B1, CYP7A1 (SantaCruz) or against GAPDH (SigmaAldrich). After overnight incubation membranes were washed 3 times in 1X TBST and incubated with a corresponding 1:2000 HRP-conjugated secondary antibody for 2 hours. Chemiluminescent signals produced by HRP enzymes were detected using enhanced chemiluminescent substrates (Biorad Clarity Western ECL Substrate) and imaged under a Carestream Gel Logic 2200 Pro camera for 8 minute exposure. Expression of protein was quantified normalized against GAPDH expression. Where possible the same blot was stripped and re-probed with different antibodies.

2.10 Statistical Analysis

A Student's *t*-test was applied to pairwise comparison for normally distributed data. A $p = .05$ or lower was considered statistically significant.

3. RESULTS

3.1 Altered AKR1D1 expression with CDCA or CA treatment HepG2 Cells.

It remains to be determined how individual bile acid synthesis can be regulated. While it is well established that AKR1D1 is responsible for the production of CDCA, its regulation is not fully understood. To examine the effects of individual bile acids at physiological concentrations on endogenous expression of vital bile acid synthesis enzymes in HepG2 cells, cells were treated with either 25uM CDCA, 25uM

CA or a DMSO negative control for 30 hours followed by mRNA and protein expression evaluation. As shown in Figure 1A, RT-PCR analysis indicated that human AKR1D1 mRNA expression was significantly decreased by nearly 65% in HepG2 cells treated with CDCA ($p < .01$) as compared to the negative control cells treated with DMSO. In HepG2 cells treated with CDCA, CYP8B1 mRNA expression was not significantly altered as compared to DMSO treated control cells. It should be noted that CYP7A1 expression was also measured as a positive control. Consistent with published reports, CYP7A1 expression significantly decreased when treated with CDCA ($p < .05$) (Fig. 1A).

Protein expression of AKR1D1 in the presence of CDCA treatment in HepG2 cells was also evaluated. Consistent with mRNA data, AKR1D1 protein expression was also significantly decreased ($p < .01$) in HepG2 cells with CDCA treatment by nearly 15% when compared to controls (Fig. 1B). Expression of CYP8B1 was not significantly altered with CDCA treatment, however, as expected, CYP7A1 expression significantly decreased ($p < .05$). Taken together, these results evidence that CDCA regulate AKR1D1, and not CYP8B1, expression in a negative feedback manner at physiological concentrations.

With conclusive results that indicate that CDCA regulates the expression of AKR1D1, we next investigated the effects of CA on the expression of AKR1D1, CYP8B1 and CYP7A1. HepG2 cells were treated with 25uM CA or a DMSO control for mRNA and protein quantification. Surprisingly, HepG2 cells exhibited a nearly two-fold increase in AKR1D1 expression when treated with CA ($p < .05$) (Fig. 2A). Interestingly, no significant change was observed in CYP8B1 or CYP7A1 expression

(Fig. 2A). To confirm the resulted from the collected mRNA data, protein expression was analyzed in response to CA treatment. Consistent with the mRNA results, AKR1D1 expression in HepG2 cells was increased by over two-fold in the presence of CA as compared to DMSO treated control cells ($p < .05$) (Fig 2B). No changes were observed in CYP8B1 or CYP7A1 expression at the protein level in response to CA treatment (Fig 2B). The data clearly states that CA regulates AKR1D1 expression in a feed-forward manner.

3.2 Altered Akr1d1 expression with CDCA or CA treatment in mice.

The previous results indicated that individual bile acids alter AKR1D1 expression *in-vitro* in HepG2 cells. Therefore, CD-1 mice were next tested to confirm the effect of individual bile acids on the bile acid synthesis pathway *in-vivo*. The CD-1 mice were injected with physiological concentrations of CDCA or CA IP (10mg/kg) and analyzed for endogenous Akr1d1, Cyp8b1 or Cyp7A1 expression. Liver tissue was harvested and mRNA and protein were isolated for RT-PCR and western blot analysis. Consistent with the *in-vitro* results, mRNA expression levels of Akr1d1 exhibited a significant decrease ($p < .05$) with CDCA treatment by nearly 72% (Fig. 3A). As anticipated, Cyp8b1 expression was unchanged in CD-1 mice in the presence of CDCA. Likewise, similar to our in-vitro data, Cyp7a1 RNA expression significantly decreased in the presence of CDCA treatment ($p < 0.05$).

As shown in Figure 3B, protein analysis also determined that Akr1d1 expression significantly decreased by over 4-fold in CDCA treated mice ($p < .05$). No change was observed in CYP8B1 expression and while there was a decreasing trend in

CYP7A1 expression, the results are not significant. Collectively, the mRNA and protein data confirm that the findings *in-vivo* were consistent with the findings *in-vitro* that CDCA regulates the expression of Akr1d1 in either a negative feedback manner.

Mice were also treated with CA to examine the changes in RNA and protein expression of Akr1d1, Cyp8b1 and Cyp7a1. Mice exhibited an increased (although not significant) trend of Akr1d1 expression with CA treatment at the mRNA level (Fig. 4A). No significant changes were observed in mRNA expression of Cyp8b1 or Cyp7a1 with CA treatment. As seen in Figure 4B, Akr1d1 protein expression was significantly increased by nearly 30% in mice with CA treatment ($p < .01$). No change was observed in Cyp8b1 or Cyp7a1 expression with CA treatment.

Collectively, the results demonstrated that individual primary bile acids regulate Akr1d1 expression. Both CDCA and CA coordinately regulated Akr1d1, but not Cyp8b1, in either a negative feedback or positive feed-forward manner, respectively, in mice, under physiological conditions.

3.3 CDCA mediated repression and CA mediated induction of AKR1D1 promoter.

The compiled results indicate that AKR1D1 expression, and not CYP8B1, is regulated by CDCA and CA *in-vitro* and *in-vivo*. Therefore, additional data was collected to examine the promoter activity of AKR1D1 in the presence of primary bile acids. To determine if AKR1D1 promoter activity was transcriptionally regulated by CDCA and CA, a human AKR1D1 promoter reporter, phAKR1D1 (-5.0 kb), was prepared and transiently transfected into HepG2 cells. Cells were treated with primary bile acid and relative luciferase activity was measured. Results confirmed that

AKR1D1 promoter activity was decreased in a dose-dependent manner by CDCA ($p < 0.05$) (Fig. 5A). Furthermore, the human AKR1D1 promoter reporter exhibited significant increases in luciferase activity with treatment of CA ($p < .05$) (Fig. 5B). Taken together the data confirm that AKR1D1 expression is transcriptionally regulated by CDCA and CA in either a negative feedback, or positive feed-forward mechanism, respectively, *in-vitro.*

3.4 FXR signaling is not involved in AKR1D1 expression.

With confirmed results *in-vitro* and *in-vivo* demonstrating that at physiological concentrations CDCA and CA regulate AKR1D1, and not CYP8B1, expression, the effects of FXR activation on AKR1D1 expression were subsequently tested. Since AKR1D1 is responsible for the synthesis of CDCA, and CDCA is a potent endogenous ligand for FXR, we investigated the relationship between FXR activation and AKR1D1 regulation. The FXRα2 nuclear receptor was transfected into HepG2 cells by transient transfection for 48 hours followed by harvesting of cells for RNA analysis to examine changes in endogenous AKR1D1 expression when FXRα2 is over-expressed in-vitro. RT-PCR results presented no significant change in endogenous expression of AKR1D1 in control cells versus cells where FXRα2 was over-expressed (Fig. 6). These results indicate that over-expression of FXR has no implications on AKR1D1 expression.

To further examine the effects of FXR activation on AKR1D1 expression, an in-vivo animal study was designed where mice were injected with the synthetic FXR agonist, GW4064 every 12 hours for 3 days at 10mg/kg. The livers were subsequently harvested for RNA isolation. RT-PCR results demonstrated that activation of the FXR

signaling pathway had no significant effect on endogenous AKR1D1 expression as compared to control mice (Fig. 7). Consistent with our in-vitro results, the results confirm that AKR1D1 is not regulated by FXR.

To ensure FXR expression was not indirectly associated with AKR1D1 expression, an FXR knockout mouse model was used to examine endogenous AKR1D1 expression. The livers of FXR knockout and wild-type control mice were analyzed for endogenous AKR1D1 RNA expression. No changes were observed between wild-type and FXR knockout mice (Fig.8). The data therefore demonstrated that FXR activation does not affect the expression of AKR1D1 and therefore FXR expression or activation does not regulate AKR1D1 expression

3.5 Increase in PPAR activity decreases expression of AKR1D1 *in-vitro* and *in-vivo*.

With conclusive data that FXR signaling is not involved in the regulation of AKR1D1, the effects of PPAR activation on AKR1D1 expression were subsequently tested in order to identify a novel activation or repression pathway. HepG2 treated with 25uM of the synthetic PPAR agonist, GW7647, were analyzed. RNA results demonstrated that, as shown in Figure 9A, AKR1D1 expression *in-vitro* was significantly repressed nearly 10-fold when treated with the PPAR agonist ($p < 0.01$). Similar to the RNA data, western-blotting results (Fig. 9B) demonstrated that protein expression of AKR1D1 also significantly decreased nearly 3-times with PPAR agonist treatment in HepG2 cells ($p < 0.05$). Taken together, the data suggests that activation of the PPAR signaling pathway decreases AKR1D1 expression *in-vitro* in HepG2 cells.

To confirm the results that were obtained *in-vitro*, which suggested that PPAR activation decreased AKR1D1 expression, the effect of AKR1D1 expression *in-vivo* in C57BL/6 mice was analyzed in response to treatment with 10mg/kg PPAR agonist GW7647. As shown in Figure 10A, mouse Akr1d1 RNA expression was significantly decreased ($p < 0.05$) by more than 2 fold with treatment of GW7647. Western blot results (Figure 10B) confirmed that protein expression of Akr1d1 was also significantly decreased nearly 2 fold with mice treated with GW7647 ($p < 0.05$). In combination with the *in-vitro* data collected, the *in-vivo* the data confirms that activation of the PPAR signaling pathway consistently represses AKR1D1 expression.

3.6 GW7647 mediated trans-repression of AKR1D1 promoter.

To further understand the mechanistic regulation of AKR1D1 by PPAR activation, transcriptional regulation of AKR1D1 was examined *in-vitro*. The human AKR1D1 promoter reporter, phAKR1D1 (-5.0 kb) was co-transfected into HepG2 cells with PPARα, PPARβ or PPARγ to identify isoform specific activation. Cells were treated with 25uM GW7647 for 30 hours followed by measurement of relative luciferase signals. The results indicate a decrease of AKR1D1 expression with all three PPAR isoforms. While a statistically significant decrease was only observed through the PPARγ isoform nuclear receptor ($p < .001$), all three isoforms displayed a decreased trend in AKR1D1 expression (Fig. 11). Therefore, it can be stated that PPAR isoforms are binding to a previously un-identified PPAR response element within the 5kb human AKR1D1 promoter region and, upon activation, repress AKR1D1 expression.

3.7 Possible involvement of PPARγ in AKR1D1 regulation.

With the possible identification of a previously un-identified PPAR response element within the 5kb promoter region of human AKR1D1, repression of AKR1D1 expression through PPAR in response to CDCA treatment was measured. HepG2 cells were transiently transfected with the phAKR1D1 (-5.0kb) promoter reporter and PPARα, PPARβ or PPARγ. Relative luciferase activity was measured after 30 hours treatment with 25uM CDCA. As shown in Figure 12, the results indicate an isoform specific transrepression of AKR1D1 by CDCA treatment, possibly through PPARγ ($p < .05$). No change was observed in AKR1D1 expression with PPARα or PPARβ co-transfection. Therefore, the data suggests that PPARγ may be involved in the transcriptional repression of AKR1D1 through CDCA treatment.

3.8 Activation of LXR signaling increases RNA and protein expression of AKR1D1 *in-vitro* and *in-vivo*.

With confirmed results that individual bile acids dictate the expression of AKR1D1, and not CYP8B1, the affects of LXR activation on AKR1D1 expression were next investigated in HepG2 cells. HepG2 cells were treated with the synthetic LXR agonist GW3965 to investigate changes in AKR1D1 expression under LXR activation. RNA was isolated and analyzed for AKR1D1 expression by RT-PCR. The results shown in Figure 13A indicated that at the mRNA level, AKR1D1 expression was significantly ($p < 0.05$) increased by over 30% by activation of the LXR signaling pathway. Protein isolated from HepG2 cells treated with either a 0.1% DMSO control or 1uM GW3965 was analyzed by western blotting. Protein results confirmed the increase, as seen at the RNA level, concluding that expression of AKR1D1 significantly increased by over 30% with treatment of the LXR agonist ($p < 0.05$) in-

vitro in HepG2 cells (Fig. 13B). The data confirms that AKR1D1 expression is increased by LXR activation *in-vitro*.

To confirm our *in-vitro* findings in an *in-vivo* model, mice were injected IP with the LXR synthetic agonist for 3 days, with injections every 12 hours. Livers were harvested and analyzed for RNA and protein expression of AKR1D1 by RT-PCR and western blot, respectively. In mice treated with the LXR synthetic agonist, no significant change was observed at the RNA level of Akr1d1 expression in comparison to vehicle treated mice (Fig. 14A). The results, however, were consistent with *in-vitro* protein expression and demonstrated that mouse Akr1d1 protein levels were significantly increased ($p < 0.05$) with LXR activation by nearly 50% (Fig. 14B). Taken together the data demonstrates that while activation of the LXR signaling pathway does not have implications on AKR1D1 expression at the transcript level, there are significant changes in protein expression. This increase of AKR1D1 protein expression, which is not seen at the RNA level, may be due to an un-identified post-translation modification.

3.9 GW3965 mediated transactivation of AKR1D1 promoter.

To further understand the mechanistic regulation of AKR1D1 by LXR activation, transcriptional regulation of AKR1D1 was examined in-vitro. The human AKR1D1 promoter reporter, phAKR1D1 (-5.0 kb) was co-transfected into HepG2 cells with LXRα or LXRβ to identify isoform specific activation. Cells were treated with GW7647 for 30 hours and relative luciferase signals were measured. AKR1D1 promoter reporter activity was significantly increased with co-transfection of LXRβ ($p < .01$), but not LXRα (Fig. 15). The results indicate that an isoform-specific

transactivation of AKR1D1 expression exists, demonstrating that LXRβ binds to an LXR response element on the 5kb human AKR1D1 promoter region and through activation by an LXR agonist induces expression of AKR1D1.

3.10 CDCA does not regulate AKR1D1 expression through LXR nuclear receptors.

With identification of a previously un-identified LXR response element in the 5kb promoter region of the human AKR1D1 gene, the effects of LXR nuclear receptor activation through CDCA treatment was analyzed. HepG2 cells were transiently transfected with phAKR1D1 (-5.0kb) and LXRα or LXRβ and treated for 30 hours with 25uM CDCA. The results showed that CDCA does not regulated the expression of AKR1D1 through binding LXRα or LXRβ and no significant change or trend was observed (Fig. 16).

4. DISCUSSION

Due to their potential to cause toxicity to hepatocytes, individual bile acid concentrations within the bile acid pool must be tightly regulated to maintain physiological homeostasis and normal healthy liver function. With respect to bile acid synthesis regulation, to date, much focus has been on CYP8B1 expression. It has been argued that expression of CYP8B1 is the determining factor of individual primary bile acid synthesis and is therefore the influential enzyme in determining the ratio of CDCA to CA in the bile acid pool. Our data, however, demonstrates that it is in fact AKR1D1 expression, and not CYP8B1, that regulates the ratio of CDCA to CA in the bile acid pool. The data confirms that the primary bile acids, CDCA and CA, regulate the expression of AKR1D1 at both the transcriptional and translational levels. This regulation of AKR1D1 is likely control bile acid synthesis in order to achieve

homeostasis within the circulating bile acid pool. Altering the expression of AKR1D1 may be one mechanism by which bile acid homeostasis is maintained in enterohepatic circulation. *In-vitro* and *in-vivo* results reveal that CDCA and CA dictate the synthesis of their own production by regulating the expression of AKR1D1 in a negative feedback manner or positive feed forward manner, respectively, to ensure bile acid homeostasis and prevent toxic accumulation.

Within the bile acid pool, when the content of the circulating bile acid ratio favors CDCA at high concentrations, CDCA signaling, in turn, results in a decreased expression of AKR1D1, not only to adjust the ratio of bile acid within the pool, but also to limit any potential pathological consequences associated with high CDCA concentrations. On the other hand, as the content of the bile acid pool favors higher CA concentrations, CA signaling results in an increase of AKR1D1 expression, without effecting CYP8B1 expression, to accelerate production of CDCA. CA is able to up-regulate expression of AKR1D1 without affecting CYP8B1 expression due to the fact that the 5β-reductase enzyme also exists prior to the formation of CA. This feed-forward mechanism of AKR1D1 regulation via CA signaling will result in a more evenly composed bile acid pool composition by balancing the CDCA to CA ratio. Since the data clearly establishes that the liver is consistently attempting to achieve homeostatic bile acid levels by adjusting the expression level of AKR1D1 under physiological conditions, we next investigated potential pathways by which AKR1D1 expression may be used to regulate bile acid synthesis.

AKR1D1 is responsible for the synthesis of CDCA, and CDCA is the endogenous ligand for FXR, therefore we investigated the relationship between FXR

activation and AKR1D1 regulation. In-vitro data demonstrated that endogenous AKR1D1 expression was not altered in HepG2 cells as a result of FXR over-expression. It was also observed that when mice were treated with the synthetic FXR agonist, AKR1D1 expression exhibited no change as compared to control mice. Likewise, in FXR knockout mice the endogenous expression of AKR1D1 displayed no change as compared to wild-type mice. Therefore it was concluded that activation of the FXR signaling pathway was not involved in the regulation of AKR1D1 expression. Consequently, greater focus was given to other signaling pathways that had implications on bile acid synthesis.

Activation of PPAR is commonly associated with lipid metabolism and fatty acid oxidation (Hunt et al., 2000). Fibrates, a common class of PPAR agonists, are synthetic ligands which, through the activation of the PPAR signaling pathway, treat hyperlipidemia to lower serum triglyceride levels and raise high-density lipoproteins (Grygiel-Górniak, 2014). Fibrates are also known to decrease insulin resistance associated with metabolic disorders (Hunt et al., 2000). Due to the fact that bile acids also serve to solubilize lipids to prevent hyperlipidemia, the relationship between bile acid synthesis and PPAR activation was subsequently investigated. In humans, it has been published that PPAR activation results in a decreased CDCA output but no change to CA production (Pineda Torra, 2002). Published reports also indicate that expression of PPAR is increased in response to physiological concentrations of CDCA in HepG2 cells (Pineda Torra, 2002).

Until now, it has only been confirmed that PPAR activation does in fact affect bile acid synthesis and bile composition, however the involvement of AKR1D1 has

not been established. With the data that has been collected, our research has consistently demonstrated that activation of PPAR results in a down-regulation of AKR1D1 expression at the translational and transcriptional level, in-vitro and in-vivo. Upon examination of isoform-specific activation of PPAR on the AKR1D1 promoter, all PPAR isoforms displayed a role in decreased expression of AKR1D1 with PPARγ more potently repressing expression. With activation of PPAR, AKR1D1 expression is decreased thereby explaining why PPAR activation results in a bile acid pool that is depleted of CDCA. Taken together, the observed results indicate that, for the first time, a PPAR response element may exist on the AKR1D1 promoter region.

In addition, it was proven that CDCA regulated expression of AKR1D1 through PPARγ binding. Activation of PPARγ has implications on glucose metabolism. There are well established links between glucose homeostasis and bile acid homeostasis (Grygiel-Górniak, 2014). It can be theorized that when bile acid levels are increased, bile acid and glucose homeostasis may be dysregualted. In such instances, we hypothesize that CDCA may bind PPARγ and decrease AKR1D1 expression. This will in-turn normalize and maintain both glucose and bile acid homeostasis. While our data does not indicate a substantial decrease in AKR1D1 expression through a CDCA and PPARγ complex as compared to CDCA alone, knowledge of the involvement of PPARγ may shed light on a means to control bile acid and glucose homeostasis by adjusting bile acid production under pathological conditions by activation of PPAR signaling through PPARγ.

Activation of the LXR signaling pathway is primarily associated with high cholesterol levels, ultimately inducing transcription of genes within the bile acid

synthesis pathway that aid to alleviate the cholesterol overload. Since bile acids are ultimately the primary means for cholesterol solubilization and removal, we next investigated a relationship between LXR activation and AKR1D1 expression. Our results indicate that activation of LXR signaling consistently increases AKR1D1 expression *in-vitro* at the transcriptional and translational levels. It has been identified that CDCA and CA differ in their roles with regards to cholesterol absorption, with CDCA inhibiting absorption and CA promoting absorption. The up-regulation of AKR1D1 expression upon LXR activation may serve as a means to increase CDCA content in the bile thereby decreasing cholesterol absorption and resulting in an increase in cholesterol metabolism. The synthetic LXR agonist, GW3965 is a full agonist of both LXRα and LXRβ with more potent activation of LXRβ. At the concentrations that the cells and mice were treated, however, activation of both isoforms was achieved. While LXRα expression is highest in the liver, LXRβ is widely distributed within the body (Y. Zhang & Mangelsdorf, n.d.). Our results indicate that despite activation of both LXRα and LXRβ, only the interaction between LXRβ and the LXRE results in a transactivation of AKR1D1 expression in HepG2 cells. This data confirms the possible presence of a previously un-identified active LXR element (LXRE) on the promoter region of AKR1D1. Follow-up testing on activation of LXR nuclear receptor via CDCA treatment determined that CDCA does not regulate AKR1D1 through LXR.

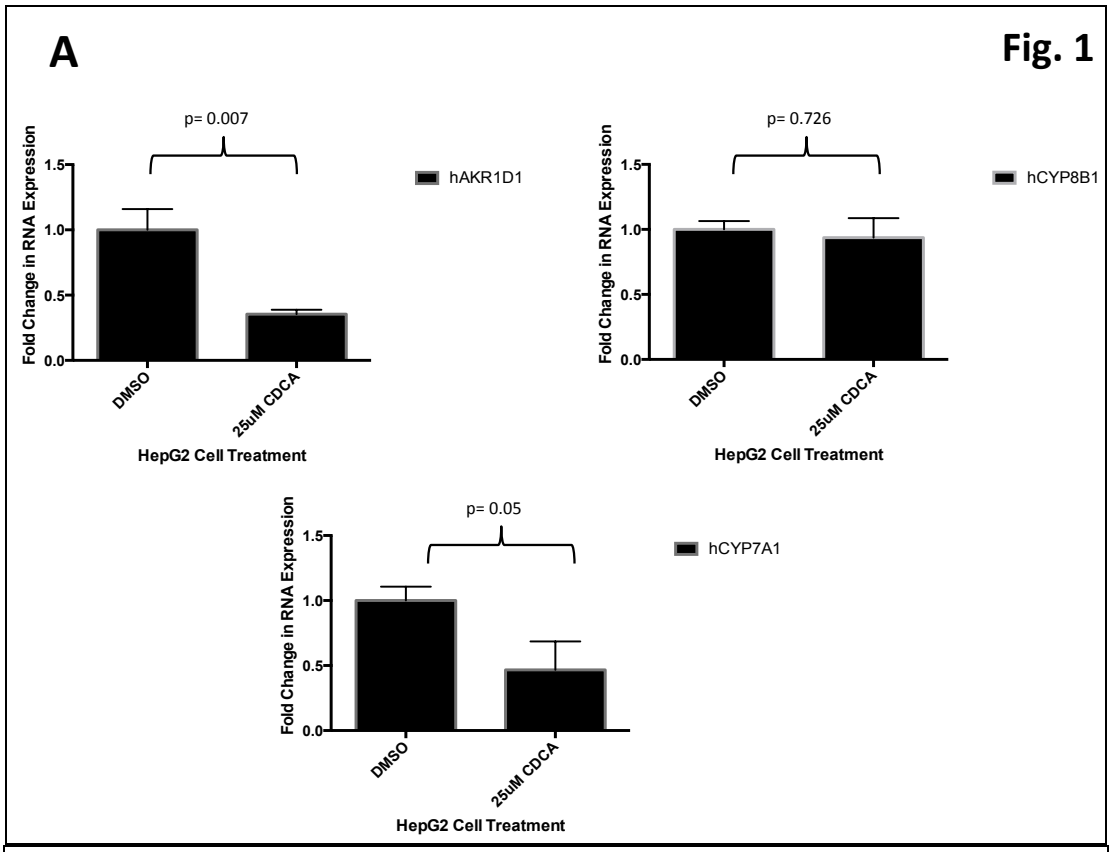
Activation of LXR also significantly increased AKR1D1 expression *in-vivo* at the translational level. The increase in AKR1D1 protein expression, which serves to increase CDCA production, may prove to be a mechanism by which LXR agonists aid

in the emulsification of cholesterol at high concentrations. The data suggestions that since expression is unchanged at the mRNA level *in-vivo* that there must be a post-translational modification that is resulting in an increase of AKR1D1 protein without change to AKR1D1 mRNA. Currently the root of this post-translational modification has not yet been identified.

Understanding the regulation of bile acid synthesis can theoretically identify mechanisms that work to control the hydrophobicity of the circulating pool to ensure proper physiological liver function. Understanding the regulation of the enzymes involved in the bile acid synthesis pathway under physiological conditions may shed light on mechanisms by which homeostasis can also be achieved in conditions of disease. Likewise, understanding the changes that occur to the individual bile acid composition within the CDCA to CA ratio during physiologically normal states and times of disease may provide insight into the regulation of the enzymes involved in bile acid synthesis. Our results demonstrate that not only bile acids, but LXR and PPAR signaling pathways as well, are involved in the regulation of AKR1D1 expression all in an effort to control CDCA production and maintain proper liver function and homeostasis.

Our results demonstrated that CDCA and CA are key regulators of AKR1D1, but not CYP8B1, expression. It is confirmed that CDCA and CA coordinately regulate AKR1D1 expression in either a negative feedback or positive feed-forward manner, respectively. Such coordinated down-regulation and up-regulation of AKR1D1 by primary bile acids represents a mechanism by which the liver maintains homeostatic bile acid levels under physiological condition. For the first time, our data suggests the

presence of LXR and PPAR response elements on the human AKR1D1 promoter, 5kb upstream from the start of transcription. With confirmation that LXR and PPAR signaling pathways are involved in regulating AKR1D1 expression a possible means to regulate bile acid production may be established. It has been proven that there is a potential cross-talk between LXR and PPAR (Yoshikawa et al., 2003). Their involvement in regulating AKR1D1 may further explain their potential cross-talk. In conclusion, CDCA and CA coordinately regulate AKR1D1, but not CYP8B1, expression, and activation of LXR and PPAR may be used to modulate bile acid production via AKR1D1 not only to maintain bile acid homeostasis, but also to ultimately aid in the treatment or alleviation of bile acid associated disorders.


A
Fig. 1
p= 0.007
Fold Change in RNA Expression
1.5
1.0
0.5
0.0
DMSO
25uM CDCA
HepG2 Cell Treatment
hAKR1D1
p= 0.726
hCYP8B1
p= 0.05
hCYP7A1

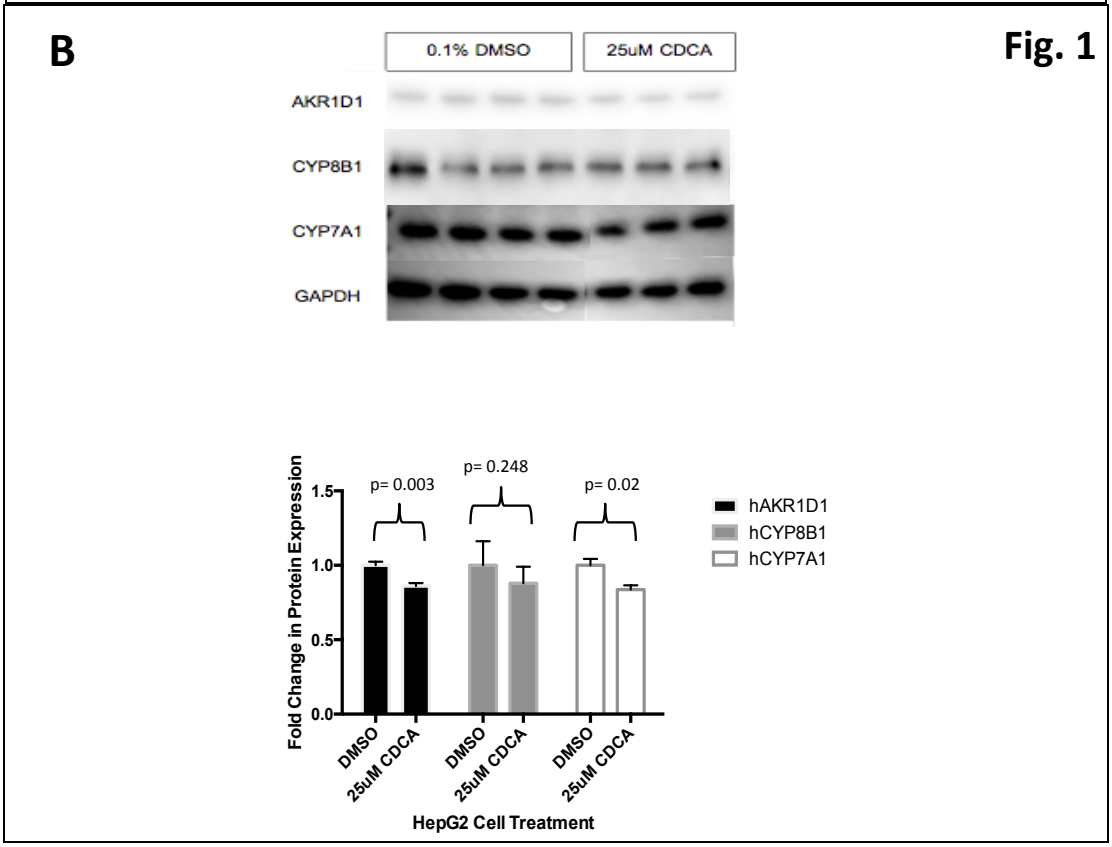

B
Fig. 1
0.1% DMSO
25uM CDCA
AKR1D1
CYP8B1
CYP7A1
GAPDH
p= 0.003
p= 0.248
p= 0.02
Fold Change in Protein Expression
hAKR1D1
hCYP8B1
hCYP7A1
HepG2 Cell Treatment

Figure 1. The effects of CDCA treatment on RNA and protein expression of AKR1D1, CYP8B1 and CYP7A1 in HepG2 Cells.

HepG2 cells were treated with 25uM CDCA in DMSO or a 0.1% DMSO vehicle control. (A) At 30 hours incubation total RNA was isolated RT-PCR quantification. The abundance of mRNA encoding AKR1D1, CYP8B1 and CYP7A1 in HepG2 cells was measured and normalized against GAPDH. The data are presented as fold changes in relative expression of the mean of six replicates and a $P< 0.05$ was considered significant (Student-t test). (B) At 30 hours post-treatment with CDCA, 20ug of cell lysate was analyzed by western blot on a 4-20% gradient gel. Expression of AKR1D1, CYP8B1 and CYP7A1 was quantified and normalized against GAPDH. The data are presented as fold changes in relative expression of the mean of at least 3 replicates and a $P< 0.05$ was considered significant (Student's *t*-test).

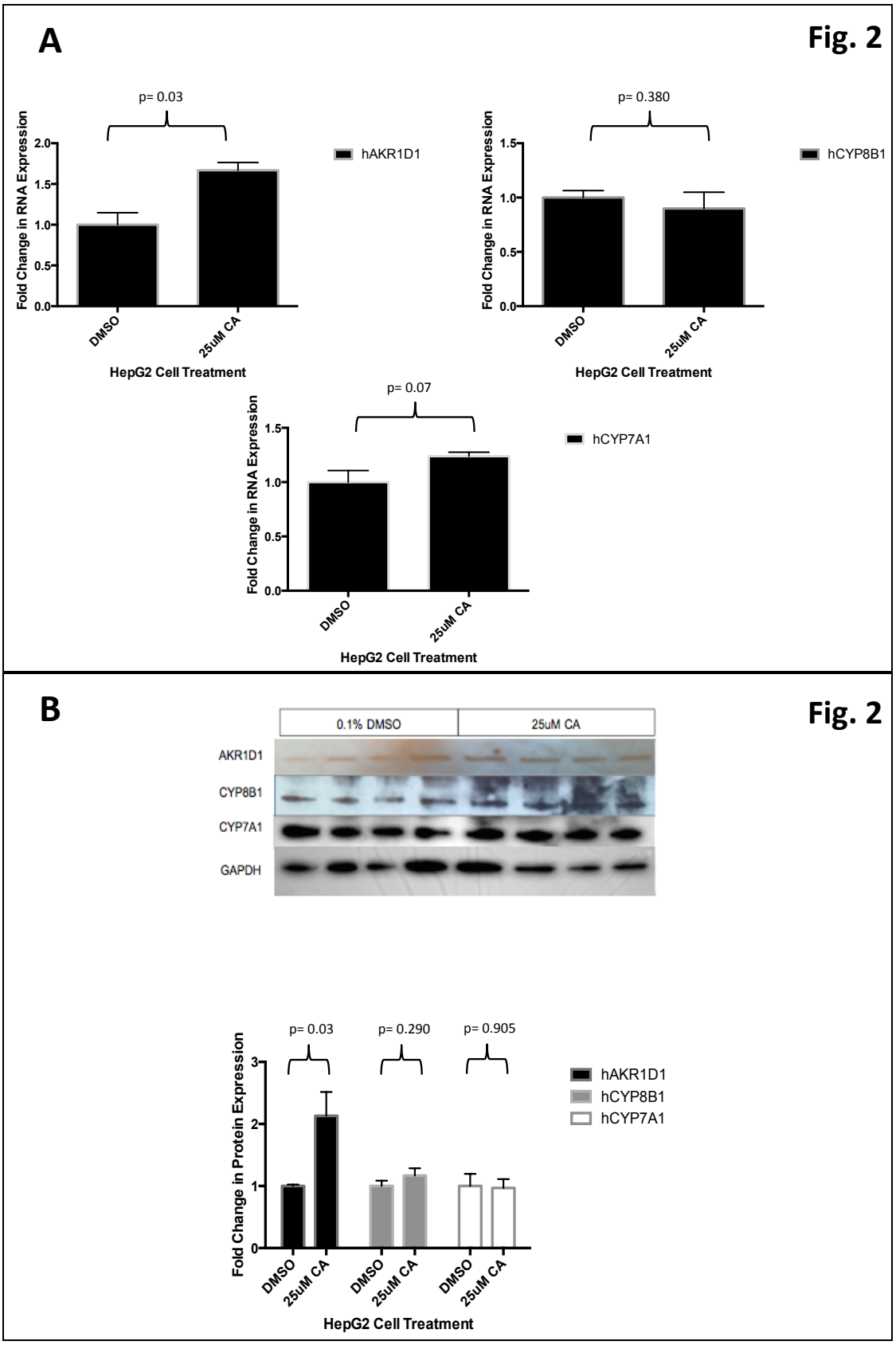
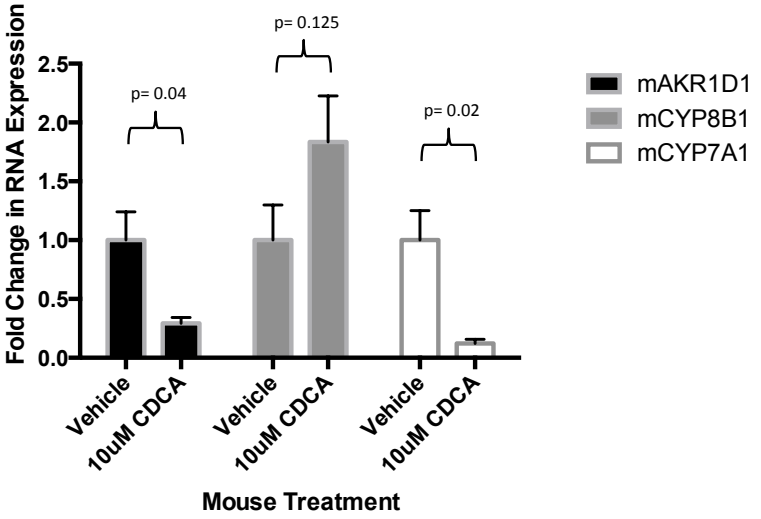

A
Fig. 2
p= 0.03
Fold Change in RNA Expression
2.0
1.5
1.0
0.5
0.0
DMSO
25uM CA
HepG2 Cell Treatment
hAKR1D1
p= 0.380
Fold Change in RNA Expression
1.5
1.0
0.5
0.0
DMSO
25uM CA
HepG2 Cell Treatment
hCYP8B1
p= 0.07
Fold Change in RNA Expression
1.5
1.0
0.5
0.0
DMSO
25uM CA
HepG2 Cell Treatment
hCYP7A1
B
Fig. 2
0.1% DMSO
25uM CA
AKR1D1
CYP8B1
CYP7A1
GAPDH
p= 0.03
p= 0.290
p= 0.905
Fold Change in Protein Expression
3
2
1
0
DMSO
25uM CA
DMSO
25uM CA
DMSO
25uM CA
HepG2 Cell Treatment
hAKR1D1
hCYP8B1
hCYP7A1

Figure 2. The effects of CA treatment on RNA and protein expression of AKR1D1, CYP8B1 and CYP7A1 in HepG2 Cells.

HepG2 cells were treated with 25uM CA in DMSO or a 0.1% DMSO vehicle control. (A) At 30 hours incubation total RNA was isolated RT-PCR quantification. The abundance of mRNA encoding AKR1D1, CYP8B1 and CYP7A1 in HepG2 cells was measured and normalized against GAPDH. The data are presented as fold changes in relative expression of the mean of six replicates and a $P < 0.05$ was considered significant (Student *t*-test). (B) At 30 hours post-treatment with CA, 20ug of cell lysate was analyzed by western blot on a 4-20% gradient gel. Expression of AKR1D1, CYP8B1 and CYP7A1 was quantified and normalized against GAPDH. The data are presented as fold changes in relative expression of the mean of at least 4 replicates and a $P < 0.05$ was considered significant (Student's *t*-test).

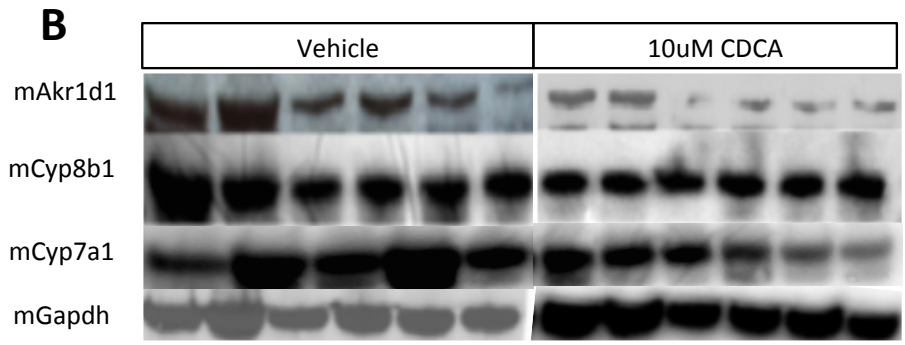
A

Fig. 3


Fold Change in RNA Expression
2.5
2.0
1.5
1.0
0.5
0.0
p= 0.04
p= 0.125
p= 0.02
Vehicle
10uM CDCA
Vehicle
10uM CDCA
Vehicle
10uM CDCA
Mouse Treatment
mAKR1D1
mCYP8B1
mCYP7A1

B

Fig. 3


Vehicle
10uM CDCA
mAkr1d1
mCyp8b1
mCyp7a1
mGapdh

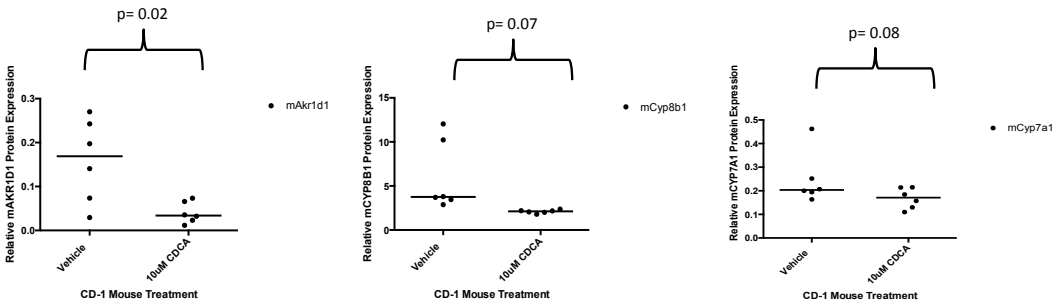
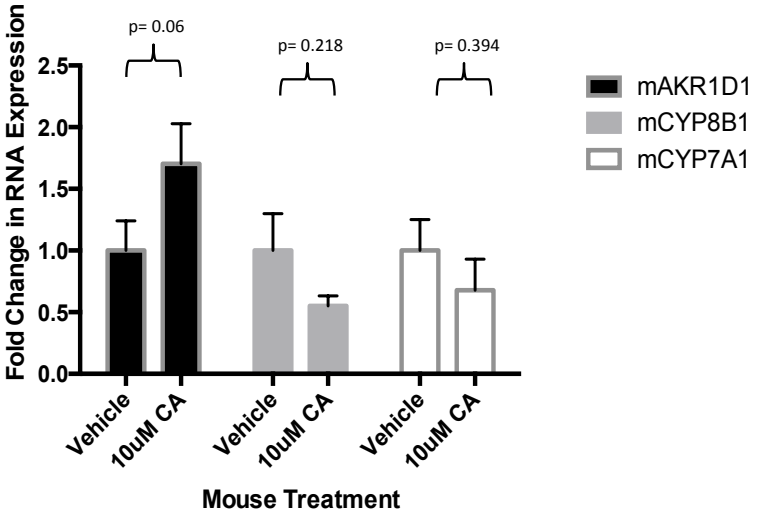

p= 0.02
Relative mAKR1D1 Protein Expression
mAkr1d1
Vehicle
10uM CDCA
CD-1 Mouse Treatment
p= 0.07
Relative mCYP8B1 Protein Expression
mCyp8b1
Vehicle
10uM CDCA
CD-1 Mouse Treatment
p= 0.08
Relative mCYP7A1 Protein Expression
mCyp7a1
Vehicle
10uM CDCA
CD-1 Mouse Treatment

Figure 3. Effect of CDCA treatment on endogenous AKR1D1, CYP8B1 and CYP7A1 expression *in-vivo*.

CD-1 mice were injected (6 per group) with propanediol vehicle or 10uM CDCA at 5mg/kg IP. Livers were harvested for RNA and protein analysis. (A) RNA was isolated from liver tissue. Endogenous AKR1D1, CYP8B1 and CYP7A1 expressions were analyzed and normalized to GAPDH. The data are presented as a fold change in relative expression of the mean of 6 mice as compared to the negative control and a $p < 0.05$ was considered significant (student *t*-test). (B) Protein was isolated from liver tissue. Endogenous AKR1D1, CYP8B1 and CYP7A1 expression were analyzed and normalized to GAPDH. The data are presented as a fold change in relative expression of the mean of 6 mice as compared to the negative control and a $p < 0.05$ was considered significant (Student's *t*-test).

A

Fig. 4


p= 0.06
p= 0.218
p= 0.394
Fold Change in RNA Expression
2.5
2.0
1.5
1.0
0.5
0.0
Vehicle
10uM CA
Vehicle
10uM CA
Vehicle
10uM CA
Mouse Treatment
mAKR1D1
mCYP8B1
mCYP7A1

B

Fig. 4

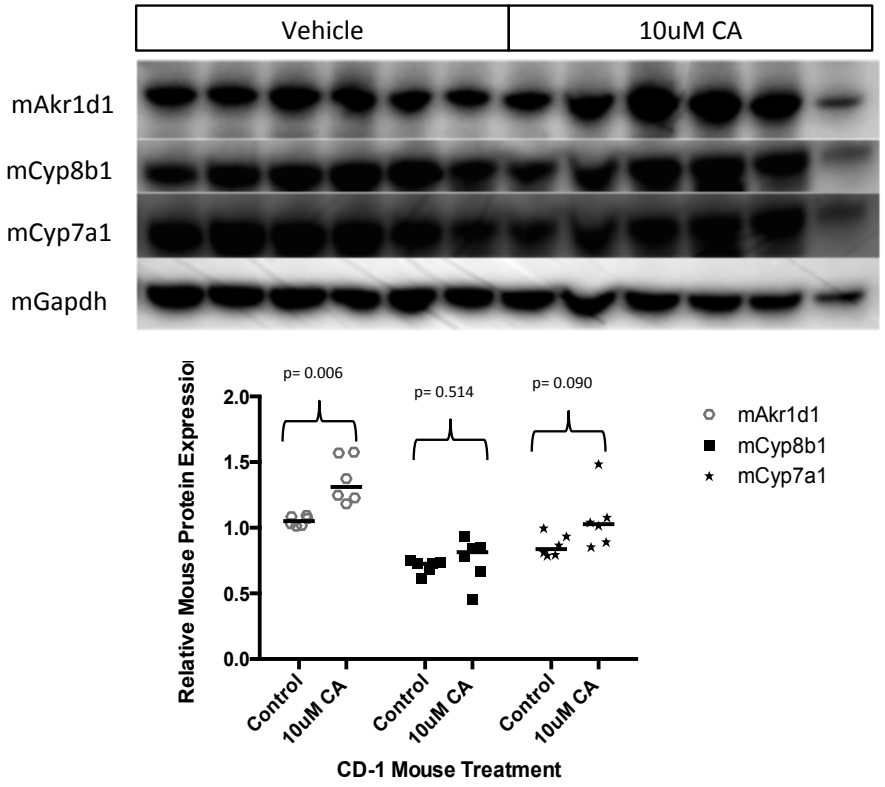
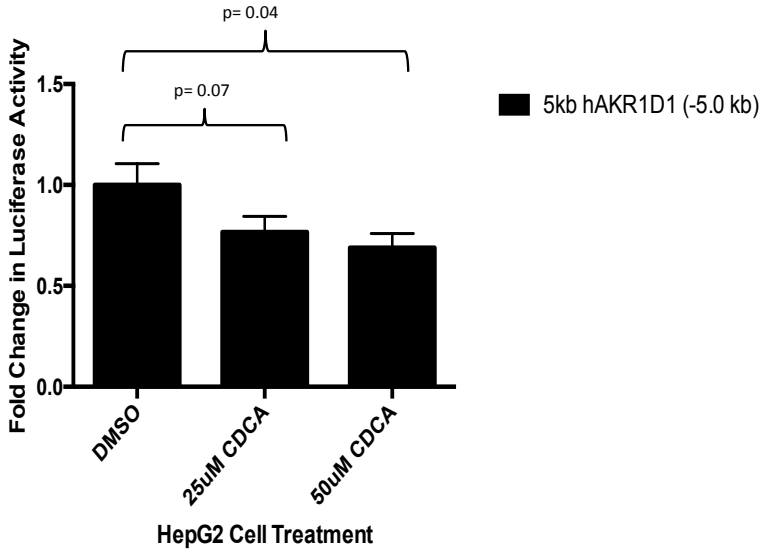

Vehicle
10uM CA
mAkr1d1
mCyp8b1
mCyp7a1
mGapdh
p= 0.006
p= 0.514
p= 0.090
Relative Mouse Protein Expressio
2.0
1.5
1.0
0.5
0.0
Control
10uM CA
Control
10uM CA
Control
10uM CA
CD-1 Mouse Treatment
mAkr1d1
mCyp8b1
mCyp7a1

Figure 4. Effect of CA treatment on endogenous AKR1D1, CYP8B1 and CYP7A1 expression *in-vivo*.

CD-1 mice were injected (6 per group) with propanediol vehicle or 10uM CA at 5mg/kg IP. Livers were harvested for RNA and protein analysis. (A) RNA was isolated from liver tissue. Endogenous AKR1D1, CYP8B1 and CYP7A1 expression were analyzed and normalized to GAPDH. The data are presented as a fold change in relative expression of the mean of 6 mice as compared to the negative control and a $p < 0.05$ was considered significant (student *t*-test). (B) Protein was isolated from liver tissue. Endogenous AKR1D1, CYP8B1 and CYP7A1 expression were analyzed and normalized to GAPDH. The data are presented as a fold change in relative expression of the mean of 6 mice as compared to the negative control and a $p < 0.05$ was considered significant (Student's *t*-test).

A

Fig. 5


p= 0.04
p= 0.07
Fold Change in Luciferase Activity
1.5
1.0
0.5
0.0
DMSO
25uM CDCA
50uM CDCA
HepG2 Cell Treatment
5kb hAKR1D1 (-5.0 kb)

B

Fig. 5

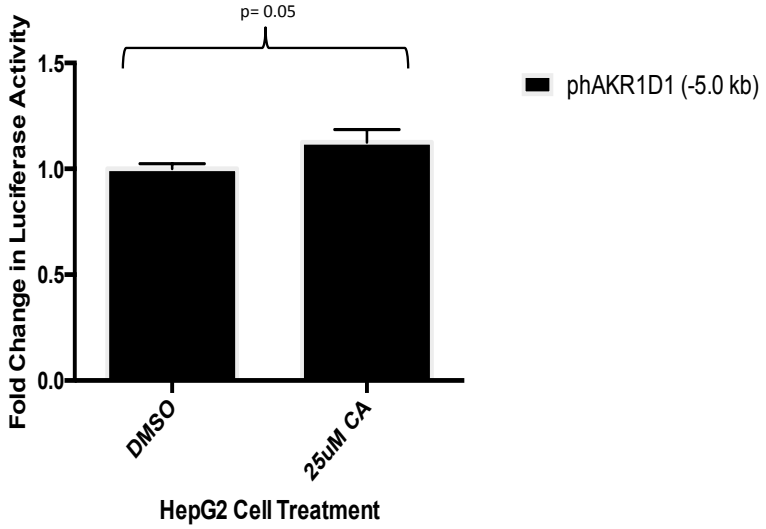

p= 0.05
Fold Change in Luciferase Activity
1.5
1.0
0.5
0.0
DMSO
25uM CA
HepG2 Cell Treatment
phAKR1D1 (-5.0 kb)

Figure 5. Alteration of human AKR1D1 promoter reporter expression with CDCA or CA treatment in HepG2 Cells.

HepG2 cells were transiently transfected with the human AKR1D1 promoter reporter plasmid phAKR1D1 (-5.0 kb) and treated with primary bile acid for 30 hours. Luciferase activation was measured by a dual-luciferase reporter assay system. (A) Luciferase activity of phAKR1D1 (-5.0 kb) in HepG2 cells with 25uM or 50uM CDCA treatment (B) Luciferase activity of phAKR1D1 (-5.0 kb) in HepG2 with 25uM CA. The data are presented as a fold change in relative expression of the mean of 3 replicates as compared to the negative control and a $p < 0.05$ was considered significant (Student's *t*-test).

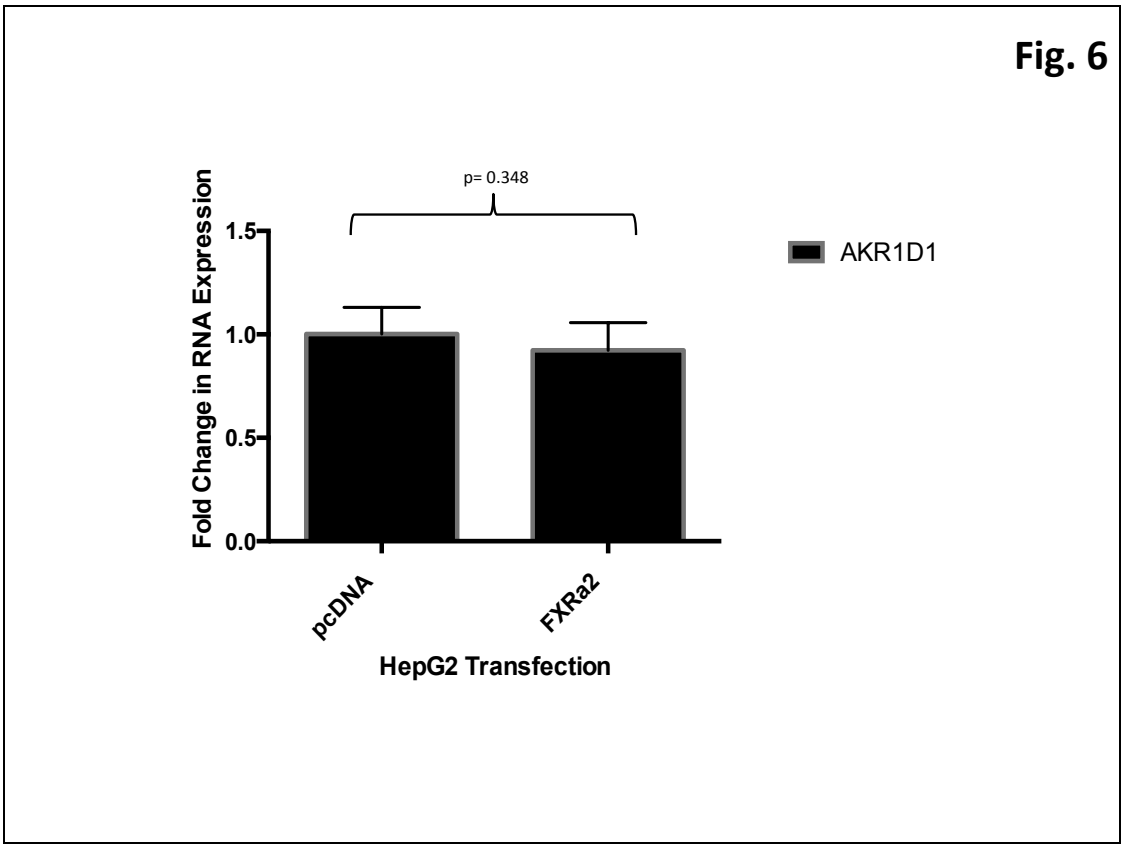


Figure 6. Over-expression of FXRα2 in HepG2 Cells.

HepG2 cells were transiently transfected with FXRα2 nuclear receptor. Following 48 hours transfection, RNA was isolated from cells and probed changes in endogenous AKR1D1 expression. The data are presented as a fold change in relative expression of the mean of 3 replicates as compared to the negative pcDNA control and a $p < 0.05$ was considered significant (Student's *t*-test).

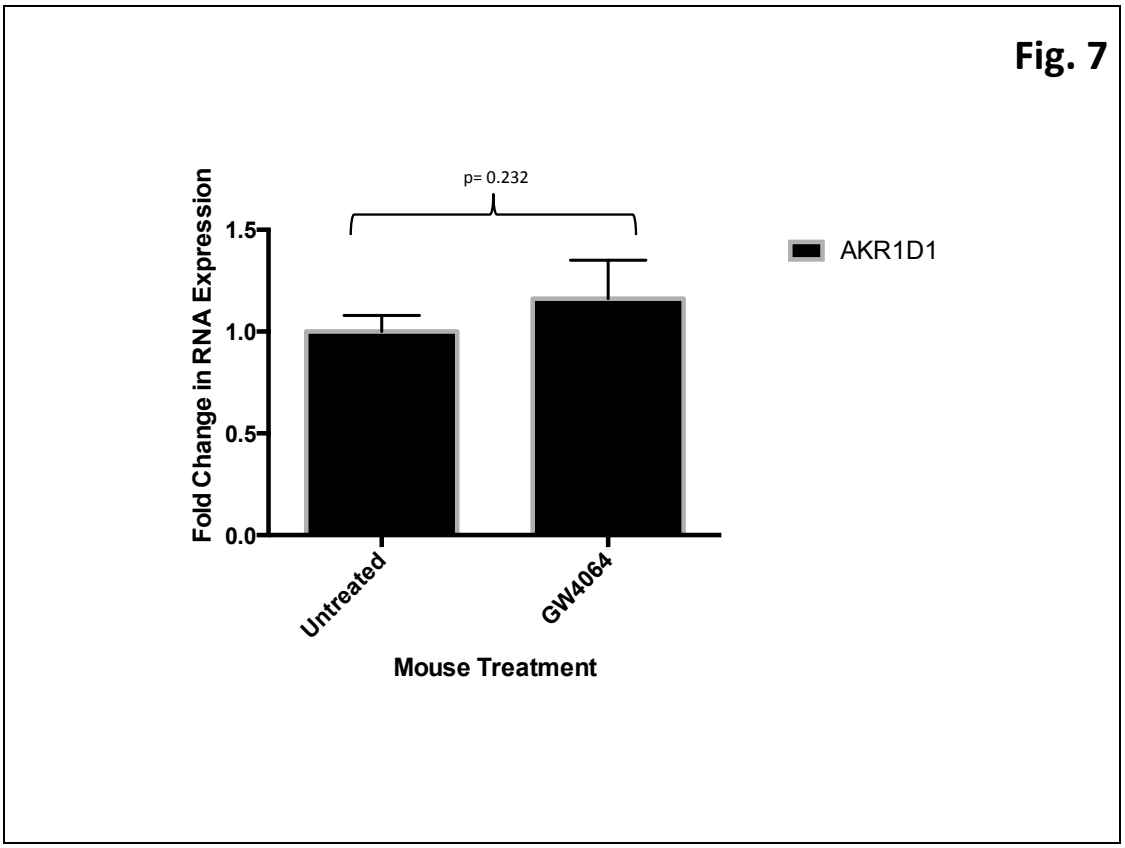


Figure 7. Endogenous mAKR1D1 RNA expression with GW4064 treatment.

Mice (6 per group) were treated with the FXR synthetic agonist GW4064 to examine changes in AKR1D1 expression. The data are presented as a fold change in relative expression of the mean of 6 replicates as compared to the negative untreated control and a $p < 0.05$ was considered significant (Student's *t*-test).

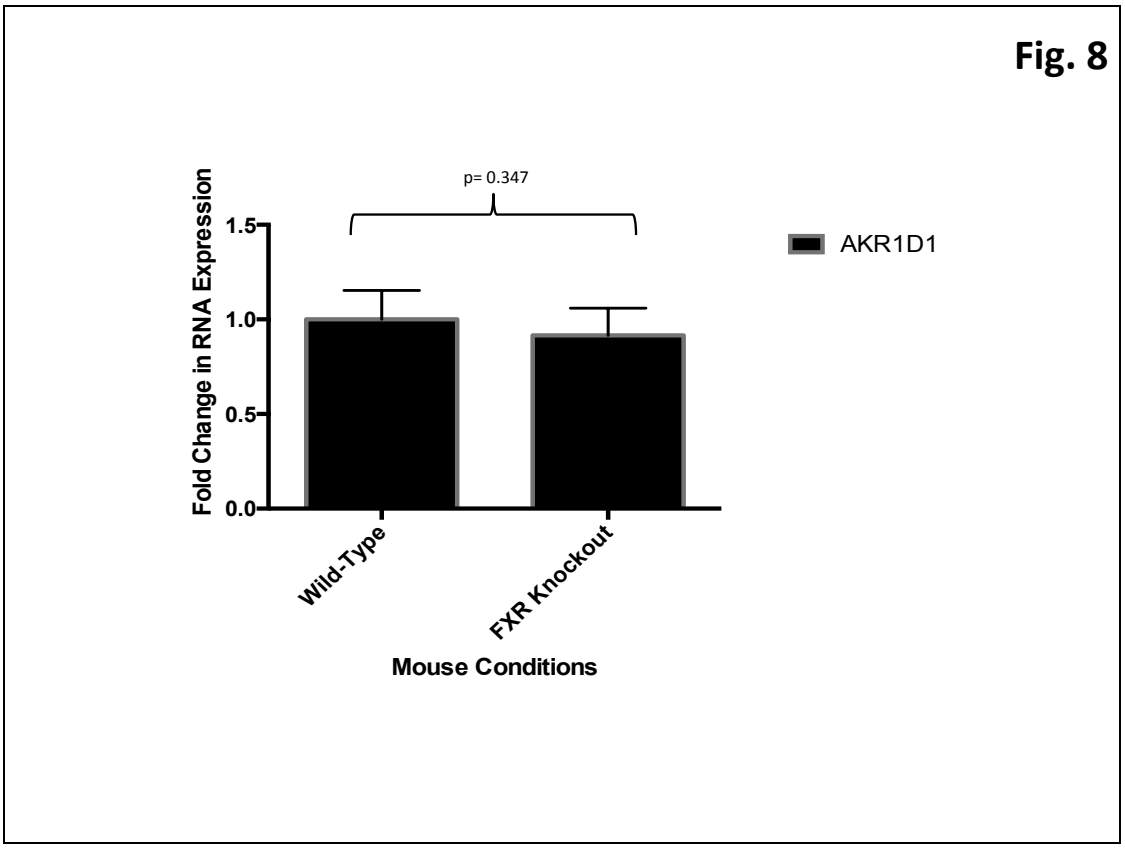
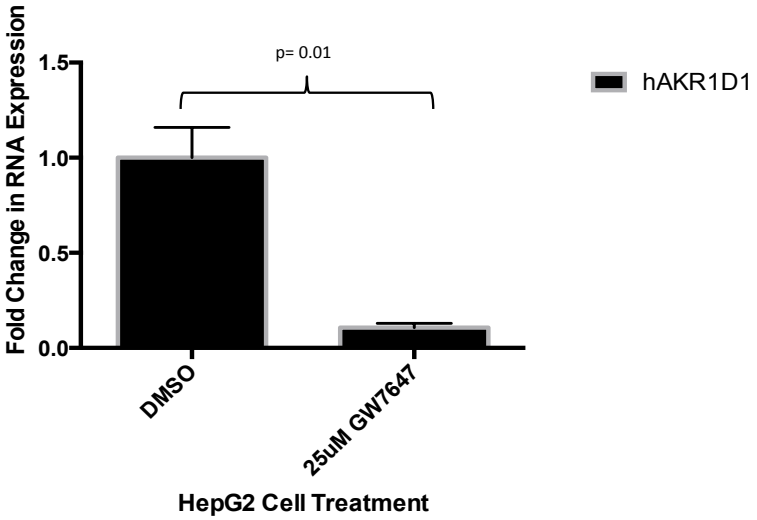


Figure 8. AKR1D1 expression in FXR -/- Mice

RNA extracted from wild-type and FXR -/- mice (6 per group) was probed for endogenous mAKR1D1 expression and normalized to GAPDH. The data are presented as a fold change in relative expression of the mean of 6 replicates as compared to the wild-type control and a $p < 0.05$ was considered significant (Student's *t*-test).

A

Fig. 9


p= 0.01
hAKR1D1
Fold Change in RNA Expression
1.5
1.0
0.5
0.0
DMSO
25uM GW7647
HepG2 Cell Treatment

B

Fig. 9

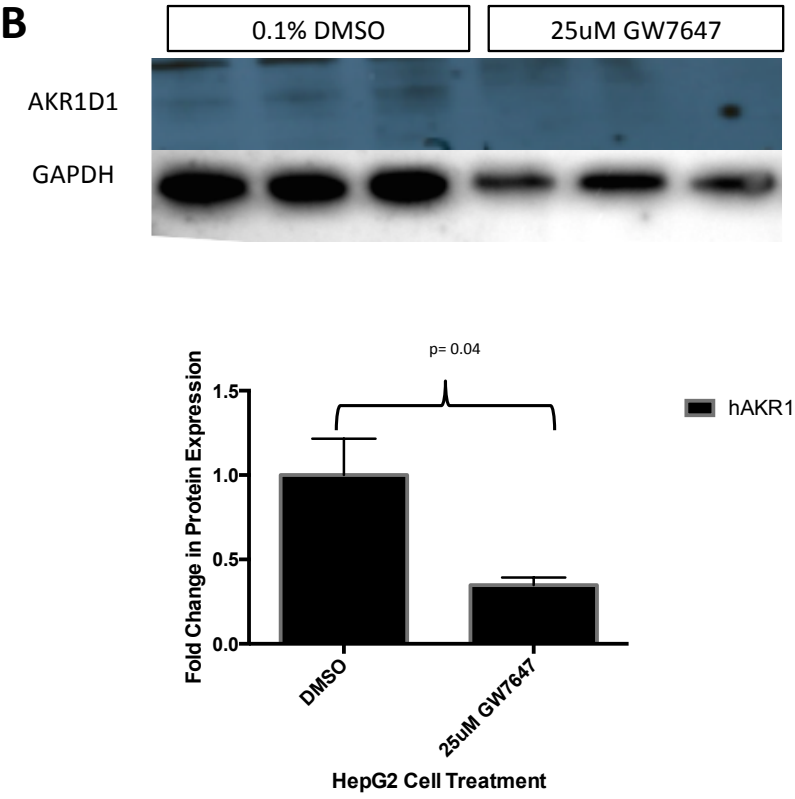
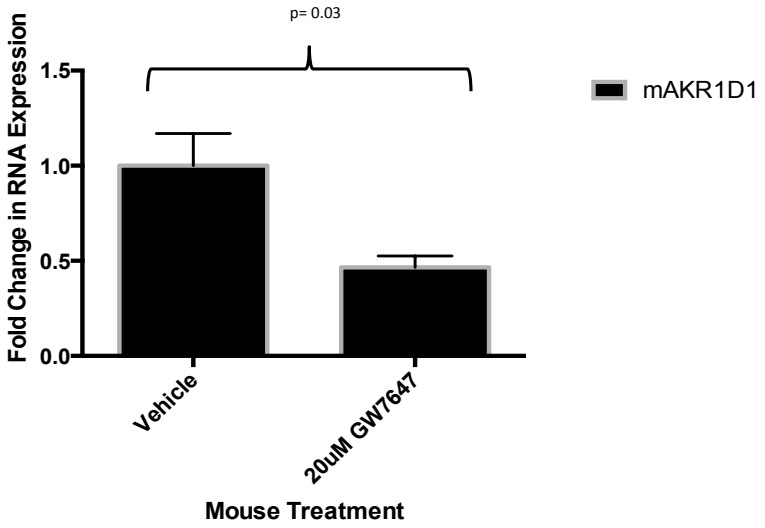

0.1% DMSO
25uM GW7647
AKR1D1
GAPDH
p= 0.04
hAKR1D1
Fold Change in Protein Expression
1.5
1.0
0.5
0.0
DMSO
25uM GW7647
HepG2 Cell Treatment

Figure 9. AKR1D1 expression in HepG2 cells treated with the PPAR synthetic agonist GW7647.

HepG2 cells were treated with 25uM GW7647 for 30 hours and RNA and protein were isolated. (A) RNA was harvested for real-time PCR analysis for the detection of AKR1D1 expression and normalized against GAPDH. The data are presented as a fold change in relative expression of the mean of 3 replicates as compared to the negative control and a $p < 0.05$ was considered significant (Student *t*-test). B) HepG2 cell lysate protein was probed for endogenous AKR1D1 expression when treated with 25uM GW7647. Endogenous AKR1D1 expression was quantified in HepG2 cells and normalized against GAPDH. The data presented are the fold change in relative expression of the mean of at least 3 replicates and a $p < 0.05$ was considered significant (Student's *t*-test).

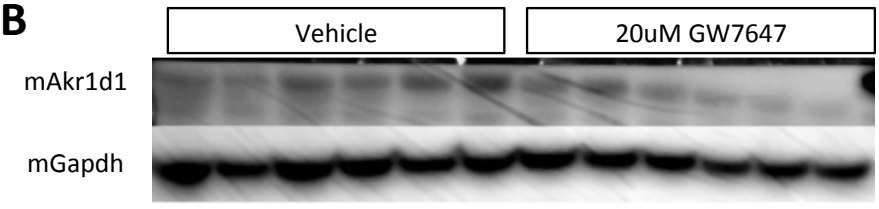
A

Fig. 10



B

Fig. 10



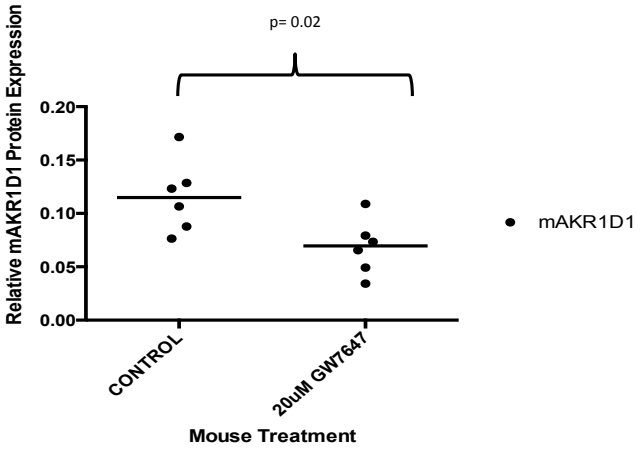


Figure 10. Endogenous AKR1D1 expression of mice treated with synthetic PPAR agonist, GW7647.

Mice were injected every 12 hours for 3 days with 20uM GW7647. Livers were harvested for RNA and protein analysis (A) RNA was isolated from liver tissue for RT-PCR quantification. AKR1D1 RNA expression as normalized against GAPDH. The data are presented as a fold change in relative expression of the mean of 6 replicates as compared to the negative control and a $p < 0.05$ was considered significant (student-t test). (B) Liver cytosolic protein was probed for endogenous AKR1D1 expression. Endogenous AKR1D1 expression was quantified and normalized against GAPDH. The data presented are the fold change in relative expression of the mean of 6 replicates and a $p < 0.05$ was considered significant (Student's *t*-test).

Fig. 11

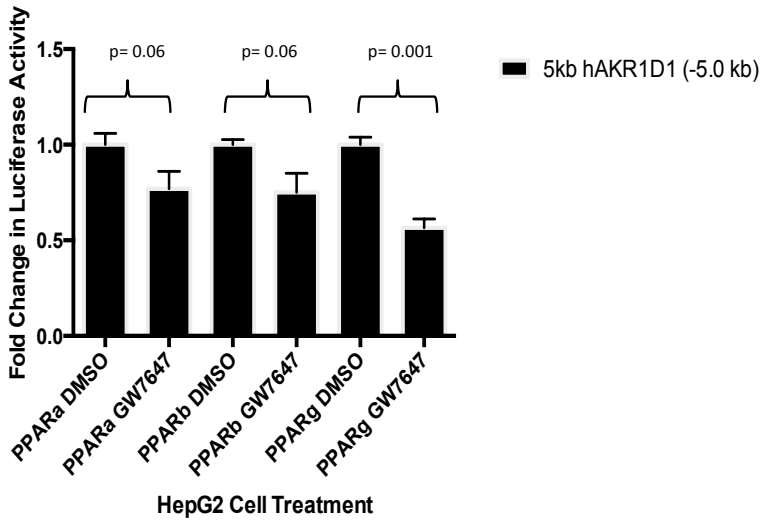


Figure 11. Transrepression of hAKR1D1 promoter reporter by PPAR isoforms in response to GW7647 in HepG2 cells.

HepG2 cells were transiently transfected with the human AKR1D1 promoter reporter phAKR1D1 (phAKR1D1 -5.0 kb) and co-transfected with PPARα, PPARβ or PPARγ and treated with either 25uM GW7647 or 0.1% DMSO control for 30 hours. Luciferase activation was measured by a dual-luciferase reporter assay system. The data are presented as a fold change in relative expression of the mean of 3 replicates as compared to the negative control and a $p < 0.05$ was considered significant (Student's *t*-test).

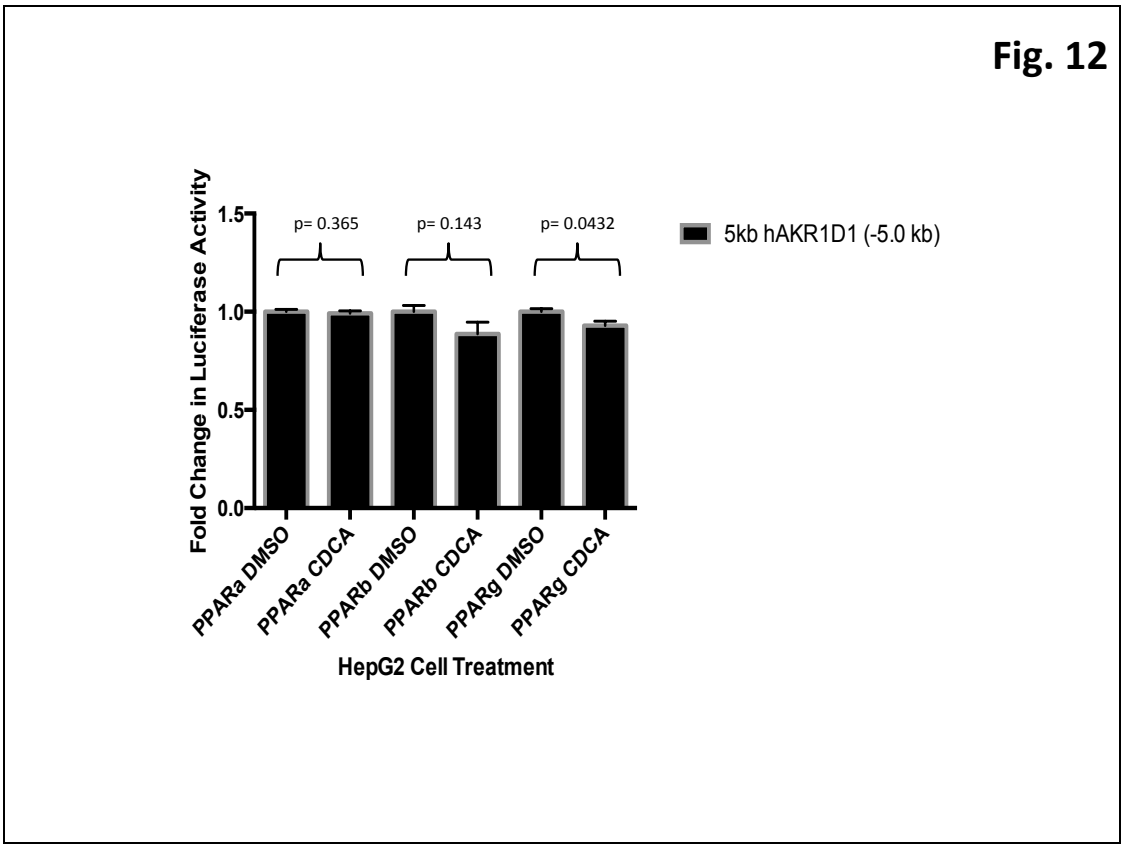
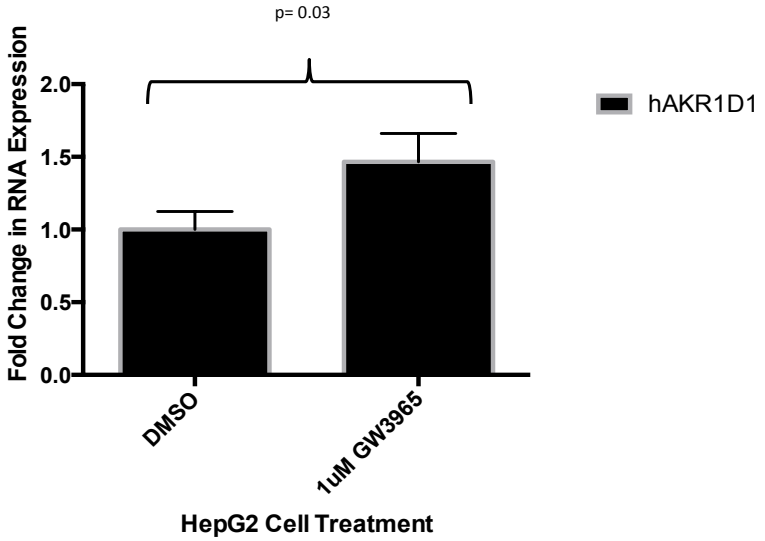


Figure 12. Isoform dependent transrepression of human AKR1D1 by PPAR in-vitro.

PPARγ mediated transrepression of human AKR1D1 promoter reporter phAKR1D1 (-5.0 kb) in the presence of 0.1% DMSO vehicle or 25uM CDCA. Luciferase activation was measured by a dual-luciferase reporter assay system. The data are presented as a fold change in relative expression of the mean of 3 replicates as compared to the negative control and a $p < 0.05$ was considered significant (Student's *t*-test).

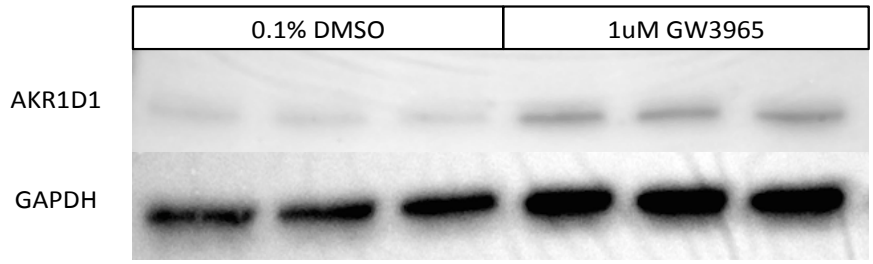
A

Fig. 13



B

Fig. 13



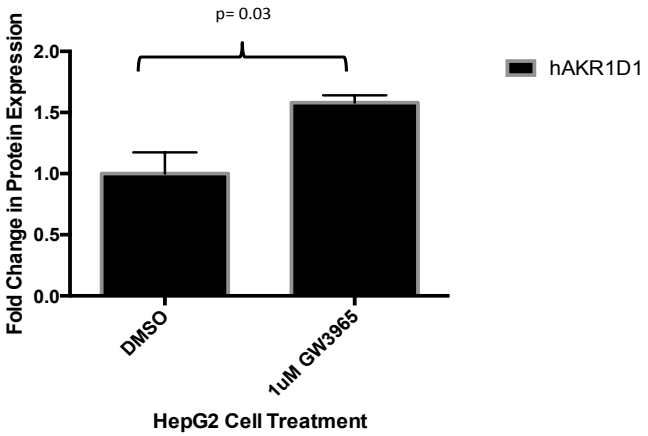
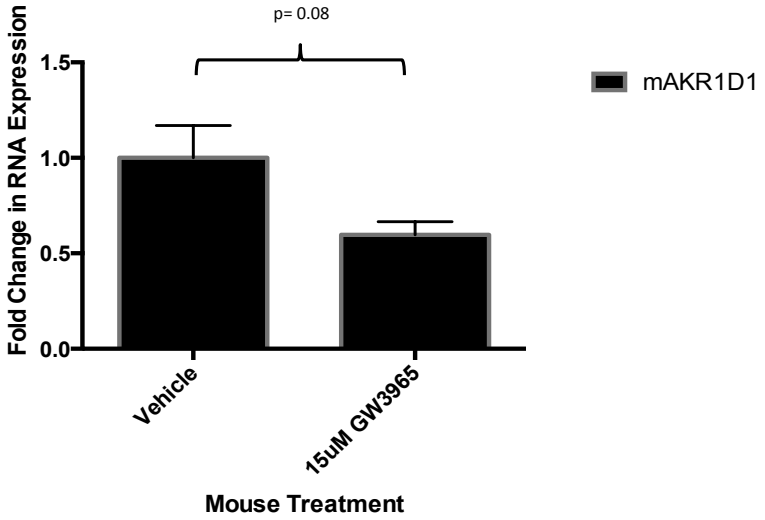


Figure 13. AKR1D1 expression in HepG2 cells treated with LXR synthetic agonist GW3965.

HepG2 cells were treated with 1uM GW3965 for 30 hours and RNA and protein were isolated. (A) RNA was harvested for real-time PCR analysis for the detection of AKR1D1 expression and normalized against GAPDH. (B) HepG2 cell lysate protein was probed for endogenous AKR1D1 expression when treated with 1uM GW3965. Endogenous AKR1D1 expression was quantified in HepG2 cells and normalized against GAPDH. The data presented are the fold change in relative expression of the mean of at-least 3 replicates and a $p < 0.05$ was considered significant (Student's *t*-test).

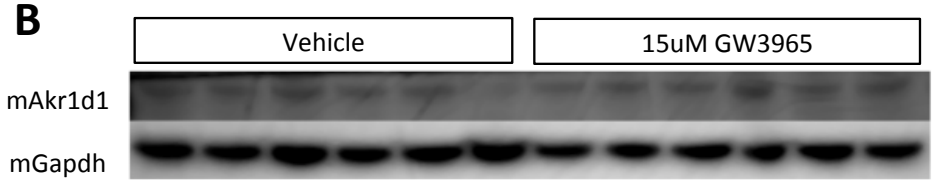
A

Fig. 14



B

Fig. 14



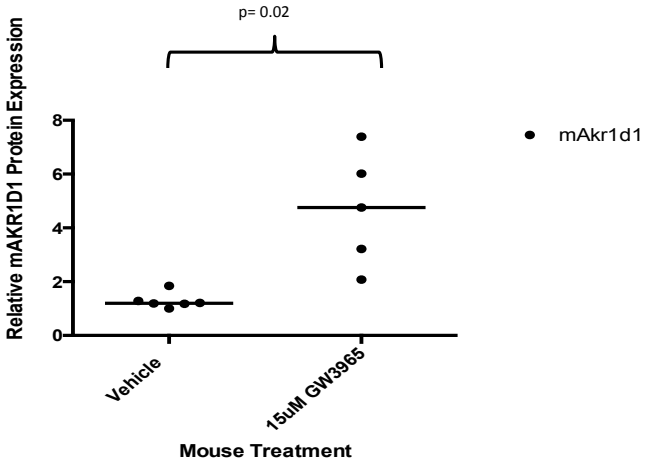


Figure 14. AKR1D1 expression in mice treated with 15uM GW3965.

Mice were injected every 12 hours for 3 days with 15uM GW3965 or a propanediol vehicle. Livers were harvested for RNA and protein analysis (A) RNA was isolated from liver tissue for RT-PCR quantification. AKR1D1 RNA expression as normalized against GAPDH. The data are presented as a fold change in relative expression of the mean of 6 replicates as compared to the negative control and a $p < 0.05$ was considered significant (student *t*-test). (B) Liver cytosolic protein was probed for endogenous AKR1D1 expression. Endogenous AKR1D1 expression was quantified and normalized against GAPDH. The data presented are the fold change in relative expression of the mean of 6 replicates and a $p < 0.05$ was considered significant (Student's *t*-test).

Fig. 15

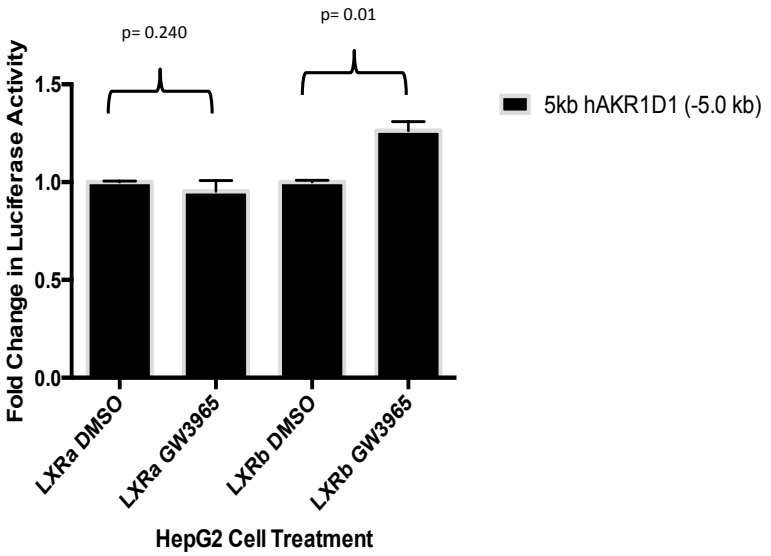


Figure 15. Isoform dependent transactivation of human AKR1D1 by LXR *in-vitro.*

HepG2 cells were transiently transfected with a human AKR1D1 promoter reporter phAKR1D1 phAKR1D1 (-5.0 kb) with either LXRa or LXRb and treated with 1uM GW3965 or 0.1% DMSO vehicle for 30 hours. Luciferase activation was measured by a dual-luciferase reporter assay system. The data are presented as a fold change in relative expression of the mean of 3 replicates as compared to the negative control and a $p < 0.05$ was considered significant (Student's *t*-test).

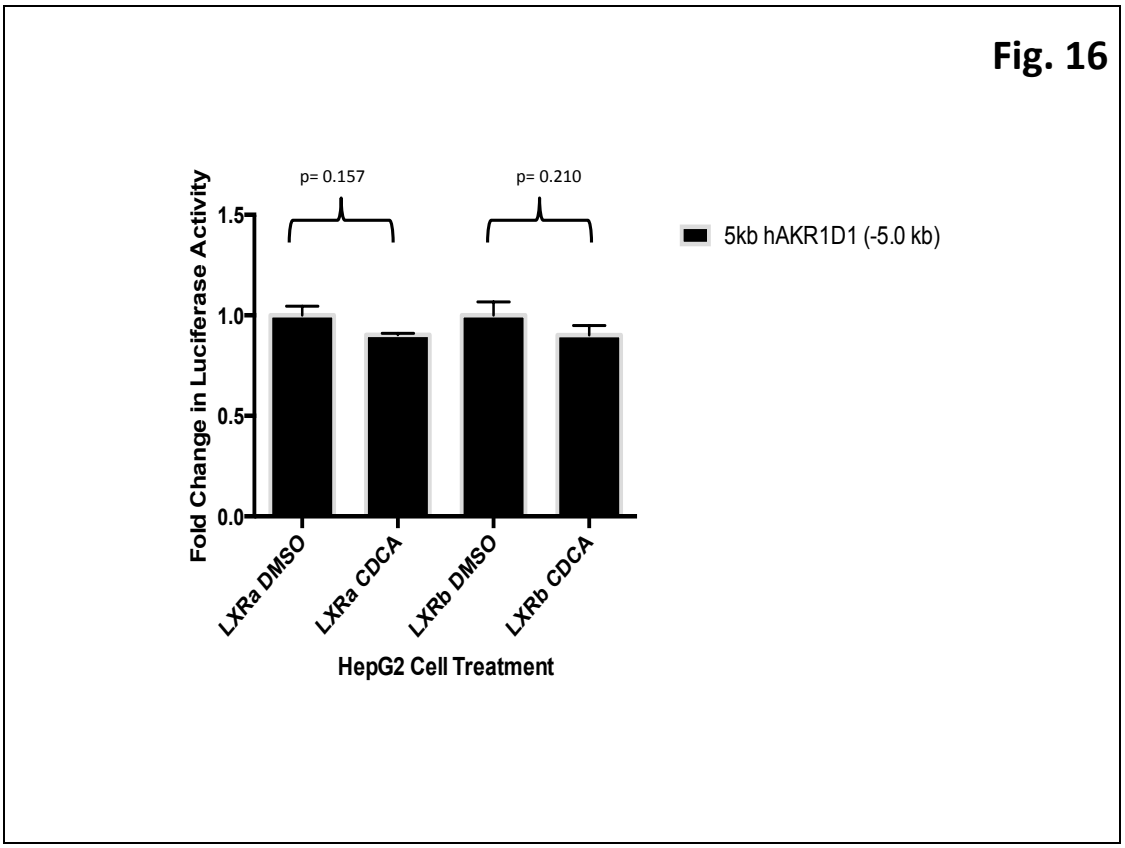


Figure 16. hAKR1D1 promoter reporter is not activated by LXRb in response to CDCA in HepG2 cells.

HepG2 cells were transiently transfected with the human AKR1D1 promoter reporter phAKR1D1 (-5.0 kb) with either LXRa or LXRb and treated with 25uM CDCA or 0.1% DMSO vehicle for 30 hours. Luciferase activation was measured by a dual-luciferase reporter assay system. The data are presented as a fold change in relative expression of the mean of 3 replicates as compared to the negative control and a $p < 0.05$ was considered significant (Student's *t*-test).

CHAPTER 3

THE ROLE OF ALDO-KETO REDUCTASE 1D1 IN TYPE II DIABETES AND HEPATOCELLULAR CARCINOMA

1. Introduction

Bile acids possess the distinguishing characteristic of being detergents and surfactants and therefore their concentrations must be tightly regulated. Not only can increases in total bile acid levels result in pathological consequences, but increases in the concentrations of individual bile acids, which result in changes to the CDCA to CA ratio, can also affect normal healthy liver function. Pathological conditions associated with dysregulation of bile acid concentrations range from cholestasis and cirrhosis to diabetes and hepatocellular carcinoma (HCC).

Type-2 diabetes (T2D) is a metabolic disorder characterized by insulin deficiency and resistance resulting in hyperglycemia as a result of genetic or environmental factors. T2D generally presents later in life and its onset is associated with obesity and hyperlipidemia. The maintenance of glucose and cholesterol levels in T2D patients is critical to avoid cardiovascular complications. Due to the increased incidence of hyperlipidemia in conditions of altered glucose homeostasis, and known role of bile acid in lipid metabolism, a link between bile acid metabolism and glucose homeostasis has been established There is evidence that bile acid metabolism is altered in conditions of T2D suggesting that insulin and glucose modulate bile acid synthesis. There are further indications that medications intended to lower cholesterol levels can also be used to improve glycemic control. Bile acid sequestrates are resins

that bind bile acid and prevent them from being reabsorbed from the gut through enterohepatic circulation (Hansen, Sonne, & Knop, 2014). With less bile acid in circulation, bile acid synthesis is increased and therefore cholesterol turn over to bile acid is increased and subsequently plasma glucose is decreased. . It is suggested that the link between bile acid and glucose homeostasis is through FXR activation indicating that glucose induces FXR expression and insulin inhibits (Duran-Sandoval et al., 2004). It has been well established that the CDCA composition of the bile acid pool is lower in patients with diabetes (Nguyen & Bouscarel, 2008), however the mechanism by which this occurs is unclear. Understanding the role of bile acid synthesis in diabetic models may identify a mechanism by which CDCA production is being regulated. While our previous data confirms that there is no correlation between FXR and AKR1D1 expression, there may be one between diabetes and AKR1D1.

Hepatocellular carcinoma (HCC) is the most prevalent type of liver cancer. It has been identified that high concentrations of CDCA are toxic to the hepatocytes and can have carcinogenic effects; therefore understanding the regulation of CDCA production may prove important for prevention or progression of HCC. Hepatocellular damage may occur as the result of infection, cirrhosis, obesity, diabetes or accumulation of bile acids (Qian & Fan, 2005). Hepatocellular damage is associated with the release of cytokines, chemokines and other molecules that amplify the pathological response to cell injury (Perez, 2009). It has been established that bile acids contribute to the formation of HCC by numerous studies that have reported spontaneous tumor development with the loss of the bile acid receptor, FXR (Kim et al., 2007; Yang et al., 2007). Not only is FXR function associated with HCC

formation, but decreased expression of BSEP has also been reported (Y. Chen et al., 2013). As bile acid concentrations exceed binding capacity of ligands necessary for excretion and regulation, toxicity and subsequent cell damage may occur with the possibility of HCC. It can therefore be suggested that at excessive concentrations bile acids may be considered carcinogenic (Jansen, 2007). It should be noted that it is not only important to regulate total bile acid synthesis, but individual concentrations should also be regulated. CDCA is considered more toxic than CA because of its characteristic increased hydrophobicity and low micellular concentration (Attili, Angelico, Cantafora, Alvaro, & Capocaccia, 1986). The emulsifying properties associated with bile acids are increased with CDCA due to the 5b-reductase activity of AKR1D1 which incorporates a 90° bend in the bile acid structure, contributing to the detergent-like characteristic (Russell, 2003).

With knowledge that high concentrations of toxic bile acids, such as CDCA, may result in severe pathological circumstances such as exacerbation of diabetic symptoms as well as the progression of HCC, we investigate the role of AKR1D1 expression in diseased conditions. Understanding the role AKR1D1 plays in the synthesis of CDCA under pathological situations may provide insight into mechanisms by which AKR1D1 can be regulated to alleviate or prevent various metabolic disorders and cancers.

3. MATERIALS AND METHODS

3.1 Chemicals and Reagents:

Taqman master-mix and RT-PCR probes were purchased from Life

Technologies. RNA Bee for RNA isolation, and propanediol were obtained from Fisher Scientific. Primary and secondary antibodies for western blotting were purchased from SantaCruz Biotechnologies. All western blotting gels, buffers, and markers were purchased through BioRad Laboratories. Complementary DNA synthesis kit was purchased through Promega. Protease inhibitor, Halt, and BSA protein quantification reagent were purchased from ThermoScientific.

3.2 Normal, Diabetic and HCC Human Liver Tissue

Human normal, HCC tumor and diabetic liver tissue were obtained from the University of Virginia, University of Pennsylvania and Ohio State University through the Cooperative Human Tissue Network. The institutional review board at the University of Rhode Island approved the use of human tissues.

3.3 Normal, diabetic and HCC Human Liver RNA Isolation and Quantitative Real-Time Polymerase Chain Reaction (RT-PCR) Analysis

100mg of human liver tissue was homogenized in 1 mL RNA-Bee for RNA extraction. 300 uL chloroform was added to each tube, which was then shaken vigorously for 30 seconds. The samples were then incubated on ice for 5 minutes before being centrifuged at 12,000 G's for 15 minutes. The supernatant was then transferred to a clean microcentrifuge tube and 0.5 mL propanediol was added. Samples were incubated at room temperature for 5 minutes and then spun at 12,000 G's for 10 minutes. The supernatant was discarded and the remaining pellet was washed with 70% ethanol in DEPC treated water and spun at 7,500 G's for 5 minutes. The supernatant was then discarded and the pellet was air dried for 10 minutes before

being reconstituted in 30 uL DEPC water. RNA was quantified by nano-drop and normalized to 2ug for reverse transcription into complementary DNA (cDNA) (Promega). Applied Biosystems 7500 System Sequence Detection Software was used for Taqman RT-PCR data analysis. Using relative quantification (RQ), transcript levels of AKR1D1 and CYP7A1 were normalized against glyceraldehyde-3-phosphate dehydrogenase (GAPDH).

3.4 Normal, diabetic and HCC Human Liver Tissue Western Blot

100mg of liver tissue was homogenized in 1 mL of Sucrose-Tris buffer containing 1% Halt. The total homogenate was spun in a micro-ultra centrifuge at 100,000g's at 4C to separate the cytosolic fraction from the membrane fraction. Cytosolic fraction protein was collected and transferred to a clean 1.5 mL microcentrifuge tube. The protein was quantified using a standard BSA assay. After quantification, protein samples were normalized to 100ug of cytosolic protein. A 1:1 dilution of protein to laemilli sample buffer containing 1% (v/v) betamercaptoethanol was incubated at 95C for 5 minutes, spun, and loaded into a 4-20% gradient SDS-PAGE gel for 1 hour at 120V. Proteins were transferred from the gel using a semi-dry transfer apparatus onto methanol-wetted PVDF membranes at 20V for 30mins. For antibody detection, membranes were blocked for 3 hours in 5% (w/v) skim milk dissolved into a 1X tris-buffered saline solution containing 0.05% Tween20 (1X TBST). Membranes were then incubated overnight in a 1:500 dilution of primary antibody in 10mL of 5% skim milk dissolved into a 1X TBST. Membranes were probed for AKR1D1, CYP7A1 (SantaCruz) or against GAPDH (SigmaAldrich). After overnight incubation membranes were washed 3 times in 1X TBST and incubated

with a corresponding 1:2000 HRP-conjugated secondary antibody for 2 hours. Chemiluminescent signals produced by HRP enzymes were detected using enhanced chemiluminescent substrates (Biorad Clarity Western ECL Substrate) and imaged under a Carestream Gel Logic 2200 Pro camera for 8 minutes. Expression of protein was quantified and normalized against GAPDH expression. Where possible the same blot was stripped and re-probed with different antibodies.

3.5 Immunohistochemistry of normal, diabetic and HCC liver tissue

Normal, diabetic and HCC human liver tissue slides were purchased from AbCam. Slides were incubated at 60C for 30mins to melt paraffin. Slides were transferred into Xylene two times for 3 minutes. Slides were rehydrated in Ethanol (100%, 95%, and 70%) for 3 minutes. Endogenous peroxidase activity was quenched to assist in secondary antibody binding by incubating slides in 3% H2O2 for 30 minutes. Slides were washing in 1X PBS 3 times for 5 minutes. Antigen retrieval was conducted to increase the affinity of the gene to the receptor in 10mM Citrate Buffer by microwaving slides at 90% power until boiling and continuing to incubate at room temperature for 30 minutes. Slides were then rinsed with 1X PBS- 0.1% Tween 20 to permeablize cells to allow antibody binding. Nonspecific binding was blocked by using 5% BSA in 1X TBS- 0.1% TX-100 for 2 hours on shaker at room temperature. Primary antibodies AKR1D1 were incubated at 1:500 in 5% BSA in 1X TBS- 0.1% TX-100 over night at 4C. Sections were then washed three times for 30minutes in 1X TBS- 0.1% TX-100. A fluorescent secondary antibody was used against the primary antibody. AlexaFluor 594 Goat Anti-Rabbit (Invitrogen A11012) was used at a dilution of 1:500 against all three primary antibodies. Secondary antibody was

incubated for 1.5 hours on a shaker at room temperature while covered. Slides were then washed 3 times for 10 minutes in 1X TBS. Sections were then mounted under a glass cover slip using Vectashield Mounting Medium with a DAPI nuclei counterstain (Vector H-1500). Images were captured under a confocal microscope at a magnification of 40X (Zeiss AxioImager M2 Imaging System).

3.6 Statistical Analysis

A Student's *t*-test was applied to pairwise comparison for normally distributed data. P .05 or lower was considered statistically significant.

4. RESULTS

4.1 AKR1D1 expression is altered in conditions of diabetes.

Bile acid homeostasis is known to play a role in glucose homeostasis. For this reason, in diabetic conditions, bile acid pools sizes are altered. To examine the role of AKR1D1 expression in livers of patients with diabetes, human livers were homogenized and RNA and protein expression of endogenous AKR1D1 was quantified as compared to healthy normal human liver tissue. The results in Figure 18A demonstrate that expression of AKR1D1 RNA was significantly decreased in livers of patients with diabetes as compared to those of normal liver tissue at the transcript level ($p < .001$). To determine if changes in RNA and protein expression were specific to AKR1D1 in diseased diabetic livers, the expression of CYP7A1 was also investigated. The data demonstrated that CYP7A1 expression was not changed at the RNA level in diabetic livers as compared to normal livers (Fig. 18B).

Western blot analysis was conducted to determine the effect of AKR1D1 and CYP7A1 expression at the protein level (Fig. 19A). Consistent with RNA results,

AKR1D1 protein expression was significantly decreased in the diseased diabetic livers as compared to normal livers ($p < .0001$) (Fig. 19B), however no change was observed in CYP7A1 protein expression.

To visually examine the localization and expression of the AKR1D1 protein in human tissue, immunohistochemistry was conducted. Images were captured on slides containing either normal human liver tissue or diabetic human liver tissue sections and were probed with AKR1D1 antibody. The results indicate that, as confirmed in the western blot data, AKR1D1 expression is decreased in diabetic human tissue as compared to the normal human tissue (Fig. 20).

Collectively the data establishes that AKR1D1 expression, and not CYP7A1, is altered in diabetics livers as compared to normal healthy livers. It is currently not clear if these results explain if in conditions of disease the liver adjusts AKR1D1 expression in order to limit CDCA production to prevent additional toxicity to an already diseased liver or if the decrease in AKR1D1 expression is in fact part of the progression of the diseased liver.

4.2 Altered AKR1D1 expression in HCC Livers.

Dysregulation of bile acid synthesis, and subsequently related inflammation and toxicity are known risk factor correlated with the progression of HCC. Due to the characteristic toxicity associated with high levels of CDCA, the role of AKR1D1 expression in human HCC livers was examined. Normal and HCC human livers were homogenized and analyzed for expression of AKR1D1 and CYP7A1. RT-PCR analysis demonstrated that expression of AKR1D1 RNA was significantly decreased in human HCC livers as compared to normal livers ($p < 0.001$) (Fig. 21A). At the

RNA level, there was no significant change in CYP7A1 expression in HCC liver tissue as compared to normal human liver tissue.

Correspondingly, when examining protein expression of AKR1D1 by western blot analysis (Fig. 22A), there was a significant decrease in HCC human liver tissue as compared to normal tissue ($p < .001$) (Fig. 22B). Western blot data reveals at expression of CYP7A1 was unchanged in HCC human liver as compared to normal human liver. Immunohistochemistry imaging confirmed western blot results by demonstrating that AKR1D1 expression was decreased in human HCC liver tissue slides as compared to normal liver tissue (Fig. 23).

In conclusion, the combined data confirms that in patients with HCC, expression of AKR1D1 is significantly downregulated. The data suggests that the liver may alter AKR1D1 expression in diseased cancerous liver tissue as an adaptive mechanism to protect the hepatocytes and liver from further damage associated with the more toxic hydrophobic primary bile acid, CDCA. Expression of CYP7A1 is unchanged indicating that changes in AKR1D1 expression are not specific to the bile acid synthesis pathway, but that AKR1D1 expression is being regulated through an alternate signaling pathway.

5. DISCUSSION

Bile acids are essential, at physiological concentrations, to aid in the emulsification and absorption of cholesterol, dietary fats and lipids. Due to their characteristic properties of being biological surfactants and detergents, increased bile acid concentrations may be toxic to the hepatocytes and liver and therefore must be tightly regulated to maintain proper physiological liver function. Uncontrolled

concentrations of toxic bile acids have the ability to cause inflammation of hepatocytes leading to apoptosis and necrosis and ultimately liver damage and the possibility cancer. Damage to the hepatocyte has the potential of activating a series of signaling pathways associated with inflammatory modulators, oxidative stress, cellular damage and death. A dysregulation in the composition of the bile acid pool may manifest itself into a number of pathological consequences. Among these are metabolic disorders such as diabetes, as well as cancerous outcomes such as hepatocellular carcinoma, HCC.

It has been established that bile acid homeostasis is essential for proper metabolic homeostasis and function (Nguyen & Bouscarel, 2008). Published data states that activation of FXR signaling decreased plasma glucose concentrations and increased insulin sensitivity in fasting diabetic mice models (Li et al., 2012). Likewise, it is known that in diabetic patients there is an increase in the size of the bile acid pool. On a study conducted on diabetes induced rats, it was established that while insulin inhibits FXR, glucose induces FXR expression (Duran-Sandoval et al., 2004). Another link between bile acid synthesis and glucose homeostasis is seen through the use of bile acid sequestrants. Bile acid sequestrants are resin molecules that bind bile acid and preventing them from partaking in enterohepatic circulation. Bile acid sequestrants are traditionally used to treat hyperlipidemia. The loss of bile acid within the bile acid pool promotes the synthesis of additional bile acids to aid in cholesterol solubilization. Recently bile acid sequestrants have been seen to aid in glucose homeostasis as well (Hansen et al., 2014). Patients taking bile acid sequestrants show an improvement in glycemic control (Staels & Fonseca, 2009). Taken together, the

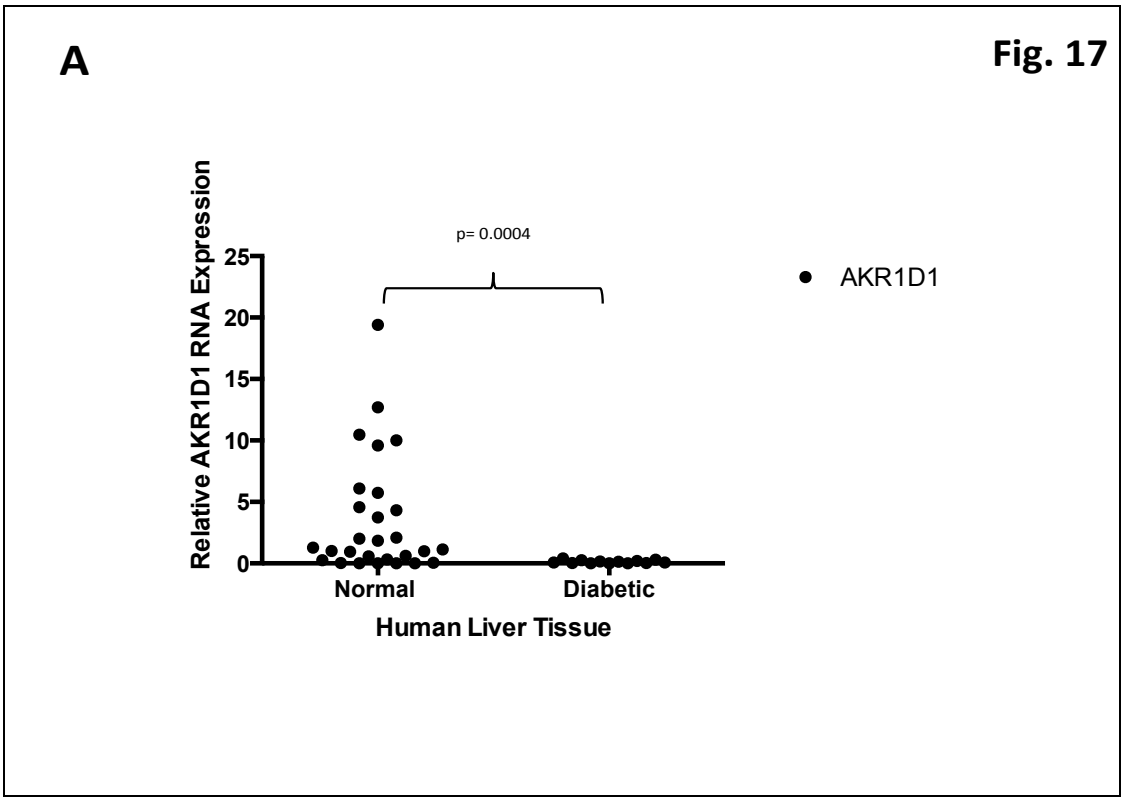
association between bile acid homeostasis and glucose homeostasis is clear however the mechanism is not yet defined.

In our study we examined the expression of AKR1D1 in diabetic human liver tissue to attempt to fill in the gap between bile acid homeostasis and glucose homeostasis through alterations in bile acid synthesis. Our results indicated that RNA and protein expression of AKR1D1 was significantly decreased in diabetic patients as opposed to normal healthy patients, with no change to CYP7A1 expression. Since AKR1D1 is responsible for the synthesis of the more hydrophobic bile acid CDCA, our data presumes a possible link between CDCA synthesis and either the prevention or pathogenesis of adverse diabetic outcomes. Consistent with our findings of a decrease in AKR1D1 expression of diabetic patients, it has been documented that the bile acid pool of diabetes-induced mice shows a significant decrease in CDCA concentrations (Li et al., 2012). With the knowledge that glucose binds FXR and aids in decreasing plasma glucose concentration (Duran-Sandoval et al., 2004), and CDCA binds FXR and aids in decreasing bile acid synthesis, our data serves to prove that the liver down-regulates AKR1D1 expression in order to prevent competitive binding of FXR in conditions of metabolic disease. Furthermore, if a down-regulation of AKR1D1 expression is associated with a preventative measure, we hypothesize that the liver is altering AKR1D1 expression in order to control further production of the more hepatotoxic CDCA to protect a liver that is already diseased due to diabetes-associated indications. While a pathway for this method has not yet been established, this novel mechanism by which the liver is attempting to regain homeostasis in times of disease would explain the decrease in AKR1D1 expression. This mechanism would

also confirm our belief that due to the toxic nature of CDCA, the liver is constantly attempting to maintain homeostasis by altering AKR1D1 expression.

Hepatocellular carcinoma (HCC) is one of the most common types of liver cancer and its advancement is linked to complications with hepatitis infections, obesity, cirrhosis, and disorders in bile acid synthesis. Excess bile acid accumulation has been linked to the progression of HCC. Due to the fact that CDCA is considered the more toxic bile acid as a result of its increased hydrophobicity, we examined the expression of AKR1D1 in patients with HCC and compared them to healthy normal human liver. Our results demonstrated that expression of AKR1D1 was severely diminished in HCC patients as compared with normal healthy livers. The down-regulation of AKR1D1 expression was documented at both the transcript and protein levels. Taken together we believe that the expression of AKR1D1 is down-regulated in HCC patients in a hepato-protective manner. Due to the toxicity associated with an accumulation of bile acids, especially CDCA, the liver may alter AKR1D1 expression as a means to protect hepatocytes from further CDCA production. Our lab previously reported a decrease in BSEP expression in HCC liver tissue (Y. Chen et al., 2013). A decrease in BSEP expression will result in bile acid that is sequestered within the liver and cannot be secreted out, exacerbating toxic conditions for the liver and hepatocytes and ultimately contributing to the pathogenesis of HCC. This data supports our hypothesis that AKR1D1 is down-regulated to suppress further CDCA production in conditions of hepatocellular stress or toxicity.

In conclusion, our data proves that in conditions of metabolic distress and HCC, the AKR1D1 expression is altered to limit the production of CDCA to prevent further toxicity.


A
Fig. 17
Relative AKR1D1 RNA Expression
p= 0.0004
25
20
15
10
5
0
Normal
Diabetic
Human Liver Tissue
AKR1D1

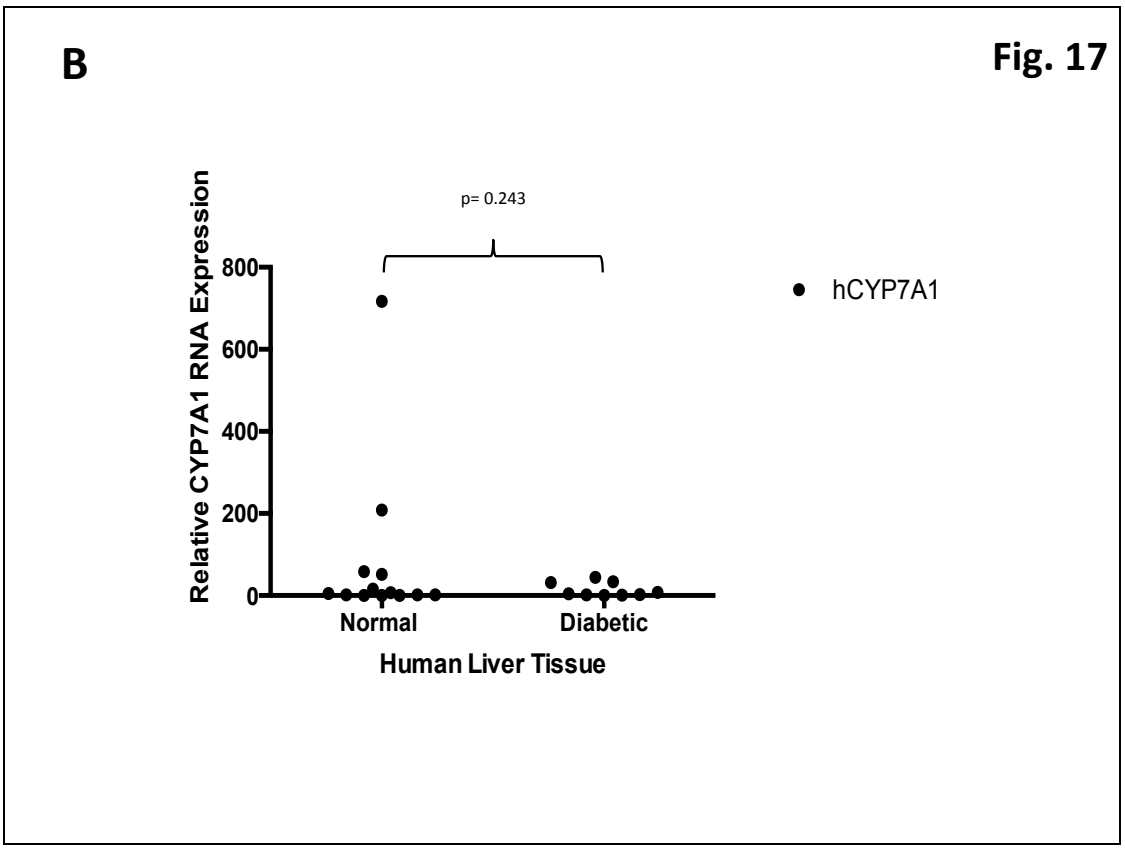
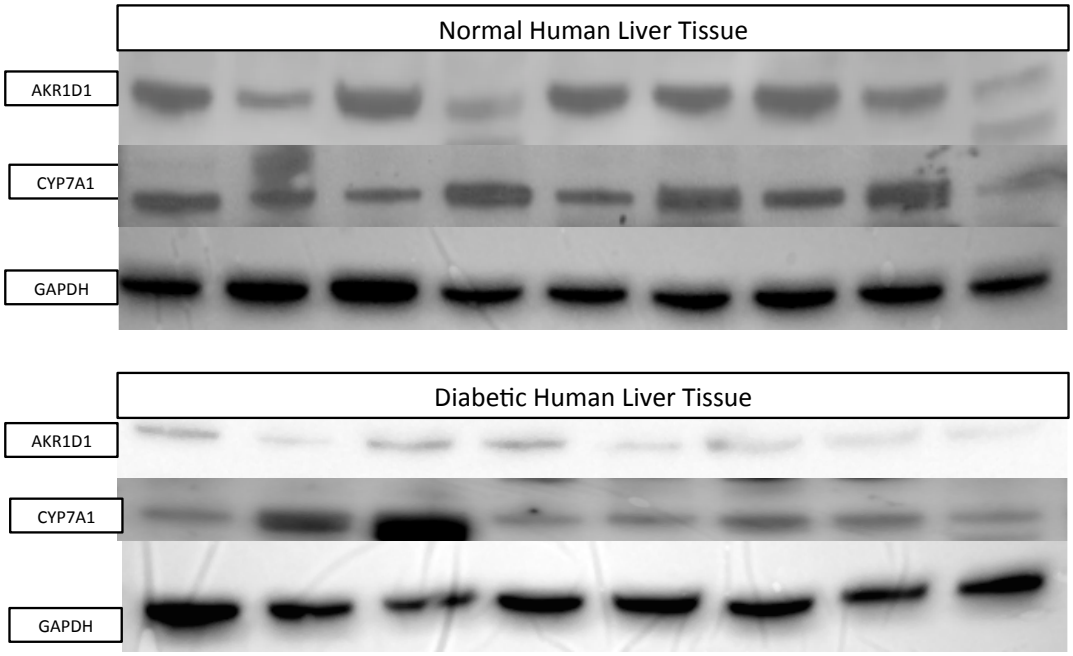

B
Fig. 17
Relative CYP7A1 RNA Expression
p= 0.243
800
600
400
200
0
Normal
Diabetic
Human Liver Tissue
hCYP7A1

Figure 17. Endogenous AKR1D1 and CYP7A1 mRNA expression in normal and diabetic human liver tissue.

RNA expression of (A) AKR1D1 and (B) CYP7A1 were measured in normal and diabetic human liver tissue. The data are presented relative expression of the mean of 20 replicates and a $p < 0.05$ was considered significant (Student's *t*-test).

A

Fig. 18



B

Fig. 18

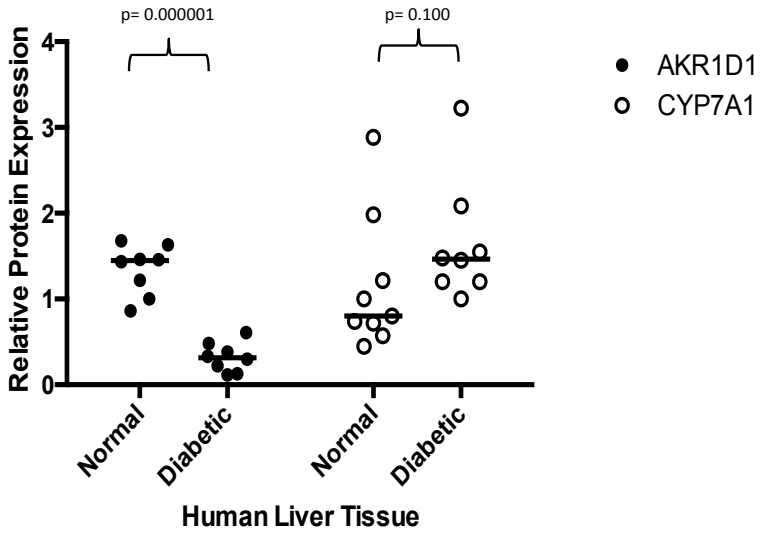


Figure 18. Endogenous AKR1D1 and CYP7A1 protein expression in normal and diabetic human liver tissue.

(A) Protein levels of AKR1D1 and CYP7A1 in normal and diabetic human liver tissue were detected by western blot. (B) Quantification of AKR1D1 and CYP7A1 protein expression in normal and diabetic human liver tissue. The data are presented relative expression of the mean of 8 replicates and a $p < 0.05$ was considered significant (Student's *t*-test).

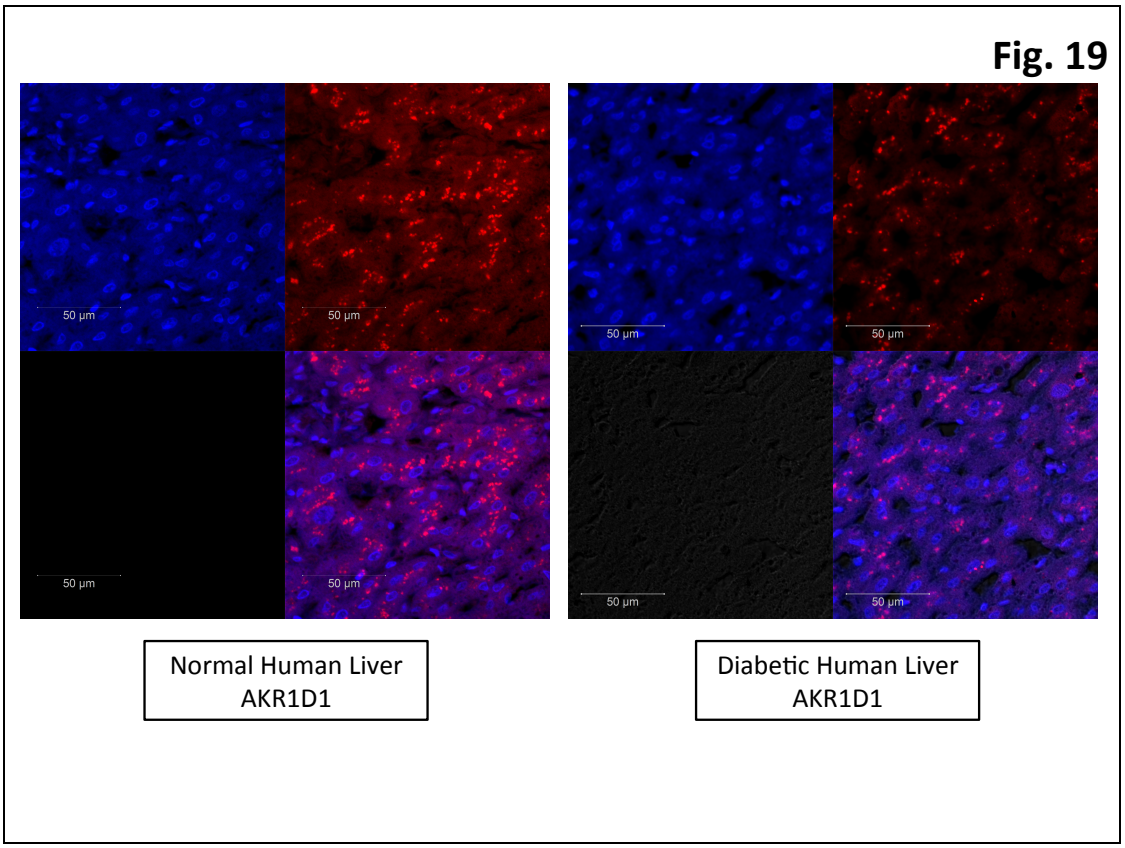
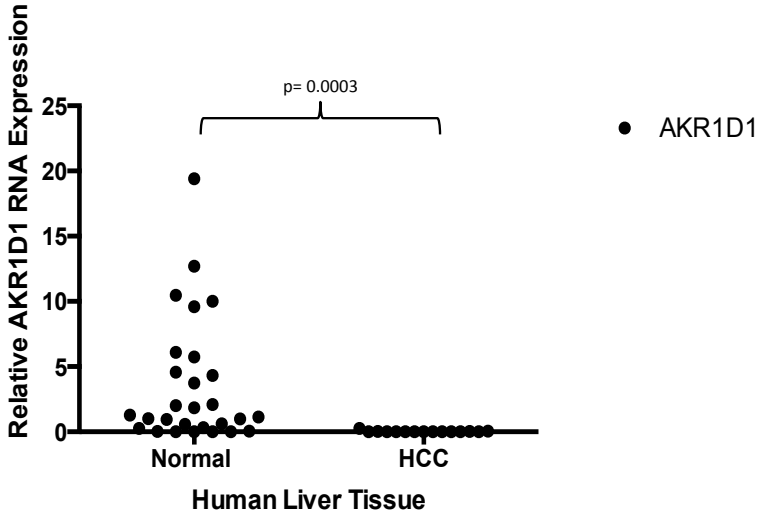


Figure 19. Expression of AKR1D1 in normal and diabetic human liver tissue.

Liver sections were subjected to immunohistofluorescent assays. Images were captured under a confocal microscope at a magnification of 40x with both fluorescent and DIC settings.

A

Fig. 20



B

Fig. 20

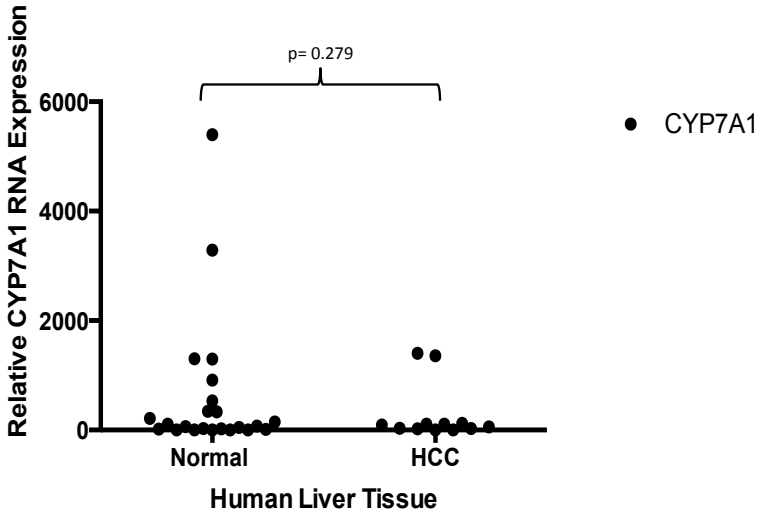
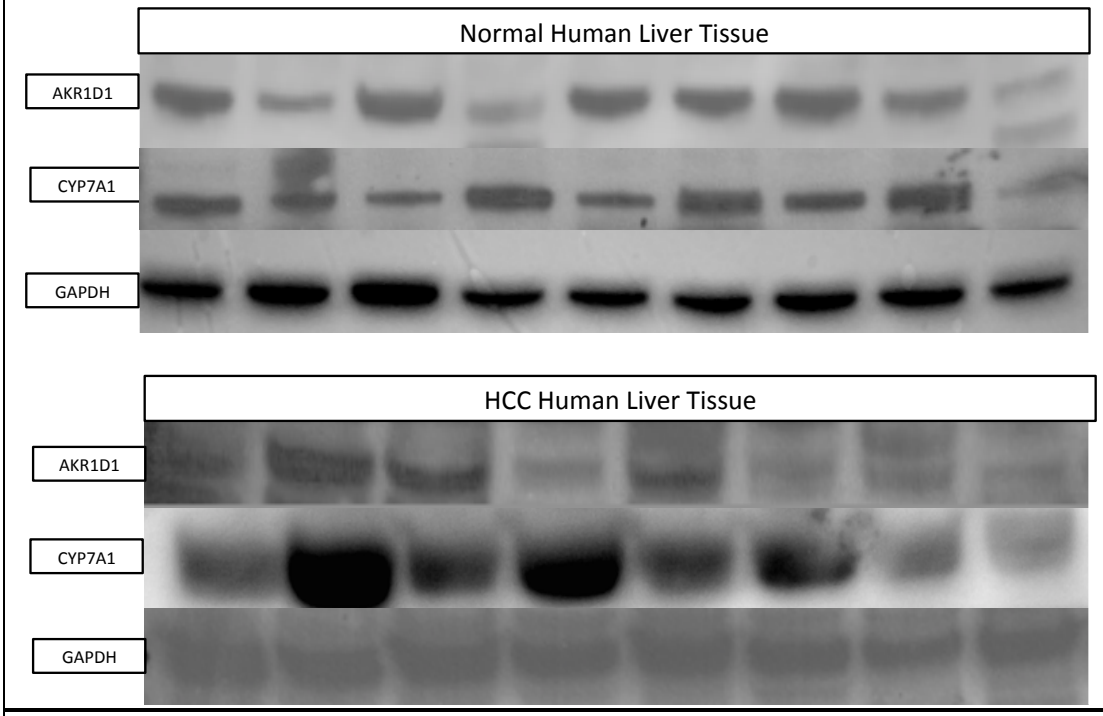


Figure 20. Endogenous AKR1D1 and CYP7A1 mRNA expression in normal and HCC human liver tissue.

RNA expression of (A) AKR1D1 and (B) CYP7A1 were measured in normal and HCC human liver tissue. The data are presented relative expression of the mean of 20 replicates and a $p < 0.05$ was considered significant (Student's *t*-test).

A

Fig. 21



B

Fig. 21

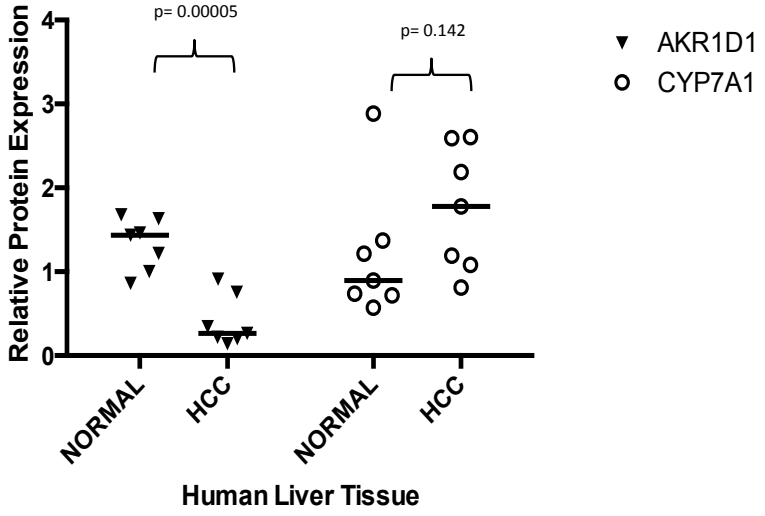


Figure 21. Endogenous AKR1D1 and CYP7A1 protein expression in normal and HCC human liver tissue.

(A) Protein levels of AKR1D1 and CYP7A1 in normal and HCC human liver tissue were detected by western blot. (B) Quantification of AKR1D1 and CYP7A1 protein expression in normal and HCC human liver tissue. The data are presented relative expression of the mean of 8 replicates and a $p < 0.05$ was considered significant (Student's *t*-test).

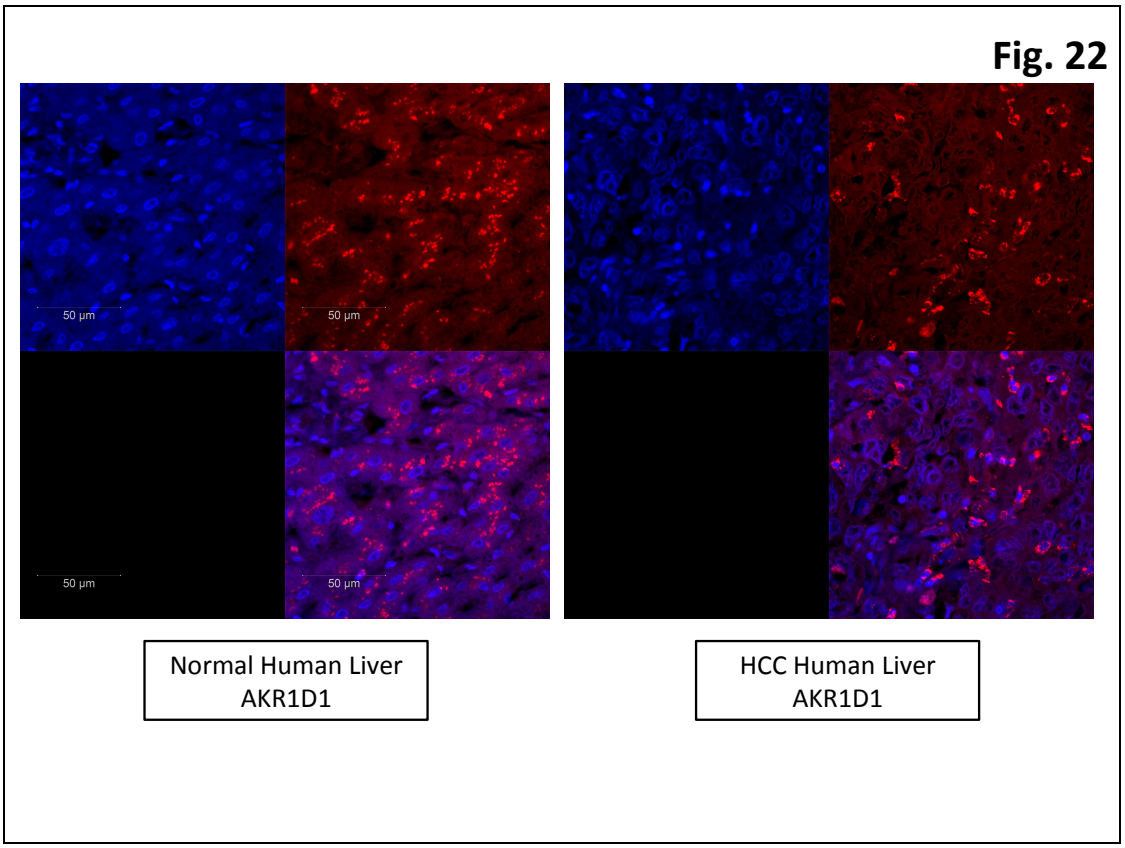


Figure 22. Expression of AKR1D1 in normal and HCC human liver tissue.

Liver sections were subjected to immunohistofluorescent assays. Images were captured under a confocal microscope at a magnification of 40x with both fluorescent and DIC settings.

CHAPTER 4

THE ROLE OF ALDO-KETO REDUCTASE 1D1 IN PREGNANCY

1. Introduction:

The correlation between bile acid concentrations in the bile acid pool and the incidence of labor is of great interest. Bile acids serves as more than biological detergents that aid in the emulsification and solubilization of cholesterol, dietary fats and lipids, they also act as hormones and are ligands for many nuclear receptors involved in various signaling pathways. AKR1D1 is a 5β-reductase enzyme that is necessary for bile acid synthesis. AKR1D1 is also involved in the regulation and metabolism of other critically important steroid hormones with known expression in the brain, uterus and placenta (M. Chen & Penning, 2014).

AKR1D1 is known to be involved in the synthesis of neuroactive steroids, including 5β-dihydroxyprogesterone (5β-DHP) in the uterus. Progesterone, a steroid hormone cholesterol derivative, is converted 5β-DHP by AKR1D1. 5β-DHP is a progesterone metabolite that inhibits contractions of the myometrium within the uterine wall during pregnancy. Therefore, during pregnancy, when estrogen levels are naturally higher, AKR1D1 expression must be consistently elevated in order to produce enough 5β-DHP to avoid pre-term uterine contractions and therefore premature labor (M. Chen & Penning, 2014), although the mechanism by which 5b-DHP inhibits contractions remains unclear. Therefore, the known data indicates that there is a possible relationship between estrogen production and AKR1D1 expression.

We hypothesize that estrogen regulates AKR1D1 expression in order to help sustain a pregnancy by increasing AKR1D1 expression.

AKR1D1 expression has also been shown to decrease towards the end of pregnancy resulting in the initiation of labor (Byrns, 2011; Sheehan et al., 2005), however the mechanism by which this occurs is unclear. This dynamic relationship between pregnancy and AKR1D1 can also be correlated to bile acid synthesis. Intrahepatic cholestasis of pregnancy (ICP) is a disorder that occurs in certain pregnant women during their third trimester (Geenes, 2009). Women with ICP have a characteristic increase in serum bile acid due to increases in pregnancy related hormones, primarily estrogen. Studies have shown that during pregnancy, expression of the bile salt export pump (BSEP) is significantly decreased as a result of increased estrogen levels (Song et al., 2014). A decrease in BSEP expression results in an increase of serum bile acid levels, indicating a correlation between ICP and increased bile acid concentrations through altered expression of BSEP (Song et al., 2014). Not only is total bile acid serum increased, but the composition of the bile acid ratio is also transformed. Characteristically, women with ICP exhibit an increase in CA concentration. Also, women presenting with ICP have a greater likelihood of experiencing pre-term labor.

In summary, due to the knowledge that AKR1D1 expression is increased during pregnancy, as are estrogen levels, we hypothesize that estrogen is transcriptionally regulating the expression of AKR1D1 during pregnancy. Taken together, investigating the expression of AKR1D1 in response to estrogen could shed greater light on the correlation between estrogen and the incidence of ICP.

2. Material and Methods:

2.1 Treatment of HepG2 Cells

HepG2 cells were seeded in 12-well plates at a cell density of 4 x 10^5 per mL in 1 mL Phenol-Red Free DMEM supplemented with 10% (v/v) Charcoal-Stripped FBS, 1% (v/v) penicillin/streptomycin and 1% (v/v) NEAA and cultured in a 5% CO_2 incubator overnight before treatment. After overnight incubation, cells were treated with 100uM E2 prepared in ethanol or a ethanol control treatment for 30 hours in 1 ml Phenol-Red Free DMEM supplemented with 1% Charcoal-Stripped FBS, 1% antibiotics and 1% NEAA. All treatments were at 0.1% of total volume medium.

2.2 Reporter Luciferase Assay

HepG2 cells were seeded in 24-well plates at a cell density of 2.5 x 10^5 in 0.5 mL Phenol-red Free DMEM supplemented with 10% (v/v) Charcoal Stripped FBS, 1% (v/v) penicillin/streptomycin and 1% (v/v) NEAA and cultured in a 5% CO_2 incubator overnight before treatment. After overnight incubation, cells were treated transiently transfected with GenJet Version II reagent. For all transfections, a standard concentration of 100ng/ul hAKR1D1 promoter reporter was used. Additionally, 10ng/ul null-Renilla luciferase plasmid was used as an internal control. Nuclear receptor expression plasmid ERa was also transfected at 100ng/ul. Twenty-four hours after transfection, cell medium was replaced with corresponding agonist treatment. HepG2 cells were treated with increasing concentrations of E2 prepared in ethanol or a control treatment of ethanol for 30 hours in 1 ml Phenol-red Free DMEM supplemented with 1% Charcoal-Stripped FBS, 1% antibiotics and 1% NEAA. All treatments were at 0.1% of total volume medium. Following 30 hours treatment, cells

were washed once with phosphate-buffered saline and lysed with 100ul of 1X passive lysis buffer (Promega) by rocking at room temperature for 15 minutes. Following cell lysis, 10ul of cell lysate was transferred into a white 96-well plate for measurement of luciferase activity. A Dual-Luciferase Reporter assay system was used which measures the reporter vector firefly luciferase activity and subsequently the renilla luminesnce of the null internal control. The luminescence signal of the reporter vector is normalized based on the null reading.

2.3 HepG2 RNA Isolation and Quantitative Real-Time Polymerase Chain Reaction (RT-PCR) Analysis

Thirty hours after treatment of HepG2 cells, cells were washed twice with 1X PBS and homogenized by pipetting with 0.25mL RNA-Bee reagent. Homogenates were then transferred to 1.5mL microcentrifuge tube. 100uL chloroform was added to each tube, which was then shaken vigorously for 30 seconds. The samples were then incubated on ice for 5 minutes before being centrifuged at 12,000 G's for 15 minutes. The supernatant was then transferred to a clean microcentrifuge tube and 0.5 mL propanediol was added. Samples were incubated at room temperature for 5 minutes and then spun at 12,000 G's for 10 minutes. The supernatant was discarded and the remaining pellet was washed with 70% ethanol in DEPC treated water and spun at 7,500 G's for 5 minutes. The supernatant was then discarded and the pellet was air dried for 10 minutes before being reconstituted in 30 uL DEPC water. RNA was quantified by nano-drop and normalized to 2ug for reverse transcription into complementary DNA (cDNA) (Promega). Applied Biosystems 7500 System Sequence Detection Software was used for Taqman RT-PCR data analysis. Using

relative quantification (RQ), transcript levels of AKR1D1 were normalized against glyceraldehyde-3-phosphate dehydrogenase (GAPDH).

2.4 HepG2 Protein Isolation and Western Blot Analysis

HepG2 cells were washed in 1X PBS and homogenized in radioimmunoprecipitation assay (RIPA) buffer with 1% (v/v) protease inhibitors Halt and 1% (v/v) phenylmethanesulfonylfluoride (PMSF). The total cell lysate was homogenized by vigorous pipetting and collected into a 1.5 mL microcentrifuge tube to be spun at 10,000 RPM at 4°C for 10 minutes. The supernatant was collected into a clean microcentrifuge tube and quantified using a standard BSA assay. After quantification, samples were normalized to 10ug of HepG2 protein lysate. A 1:1 dilution of protein to laemilli sample buffer containing 1% (v/v) betamercaptoethanol was incubated at 95°C for 5 minutes, spun, and loaded into a 4-20% gradient SDS-PAGE gel for 1 hour at 120V. Proteins were transferred from the gel using a semi-dry transfer apparatus onto methanol-wetted PVDF membranes at 20V for 30mins. For antibody detection, membranes were blocked for 3 hours in 5% (w/v) skim milk dissolved into a 1X tris-buffered saline solution containing 0.05% Tween20 (1X TBST). Membranes were then incubated overnight at 4°C in a 1:500 dilution of primary antibody in 10mL of 5% skim milk dissolved into a 1X TBST. Membranes were probed for AKR1D1 (SantaCruz) or against GAPDH (SigmaAldrich). After overnight incubation membranes were washed 3 times in 1X TBST and incubated with a corresponding 1:2000 HRP-conjugated secondary antibody for 2 hours. Chemiluminescent signals produced by HRP enzymes were detected using enhanced chemiluminescent substrates (Biorad Clarity Western ECL Substrate) and imaged

under a Carestream Gel Logic 2200 Pro camera for 8 minutes. Expression of protein was quantified and normalized against GAPDH expression. Where possible the same blot was stripped and re-probed with different antibodies.

2.5 Pregnancy Study

Fourteen C57BL/6 female mice were bred in house and randomly separated into 3 groups at 6-8 weeks of age. Five mice were separated as control mice and the remaining mice were bred overnight to ensure consistent timing of pregnancy. For mice sacrificed during pregnancy, livers were harvested at day 17 of pregnancy. The remaining mice were sacrificed 3 days after giving birth. Livers of all 14 mice were harvested for subsequent RNA extraction.

2.6 Mouse Liver RNA Isolation and Quantitative Real-Time Polymerase Chain Reaction (RT-PCR) Analysis

In all mouse experiments, 100mg of mouse liver was homogenized in 1 mL RNA-Bee for RNA extraction. 300 uL chloroform was added to each tube, which was then shaken vigorously for 30 seconds. The samples were then incubated on ice for 5 minutes before being centrifuged at 12,000 G's for 15 minutes. The supernatant was then transferred to a clean microcentrifuge tube and 0.5 mL propanediol was added. Samples were incubated at room temperature for 5 minutes and then spun at 12,000 G's for 10 minutes. The supernatant was discarded and the remaining pellet was washed with 70% ethanol in DEPC treated water and spun at 7,500 G's for 5 minutes. The supernatant was then discarded and the pellet was air dried for 10 minutes before being reconstituted in 30 uL DEPC water. RNA was quantified by nano-drop and normalized to 2ug for reverse transcription into complementary DNA

(cDNA) (Promega). Applied Biosystems 7500 System Sequence Detection Software was used for Taqman RT-PCR data analysis. Applied Biosystems 7500 System Sequence Detection Software was used for Taqman RT-PCR data analysis. Using relative quantification (RQ), transcript levels of AKR1D1 were normalized against glyceraldehyde-3-phosphate dehydrogenase (GAPDH).

2.7 Statistical Analysis

A Student's *t*-test was applied to pairwise comparison for normally distributed data. P 0.05 or lower was considered statistically significant.

3. Results

3.1 Altered endogenous AKR1D1 expression in estrogen treated HepG2 cells.

AKR1D1 is most abundantly expressed in the liver; therefore we examined the effects of estrogen on hepatic AKR1D1 expression in HepG2 cells. HepG2 cells were seeded in phenol-red free medium supplemented with charcoal stripped FBS in order to limit exogenous estrogenic effects, and thus, false positive results. Cells were treated with estrogen and isolated for RNA and protein analysis. As seen in Figure 23A, RT-PCR data reveals that AKR1D1 showed an increased trend in expression with E2 treatment (results not significant). Protein data (Figure 23B) revealed that endogenous AKR1D1 expression was significantly increased with treatment of estrogen ($p < .01$).

3.2 Estrogen mediated transactivation of the human AKR1D1 promoter.

With confirmation that endogenous hepatic AKR1D1 expression was altered with estrogen treatment, we next investigated the activity of the AKR1D1 promoter in response to estrogen *in-vitro*. The human AKR1D1 promoter reporter, phAKR1D1 (-

5.0 kb) was transiently transfected with estrogen receptor alpha (ERα) into HepG2 cells. Twenty-four hours after transfection cells were treated with 100nM estrogen. Thirty hours after treatment, luciferase activity of AKR1D1 was quantified. As shown in Figure 24, while no significant change was observed on the AKR1D1 promoter in the absence of the ERα nuclear receptor, the presence of ERα significantly increased the expression of the human AKR1D1 promoter reporter ($p < .01$) as compared to DMSO. The results indicate the possible presence of a previously unidentified estrogen response element (ERE) on the promoter region of AKR1D1, which is resulting in the transactivation of AKR1D1 expression.

3.3 AKR1D1 is dose-dependently regulated by estrogen.

With confirmation of the ERE on the 5.0kb human AKR1D1 promoter, we next investigated the effect of various concentrations of estrogen on AKR1D1 expression *in-vitro*. HepG2 cells were co-transfected with phAKR1D1 (-5.0 kb) and ERα and treated with increasing concentrations of estrogen for 30 hours. As seen in Figure 25, AKR1D1 expression is altered in response to different concentrations of estrogen. The results indicate that AKR1D1 expression peaks at 40nM E2 and 400nM E2 and decreases with 80nM E2 and over 1ug E2 treatment *in-vitro*. While a definitive trend has not yet been established, the data suggests a highly dynamic relationship between AKR1D1 and estrogen.

3.4 Hepatic AKR1D1 Expression is Up-Regulated During Pregnancy

It is well established that AKR1D1 catalyzes the conversion of progesterone into its metabolite 5b-DHP in order to inhibit contractions and maintain normal pregnancy conditions. In late stages of pregnancy the production of 5b-DHP is

decreased which results in an initiation of contractions and ultimately, labor. To examine this phenomenon in-vivo, mice were separated into 3 groups and livers were harvested for RNA and protein before, during and after pregnancy. As seen in Figure 26A, the results indicate that transcriptionally, AKR1D1 expression is significantly increased during pregnancy by over eight fold as compared to mice that are not pregnant. The expression of AKR1D1 decreases directly after birth, presumably returning back to control level over time. Translationally, AKR1D1 exhibits a trend similar to that seen in RNA expression, however the results are not significant (Figure 26B). While the role of AKR1D1 expression during pregnancy has been documented within the uterus, our data now clearly demonstrates that AKR1D1 expression within the liver is also altered during pregnancy.

4. DISCUSSION

While AKR1D1 is necessary for the synthesis of primary bile acids it is also involved in steroid metabolism. AKR1D1 plays a vital role in the metabolism of progesterone to its metabolite, 5β-dihydroxyprogesterone (5β-DHP). The role of 5β-DHP is in prevention of uterine contractions during pregnancy. AKR1D1 expression must be maintained at elevated levels during pregnancy in order to produce sufficient concentrations of the progesterone metabolite to prevent uterine contractions prior to parturition. Towards the end of pregnancy, AKR1D1 expression dramatically declines, reducing the expression of 5β-DHP and resulting in a relaxing of the uterine muscle where contractions begin to initiate labor (Byrns, 2011; Sheehan et al., 2005). Ultimately, the relationship between estrogen and AKR1D1 expression is poorly

defined therefore mechanism by which AKR1D1 expression is increased during pregnancy and dramatically reduced prior to labor is not clearly understood.

To examine the effects of pregnancy on the expression of AKR1D1 *in-vivo*, AKR1D1 expression was measured in mice before, during and after pregnancy. Our results indicate that AKR1D1 expression is increased in mice during pregnancy and is decreased after birth *in-vivo*. After investigation of the endogenous expression of AKR1D1 in response to estrogen in-vitro, we were able to confirm our hypothesis that estrogen increases AKR1D1 expression. This would explain the increased AKR1D1 expression in our animal study as well as the increase in AKR1D1 expression necessary for a full term pregnancy. It is well known that during pregnancy estrogen levels are elevated (Pařízek et al., 2014). For the first time, our results indicate that it may be through increased estrogen levels during pregnancy that regulate the expression of AKR1D1 in order to produce 5β-DHP and prevent contractions. To further understand the transcriptional regulation of AKR1D1 by estrogen, phAKR1D1 (-5.0 kb) promoter reporter was co-transfected with ERα and treated with estrogen. For the first time we were able to reveal the possible presence of a previously un-identified estrogen response element (ERE) within the 5.0 kb AKR1D1 promoter region. Due to the fact that initial testing of the ERE on AKR1D1 in response to estrogen was under elevated concentrations of estrogen at 100nM, we next examined if the expression of AKR1D1 in response to estrogen was dose-dependent. Our results revealed a highly complex and dynamic relationship between AKR1D1 and estrogen. While even the smallest concentrations of estrogen up-regulate AKR1D1 promoter activity, increased concentrations varied with altered expression. The intricate

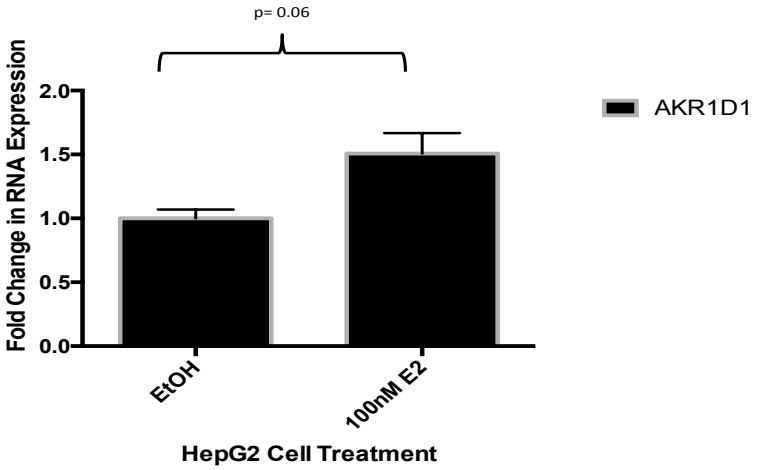
alterations in AKR1D1 expression in response to varying concentrations of estrogen paint the picture of an atypical dose response curve. The initial peak illustrated in Figure 25 may represent changes in AKR1D1 expression under physiologically normal concentrations of estrogen while the second peak may represent more pathological circumstances. Overall, the relationship between AKR1D1 and estrogen depicted in Figure 25 may correlate to the fluctuating concentrations exhibited during normal pregnancy.

It has been suggested that excessively high estrogen levels are what ultimately inhibits the conversion of progesterone to 5β-DHP by AKR1D1 (Byrns, 2011). Since our data revealed a decreased trend at high concentrations but not a decrease as compared to the control, it can be speculated that changes in AKR1D1 expression towards the end of pregnancy may be dependent on a number of other factors as well. It can be considered that any number of hormones, which are increased during pregnancy, may be competing with estrogen binding on the AKR1D1 promoter. Estrogen receptors are highly promiscuous with respect to ligand selectivity (Ng, Perkins, Tong, & Hong, 2014) and therefore the binding of a hormone which could compete with estrogen may result in a decrease of AKR1D1 expression and therefore a decrease of the 5β-DHP metabolite. Furthermore, it is possible that elevated concentrations of estrogen do, in fact, decrease AKR1D1 expression prior to parturition, however the specific element responsible for the decrease is located on a region beyond the 5.0kb region that has been cloned. Currently, further testing of additional pregnancy related hormones on an extended promoter region is necessary prior to understanding the decrease in AKR1D1 prior to parturition.

In summary, our data collectively demonstrates that AKR1D1 is transcriptionally regulated by estrogen through a previously un-identified estrogen response element within the 5.0 kb promoter region. The increase in AKR1D1 expression in response to estrogen serves to fill the gap in knowledge pertaining to the elevated AKR1D1 expression necessary to maintain pregnancy. As hormone levels are increased during pregnancy, estrogen serves to increase AKR1D1 expression in order to sufficiently catalyze the conversion of progesterone to 5β-DHP to prevent uterine contractions. With the identification of an estrogen response element on the AKR1D1 promoter and the known promiscuity of the estrogen receptor ligand selectivity, further testing on AKR1D1 expression is necessary in order to identify the mechanism by which AKR1D1 expression is substantially decreased prior to parturition. Furthermore, with enhanced understanding of the relationship between estrogen and AKR1D1, a link may be established between AKR1D1 and ICP in order to fully understand the role of AKR1D1 in pregnancy.

A

Fig. 23



B

Fig. 23

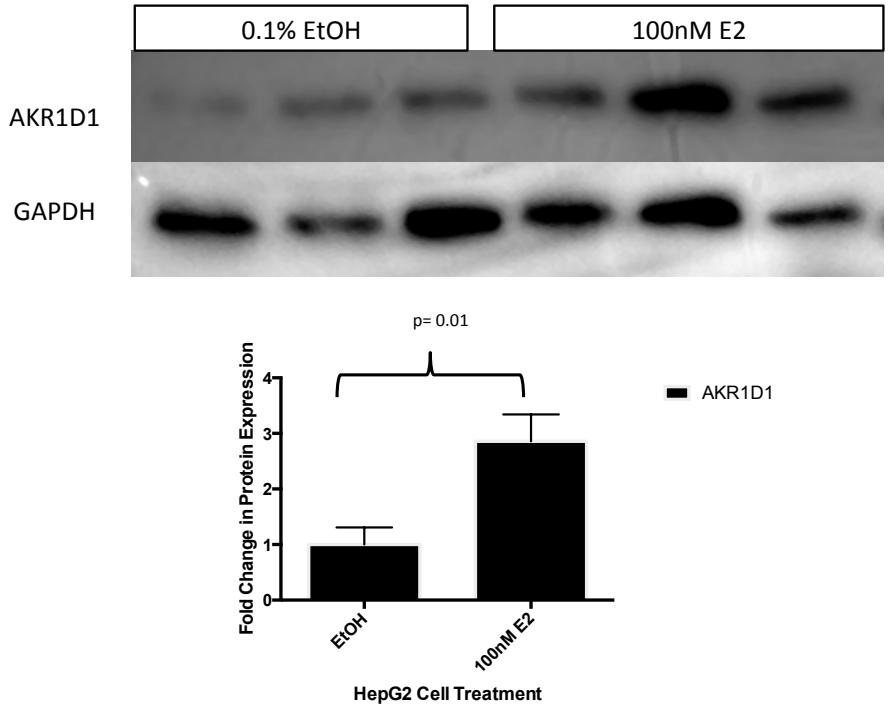


Figure 23. Estrogen up-regulates AKR1D1 expression *in-vitro* in HepG2 cells.

HepG2 cells were treated with 100uM E2 or an EtOH negative control for 30 hours prior to RNA and protein extractions. A. RT-PCR analysis of AKR1D1 expression in response to estrogen treatment. B. Western blot analysis of AKR1D1 expression in response to estrogen treatment. The data are presented as a fold change in relative expression of the mean of 3 replicates as compared to the negative control and a $p < .05$ was considered significant (Student's *t*-test).

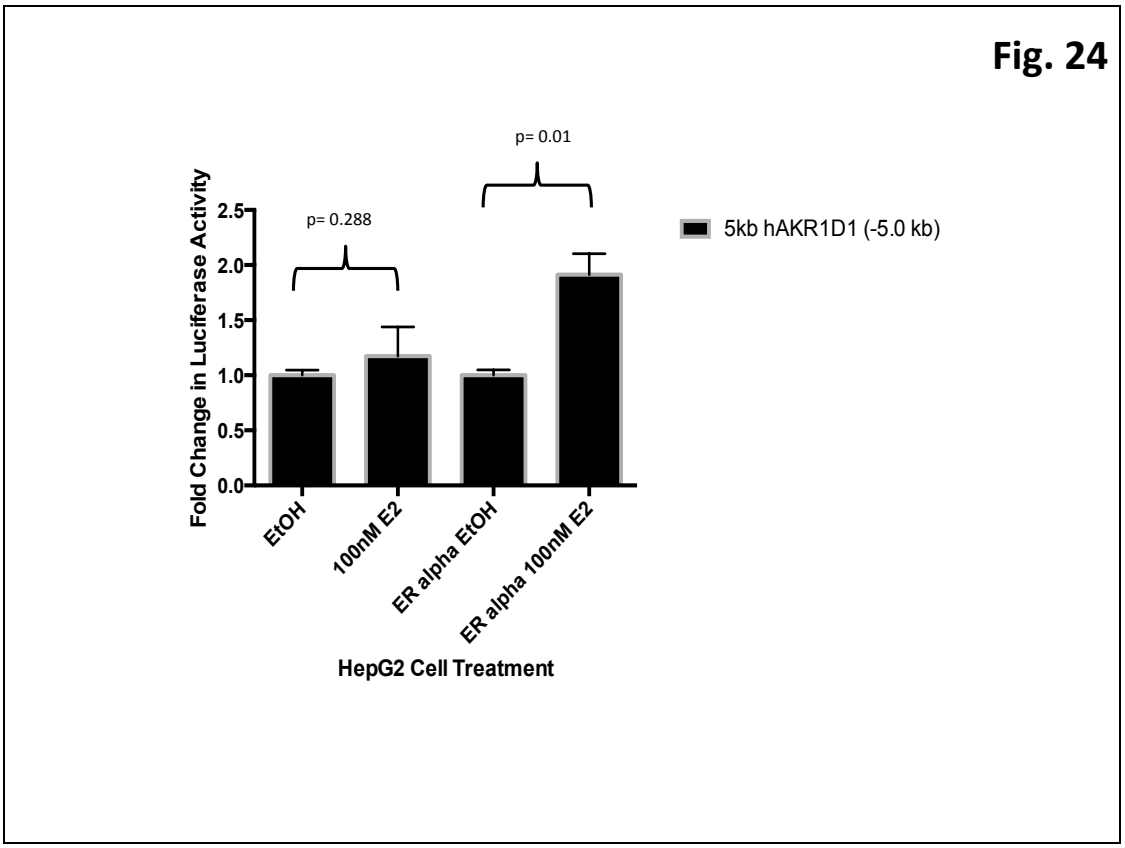


Figure 24. Alteration of human AKR1D1 promoter reporter expression with E2 treatment in HepG2 Cells with or without ERα.

HepG2 cells were transiently transfected with the human AKR1D1 promoter reporter plasmid phAKR1D1 (-5.0 kb) with or without co-transfection of ERα and treated with estrogen for 30 hours. Luciferase activation was measured by a dual-luciferase reporter assay system. The data are presented as a fold change in relative expression of the mean of 3 replicates as compared to the negative control and a $p < 0.05$ was considered significant (Student's *t*-test).

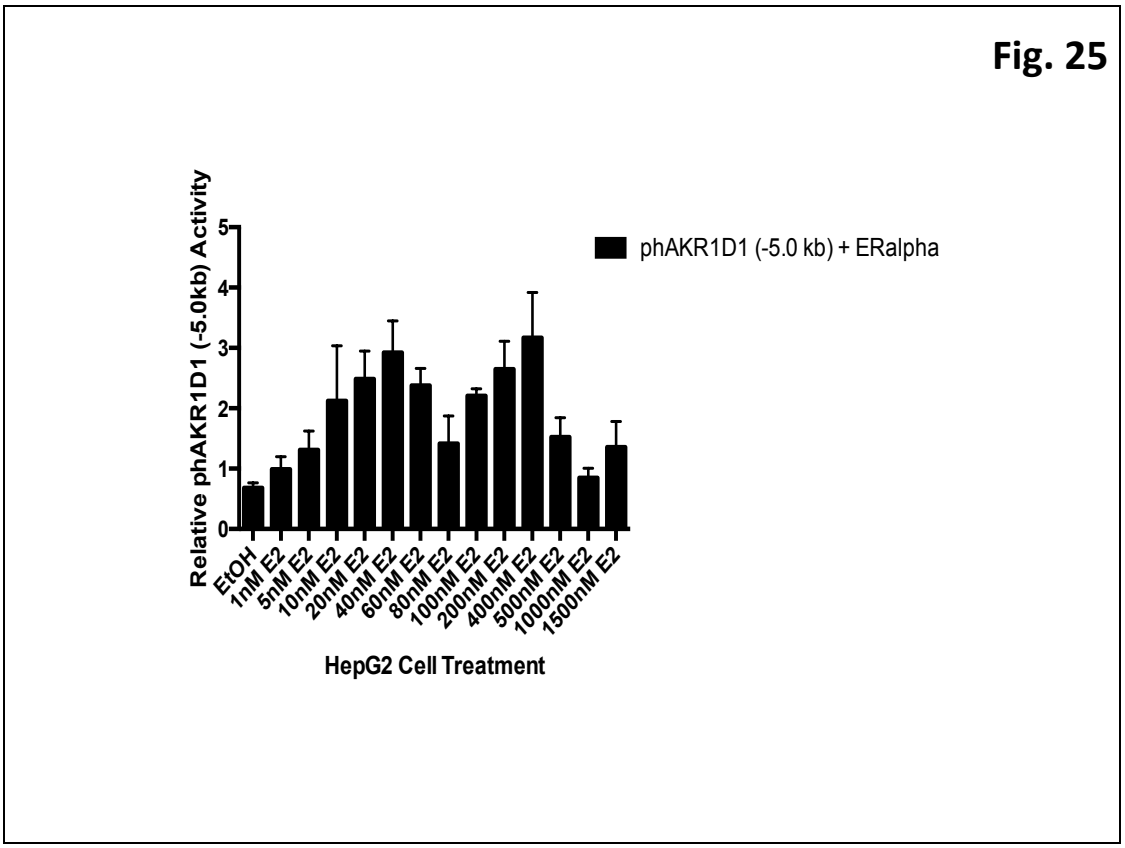
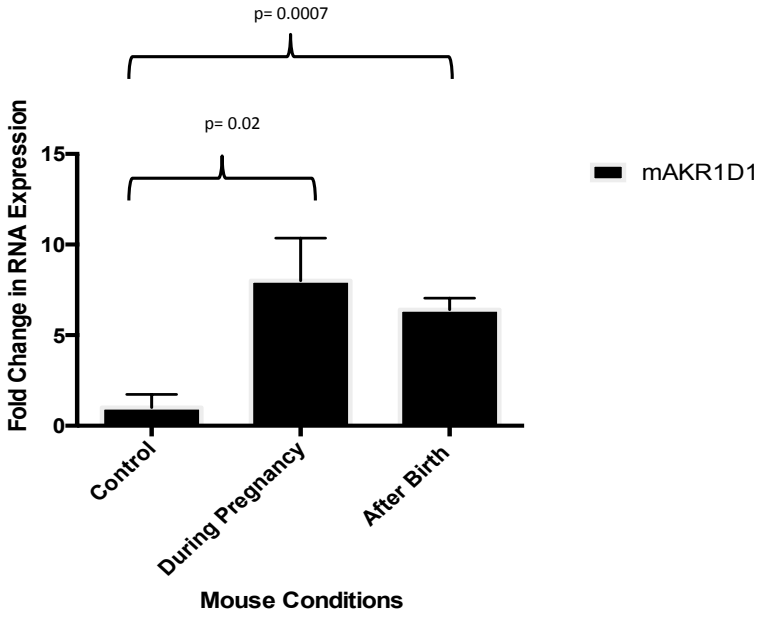


Figure 25. Alteration of human AKR1D1 promoter reporter expression with increasing E2 treatment in HepG2 Cells with or without ERα.

HepG2 cells were transiently transfected with the human AKR1D1 promoter reporter plasmid phAKR1D1 (-5.0 kb) and ERα and treated with increasing concentrations of estrogen for 30 hours. Luciferase activation was measured by a dual-luciferase reporter assay system. The data are presented as a fold change in relative expression of the mean of 3 replicates as compared to the negative control and a $p < 0.05$ was considered significant (Student's *t*-test).

A

Fig. 26



B

Fig. 26

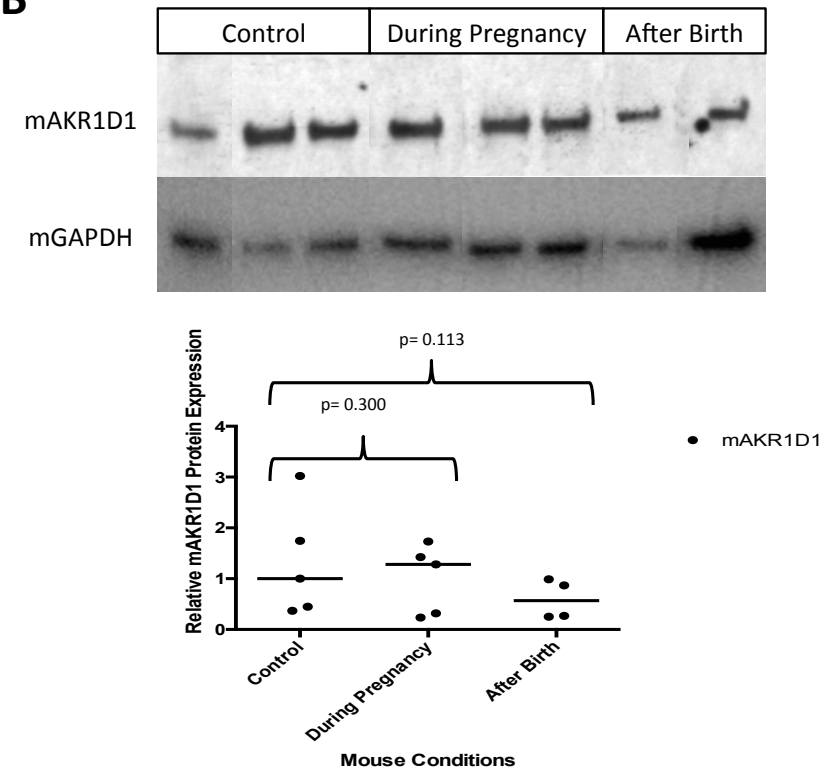


Figure 26. Altered AKR1D1 expression, before, during and after pregnancy.

Female mice were separated into 3 groups (6 mice per group) before, during and after pregnancy for liver isolation. RNA and Protein expression of AKR1D1 was measured. (A) RNA was isolated from the liver for RT-PCR quantification. AKR1D1 RNA expression was normalized against GAPDH. The data are presented as a fold change in relative expression of the mean of 5 replicates as compared to the negative control and a $p < .05$ was considered significant (student *t*-test). (B) Protein expression of endogenous AKR1D1 was detected and quantified by western blot. The data are presented as a fold change in relative expression of the mean of 5 replicates as compared to the negative control and a $p < .05$ was considered significant (Student's *t*-test).

CHAPTER 5

SUMMARY

The maintenance of cholesterol and bile acid levels are critical for the prevention of cardiovascular and metabolic diseases. Bile acids are the end product of cholesterol catabolism and serve to solubilize, transport and eliminate cholesterol, lipids and fat-soluble vitamins from the liver and intestines. Recently it has been discovered that the role of bile acids is greater than to serve as biological detergents and emulsifiers, but are also to serve as intricate signaling hormones responsible for the regulation of numerous metabolic and regulatory processes such as lipid, drug, and glucose metabolism. Impairment in the efficacy of bile acid signaling could lead to the occurrence or exacerbation of adverse metabolic disorders including obesity, diabetes, cholestasis, or liver injury, cancer and even pre-term birth in pregnant women.

One underlying trend that is seen in many bile acid related cardiovascular, metabolic and gestational disorders is an imbalance in the bile acid pool composition and size. Depending on the concentrations of individual bile acids, the total bile acid pool composition can be either hydrophobic or hydrophilic in nature. The ratio of hydrophobic to hydrophilic bile acids in the bile acid pool greatly affects rates of absorption and excretion, and therefore the concentrations must be tightly regulated. The size of the bile acid pool must also be regulated because while at physiological concentrations bile acids are not harmful to the body, due to their detergent-like properties, bile acid accumulation can be toxic. Deregulation of bile acid synthesis

could lead to changes in bile acid pool composition or size may lead to pathological consequences although little is known regarding the underlying mechanisms in physiological and pathological conditions.

The objective of these studies was to investigate the regulation of bile acid synthesis with respect to aldo-keto reductase 1D1 (AKR1D1), a 5β-reductase enzyme responsible for the synthesis of the hydrophobic primary bile acid, chenodeoxycholic acid (CDCA). We will also examined 12-alpha hydroxylase (CYP8B1), the enzyme responsible for the production of the hydrophilic bile acid, cholic acid (CA). While it is understood that AKR1D1 is responsible for the production of CDCA, and CYP8B1 is responsible for the production of CA, little is known regarding their individual regulation. Likewise, little is known regarding their roles in regards to the regulation and maintenance of the CDCA to CA composition ratio. To date, much attention has been given to CYP8B1 expression with claims that CYP8B1 is the rate-limiting enzyme for the production of either CDCA or CA. It can be argued that since AKR1D1 is responsible for the production of CDCA, and CDCA is the more toxic bile acid, which has the potential to cause or exacerbate pathological outcomes, insight into the regulation of AKR1D1 is vital. Furthermore, due to the characteristic involvement of AKR1D1 in the synthesis of steroid hormones, such as progesterone, additional understanding of the contribution of AKR1D1 in processes such as pregnancy and disorders such as intrahepatic cholestasis of pregnancy is fundamental.

Understanding the physiological mechanisms by which AKR1D1 regulates CDCA synthesis, and likewise, CDCA regulates AKR1D1 synthesis, will ultimately reveal a more in-depth understanding of the regulation of the bile acid synthesis

pathway. Additionally, in understanding the regulation of the bile acid synthesis pathway, which generates both hydrophobic and hydrophilic bile acids, novel mechanistic signaling pathways may be revealed in which the ratio of bile acid's composition may be controlled to treat and prevent cardiovascular and metabolic disorders.

In this dissertation, we first provided an overall summary characterizing the role of cholesterol and bile acid and the association between them that is essential for normal liver function. Within this summary of current knowledge pertaining to bile acid synthesis and homeostasis was an introduction of key enzymes that are involved in the formation of primary bile acids, mainly, AKR1D1 and CYP8B1 as well as certain essential nuclear receptors and signaling pathways. Next, we investigated the regulation of AKR1D1 by primary bile acids, CDCA and CA, and identified possible signaling pathways that may be associated, responsible or manipulated for such regulation. We then examined the role of AKR1D1 in conditions of metabolic disease and liver cancer and introduced findings that are related to further understanding the role of AKR1D1 in physiological and pathological conditions. Finally, we investigated the role of AKR1D1 during pregnancy.

Within the combined research is data demonstrating the role and regulation of AKR1D1 in physiological and pathological conditions ranging from bile acid synthesis, steroid metabolism and various diseases and disorders. Our data exposes various possible mechanisms by which AKR1D1 expression is regulated through bile acid and various signaling pathways. Specifically, the regulation of AKR1D1 by CDCA and CA was established. This phenomenon confirms that the liver is

consistently altering AKR1D1 expression in an effort to control the synthesis of CDCA or CA in order to maintain physiologically normal bile acid concentrations within the bile acid pool. By doing this, AKR1D1, and not CYP8B1, may be responsible for the regulation of the CDCA to CA ratio and ultimately the hydrophobicity or hydrophilicity of the bile acid pool.

Additionally, for the first time, the possible presence of various response elements were identified within the promoter region of the human AKR1D1 gene, 5kb upstream from the start of transcription. Within the promoter region exist possible response elements for PPAR, LXR and ER. Isoforms of PPAR bind the PPAR response element on the AKR1D1 promoter and under agonist activation represses the transcription of AKR1D1 resulting in a decrease of gene expression. On the other hand, LXRβ binds the LXR response element on the AKR1D1 promoter region and under activation by LXR agonist's increases the transcription of AKR1D1. Likewise, ERα binds the estrogen response element on the AKR1D1 promoter region and in response to estrogen increases AKR1D1 expression. The suggested presence of these previously un-identified response elements expose novel pathways by which AKR1D1 expression can be regulated and manipulated to control bile acid synthesis.

Moreover, the data presented in this dissertation serves to fill in gaps of knowledge pertaining to the occurrence or exacerbation of bile acid related metabolic disorders and cancer. The decrease in AKR1D1 expression that was observed in diabetic patients serves to explain the decrease in CDCA expression in diabetic livers. We propose that this decrease represents a mechanism by which the liver alters AKR1D1 expression in an effort to protect the liver from further toxicity in the

presence of CDCA and assist the liver in regaining physiological control of bile acid concentrations. Similarly, while it is known that an accumulation of bile acids can result in hepatocyte inflammation, damage and the progression of HCC, our results indicate that HCC patients presented with a decrease in AKR1D1 expression. We theorize that this decrease is a hepato-protective mechanism by which AKR1D1 expression is altered to minimize CDCA production in an already diseased liver.

Finally, our experiments examined the role of AKR1D1 in response to estrogen. Our results indicate that AKR1D1 expression is increased during pregnancy, possibly through activation of the estrogen response element located on the promoter region of AKR1D1 and decreased towards the end prior to labor. These results are consistent with the knowledge that elevated AKR1D1 expression is required in order to catalyze the production of the progesterone metabolite necessary to prevent uterine contractions. Furthermore our results uncovered the possible existence of a previously un-identified estrogen response element on the promoter region of AKR1D1. Our data demonstrates a highly complex relationship between AKR1D1 and estrogen.

In conclusion, the presented data represents a comprehensive understanding of the transcriptional regulation of the 5β-reductase enzyme, AKR1D1. Within this dissertation we expose novel mechanism and pathways by which AKR1D1 may be regulated in physiological, as well as pathological, conditions. The evidence provided suggests that the liver is consistently attempting to maintain bile acid homeostasis through altered expression of AKR1D1. Furthermore, we have laid the foundation for future manipulation of AKR1D1 expression in order to regulate the bile acid pool ratio

to therapeutically alleviate or prevent various bile acid and steroid related disorders and diseases.

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