Characterizing a Novel Ligand for the Farnesoid X Receptor using Transgenic Zebrafish

by

Selina Costa

A thesis submitted in conformity with the requirements for the degree of Master of Science
Department of Molecular Genetics
University of Toronto

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Department of Molecular Genetics
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Abstract

Nuclear receptors (NRs) are ligand activated transcription factors that are involved in variety of processes, such as metabolism, development, and reproduction. The farnesoid x receptor (FXR) is a NR that is involved in maintaining lipid homeostasis in the liver. When FXR is downregulated in the liver, diseases such as fatty liver disease can arise. Using the ligand-trap system in zebrafish, a novel ligand for FXR, 8215 Ethyl, was identified. This ligand was characterized and it was discovered that 8215 Ethyl induces FXR expression robustly and selectively in the liver at non-toxic concentrations. 8215 Ethyl is also able to induce activation of the NRs PPARγ and RORγ in zebrafish. FXR, PPARγ, and RORγ are regulated by 8215 Ethyl through the direct binding of the ligand to the ligand binding domains of these NRs. These findings indicate that 8215 Ethyl may have therapeutic potential to treat diseases associated with downregulation of FXR.
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# Table of Contents

Acknowledgments............................................................................................................................. iii

Table of Contents ............................................................................................................................... v

List of Tables ................................................................................................................................... viii

List of Figures ................................................................................................................................... ix

Chapter 1 Introduction ......................................................................................................................... 1

1 Introduction .................................................................................................................................. 1

1.1 Nuclear Receptor Structure and Function ................................................................................... 1

1.1.1 Background ............................................................................................................................... 1

1.1.2 Nuclear Receptor Structure ...................................................................................................... 2

1.1.3 Nuclear Receptors as Drug Targets .......................................................................................... 5

1.2 NRs Involved in Lipid Metabolism in the Liver ............................................................................ 5

1.2.1 Overview .................................................................................................................................. 5

1.2.2 Liver X Receptor ..................................................................................................................... 6

1.2.3 Farnesoid X Receptor ............................................................................................................. 8

1.3 Using the Ligand Trap System in Zebrafish to Identify and Characterize Novel Ligands for Human Nuclear Receptors ......................................................................................................................... 11

1.3.1 Traditional Methods of High Throughput Screening for Novel Ligands ............................... 11

1.3.2 Using Zebrafish as an In Vivo Model for HTS ....................................................................... 12

1.3.3 The Ligand-Trap System ......................................................................................................... 13

1.4 Project Objectives ....................................................................................................................... 15

Chapter 2 Methods and Results ......................................................................................................... 16

2 Methods and Results ....................................................................................................................... 16

2.1 Methods .................................................................................................................................... 16

2.1.1 Zebrafish Embryo Ligand Treatment ..................................................................................... 16

2.1.2 Quantification of GFP Signal in Zebrafish .......................................................................... 17
<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.1.3 Luciferase Reporter Assay</td>
<td>17</td>
</tr>
<tr>
<td>2.1.4 Bacterial Expression and Purification of NRs</td>
<td>18</td>
</tr>
<tr>
<td>2.1.5 SDS-Page and Coomassie Blue Staining</td>
<td>19</td>
</tr>
<tr>
<td>2.1.6 Western Blot</td>
<td>19</td>
</tr>
<tr>
<td>2.1.7 Preparation of Ligand for Mass Spectrometry Analysis</td>
<td>20</td>
</tr>
<tr>
<td>2.1.8 Liquid Chromatography Mass Spectrometry</td>
<td>21</td>
</tr>
<tr>
<td>2.2 Results Part I – LXRα Drug Screen</td>
<td>21</td>
</tr>
<tr>
<td>2.2.1 Validation: Homozygous LXRα Zebrafish Respond to Exogenously Added Ligands</td>
<td>21</td>
</tr>
<tr>
<td>2.2.2 Screening the CCBN Library for Novel LXRα Ligands</td>
<td>26</td>
</tr>
<tr>
<td>2.3 Results Part II – Characterization of a Novel FXR Ligand</td>
<td>26</td>
</tr>
<tr>
<td>2.3.1 Validation: FXR Zebrafish Respond to Exogenously Added Ligands</td>
<td>26</td>
</tr>
<tr>
<td>2.3.2 FXR Zebrafish Respond to a Newly Identified Ligand</td>
<td>28</td>
</tr>
<tr>
<td>2.3.3 8215 Ethyl May Act Tissue Selectively</td>
<td>32</td>
</tr>
<tr>
<td>2.3.4 Testing Analogous Compounds to 8215 Ethyl in FXR Zebrafish</td>
<td>36</td>
</tr>
<tr>
<td>2.3.5 Characterizing 8215 Ethyl in Mammalian Cells</td>
<td>40</td>
</tr>
<tr>
<td>2.3.6 Determining Specificity of 8215 Ethyl in Transgenic Zebrafish</td>
<td>42</td>
</tr>
<tr>
<td>2.3.7 Bacterial Expression and Purification of FXR</td>
<td>45</td>
</tr>
<tr>
<td>2.3.8 Mass Spectrometry to confirm direct binding of 8215 Ethyl to NRs</td>
<td>47</td>
</tr>
<tr>
<td>Chapter 3 Discussion and Future Directions</td>
<td>51</td>
</tr>
<tr>
<td>3 Discussion and Future Directions</td>
<td>51</td>
</tr>
<tr>
<td>3.1 Discussion</td>
<td>51</td>
</tr>
<tr>
<td>3.1.1 LXRα Screen</td>
<td>51</td>
</tr>
<tr>
<td>3.1.2 Characterization of a Novel Ligand for FXR</td>
<td>53</td>
</tr>
<tr>
<td>3.2 Future Directions</td>
<td>57</td>
</tr>
<tr>
<td>3.3 Conclusion</td>
<td>58</td>
</tr>
</tbody>
</table>
List of Tables

Chapter 2:

Table 2-1: Summary of analogous compounds tested…………………………………………...38
List of Figures

Chapter 1:

Figure 1-1: Basic Structure of a Nuclear Receptor.................................................................4
Figure 1-2: Basic Structure of the NR DNA Binding Domain..................................................4
Figure 1-3: The Ligand-Trap System.........................................................................................14

Chapter 2:

Figure 2-1: LXR\(\alpha\) transgenic zebrafish respond to exogenously added ligand....................23
Figure 2-2: GFP expression in LXR\(\alpha\) embryos treated with T0901317.................................23
Figure 2-3: Transgenic LXR\(\alpha\) embryos respond to GW3965................................................24
Figure 2-4: Transgenic LXR\(\alpha\) zebrafish developmental time course.....................................25
Figure 2-5: FXR transgenic zebrafish respond to exogenously added ligand............................27
Figure 2-6: 8215 Ethyl is structurally unrelated to OCA..........................................................29
Figure 2-7: Transgenic FXR zebrafish respond to a novel ligand, 8215 Ethyl.............................29
Figure 2-8: Dose response curve of GFP expression in FXR embryos treated with agonists......30
Figure 2-9: Toxicity of OCA and 8215 Ethyl in zebrafish embryos...........................................31
Figure 2-10: Comparison of proportion of embryos with GFP+ livers in different treatments...33
Figure 2-11: More embryos treated with 8215 Ethyl have liver specific responses...............34
Figure 2-12: GFP expression in the liver is brighter in embryos treated with 8215 Ethyl..........35
Figure 2-13: Side group changes for structure-activity relationship study...............................37
Figure 2-14: 8215 Ethyl is the best agonist for FXR activation in zebrafish...............................37
Figure 2-15: Western blot confirming FXR expression in HepG2 Cells.................................41
Figure 2-16: HepG2 luciferase reporter assay.................................................................41
Figure 2-17: PPARγ zebrafish embryos respond to 8215 Ethyl........................................43
Figure 2-18: RORγ zebrafish embryos respond to 8215 Ethyl........................................43
Figure 2-19: LXRα zebrafish embryos do not respond to 8215 Ethyl..............................44
Figure 2-20: Confirmation of FXR protein purification....................................................46
Figure 2-21: LC-MS spectra for OCA and 8215 Ethyl....................................................49
Figure 2-22: LC-MS spectra for samples extracted from OCA treated NRs....................50
Figure 2-23: LC-MS spectra for samples extracted from 8215 Ethyl treated NRs............50
Chapter 1
Introduction

1 Introduction

1.1 Nuclear Receptor Structure and Function

1.1.1 Background

Nuclear receptors are ligand inducible transcription factors that are involved in a wide breadth of roles, such as development, metabolism, reproduction, and cellular differentiation (Aranda & Pascual, 2001). There are 48 NRs in humans, approximately half of which have identified endogenous ligands. The other half are considered “orphan” NRs, as no natural ligand has been identified (Gronemeyer et al., 2004). NRs bind to specific sequences in the 5' regulatory sequence of target genes, called hormone response elements (HREs) (Aranda & Pascual, 2001). HREs are typically found in enhancer elements far from the transcriptional start site and consist of consensus sequences of 5-6 nucleotides in length separated by a variable spacer sequence (Kumar & Thompson, 1999; Sever & Glass 2013). These consensus sequences can be arranged individually, or as inverted or direct repeats. The combination of the sequence, orientation, and spacing between repeats, as well as surrounding features, leads to specificity of NR binding (Pardee et al. 2011). The activity of NRs is regulated through the binding of small molecules to the ligand binding domain (LBD). Once bound by ligand, the NR undergoes a conformational change which encourages the recruitment of coregulators, which can induce or inhibit the transcription of target genes (Aranda & Pascual, 2001). Coregulators can also influence the tissue specific actions of NRs (Gallastegui et al. 2013).

There are four classes of NRs that are defined based on their dimerization and DNA binding properties (Mangelsdorf et al., 1995; Sever & Glass, 2013). Class I NRs are the steroid hormone receptors. Receptors in this class are cytoplasmic and homodimerize once bound by ligand. Homodimerization exposes the nuclear localization signal and the NRs enter the nucleus and bind to specific HREs to induce transcription of target genes. The HREs of class I NRs are generally organized as inverted repeats, allowing for the DNA binding domain of both receptors that are part of the homodimer to bind to the DNA.
Class II NRs are generally nuclear and found as heterodimers with the retinoid-X-receptor (RXR) (Mangelsdorf et al., 1995; Sever & Glass, 2013). When no ligand is present, the heterodimer binds to the HRE complexed with corepressors, repressing expression of target genes. Once ligand-bound, a conformational change leads to the dissociation of corepressors, which are replaced by coactivators. The HRE of class II NRs are generally organized as direct repeats to allow for the DNA binding domain of both receptors that make up the heterodimer to bind the DNA.

Class III NRs function similarly to class I receptors, but bind to HREs organized as direct repeats (Mangelsdorf et al., 1995; Sever & Glass, 2013). Class IV receptors act as monomers and bind DNA at half-site HREs. Class III and IV NRs are less understood, and comprise the majority of orphan NRs.

1.1.2 Nuclear Receptor Structure

NRs have a highly conserved modular structure containing an N-terminal domain (NTD), DNA binding domain (DBD), hinge region, and ligand binding domain (LBD). Each region is distinct and can act independently of the others (Bain et al., 2007) (Figure 1-1).

The NTD is the least conserved region in terms of both size and sequence (Krust et al., 1986). This region contains a ligand independent activation domain (AF-1) which is involved in transcriptional activation of target genes (Bain et al. 2007). The AF-1 can also act synergistically with the AF-2 (see below) of the LBD, further increasing transcriptional activation (Takimoto et al., 2003). The NTD is usually highly disordered but is able to form secondary and tertiary structures in the presence of interacting molecules such as coregulators, other transcription factors, and general transcriptional machinery (Bain et al., 2007).
The DBD is the most highly conserved region of NRs and is responsible for binding to HREs in target genes (Bain et al., 2007) (Figure 1-2). The DBD is made up of 2 zinc finger domains that are stabilized by zinc atoms coordinating with 4 cysteine residues. There are also 2 alpha helices which play a role in binding. The N-terminal helix interacts directly with the HRE in an area called the P-box, while the C-terminal helix is positioned perpendicularly and helps maintain DBD stability. Interactions between the P-box and the HRE lead to conformational changes in the D-box, located in the C-terminal zinc finger (Bauman et al., 1993). This allows for homodimerization of the DBD in class I NRs. In class II NRs that heterodimerize with RXR, a third C-terminal helix called the C-terminal extension (CTE) unfolds to reveal the T-box and A-box (Holmbeck et al., 1998; Bain et al., 2007). The T-box is required for heterodimerization and the A-box binds to DNA, further increasing affinity and stability.

As implied, the hinge region is a flexible and variable length amino acid sequence that connects the DBD to the LBD (Bain et al., 2007). It also tends to have phosphorylation sites that increase NR transactivation (Knotts et al., 2001; Lee et al., 2006).

The LBD contains the ligand binding pocket where ligands bind. This binding generally results in a conformational change that influences the binding of coregulators (Bain et al., 2007). The LBD usually contains 12 alpha helices that are arranged in 3 anti-parallel layers (Li et al., 2003). Helices 3, 7, and 10 form the ligand binding pocket with the ligand sitting behind helix 3 and in front of helices 7 and 10. The shape, size, hydrophobicity, and specific accessible residues of the ligand binding pocket determine ligand specificity for a given NR. The LBD also contains a ligand dependent AF-2, which is important in the transactivation of target genes (Bain et al., 2007). In the presence of ligand, several of the helices, including helix 12, form a hydrophobic groove, which is the structural component of the AF-2 (Bourguet et al., 1995). When the receptor is not ligand bound, helix 12 is positioned away from the LBD, allowing corepressors to bind. This binding of corepressors sterically blocks helix 12 from moving to the active position, preventing formation of the hydrophobic groove (Bain et al. 2007). Once ligand-bound, there is a conformational change in the LBD that brings helix 12 into position, which disrupts corepressor binding and allows for coactivators to bind to the AF-2 (Shiau et al., 1998).
Figure 1.1. Basic structure of a nuclear receptor. Nuclear receptors are modular proteins that contain independently functioning domains. These domains are the N-terminal domain, DNA binding domain, hinge region, and ligand binding domain.

Figure 1-2. Basic structure of the NR DNA binding domain. The DBD of a nuclear receptor consists of two zinc finger domains and 2 helices. The D-box is located within the second zinc finger domain. Some NRs also contain a third helix called the C-terminal extension (CTE) that allows for heterodimerization of the protein. Figure adapted from Bain et al., 2007.
1.1.3 Nuclear Receptors as Drug Targets

NRs are ideal drug targets because they are naturally regulated by small hydrophobic molecules that are the typical size of drugs (Huang et al. 2010). NRs also control many systemic pathways, allowing a variety of different diseases to be targeted. NRs can be targeted by agonists, which generally increase transactivation, or antagonists, which block transactivation. The high level of NR involvement in many central pathways also presents a challenge in targeting NRs with novel drugs, as the modulation of NRs can impact many genes in many different pathways, some of which may lead to negative side effects. It is therefore important to find ligands that selectively modulate a subset of desirable genes, or in a subset of tissues, thereby leading to fewer side effects.

The selective estrogen receptor modulators (SERMS) are an example of a class of drugs that can selectively modulate estrogen receptor alpha (ERα) in a subset of tissues (Nilsson et al. 2005). Some SERMS act as agonists in some tissues (eg. skeletal tissue), while acting as an antagonist in others (eg. breast tissue). One of the first SERMS developed was tamoxifen, which has been used to treat breast cancer as it blocks the binding of estrogen to ERα in breast tissue, while preventing undesirable side effects elsewhere, such as decreases in bone mineral density. This class of drugs is also useful for treating other diseases, including post-menopausal osteoporosis, and infertility. Selectively acting ligands for other NRs, such as the PPARs and AR, have also been identified, indicating that other NRs should also be amenable to selective modulation (Chen 2008).

1.2 NRs Involved in Lipid Metabolism in the Liver

1.2.1 Overview

NRs play a major role in a variety of processes that are central in healthy liver function (Wagner et al., 2011). Of these processes, one of the most important is maintaining energy homeostasis through lipid metabolism (Tanaka et al., 2017). The NR1 family of NRs, which include the peroxisome proliferator-associated receptors (PPARs), liver x receptors (LXRs), farnesoid x
receptor (FXR), constitutive androgen receptor (CAR), and pregnane x receptor (PXR), play important roles in nutrient control with central roles in hepatic lipid metabolism (Evans & Mangelsdorf, 2014). Lipid metabolism can be broken down into two states: fasting and fed (Tanaka et al., 2017). In the fasting state, fatty acids (FA) are used as an energy source. In the liver, FAs bind to PPARα and encourage transcription of genes related to breaking down FAs into energy. In the fed state, FXR, LXRα, PPARβ/δ and PPARγ are involved in absorbing nutrients from food in the gut and distributing these to peripheral tissues. Bile acids bind to FXR in the intestine, which promotes nutrient absorption, while hepatic FXR is important for lipoprotein clearance. Excess cholesterol is removed from the liver using reverse cholesterol transport which is under control of LXRα and FXR. After eating, PXR and CAR are involved in removing toxic dietary metabolites, including excess lipid. Dysregulation of these NRs can lead to changes in lipid homeostasis, which can then lead to the formation, or progression, of liver disease (Wagner et al., 2011).

1.2.2 Liver X Receptor

1.2.2.1 Overview

The liver x receptor (LXR) exists in two isoforms in humans: LXRα and LXRβ. LXRα is expressed highly in the liver, kidney, intestine, and macrophages, while LXRβ is ubiquitously expressed (Shinar et al., 1994; Willey et al., 1995). LXRα is a class II NR as it forms a heterodimer with RXR to activate transcription of downstream genes (Willy et al., 1995). The LXR response element (LXRE) consists of the core sequence AGGTCA separated by 4 nucleotides in direct repeats, described as a DR4 RE. Natural ligands for LXRα include several oxysterols, which are oxidized derivatives of cholesterol (Peet et al., 1998). The most potent of these for LXRα are 24(S)-hydroxycholesterol, 22(R)-hydroxycholesterol, and 24(S), 25-epoxycholesterol.

LXRα acts as a cholesterol sensor as it targets genes that are intimately linked to the reverse cholesterol transport (RCT) pathway (Hong & Tontonoz, 2014). RCT is a process where excess cholesterol in peripheral tissues is returned to the liver by high density lipoproteins (HDL) for
excretion in the bile. Target genes of LXRα include the ABC family of transporters, apolipoprotein E (APOE), lipoprotein remodeling enzymes, sterol regulatory element-binding protein 1 (SREBP-1c), and cholesterol 7 alpha-hydroxylase (CYP7A1) (Peet et al., 1998; Repa et al., 2000; Repa et al., 2002; Laffitte et al., 2001; Laffitte et al., 2003). The RCT pathway begins when ABCA1 and ABCG1 actively transport cholesterol out of peripheral tissues, such as macrophages (Hong & Tontonoz, 2014). The cholesterol is accepted by HDL, which transports it back to the liver. The cholesterol is actively taken up by the liver, and then is either secreted by ABCG5 or ABCG8, or converted into bile acids by CYP7A1. Bile acids are secreted to the bile duct and then either removed via the intestine, or recycled back to the liver. Mice that lack the LXRα gene have increased plasma cholesterol levels, as well as a buildup of cholesterol in the liver (Peet et al., 1998). They also have enlarged livers, which eventually leads to liver failure. There was also a gross dysregulation of important regulatory proteins involved in sterol and fatty acid synthesis, such as SREBP-1c. Finally, there was a downregulation in CYP7A1, leading to a decrease in bile acid synthesis. Taken together, these studies have shown that LXRα is important in regulating cholesterol homeostasis.

1.2.2.2 Diseases Associated with LXRα

Atherosclerosis is characterized by an accumulation of cholesterol-loaded macrophages along artery walls (Hong & Tontonoz, 2014). This disease is associated with the downregulation of LXRα in macrophages, which prevents them from ridding themselves of excess cholesterol, leading to the development of atherosclerotic plaques in the artery. In mouse models of atherosclerosis (LDLR−/− and apoE−/− mice), oral administration of a synthetic LXRα agonist, GW3965, not only stops the progression of atherosclerotic plaques, but also reverses them (Joseph et al., 2002). The macrophages in these mice had increased ABCA1 and ABCG1 mRNA levels compared to vehicle treated control mice, suggesting that LXRα upregulation leads to an increase in these transporters, allowing the macrophages to continue RCT as normal.
Although LXRα appears to be an ideal drug target for the treatment of atherosclerosis, there may be some undesirable side effects when using LXRα agonists. Wildtype mice treated with synthetic LXRα agonist T0901317 had increases in plasma triglyceride and phospholipid levels caused by the induction of hepatic SREBP-1c (Schultz et al., 2000). It is possible that identifying a ligand that targets LXRα selectively in peripheral tissues like macrophages, without targeting the liver, may be the best option for the treatment of atherosclerosis (Hong & Tontonoz, 2014). This was demonstrated in mouse models of atherosclerosis that were treated with ATI-111 (Peng et al., 2011). In these mice, there was a high activation of LXRα target genes in the intestine and macrophages, while these same target genes were only slightly increased in the liver. Compared to vehicle treated mice, these mice did not have an increase in liver lipid levels, plasma cholesterol and triglyceride levels were decreased, and their atherosclerotic lesions were reduced. Although ATI-111 appears to be a promising LXRα agonist, there have not been any further studies to determine the therapeutic potential of this compound. However, this indicates that selective LXRα agonists could be developed as therapeutic compounds for atherosclerosis.

1.2.3 Farnesoid X Receptor

1.2.3.1 Overview

The farnesoid x receptor (FXR) is a bile acid sensor that contributes to cholesterol homeostasis (Wang et al. 1999). The natural ligands of FXR include chenodeoxycholic acid (CDCA), lithocholic acid (LCA), and deoxycholic acid (DCA) (Makishima et al., 1999; Parks et al., 1999; Wang et al., 1999). FXR is a class II NR which forms a heterodimer with RXR to induce transcription of target genes (Forman et al., 1995). The FXR response element (FXRE) is made up of 2 core sites that are separated by 1 nucleotide arranged in inverted repeats (IR1 RE). FXR is expressed in tissues where bile acid transport and synthesis occurs, including the liver and intestine (Forman et al., 1995).

The main role of FXR is to act as a bile acid sensor in the liver (Wang et al., 1999). This is achieved through the indirect inhibition of CYP7A1 expression, which is the first and rate limiting enzyme in bile acid synthesis (Goodwin et al., 2000). FXR directly induces SHP-1
expression, which represses LRH-1 activation of CYP7A1, leading to an overall decrease in bile acid synthesis. The ability of FXR to regulate bile acid levels through the use of a negative feedback loop is important as high bile acid levels can be toxic to cells as it causes mitochondrial damage and necrosis (Jiao et al. 2015). FXR also inhibits the de novo synthesis of triglycerides in the liver through indirect inhibition of SREBP-1c (Watanabe et al., 2004). Similar to the CYP7A1 bile acid synthesis pathway, FXR directly induces SHP-1 expression, which inhibits LRH-1 activation of SREBP-1c, leading to a decrease in hepatic triglyceride levels. Both of these pathways act in opposition to LXRα regulated pathways, suggesting that FXR and LXRα work together to maintain cholesterol homeostasis (Goodwin et al., 2000; Watanabe et al., 2004). FXR also directly inhibits the activity of LXRα in the presence of FXR ligands, further decreasing SREBP-1c and CYP7A1 expression (Wang et al., 1999). Therefore, lipid homeostasis is a delicate balance between LXRα and FXR activities. FXR also has a role in bile acid transport. In the intestine, FXR induces expression of the intestinal bile acid binding protein (I-BABP), which is involved in the active transport of bile acids from the intestine back to the liver (Makishima et al., 1999).

The role of FXR as a bile acid sensor is apparent in mice that lack FXR (Sinal et al. 2000). These mice were fed a diet that consisted of 1% cholic acid to mimic a high cholesterol diet. FXR null mice fed a high cholesterol diet had severe hepatotoxicity with large lipid vacuoles in the liver. These mice also had larger livers and their hepatic triglyceride and cholesterol levels were higher than wild type mice fed the same diet. The livers of these mice had an upregulation of CYP7A1 and a downregulation of SHP. FXR null mice fed a control (not cholic acid supplemented) diet had elevated serum cholesterol, phospholipid, and triglyceride levels. This indicates that FXR has a central role in lipid homeostasis.

1.2.3.2 Diseases Associated with FXR

Non-alcoholic fatty liver disease (NAFLD) is characterized by an accumulation of triglycerides in hepatocytes in the absence of alcohol consumption, viral infection, or other causes of liver disease (Jiao et al. 2014). The severity of NAFLD can range from simple steatosis, to
steatohepatitis, to more severe cases of cirrhosis, hepatocellular carcinoma, and liver failure. The cause of NAFLD is not completely understood, but it appears to be a result of dysfunctional FXR regulation as a result of external stresses (Jiao et al., 2014). In cases of NAFLD associated with obesity, chronic inflammation of the liver leads to an upregulation of Yin Yang 1 (YY1) by inflammatory cytokines (Lu et al., 2014). YY1 inhibits the transcription of FXR by binding to intron 1, leading to a downregulation of FXR in the liver. Overall, this leads to liver steatosis and NAFLD. Obese mice with hepatic YY1 overexpression have enlarged livers and increased triglyceride levels, as well as steatohepatitis (Lu et al., 2014).

Endoplasmic reticulum (ER) stress caused by ageing is also implicated in the development of NAFLD (Xiong et al., 2014). In response to ER stress, the transcription factor hepatocyte nuclear factor 1-alpha (HNF1α) is downregulated. HNF1α has a binding site in the promoter of FXR and acts as a transcriptional activator. Therefore, this downregulation of HNF1α due to ER stress leads to a downregulation of FXR. Old mice have decreased FXR expression and increased hepatic bile acid levels compared to young and middle aged mice (Xiong et al., 2014). When these old mice are treated with the ER stress repressor 4-phenyl butyric acid (PBA), they have increased FXR expression in the liver. Treating young mice with tunicamycin, which induces ER stress, also leads to an old mouse phenotype of decreased FXR expression and increased hepatic triglyceride levels. Old mice also had decreased binding affinity of HNF1α for the FXR promoter as compared to young mice, leading to a downregulation of FXR in the liver. These results suggest that factors such as obesity and ageing can lead to downregulation of FXR, which in turn leads to hepatic steatosis. It is therefore logical that upregulating hepatic FXR expression might reverse these effects, preventing or reversing hepatic steatosis, and therefore NAFLD.

Agonists have been developed for FXR that have clinical potential. One of these agonists is obeticholic acid (OCA) (Pellicciari et al., 2002). OCA was derived from the structure of the natural FXR ligand CDCA and has potent and selective FXR activity. It has been shown that OCA treatment in a rat model of cholestasis leads to decreased liver injury and increased bile flow, indicating that OCA may have therapeutic potential. OCA has been tested in a phase III clinical trial in patients with NAFLD (Neuschwander-Tetri et al., 2015). In this trial, patients
with NAFLD and developing non-alcoholic steatohepatitis (NASH) were randomized to receive either 25mg daily OCA or placebo. A higher percentage of patients in the OCA treatment group had improved liver histology, including decreased fibrosis, hepatocellular ballooning, steatosis, and lobular formation. Serum liver enzyme biochemistry, an indicator of liver function, was also improved with OCA treatment. However, there were also adverse side effects that were associated with OCA treatment. A large proportion of patients in the OCA group had severe pruritus. OCA treatment also led to an increase in serum cholesterol and a decrease in HDL. In some patients, this increase was concerning and lipid lowering drugs were used in combination with OCA to reduce cholesterol levels. However, some patients had persistent and unchanging increases in cholesterol levels and had to discontinue OCA treatment. There was also no increase in the number of patients in the OCA group who were “cured” of NASH. Although clinical trials have demonstrated that OCA could potentially be used therapeutically for the treatment of NAFLD, this is not without side effects. Activation of FXR blocks the conversion of cholesterol to bile acids, which could increase serum and liver cholesterol levels. FXR activation may also promote RCT out of tissues, into the serum.

It has been demonstrated that OCA is also an agonist for the G protein-coupled bile acid receptor (GPBAR1) (Festa et al., 2017). This receptor is implicated in some of the side effects of OCA, including pruritus (Alemi et al., 2013) and cholesterol gallstone formation due to increased cholesterol levels (Vassileva et al., 2006). It has been proposed that using an FXR specific agonist, such as a derivative of OCA, or identification of a structurally unrelated FXR agonist, could lead to better therapeutic outcomes in patients with NAFLD (Festa et al., 2017).

1.3 Using the Ligand Trap System in Zebrafish to Identify and Characterize Novel Ligands for Human Nuclear Receptors

1.3.1 Traditional Methods of High Throughput Screening for Novel Ligands

High throughput screening (HTS) is typically used to identify novel ligands of NRs from a large number of compounds (Rauncy & Lasker, 2010). To qualify as HTS, a system must demonstrate robustness, sensitivity, specificity, and reproducibility in identifying novel ligands with speed
and confidence. The most common methods of HTS are via in vitro assays. In general, these assays use transient transfection of a receptor and a reporter gene, such as luciferase, with an HRE in the reporter gene promoter. These assays allow for the identification of NR ligand binding and transactivation. However, due to metabolic inactivation in vivo, failure to reach target tissues, or off target toxicity, a large number of compounds discovered using in vitro screens fail when tested in humans (Delvicchio et al., 2011). These types of systems do not predict the in vivo activity of a ligand in terms of delivery, stability, and tissue- or cell type-specificity (Tiefenbach et al., 2010). These screens also only test ligands in a single cell type, ignoring tissue-specific interactions, ligands, cofactors, and conditions. Therefore, using an in vivo model for HTS of ligands for NRs would be beneficial for the identification of novel ligands that are stable, bioavailable, non-toxic and tissue-selective.

1.3.2 Using Zebrafish as an In Vivo Model for HTS

The zebrafish (Danio rerio) has become an attractive model for HTS (Delvicchio et al., 2011). Zebrafish embryos are small, transparent, and develop externally, allowing for development to be observed, and fluorescent reporters to be used. Large numbers of embryos are easily obtainable as each mating pair can produce 100-300 eggs per day. Zebrafish are also DMSO tolerant and can absorb compounds from the surrounding water, allowing for easy drug treatment. NRs and their cofactors are also highly conserved between zebrafish and humans. Wildtype zebrafish can be used in many different stages of drug discovery and development. They can be used for toxicology studies to predict if compounds will be toxic in humans (Eimon & Rubinstein, 2009). The use of morpholino and CRISPR knockouts in zebrafish can also be used to confirm drug targets (Ito et al., 2010). Zebrafish are also good for following up on drug hits by doing structure-activity relationship studies to identify better or less toxic compounds related to an original hit (Delvicchio et al., 2011). Using transgenic zebrafish that contain fluorescent reporters can further increase screening output, specificity, and complexity.
1.3.3 The Ligand-Trap System

Our lab has developed a zebrafish HTS platform referred to as the ligand-trap (LT) system that allows chemical library screens to identify novel ligands for NRs (Tiefenbach et al., 2010) (Figure 1-3). The ligand-trap system is a binary expression system which uses a bacterial LexA DBD and corresponding ColE1 promoter driving GFP expression. A zebrafish heat shock promoter (hsp70) leads to heat-inducible expression of a LexA DBD-human NR LBD fusion protein. When ligand-bound, and in the presence of necessary cofactors, this fusion protein drives eGFP expression. The system also features a positive amplification loop in the form of an additional ColE1 promoter site that drives expression of a LexA-Gal4 fusion protein, which then also drives eGFP expression. This allows for increased duration and intensity of GFP expression. The fusion protein also contains a triple affinity tag that can be used to purify the protein from responsive fish tissues for further analysis of interacting small molecules and protein cofactors.

An advantage of this system is that the transgene is ubiquitously expressed, allowing for GFP expression to be seen only where the fusion protein is being bound by active ligands and cofactors. This system also allows us to detect responses to compounds that act more spatially or temporally specific. It has been demonstrated that in zebrafish, this system able to respond to exogenously added ligands in a dose dependent manner, and demonstrates tissue specificity in response to different ligands, making it an ideal system for HTS of novel ligands for NRs (Tiefenbach et al., 2010). Tissue specificity in response to different ligands may arise from many mechanisms, including differential transport of ligand into tissues, differing metabolism of tissues, or cell-specific conditions or cofactors.
Figure 1-3. The ligand-trap system. Heat shock induces transcription of a LexA DBD-human NR LBD fusion protein which drives eGFP expression. The fusion protein can be purified using a triple affinity tag. The construct also contains a positive feedback system which allows for GFP to be expressed brighter, and for longer.
1.4 Project Objectives

My first project objective was to perform a drug screen using transgenic zebrafish embryos to identify novel ligands for LXRα. 3580 compounds were screened, but no promising hits came out of the screen. My second project objective was to characterize a novel ligand for FXR that was previously identified by our lab. I first characterized the activity, specificity, and toxicity of this novel ligand in transgenic FXR LT zebrafish. I also characterized the activity of the novel ligand in a mammalian cell culture system. The ability of this novel ligand to directly bind to FXR and other NRs was also determined using liquid chromatography mass spectrometry.

This project will help to further validate the use of an in vivo zebrafish model and the ligand-trap system to identify and characterize novel ligands for NRs. The characterization of a novel FXR ligand will lead to a new research tool for studying the biology of FXR. A novel ligand for FXR may also have therapeutic potential and could lead to a new treatment option for diseases associated with downregulation of FXR.
2 Methods and Results

2.1 Methods

2.1.1 Zebrafish Embryo Ligand Treatment

2.1.1.1 LXRα Drug Screen

Homozygous zebrafish containing human LXRα and screening conditions were established by Dr. Michael Long. LXRα zebrafish were in-crossed and embryos were collected and maintained at 28.5°C on a 12/12-hour light/dark cycle. Embryos 2 days post fertilization (dpf) were dechorionated manually and arrayed with 3-5 embryos per well in 96 well plates (Corning microplates, #3651). Embryos were treated with compounds from a Canadian Chemical Biology Network (CCBN) library at a concentration of 10μM in 1% DMSO and 200μL fish water (0.075g/L NaHCO3, 0.018g/L sea salt, 0.0084g/L CaSO4 2H2O). 1% DMSO and 2.5μM T0901317 (Cayman Chemicals, #293754-55-9) were included as controls. Embryos were pre-incubated in the compounds for 1 hour at 28.5°C in the dark before heat shock in a water bath at 37°C for 45 minutes. After heat shock, embryos were left for 16 hours at 28.5°C in the dark. Images were taken using an isocytate laser scanning cytometer (Molecular Devices ImageXpress Velos), and were manually analyzed for GFP expression. Higher magnification images were taken using a SteREO Lumar V12 fluorescence microscope (Zeiss) and Northern Eclipse software.

2.1.1.2 FXR Embryo Treatments

Zebrafish containing human FXR were established by Dr. Jason Burgess. FXR zebrafish were outcrossed to wildtype zebrafish and embryos were collected and maintained as described above. Embryos were arrayed in 6 well plates (Sarstedt, 83.3920.005) and treated with 8215 Ethyl (Enamine, Z21528215) in 1% DMSO and 3ml fish water. 1% DMSO and 10μM OCA were used as controls (ApexBio B4888). Embryos were pre-incubated in compound for 30 minutes at 28.5°C in the dark before heat shock in a water bath at 37°C for 30 minutes. Embryos were then
left for 16 hours at 28.5°C in the dark. GFP expression was analyzed using images from the Isocye and an AxioZoom V16 fluorescent microscope (Zeiss).

2.1.2 Quantification of GFP Signal in Zebrafish

GFP expression levels were initially quantified using images from the laser scanner. Embryos were traced in ImageJ and the integrated density was measured as relative light units. To quantify GFP expression in LXRα embryos, the entire embryo was traced. To quantify GFP expression in FXR embryos, the head and body, excluding the yolk sac was traced.

2.1.3 Luciferase Reporter Assay

HepG2 cells were maintained in DMEM (Wisent, 319-005-CL) containing 10% FBS (Sigma-Aldrich, F1051-500ML) and 2% penicillin-streptomycin (Sigma-Aldrich, P4333-100ML). Cells were seeded in 48-well plates (Starstedt, 83.3923.005) and grown to ~80% confluence. Each well was transfected with 125ng UAS-Luciferase, 12.5ng pSL9-Renilla Luciferase, and 50ng CMV-Gal4-hFXR or CMV-Gal4 using 1.3μl lipofectamine 2000 (Invitrogen, 11668019) in serum-free DMEM. Media was changed after 24 hours to media that contained 0.1% DMSO, OCA, or 8215 Ethyl. Each ligand treatment was performed in triplicate. Cells were lysed (Promega cell culture lysis buffer, E1500) and incubated for 5 minutes at room temperature on a shaker. The plate was centrifuged at 1500rpm at 4°C for 2 minutes to remove cellular debris. Lysates were arrayed into white-bottomed 96-well plates (Corning, 3922). Luciferase and renilla luciferase activities were measured using a dial-luciferase reporter assay system (Promega, E1910) and Renilla luciferase assay system (Promega, E2810) using a luminometer (EG&G Berthold microplate luminometer LB96V microlumat plus).
2.1.4 Bacterial Expression and Purification of NRs

2.1.4.1 Bacterial Expression of FXR

The human FXR ligand binding domain was cloned from the ligand-trap MN24 vector into the p15TV-L vector using NdeI and XhoI restriction enzymes (Chun Hu). BL21-codon plus E. coli were transformed and grown in 40ml Luria-Bertani (LB) broth (BioShop, LBL405.1) with 100μg/ml ampicillin (BioShop, AMP201.5) overnight. Starter culture was transferred to 1L Terrific Broth (BioShop, TER409.1) with 100μg/ml ampicillin at 37°C at 200rpm until OD$_{600}$=~1. Protein expression was induced by adding 0.5mM isopropyl β-D-1-thiogalactopyranoside (IPTG) (BioShop, IPT001.5) and the temperature was decreased to 20°C. The cell culture was grown for an additional 6 hours. Cells were harvested by centrifugation at 7000rpm for 10 minutes at 4°C. Media was removed and cell pellets were stored overnight at -20°C.

2.1.4.2 Bacterial Expression of LXRα

The human LXRα ligand binding domain was cloned from the ligand-trap MN24 vector into the p15TV-L vector using AseI and XhoI restriction enzymes. BL21-codon plus E. coli were transformed and grown in 40ml LB broth overnight. Starter culture was transferred to 1L terrific broth with ampicillin at 37°C at 200rpm until OD$_{600}$=~1. Protein expression was induced by adding 0.5mM IPTG and the temperature was decreased to 22°C. The culture was grown for an additional 16 hours. Cells were harvested by centrifugation at 7000rpm for 10 minutes at 4°C. Media was removed and cell pellets were lysed immediately.

2.1.4.3 Protein Purification

Cell pellets were resuspended in lysis buffer (500mM NaCl, 5% glycerol, 10mM Tris pH 7.5, 5mM imidazole, 0.5% CHAPS, 1 SIGMAFAST protease inhibitor cocktail tablet EDTA-free) and then sonicated (10 seconds on, 10 seconds off, 5 minutes, 30% amplitude) to ensure full cell lysis. Lysate was centrifuged at 7000rpm for 10 minutes at 4°C and the supernatant was removed for purification. 1ml Ni-NTA resin (Qiagen, #30210) was loaded into a 100ml column and pre-
equilibrated with 10ml binding buffer (500mM NaCl, 5% glycerol, 10mM Tris pH 7.5, 5mM imidazole). Lysate was loaded into the column, and flow through was collected. The column was washed with 50ml wash buffer (500mM NaCl, 5% glycerol, 10mM Tris pH 7.5, 30mM imidazole) and then protein was eluted batch wise using 4-6 successive 1ml elutions (500mM NaCl, 5% glycerol, 10mM Tris pH 7.5, 250mM imidazole). Protein concentration was determined using Bradford reagent (Sigma-Aldrich, B6916). Protein was flash frozen in liquid nitrogen and then stored at -80°C. RORγ and PPARγ proteins were expressed and purified by Jiabao Liu.

2.1.5 SDS-Page and Coomassie Blue Staining

Samples from the pellet, lysate, flow-through, wash, and elution fractions were prepared by adding 4X SDS gel-loading buffer (200mM Tris pH 6.8, 400mM DTT, 8% SDS, 0.4% bromophenol blue, 40% glycerol) and boiling for 5 minutes. Samples were run on a 12% SDS-Page gel (5ml resolving gel: 1.6ml H2O, 2.0ml 30% acrylamide mix, 1.3ml 1.5M Tris pH 8.8, 50μl 10% SDS, 50μl 10% ammonium persulfate, 2μl TEMED; 1ml stacking gel: 680μl H2O, 170μl 30% acrylamide mix, 130μl 1M Tris pH 6.8, 10μl 10% SDS, 10μl 10% ammonium persulfate, 1μl TEMED) for 20 minutes at 120V, followed by 30 minutes at 200V. The gel was placed in a glass container and covered with water, then microwaved for 10 seconds 3 times, replacing the water each time. Water was then replaced by Coomassie blue stain (80mg Coomassie brilliant blue, 3ml HCl, 1L H2O) and microwaved for 10 seconds. The gel was incubated on a shaker for 30 minutes at room temperature and then replaced with destaining solution (45% methanol, 10% acetic acid in water) overnight.

2.1.6 Western Blot

Samples from the lysate, flow through, wash, and elution fractions were prepared and run on a 12% SDS-PAGE gel as described above, and then transferred to a membrane at 95V for 1 hour. The membrane was blocked with 5% skim milk powder (BioShop, SKI400.250) in PBS for 5 minutes, followed by anti-FLAG primary antibody (1:10000) (Sigma-Aldrich, F3165) overnight at 4°C. The membrane was washed 3X 30 minutes in PBS and then incubated with anti-mouse
secondary antibody (1:20000) (GE Healthcare, NA931) for 1 hour at room temperature. Antibody signal was detected using super signal west dura extended duration substrate (ThermoFisher, 34075).

2.1.7 Preparation of Ligand for Mass Spectrometry Analysis

2.1.7.1 Protein-Ligand Treatment

Purified FXR, LXRα, RORγ, and PPARγ proteins were treated with a 1:10 protein:ligand molar ratio of OCA or 8215 Ethyl. The protein-ligand mixtures were incubated for 1 hour at 4°C and then centrifuged at 12000rpm for 2 minutes to remove any denatured protein.

2.1.7.2 Desalting

A PD-10 desalting column (GE Healthcare, 17085101) was equilibrated with 25ml water, and samples passed through. The protein-ligand complexes were eluted 1ml at a time using PBS. The elution fraction with the highest concentration of protein as determined by Bradford reagent was used.

2.1.7.3 Phenol-Chloroform Extraction

A 2:1 chloroform:methanol mixture was made in an amber vial. The ligand sample was added, vortexed, and incubated at room temperature for 15 minutes. 1.25ml of both chloroform and PBS were added, vortexed, and then centrifuged for 10 minutes at 1500rpm at 4°C. The upper (aqueous) phase was removed and this was repeated once. 1ml of pre-equilibrated chloroform, methanol, and PBS (1:1:0.8) was added and centrifuged as above. The lower (organic) phase was transferred to a new amber vial and evaporated using a rotary evaporator. The dried sample was stored at -20°C and resuspended in methanol prior to LC-MS analysis.
2.1.8 Liquid Chromatography Mass Spectrometry

Dried samples were resuspended in 100μl methanol and filtered through a 0.22mm filter (Sigma-Aldrich, M9535). Liquid chromatography-mass spectrometry (LC-MS) was performed by biozone (Centre for Applied Bioscience and Bioengineering at University of Toronto). Sample was injected into a Thermo Scientific 23 Q-Exactive MS coupled with a Dionex Ultimate 3000 U HPLC. A phenyl-hexyl column was used for LC separation (Agilent Zorbax Eclipse Plus Phenyl-Hexyl 3.0x50mm 1.8micron). OCA and 8215 were eluted using 75% methanol in water with 0.1% formic acid (solvent A) and isopropanol (solvent B) at a flow rate of 200μl/minute. The following conditions were used to detect OCA and 8215 Ethyl: 70,000 resolution in positive and negative ionization mode, 300-500m/z, 320°C capillary temperature.

2.2 Results Part I – LXRα Drug Screen

2.2.1 Validation: Homozygous LXRα Zebrafish Respond to Exogenously Added Ligands

Homozygous transgenic LXRα zebrafish were previously established by Dr. Michael Long. This transgenic line contains the MN24 ligand trap vector with the LXRα LBD. I first validated that these fish respond to exogenously added ligand in a consistent manner that would be appropriate for HTS. LXRα zebrafish were treated with increasing concentrations of the known agonist T0901317 (figure 2-1). GFP can be detected in this fish line at 1μM as scattered dots in the yolk sac and the gills. At 2.5μM, GFP expression can be seen in the hindbrain, forebrain, gills, and epithelial tissue. When treated with 5μM and higher, GFP expression is stronger in these same tissues. The response of LXRα embryos to T0901317 at concentrations of 0.1-75μM was quantified and plotted to create a dose response curve (figure 2-2). GFP expression increased between 0.1-5μM where the maximum response was reached. The EC50 of T0901317 in this fish line is 2.5μM. To validate that the LXRα fish line responds to multiple LXRα agonists, fish were also treated with GW3965 (figure 2-3). Fish responded to this compound when treated at 5μM, with expression seen in the mouth and gills. The signal increased as the concentration increased, and at 20μM expression could also be seen in the brain and epithelial tissue.
To determine the optimal developmental time point for screening the LXRα fish line, embryos were treated every 24 hours with 10μM T0901317 (figure 2-4). Embryos treated prior to 2dpf did not survive for GFP analysis. DMSO treated embryos at all stages did not have a GFP response. Embryos treated at 2dpf had a strong response to T0901317 in the brain, heart, and epithelium. Treatment at 3 and 4 dpf had a weaker response in these tissues. At 5dpf, GFP expression was only seen as a few dots in the head. When treated at 6 and 7 dpf GFP expression was not detected. It appears that 2dpf is an appropriate time point for screening as it produced the brightest GFP response to T0901317 treatment. Although LXRα is expressed in the liver, it is not beneficial to wait until liver development is complete, as GFP expression was not seen at these stages.
Figure 2-1. LXRα transgenic zebrafish respond to exogenously added ligand. Transgenic LXRα zebrafish respond to a known LXRα agonist T0901317 in a dose dependent manner.

Figure 2-2. GFP expression in LXRα embryos when treated with T0901317. Transgenic LXRα embryos were exposed to increasing concentrations of T0901317. GFP response increases until the maximum response is reached at 5μM. The EC50 of T0901317 in this fish line is 2.5μM. Error bars indicate variation between individual embryos in a single treatment.
Figure 2-3. Transgenic LXRα embryos respond to GW3965. GFP expression is seen at concentrations of 5μM GW3965 and higher. At 5μM and 10μM, GFP expression is seen in the mouth and gills. At 20μM, expression is stronger and seen in the brain and epithelial tissue as well. Fluorescence seen in the yolk is due to non-specific light absorption/emission.
Figure 2-4. Transgenic LXRα zebrafish developmental time course. Transgenic LXRα zebrafish were treated with 10μM T0901317 every 24 hours between 2dpf-7dpf. Embryos treated at 2dpf have a strong response to T0901317 in the brain, heart, and epithelium.
2.2.2 Screening the CCBN Library for Novel LXRα Ligands

The CCBN library contains 3580 compounds that include FDA approved drugs, natural compounds, pharmacologically active drugs, and drugs involved in clinical trials. The library was screened using 2dpf LXRα embryos using the conditions used to validate the fish line. All compounds were screened and 0 compounds were identified to be LXRα agonists.

2.3 Results Part II – Characterization of a Novel FXR Ligand

2.3.1 Validation: FXR Zebrafish Respond to Exogenously Added Ligands

Transgenic FXR zebrafish were previously established by Dr. Jason Burgess and Chun Hu. The transgenic fish contain the MN24 ligand trap vector containing the FXR LBD. I first validated that the FXR fish could respond to exogenously added FXR agonists. Transgenic FXR fish were treated with obeticholic acid (OCA), which is a known agonist of FXR (figure 2-5). Embryos treated with 5µM OCA responded primarily in the brain, and occasionally in the heart, epithelium, and liver. At 10µM, this response becomes brighter in the same tissues. This GFP expression confirms that the transgenic FXR zebrafish can respond to exogenously added human FXR ligands. However, due to heterogeneity of LT insert positions and numbers in this line, the embryos do not have a consistent pattern or level of GFP expression when treated with the same concentration of ligand, so large numbers of embryos need to be used to draw conclusions.
Figure 2-5. FXR transgenic zebrafish respond to exogenously added ligand. Transgenic FXR embryos were treated with 5μM and 10μM OCA. These embryos responded at 5μM with GFP expression seen in the brain, heart, liver, and epithelium. Embryos treated with 10μM OCA had stronger expression in these same tissues.
2.3.2 FXR Zebrafish Respond to a Newly Identified Ligand

FXR LT fish screening was conducted by Dr. Jason Burgess. In this screen, a novel ligand for FXR, 8215 Ethyl, was identified (figure 2-6). This compound is structurally unrelated to OCA, which is a derivative of the natural FXR ligand CDCA. To characterize 8215 Ethyl, transgenic FXR zebrafish were treated with increasing concentrations. When treated with 5μM, embryos exhibit GFP expression primarily in the liver, and occasionally in the brain and epithelium (figure 2-7). Embryos treated with 10μM have a brighter response, and GFP expression is seen primarily in the brain, liver, and epithelium. The level of expression when zebrafish were treated from 1-100μM OCA or 8215 Ethyl was quantified and a dose response curve was constructed (figure 2-8). Embryos treated with OCA begin to respond at 1μM, and reach the maximum response at 25μM. Embryos treated with 8215 Ethyl begin to respond at 5μM and reach the maximum response at 10μM. The EC₅₀ of both 8215 Ethyl and OCA is 5μM.

The toxicity of these two compounds was determined by counting the number of embryos that had abnormal developmental phenotypes, such as heart edema, stunted growth, or curved spines. The toxicity of 8215 Ethyl increases in a dose dependent manner, and reaches 100% toxicity at 40μM (figure 2-9). Toxicity is very low in embryos treated with OCA, as no abnormal phenotypes are seen in embryos until they are treated with 50μM. Although toxicity is high at high concentrations of 8215 Ethyl, at 5μM, where a GFP response is seen, the toxicity is lower (7%), and is not significantly higher than toxicity when treated with OCA at 5μM (p>0.05). FXR transgenic zebrafish respond to 8215 Ethyl at concentrations where it is not more toxic to the zebrafish than OCA treatment.
Figure 2-6. The chemical structures of OCA and 8215 Ethyl are structurally unrelated.

Figure 2-7. Transgenic FXR zebrafish respond to a novel ligand, 8215 Ethyl. Embryos treated with 5μM 8215 Ethyl had GFP expression in the liver, brain, and epithelium. Embryos treated with 10μM 8215 Ethyl had GFP expression in the brain, liver, heart, and epithelium.
Figure 2-8. Dose response curve of GFP expression in FXR embryos treated with agonists. Zebrafish begin to respond to OCA at 1μM and the expression increases until the maximum response is reached at 25μM. Zebrafish begin to respond to 8215 Ethyl at 5μM and the expression increases until the maximum response is reached at 10μM. The EC₅₀ of both compounds is 5μM. Error bars indicate variation between two treatments of OCA and three treatments of 8215 Ethyl.
Figure 2-9. Toxicity of OCA and 8215 Ethyl in zebrafish embryos. The toxicity of 8215 Ethyl increases rapidly as concentration increases, until it reaches 100% at 40μM. Toxicity of OCA is much lower with no abnormal phenotypes seen until 50μM. Error bars indicate variation between two treatments of OCA and three treatments of 8215 Ethyl.
2.3.3 8215 Ethyl May Act Tissue Selectively

It was observed that embryos treated with 8215 Ethyl had more GFP expression in the liver compared to embryos treated with OCA (figure 2-10). 55% and 43% of GFP+ embryos had GFP expression in the liver when treated with 5μM and 10μM 8215 Ethyl, respectively. When treated with 5μM and 10μM OCA, 24% and 26% of GFP+ embryos had GFP expression in the livers, respectively. Although the proportion of embryos with liver GFP expression is higher in embryos treated with 8215 Ethyl, it was not significantly higher. However, 8215 Ethyl did lead to a significant increase in embryos that had liver-only GFP responses (figure 2-11). When treated with 5μM 8215 Ethyl, 45% of GFP+ embryos had a liver-only response. This GFP expression becomes broader when treated with 10μM 8215 Ethyl, as only 13% of GFP+ embryos had a liver specific response. Embryos treated with 5μM and 10μM OCA do not have liver-only responses. The proportion of embryos that had a liver specific response when treated with 5μM 8215 Ethyl was significantly higher than in any other treatment group (p<0.01). To further characterize the liver responses, embryos were treated with 1μM-50μM 8215 Ethyl and OCA, and GFP expression in the liver was quantified to create a dose response curve (figure 2-12). At 5μM and higher, 8215 Ethyl had a brighter GFP response in the liver compared to OCA treatment. This suggests that 8215 Ethyl may be able to specifically target the liver at lower, less toxic, concentrations, while OCA targets the embryo more broadly.
Figure 2-10. Comparison of proportion of embryos with GFP+ livers at different treatment concentrations. The percentage of embryos with GFP expression in the liver is slightly increased in the 5μM 8215 Ethyl treatment group. However, this is not a significant increase. Error bars indicate variation of embryos within a single treatment group.
Figure 2-11. More embryos treated with 8215 Ethyl have liver specific responses. A) An example of an embryo treated with 5μM 8215 Ethyl with liver specific expression. B) Significantly more embryos treated with 5μM 8215 Ethyl had liver specific responses than in any other treatment group (p<0.01). Error bars indicate variation of embryos within a single treatment group.
Figure 2-12. GFP expression in the liver is brighter in embryos treated with 8215 Ethyl. At 5μM and higher, GFP expression in the liver is brighter when treated with 8215 Ethyl compared to OCA. Error bars indicate variation between two treatments of OCA and three treatments of 8215 Ethyl. Trend line is predictive only between 1μM and 5μM.
2.3.4 Testing Analogous Compounds to 8215 Ethyl in FXR Zebrafish

To determine whether 8215 Ethyl activity could be improved, or toxicity could be reduced, structure-activity relationship studies were done using 2dpf embryos. These embryos were treated with compounds that had changes to 2 possible side chains, but kept the same backbone structure as 8215 Ethyl (figure 2-13). In total, 7 analogous compounds were tested (table 2-1). Four of these compounds (0070 Allyl, 0111 Backbone, 6719 Methyl, and 0072 No side chain) led to a GFP response in transgenic FXR embryos. The other three compounds led to no GFP response. 8215 Ethyl and 0072 No side chain were the only compounds that led to a significant increase in GFP expression over DMSO treated embryos (figure 2-14). However, 0072 No side chain is highly lethal in zebrafish, with 51% of embryos dying before GFP analysis could be completed, and 100% of surviving embryos having abnormal developmental phenotypes. It was determined that 8215 Ethyl is the best agonist of the analogous compounds tested. It was also concluded that the ethyl group on side chain A is important for the robust activation of FXR.
Figure 2-13. Side group changes for structure-activity relationship study. Changes to 8215 Ethyl occurred at side chains A, B, or both, while the backbone structure remained the same.

Figure 2-14. 8215 Ethyl is the best agonist for FXR activation in zebrafish. GFP expression in the 8215 Ethyl group is significantly higher than GFP expression in any other group except 0072 No side chain (p<0.05). However, 0072 No side chain is highly lethal in zebrafish. Error bars indicate variation between individual embryos in a single treatment group.
<table>
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<th>Compound Structure</th>
<th>GFP Expression</th>
<th>Transgenic FXR Zebrafish</th>
<th>Comments</th>
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<td>++</td>
<td><img src="image2" alt="Image" /></td>
<td>GFP expression in brain, liver, epithelium</td>
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<td>GFP expression in eye, epithelium</td>
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<tr>
<td>6719 Methyl</td>
<td><img src="image7" alt="Structure Image" /></td>
<td>+</td>
<td><img src="image8" alt="Image" /></td>
<td>GFP expression in epithelium</td>
</tr>
</tbody>
</table>
| 0072 No Side Chain | ![Structure Image](image9) | ++             | ![Image](image10)        | GFP expression in eye  
High lethality (51%) |
| 0041 No Ester | ![Structure Image](image11) | -              | ![Image](image12)        |                     |
| 9248 Methyl Carboxyl | ![Structure Image](image13) | -              | ![Image](image14)        |                     |
| 7121 Butyl | - | ![Image](image.png) |

**Table 2-1. Summary of analogous compounds tested.** Seven compounds with the same backbone structure as 8215 Ethyl were tested in transgenic FXR embryos.

++ = significantly increased GFP expression, + = GFP expression, - = No GFP expression.
2.3.5 Characterizing 8215 Ethyl in Mammalian Cells

Luciferase reporter assays using HepG2 cells were done to see if 8215 Ethyl also functions in mammalian cells. Cells were co-transfected with CMV-Gal4-hFXR LBD, UAS-Luciferase, and pSL9-Renilla luciferase and then treated with increasing concentrations of 8215 Ethyl and OCA. Western blot analysis confirmed expression of the CMV-Gal4-hFXR LBD fusion protein (figure 2-15).

HepG2 cells begin responding to OCA at 250nM, and this response increases in a dose dependent manner until the maximum response is reached at 7.5μM (figure 2-16). The EC\textsubscript{50} of OCA in HepG2 cells using this assay is 2.5μM. When HepG2 cells are treated with 8215 Ethyl, there is no measurable increase in luciferase reporter activity. To ensure that 8215 Ethyl was not simply binding serum proteins preventing it from entering the cells, the luciferase reporter assay was repeated by treating the cells with OCA and 8215 Ethyl in media that did not contain FBS (figure 2-16). 8215 Ethyl failed to induce luciferase activity in this assay as well, while OCA did lead to a dose dependent increase. This indicates that 8215 Ethyl is inactive in this assay using these cellular conditions.
Figure 2-15. **Western blot confirming FXR expression in HepG2 Cells.** HepG2 cells were transfected with Gal4-FXR LBD and then lysed. Anti-FLAG antibody was used to detect FXR protein expression.

Figure 2-16. **HepG2 luciferase reporter assay.** A) Dose response curve of HepG2 cells treated with increasing concentrations of 8215 Ethyl and OCA. Cells treated with OCA demonstrated a dose dependent increase in luciferase activity with an EC$_{50}$ of 2.5μM. Cells treated with 8215 Ethyl did not demonstrate an increase in luciferase activity at any concentration. B) Relative luciferase activity in HepG2 cells when drug treatment was done in serum-free media. There is a dose dependent increase of luciferase activity in OCA treated cells, but not in 8215 Ethyl treated cells. Error bars indicate variation between three replicates of the same treatment.
2.3.6 Determining Specificity of 8215 Ethyl in Transgenic Zebrafish

To determine if 8215 Ethyl activates FXR selectively, PPARγ, RORγ, and LXRα transgenic zebrafish lines were also tested. PPARγ and RORγ embryos were treated using the same protocol as FXR embryos, as optimized by Dr. Jiabao Liu.

Somewhat unexpectedly, PPARγ and RORγ embryos treated with 10μM 8215 Ethyl both exhibit increased GFP expression compared to DMSO treated embryos. PPARγ embryos had weak expression in epithelial tissue (figure 2-17). This expression is not as bright as treatment with the PPARγ agonist rosiglitazone, which leads to broad epithelial GFP expression. Vehicle treated RORγ embryos have basal GFP expression in the hindbrain and heart. Treatment of RORγ embryos with 8215 Ethyl leads to an increase of basal GFP expression in the brain, heart, and epithelial tissue (figure 2-18). This increase appears to be greater than the increase induced by the known RORγ agonist OBT. LXRα embryos treated with 8215 Ethyl do not have any GFP expression (figure 2-19). This suggests that 8215 Ethyl is an agonist for not only FXR, but also PPARγ and RORγ.
**Figure 2-17. PPARγ embryos respond to 8215 Ethyl.** Vehicle treated PPARγ embryos do not have any GFP expression. When treated with 8215 Ethyl, these embryos have weak GFP expression in the epithelial tissue. Embryos treated with PPARγ agonist rosiglitazone have broad epithelial GFP response that is brighter than 8215 Ethyl treated embryos. Thus, 8215 Ethyl either has relatively low affinity for PPARγ, or is only a partial agonist.

**Figure 2-18. RORγ embryos respond to 8215 Ethyl.** Vehicle treated RORγ embryos have basal GFP expression in the brain and heart. Treatment with 8215 Ethyl expands this GFP expression to the rest of the head and the epithelial tissue. This increase in GFP expression appears to be better than the GFP increase seen when RORγ embryos are treated with the known RORγ agonist OBT.
Figure 2-19. LXRα embryos do not respond to 8215 Ethyl. Vehicle treated LXRα embryos do not have GFP expression. Embryos treated with 8215 ethyl also do not have any GFP expression. Treatment with the LXR agonist T0901317 leads to GFP expression in the brain.
2.3.7 Bacterial Expression and Purification of FXR

To confirm that 8215 acts as an FXR agonist by binding to the ligand binding domain, FXR LBD protein was expressed and purified. *E. coli* cells were used as a host for protein expression by transforming cells with a p15TV-L vector containing the FXR LBD. This vector allows for IPTG inducible protein expression and contains a 5’ 6X-His tag that allows for protein purification. Protein expression conditions were optimized based on the protocol used by Downes *et al.* (2013). Protein expression and successful purification was confirmed by Western blot and SDS-PAGE (figure 2-20). Once FXR protein was successfully purified, it was used to determine whether 8215 Ethyl was binding directly to FXR.
Figure 2-20. Confirmation of FXR protein purification. A) SDS PAGE gel showing purified FXR protein. B) Western blot confirming the eluted protein is FXR.

IN = input, FT = flow through, W = wash, E = elution
2.3.8 Mass Spectrometry to confirm direct binding of 8215 Ethyl to NRs

To determine whether 8215 Ethyl was binding directly to the FXR LBD in zebrafish, purified protein was incubated with 8215 Ethyl. The unbound ligand was removed from the sample, and the protein-ligand complex was then denatured in chloroform/methanol, and the ligand extracted with chloroform. The spectra of pure OCA and 8215 Ethyl were first profiled by injecting 1mM of each compound into the mass spectrometer. LC-MS analysis in negative ionization mode detected a peak corresponding to the mass of OCA (419.3180Da) and associated hydrogen adducts ([M-H+]\(^+\)) (figure 2-21). The extracted ion chromatograph (OCA, 419.3180[M-H+]\(^+\)) has a peak corresponding to a retention time of 7.25 minutes. LC-MS analysis in positive ionization mode detected a peak corresponding to the mass of 8215 Ethyl (355.1120 Da) and associated hydrogen adducts ([M+H+]\(^+\)) (figure 2-21). The extracted ion chromatograph (8215 Ethyl, 355.1120 [M+H+]\(^+\)) has a peak corresponding to a retention time of 7.27 minutes.

LC-MS analysis of extract from OCA treatment of FXR protein detected a peak in negative ionization mode at 419.3179, corresponding to the mass of OCA (figure 2-22). The extracted ion chromatograph (OCA, 419.3180[M-H+]\(^+\)) shows a peak with a retention time of 7.19 minutes. Analysis of the extract from OCA treated PPARγ, RORγ, and LXRα protein did not detect a peak corresponding to this mass and the extracted ion chromatograph does not show a peak. This indicates that OCA binds FXR directly and selectively.

LC-MS analysis of extract from 8215 Ethyl treatment of FXR protein detected a peak in the positive ionization mode at 355.1114, corresponding to the expected mass of 8215 Ethyl (figure 2-23). The extracted ion chromatogram (8215 Ethyl, 255.1122[M+H+]\(^+\)) has a retention time of 7.32 minutes. Analysis of extract from 8215 Ethyl treatment of PPARγ and RORγ also detected a peak corresponding to this mass (355.1109 and 355.1116, respectively). The extracted ion chromatograph of these samples showed peak retention times at 7.31 and 7.36 for RORγ and PPARγ, respectively. Analysis of extract from LXRα protein treated with 8215 Ethyl did not detect a peak corresponding to the correct mass, and the extracted ion chromatograph did not
have a peak. These results indicate that 8215 Ethyl binds to FXR, RORγ, and PPARγ, but does not bind to LXRα.
Figure 2-21. LC-MS spectra for OCA and 8215 Ethyl. A) LC-MS analysis under negative ionization mode. \( m/z \) values for the hydrogen adduct ([M-H]+) of OCA. B) Extracted chromatogram from OCA, 419.3180 [M-H]+. C) LC-MS analysis under positive ionization mode. \( m/z \) values for the hydrogen adduct ([M+H]+) of 8215 Ethyl. D) Extracted chromatogram from 8215 Ethyl, 355.1120 [M+H]⁺.
Figure 2-22. LC-MS spectra for samples extracted from OCA treated NRs. A) LC-MS analysis under negative ionization mode. m/z values for the hydrogen adduct ([M-H]+) of OCA. B) Extracted chromatogram from OCA, 419.3180 [M-H]+. In both A) and B) a peak is detected in extract from treated FXR protein, but not from treated LXRα, RORγ, or PPARγ protein.

Figure 2-23. LC-MS spectra from samples extracted from 8215 Ethyl treated NRs. A) LC-MS analysis under positive ionization mode. m/z values for the hydrogen adduct ([M+H]+) of 8215 Ethyl. B) Extracted chromatogram from 8215 Ethyl, 355.1122[M+H]+. In both A) and B) a peak is detected in extract from treated FXR, RORγ, and PPARγ protein, but not from treated LXRα protein.
Chapter 3
Discussion and Future Directions

3 Discussion and Future Directions

3.1 Discussion

3.1.1 LXRα Screen

3.1.1.1 Optimization of the LXRα Screen

To perform a drug screen using zebrafish, the screening protocol must allow for optimal GFP expression when the embryos are exposed to drug. Expression needs to be sufficiently strong and consistent for the identification of both strong and weak agonists. It was first determined that the transgenic LXRα embryos could respond to exogenously added ligands by treating embryos with a well known and potent agonist T0901317 (Schultz et al. 2000). T0901317 treatment led to robust GFP expression in the transgenic LXRα zebrafish, with an EC_{50} of 2.5µM. This is higher than the reported EC_{50} in HEK293 cells of 20nM (Schultz et al. 2000). This is likely due to the increased complexity of the zebrafish compared to cell culture. Zebrafish are multicellular and ligands must penetrate multiple cell layers to get to the tissues where they are active, compared to a cell culture which is a single cell layer. Ligands may also be deactivated at a higher rate in zebrafish due to increased metabolic requirements of zebrafish compared to single cells, meaning that the ligands are more stable in cell culture assays, and a lower concentration is needed to see a response. To ensure that transgenic LXRα zebrafish were responsive to other known agonists, embryos were also treated with the agonist GW3965, another potent LXRα agonist (Collins et al. 2002). This compound also led to GFP expression in the zebrafish, demonstrating that this fish line has the ability to respond to multiple LXRα agonists.

Many conditions were optimized prior to beginning the screen, including heat shock duration and temperature, drug pre-incubation time, and agonist concentration. Importantly, the developmental stage of screening also had to be optimized to ensure that GFP would be strongly induced, and assess tissue-selective responses. It was determined that treating embryos with compound at 2dpf and observing GFP signal at 3dpf would be best for the screen. At this stage, GFP is seen robustly in the brain, heart, and epithelium. At 3dpf, when the GFP signal is the
strongest, the zebrafish liver is in the middle of development (Ting Tao et al. 2009). The liver has completed the “budding” stage where cells differentiate and the liver is mostly formed. At 72hpf, the liver is undergoing the “growth” stage of development where it is getting larger and changing shape due to rapid cell proliferation. It is therefore possible that compounds that are active in the liver could be detected at this time point. After optimization, there was high confidence that my zebrafish line and the screening conditions were optimized to detect any novel hits that were present in the screening library.

3.1.1.2 No Novel Hits Were Identified in the LXRα Screen

The CCBN library is a chemically diverse library which contains compounds that are FDA approved, pharmacologically active, involved in chemical trials, and derived from natural sources. At the completion of the screen, 0 compounds were identified that induced GFP expression in the zebrafish. This was surprising, as prior screens with 6 other NR LT lines yielded multiple hits with this library, and LXRα is known to have a ligand binding pocket that is capable of binding compounds that differ significantly in shape and size (Schultz et al., 2000; Collins et al., 2002). However, the ligand binding pocket of LXRα only has a few available residues for hydrogen bond formation, which may limit the types of compounds that are able to properly bind (Svennson et al., 2003). It has been demonstrated that potent LXRα agonists require a single oxygen on an accessible side chain to act as a hydrogen bond acceptor, which may reduce the number of compounds that can successfully bind (Janowski et al., 1999). It is also possible that agonists were present in the library, but it was not possible to detect them. The library was screened at a relatively high concentration (10μM), and there was a lot of lethality. It is possible that any present agonists were lethal at this concentration, and that the GFP expression was not detected before embryo death. Retesting these compounds at lower sub-lethal concentrations might reveal activity. Finally, my screen was only able to detect LXRα agonists. Potential antagonists were not possible to identify as my fish line does not exhibit basal LXRα activity in response to endogenous ligands or cofactors. It may be possible to identify antagonists for LXRα if embryos were co-treated with an agonist such as T0901317 to identify compounds that could compete with T0901317 for the binding pocket and thereby decreasing the GFP signal.
3.1.2 Characterization of a Novel Ligand for FXR

3.1.2.1 FXR Screen

The FXR screen was completed previously by Dr. Jason Burgess (unpublished data). The screen was done with a library that was provided by Otava Chemicals that contained ~400 compounds computationally predicted to fit the ligand binding pocket of FXR. In this screen, the compound 0070 Allyl was identified as a ligand that could induce GFP expression weakly in transgenic FXR zebrafish. This hit was validated and structurally related compounds to 0070 Allyl were tested (Jason Burgess, unpublished data). A related compound, 8215 Ethyl, was found to induce GFP expression in FXR transgenic zebrafish in the brain and liver that appeared to be as bright as OCA treatment. This compound was chosen for follow up.

3.1.2.2 8215 Ethyl Activates FXR in the Liver of Transgenic Zebrafish

Transgenic FXR zebrafish that incorporate the ligand-trap system were used to characterize the activity of 8215 Ethyl. The pattern of expression in zebrafish treated with 8215 Ethyl differed slightly from embryos treated with OCA. 8215 Ethyl treated embryos had GFP expression primarily in the liver, and occasionally in the brain and epithelium, while OCA treated embryos had GFP expression primarily in the brain, and occasionally in the heart, epithelium, and liver. Some of the tissues that were GFP+ in the zebrafish were expected, such as the liver and the brain, as FXR is known to be expressed in these tissues in humans (Forman et al., 1995; Huang et al., 2016). However, some of the tissues that had GFP expression were unexpected, such as the heart and epithelium, as FXR is not known to be expressed in these tissues in humans or zebrafish. As the embryos are treated early in development, it is possible that FXR is expressed in these tissues early in development, and later becomes more restricted to specific tissues in adults.

The differential expression patterns in zebrafish treated with OCA and 8215 Ethyl was interesting with 8215 Ethyl treated embryos displaying an overall higher level of expression, and more tissue specific expression, in the liver. This is a promising result as it suggests that 8215 Ethyl, and perhaps other related compounds, may selectively target the liver at lower doses, thereby reducing systemic toxicity. Selectively targeting FXR to liver tissues at relatively lower
doses may help with side effects that are seen when using other FXR agonists, such as OCA. FXR is known to have a small role in metabolism and NAFLD in macrophages, adipocytes, and enterocytes, in addition to hepatocytes and hepatic stellate cells (Musso et al., 2016). The agonism of FXR in these other tissues may aid in the development of unwanted and adverse side effects. One major unwanted side effect of OCA activation of FXR is increased plasma lipid levels (Neuschwander-Tetri et al., 2015). The selective activation of FXR in the liver at a lower dose may also be beneficial in NAFLD treatment as it may also reduce the inhibition of SREPB-1c and CYP7A1, such that there is adequate inhibition of lipogenesis without major inhibition of bile acid synthesis.

Another advantage of targeting liver tissue-specifically is that there could be a reduction in activation of the G protein-coupled bile acid receptor 1 (GPBAR1). GPBAR1 is expressed in many tissues, including the liver, intestine, lungs, stomach, spleen, colon, and white adipose tissue (Maruyama et al., 2006). OCA is known to be an agonist for both FXR and GPBAR1, and GPBAR1 activation has been implicated in some of the side effects of OCA (Vassileva et al., 2006; Alemi et al., 2013; Festa et al., 2017). There is the potential that if 8215 Ethyl is active selectively in the liver at lower doses, GPBAR1 will also only become more active in the liver, which might mitigate some of the side effects that are seen with OCA treatment. It is equally possible that 8215 Ethyl has no effect on GPBAR1 in any tissue. Importantly, these results also validate that this whole animal screening approach is able to identify tissue-selective ligands (Tiefenbach et al. 2010).

Tissue specificity in response to different ligands may arise from many mechanisms. Different tissues may have different transport proteins which are able to transport ligands into tissues with differing efficiencies. Some tissues may also deactivate ligands before they have a chance to bind the LBD, or may actively transport the ligand out of the tissue. Different cell types also have different conditions and cofactors present that can affect the binding efficiency of ligands to the LBD, or affect the stability of the ligand in the tissue.
3.1.2.3 8215 Ethyl Does Not Induce FXR Activity in a Mammalian Cell Reporter Assay

Luciferase reporter assays were completed using HepG2 cells that were transfected with a Gal4-UAS luciferase binary reporter system. OCA was able to activate FXR in this system, leading to a strong induction of luciferase activity in a dose dependent manner. The EC$_{50}$ of OCA in this cell line, using this system is 2.5µM. This is considerably higher than the reported EC$_{50}$ in HuH7 cells of 85nM (Pellicciari et al., 2002). A dose dependent trend of luciferase induction was not seen when HepG2 cells were treated with 8215 Ethyl. Initially, it was thought that 8215 Ethyl may be binding to proteins in the cell culture serum, and not crossing the cell membrane. The experiment was repeated without serum in the culture media during drug treatment, but luciferase induction was still absent.

It is unknown why 8215 Ethyl induces FXR activity in zebrafish, but not HepG2 cells. One possible reason is that HepG2 cells are a hepatocellular carcinoma cell line, and are not completely indicative of all cell types or states in the liver. The liver is a fully functioning organ that is complex and made up of many different cells that have a unique microenvironment. HepG2 cells are a simplified in vitro model that is comparatively simple, and represent only a single, cancerous cell type. It is known that cellular composition differs in HepG2 cells compared to the liver (Castell et al., 2006). Many metabolic enzymes found in the liver are absent from HepG2 cells, and other enzymes are present as non-liver isozymes. Thus, the tissue-selectivity exhibited by 8215 Ethyl in zebrafish, as opposed to OCA, may reflect a preference for specific liver cell types, cofactors, or conditions. It will be important to repeat these experiments in other liver cell lines, as well as in a mammalian animal model to determine whether 8215 Ethyl induces FXR activity in a mammalian system. These results also further support the value of first screening in an in vivo model, such as zebrafish, rather than an in vitro model.

3.1.2.4 8215 Ethyl Induces PPARγ and RORγ Activity in Zebrafish

To determine whether 8215 Ethyl induces FXR activity exclusively, PPARγ, RORγ, and LXRα transgenic embryos were treated. Expression was seen in both PPARγ and RORγ embryos, but
not in LXRα embryos. PPARγ-induced GFP expression was weak and limited to epithelial tissue. PPARγ is expressed in the liver in small amounts in hepatic stellate cells, and plays a role in triglyceride homeostasis by regulating triglyceride clearance and lipogenesis (Zardi et al., 2013). The activation of PPARγ in the liver has a role in preventing liver fibrosis, which is a common characteristic of NAFLD (Galli et al., 2002). Although there are no identified ligands that are dual FXR/PPARγ agonists, the PPARγ ligand binding pocket is large, and there is a lot of diversity in the ligands that have been found to bind to PPARγ (Nolte et al., 1998). In RORγ zebrafish embryos, 8215 Ethyl increased basal GFP expression slightly, leading to broader expression in the brain, heart, and epithelium. RORγ plays a large role in autoimmune diseases, but also plays a smaller role in lipid metabolism by regulating pathways involved in the metabolism of bile acids, steroids, and xenobiotics (Kang et al., 2007; Kumar et al., 2010). These results suggest that 8215 Ethyl is an agonist for FXR, PPARγ, and RORγ, but it is still unknown what the effect of this in a model of NAFLD will be. It is likely that these three NRs are activated to different extents by 8215 Ethyl. It will be important to identify how 8215 Ethyl interacts with these receptors to identify whether these additional interactions will have detrimental effects. Completing a titration of 8215 Ethyl on the PPARγ and RORγ lines would also be useful to identify whether 8215 ethyl can induce GFP expression in FXR fish at concentrations where the other NRs are inactive, and to identify if a liver-only response can be seen in the these NR lines. Other NR lines should also be tested to see if 8215 Ethyl interacts with additional NR LBDs. Testing the 8215 Ethyl analogous compounds on the PPARγ and RORγ lines would also be useful to identify if one of these compounds is FXR specific.

3.1.2.5 8215 Ethyl Directly Binds to FXR, PPARγ, and RORγ In Vitro

To determine whether 8215 Ethyl was directly binding to the NRs, FXR, PPARγ, RORγ, and LXRα LBDs, they were expressed in bacteria and purified. The purified proteins were co-incubated with OCA or 8215 Ethyl, unbound ligand removed and bound ligand extracted. The sample was then analyzed using LC-MS to determine whether the ligand was present, which would indicate that it was previously bound to the NR LBD. Using this method, it was confirmed that 8215 Ethyl binds directly to the FXR, PPARγ, and RORγ LBD, but not to the LXRα LBD. These results indicate that the induction of GFP in the zebrafish embryos was due to the binding
of 8215 Ethyl to the LBD of these receptors. This also confirms that 8215 Ethyl is a FXR, PPARγ, and RORγ agonist. Although NR ligands typically bind within the LBD ligand binding pocket, the relatively promiscuous actions of 8215 Ethyl suggest the possibility that it may bind to one or more of these receptors in a non-conventional manner. The best way to determine this would be via structural studies.

3.2 Future Directions

Additional studies characterizing 8215 Ethyl will help to understand how this compound interacts with FXR and other NRs. It will first be important to determine whether this compound can induce FXR activity in a mammalian model, either in vivo or in vitro. It is also necessary to determine whether 8215 Ethyl is also a GPBAR1 agonist.

Completing more structure-activity relationship studies in a more systematic way would be interesting to determine if related compounds can more specifically target FXR, decrease toxicity, increase liver specificity, or decrease the dose required to see liver activity. To determine if 8215 Ethyl and OCA have different effects on FXR activity, qPCR or RNA-seq could be done using different zebrafish tissues after treatment to see how these ligands affect the transcription of target genes. FXR protein from fish tissue could also be affinity purified for use in protein pull down assays to determine whether these compounds lead to different coregulators associating with FXR in vivo. If so, these may be differentially expressed in responding or non-responding cells.

It would ultimately be interesting to determine the therapeutic potential of 8215 Ethyl in animal models. This can be done in both fish and mammalian models, such as mice. Fish models of NAFLD can be created simply and cheaply by feeding zebrafish larvae a high fat diet (Dai et al., 2015). This could be an ideal first model for the testing of 8215 Ethyl as a therapeutic compound. Mouse models of NAFLD have also been developed (Lai et al., 2015) that can also be used to test the potential effectiveness of 8215 Ethyl as a therapeutic compound.
3.3 Conclusion

My project determined that a novel FXR ligand, 8215 Ethyl, is an agonist for the nuclear receptors FXR, RORγ, and PPARγ. This agonism was confirmed through the use of transgenic zebrafish that incorporate the ligand trap system, as well as demonstrating that 8215 Ethyl binds to the LBD of these NRs in vitro. Interestingly, it appears that 8215 Ethyl activates FXR in the liver-specifically at low doses, where toxicity is also low. This was not seen in embryos treated with similar concentrations of OCA. This project also confirmed that the ligand trap system in zebrafish can be used to characterize novel ligands for NRs. Differential tissue expression patterns were seen in zebrafish when treated with different ligands. The ligand trap system also correlated well with in vitro protein binding experiments, as the same NRs were concluded to be bound and activated by 8215 Ethyl. Overall, this research is a good first step in the characterization of 8215 Ethyl as a potential research tool and therapeutic compound. Further research that focuses on mammalian models will need to be completed to determine whether 8215 Ethyl, or related compounds, have therapeutic potential.
References


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Figure 1-2: Adopted from Bain et al. 2007.