Characterization of a lead compound targeting LXRβ for the treatment of glucocorticoid-induced diabetes

By

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A thesis submitted in conformity with the requirements for the degree of Master of Science

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Abstract

A known cause of diabetes is the long-term use of glucocorticoid drugs (GCs) which are commonly used to treat chronic inflammation. Our lab has found that co-treatment of GCs with an LXRβ antagonist can decrease GC-induced diabetes. Herein, we characterize the pharmacokinetics of a pre-clinical lead candidate drug (LO520) targeting LXRβ that could be used along with GC therapy to prevent the diabetogenic side effects of GCs. LO520 demonstrated receptor specificity towards LXRβ in both murine and human LXR plasmid-based expression systems and significantly reduced plasma glucose levels when dosed in the presence of GC drug (dexamethasone, Dex) without altering the desired immunosuppressive effect. LO520 decreased cholesterol transporter mRNA expression in macrophages but did not decrease cholesterol efflux. Future studies will need to investigate the molecular mechanisms of LO520 in regulating gluconeogenesis and whether the use of LO520 will impact reverse cholesterol transport in vivo and the development of atherosclerosis.
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<td>ACC</td>
<td>Acetyl-CoA carboxylase</td>
</tr>
<tr>
<td>ACTH</td>
<td>Adrenocorticotropic hormone</td>
</tr>
<tr>
<td>AIM/Spα</td>
<td>Apoptosis inhibitor 6</td>
</tr>
<tr>
<td>ANOVA</td>
<td>Analysis of variance</td>
</tr>
<tr>
<td>AP1</td>
<td>Activating protein 1</td>
</tr>
<tr>
<td>Apo</td>
<td>Apolipoprotein</td>
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<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
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<tr>
<td>AUC</td>
<td>Area under the curve</td>
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<tr>
<td>COX2</td>
<td>Cyclooxygenase-2</td>
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<tr>
<td>CRH</td>
<td>Corticotropin releasing hormone</td>
</tr>
<tr>
<td>Ct</td>
<td>Threshold cycle</td>
</tr>
<tr>
<td>Cyp7a1</td>
<td>Cholesterol 7-alpha-hydroxylase</td>
</tr>
<tr>
<td>DEX</td>
<td>Dexamethasone</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco’s modified eagle medium</td>
</tr>
<tr>
<td>DR</td>
<td>Direct repeat</td>
</tr>
<tr>
<td>FAF BSA</td>
<td>Fatty acid free bovine serum albumin</td>
</tr>
<tr>
<td>FAS</td>
<td>Fatty acid synthase</td>
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<tr>
<td>FBS</td>
<td>Fetal bovine serum</td>
</tr>
<tr>
<td>FOXO1</td>
<td>Forkhead box protein O1</td>
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<td>G6Pc</td>
<td>Glucose-6-phosphatase, catalytic subunit</td>
</tr>
<tr>
<td>GC</td>
<td>Glucocorticoid</td>
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<tr>
<td>GR</td>
<td>Glucocorticoid receptor</td>
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<td>GRE</td>
<td>Glucocorticoid response element</td>
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<td>HDL</td>
<td>High density lipoprotein</td>
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<tr>
<td>HEK293</td>
<td>Human embryonic kidney 293 cells</td>
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<tr>
<td>HEPES</td>
<td>4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid</td>
</tr>
<tr>
<td>HPA</td>
<td>Hypothalamic-pituitary-adrenal</td>
</tr>
<tr>
<td>hr</td>
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</tr>
<tr>
<td>HRE</td>
<td>Hormone response element</td>
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<tr>
<td>HSP</td>
<td>Heat shock protein</td>
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<tr>
<td>I.p.</td>
<td>Intraperitoneal</td>
</tr>
<tr>
<td>I.v.</td>
<td>Intravenous</td>
</tr>
<tr>
<td>IC50</td>
<td>Half maximal inhibitory concentration</td>
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<tr>
<td>KO</td>
<td>Knockout</td>
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<tr>
<td>LBD</td>
<td>Ligand binding domain</td>
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<tr>
<td>LC/MS/MS</td>
<td>Liquid Chromatography Tandem Mass Spectrometry</td>
</tr>
<tr>
<td>LDL</td>
<td>Low density lipoprotein</td>
</tr>
<tr>
<td>LDLR</td>
<td>Low density lipoprotein receptor</td>
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<tr>
<td>LXR</td>
<td>Liver X receptor</td>
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<tr>
<td>LXRE</td>
<td>Liver X receptor response element</td>
</tr>
<tr>
<td>Min</td>
<td>Minutes</td>
</tr>
<tr>
<td>NF-κB</td>
<td>Nuclear factor kappa-light-chain-enhancer of activated B cells</td>
</tr>
<tr>
<td>NCOR</td>
<td>Nuclear receptor corepressor</td>
</tr>
<tr>
<td>NOS2</td>
<td>Nitric oxide synthase 2</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
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<tr>
<td>--------------</td>
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</tr>
<tr>
<td>NR</td>
<td>Nuclear receptor</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>Pepck</td>
<td>Phosphoenolpyruvate carboxykinase</td>
</tr>
<tr>
<td>PGC1α</td>
<td>Peroxisome proliferator activated receptor gamma coactivator 1 alpha</td>
</tr>
<tr>
<td>PK</td>
<td>Pharmacokinetic</td>
</tr>
<tr>
<td>PMA</td>
<td>Phorbol-12-myristate-13-acetate</td>
</tr>
<tr>
<td>PUFA</td>
<td>Polyunsaturated fatty acids</td>
</tr>
<tr>
<td>PXR</td>
<td>Pregnane X receptor</td>
</tr>
<tr>
<td>QPCR</td>
<td>Quantitative polymerase chain reaction</td>
</tr>
<tr>
<td>RLU</td>
<td>Relative luciferase unit</td>
</tr>
<tr>
<td>RXR</td>
<td>Retinoid X receptor</td>
</tr>
<tr>
<td>SCD-1</td>
<td>Stearoyl-coA desaturase-1</td>
</tr>
<tr>
<td>SMRT</td>
<td>Silencing mediator for retinoid or thyroid-hormone receptors</td>
</tr>
<tr>
<td>SREBP-1c</td>
<td>Sterol regulatory element-binding protein-1c</td>
</tr>
<tr>
<td>ssFBS</td>
<td>Super-striped fetal bovine serum</td>
</tr>
<tr>
<td>STAT</td>
<td>Signal transducer and activator of transcription</td>
</tr>
<tr>
<td>T09</td>
<td>T0901317</td>
</tr>
<tr>
<td>TAT</td>
<td>Tyrosine aminotransferase</td>
</tr>
<tr>
<td>TNFα</td>
<td>Tumor necrosis factor alpha</td>
</tr>
<tr>
<td>Veh</td>
<td>Vehicle</td>
</tr>
<tr>
<td>VLDL</td>
<td>Very low density lipoprotein</td>
</tr>
<tr>
<td>WBC</td>
<td>White blood cell</td>
</tr>
<tr>
<td>WT</td>
<td>Wildtype</td>
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Chapter 1: Literature Review

1.1 Statement of Research Problem

Diabetes is a metabolic disease characterized by high blood-glucose levels (hyperglycemia) resulting from defects in insulin secretion and/or insulin action (American Diabetes Association 2010). It was estimated that in 2017 there were 451 million people with diabetes worldwide, and by 2045, this value is expected to increase to 693 million (Cho et al. 2018). The cause of diabetes is multifactorial as risk factors include lifestyle choices (diet and physical activity), environmental factors and genetic predisposition to the disease (American Diabetes Association 2010). A lesser known yet still prevalent risk factor to the development of diabetes is the chronic use of potent anti-inflammatory and immunosuppressive drugs called glucocorticoids (GC) (Suh & Park, 2017). The relationship between the development of diabetes from glucocorticoids is striking as 18.6% of patients that use glucocorticoids develop GC-induced diabetes and that 32.3% of patients using GCs develop GC-induced hyperglycemia (Liu et al. 2014).

The physiological actions of GCs are mediated by the ligand-dependent transcription factor glucocorticoid receptor (GR, NR3C1), which is a member of the nuclear receptor superfamily. Chronic GC exposure leads to overstimulation of their role in various physiological processes including increased hepatic glucose production, muscle atrophy, lipolysis and inhibition of insulin release from pancreatic β-cells, among other mechanisms (Delaunay et al. 1997; Gesina et al. 2004; Vegiopoulos & Herzig 2007; Odedra et al., 1983; Munck 1971). Although GC drugs can be discontinued to resolve their diabetogenic side effects, the fact that GCs are extremely potent and effective anti-inflammatory drugs makes them essential for the treatment of severe inflammatory conditions.

Similar to GR, liver X receptors (LXRs) are also ligand-activated transcription factors which are tightly associated with metabolism. Studies in our lab have shown that when treated with a synthetic GC called dexamethasone (Dex), LXRα/β-/- mice did not develop hyperglycemia (Patel et al. 2011). Both wildtype and LXRα/β-/- mice demonstrated significant
spleen atrophy with chronic Dex treatment demonstrating that mice lacking LXRα/β exhibit dissociated glucocorticoid actions by being resistant to the glycemic response of Dex but sensitive to lymphoid organ atrophy (Patel et al. 2011). GR target genes involved in gluconeogenesis (Phosphoenolpyruvate carboxykinase, Pepck, and Glucose-6-phosphatase, catalytic subunit, G6pc) and their transcriptional regulators (Peroxisome proliferator activated receptor gamma coactivator 1 alpha, Pgc1α and Forkhead box protein O1, Foxo1) were increased in the presence of Dex for wildtype mice but not LXRα/β-/- mice (Patel et al. 2011). Using primary mouse hepatocytes, it was determined that the LXRβ isoform was responsible for mediating Pepck gene upregulation and glucose production in response to Dex (Patel et al. 2011). In contrast, the expression of another GR target gene, Tat, was induced equally by Dex in WT, LXRα-/-and LXRβ-/- cells showing that GC-sensitive gene regulation by LXR is gene selective. Taken together, these results show that once LXRβ is knocked out, mice treated with glucocorticoids demonstrate the desired effects of immunosuppression without the detrimental side effects of hyperglycaemia and hepatic steatosis normally observed in WT mice with GC-induced type 2 diabetes.

To assess the physiological role of LXRβ antagonism and determine whether GC signaling would be similarly affected compared to LXRβ-/- mice, our lab tested the effect of an LXR dual antagonist (GSK2033) in LXRα-/- mice (Patel et al., 2017). Similar to GC-treatment of LXRβ-/- mice, antagonism of LXRβ by GSK2033 in LXRα-/- mice resulted in resistance to GC-induced hyperglycemia and hepatosteatosis while preserving the anti-inflammatory effects of Dex in mice. The limitation of GSK2033 is that it has a short half-life (10 minutes) and lacks LXR isoform selectivity. In collaboration with Dr. Arturo Orellana’s lab at York University (Toronto, ON), we created a novel LXRβ specific antagonist, LO520. My thesis aimed to characterize the in vitro and in vivo activity of LO520 to develop a pre-clinical lead candidate targeting LXRβ that could be used as a co-therapy with GCs to prevent the diabetogenic side effects of GC drugs.
1.2 Background

1.2.1 Glucocorticoids (GC)

Glucocorticoids (cortisol in humans, and corticosterone in rodents) are endogenous stress hormones necessary for life as they regulate numerous physiological processes in an effort to maintain energy homeostasis (Oakley & Cidlowski, 2011). As GC exposure is meant to be “short-term” due the presence of negative feedback, chronic GC exposure leads to overstimulation of their role in various physiological processes. In terms of metabolism, overstimulation of GC release leads to an increase in peripheral glucose via muscle and fat breakdown as well as hepatic gluconeogenesis (Exton, 1979; Rebuffé-Scribe et al., 1988). Due to their powerful anti-inflammatory and immunosuppressive actions however, synthetic GCs (such as dexamethasone, Dex) are commonly used to treat inflammatory and autoimmune diseases such as asthma and multiple sclerosis and are commonly prescribed to prevent organ transplant rejection (Oakley & Cidlowski, 2011).

1.2.2 Glucocorticoids as a contributing factor to the diabetes epidemic

The prevalence of GC use varies by age and sex, with the highest prevalence in the elderly (>65 yrs) and in women with population averages hovering around 1-2% (Overman et al., 2013). For example, oral glucocorticoid use was 1.2% from 1999 to 2008 in the USA, chronic GC use was 1.4% in France from 2007 to 2013, and 3% of the Danish population redeemed at least one prescription for a systemic GC annually between 1999 and 2015 (Bénard-Laribièrè et al., 2017; Laugesen et al., 2017; Overman et al., 2013). It has been shown that 18.6% of patients that use glucocorticoids develop GC-induced diabetes and that 32.3% of patients develop GC-induced hyperglycemia (Liu et al., 2014). A case control study by Gurwitz et al. (1994) looked at GC-treated patients and the development of new onset diabetes. The magnitude of risk for developing diabetes increased substantially with increasing glucocorticoid dose (Gurwitz et al., 1994). Hwang and Weiss (2014) noted the severity of GC-induced diabetes as they found that in their 550-bed teaching hospital, 40% of the inpatient consults to the Endocrine Consult Service were due to either type 2 diabetes exacerbated by steroid use or new onset steroid-induced diabetes. In order to lessen the prevalence of development as well as the mortality rate by diabetes, researchers have been looking at many factors contributing to the development of
diabetes and targeting various molecular pathways in order to elucidate their roles in the pathophysiology of this disease. The significance for this research is of great magnitude since the global healthcare expenditure for people with diabetes was estimated to be USD 850 billion in 2017 and mortality caused by diabetes was the seventh leading cause of death in the United States in 2015 (Cho et al., 2018). Finally, stress is reported as a contributor to the development of diabetes as seen in a cross-sectional study done by Mooy and colleagues, where there was a positive correlation between chronic psychological stress and undetected type 2 diabetes and visceral adiposity (Mooy et al., 2000). A retrospective cohort study done by Plummer and colleagues (2016) reported that 17% of patients in a hospital intensive care unit (ICU) during 2004 and 2011 were identified with stress-induced hyperglycemia. The risk of diabetes in patients with stress-induced hyperglycemia was roughly double those without, thereby further supporting the role of stress on diabetogenesis.

1.2.3 Molecular Mechanisms of Glucocorticoid Actions & the Glucocorticoid Receptor

Glucocorticoid secretion is controlled by the hypothalamic-pituitary-adrenal (HPA) axis whereby endogenous and exogenous signals prompt the hypothalamus to release corticotropin-releasing hormone (CRH), which acts on the anterior pituitary to stimulate the secretion of adrenocorticotropic hormone (ACTH). ACTH then acts on the adrenal cortex resulting in GC production and secretion from the zona fasciculata (Vegiopoulos & Herzig, 2007) (Figure 1.1). GCs work to maintain homeostasis in a plethora of physiological processes including immune function, reproduction, metabolism and skeletal growth (Hoes et al., 2011; Vegiopoulos & Herzig, 2007). They also work through negative feedback thereby regulating their duration of action by inhibiting CRH and ACTH release by the hypothalamus and anterior pituitary (Figure 1.1).

The physiologic actions of GCs are mediated by the ligand-dependent transcription factor glucocorticoid receptor (GR, NR3C1), which is a member of the nuclear receptor superfamily. GR is expressed in nearly every cell in the body and is involved in various pathways including energy homeostasis, development and immune suppression (Oakley & Cidlowski, 2011). GR is primarily located in the cytoplasm in the absence of a ligand (Echeverría et al., 2009). Once
bound by GC, GR undergoes a conformational change and dissociates from a large multiprotein chaperone complex associated with chaperone proteins (HSP70, HSP90, p23) and immunophilins of the FK506 family (FKBP51 and FKBP52) (Oakley & Cidlowski, 2011; Pratt, Galigniana, Morishima, & Murphy, 2004). From there, the GC-GR complex translocates into the nucleus where it activates or represses a distinct set of genes via cis-regulation (direct DNA binding) or trans-regulation (indirect protein tethering with other transcription factors bound to DNA) (Kassel & Herrlich, 2007; Phuc Le et al., 2005; Uhlenhaut et al., 2013). GR can bind directly to GREs as a homodimer and regulate the expression of target genes or it can act as a monomeric protein that interacts with other transcription factors and affect transcription (Vandevyver et al., 2013). The consensus sequence for GRE binding consists of two inverted repeat AGAACA sequences separated by three nucleotides (AGAACAnnnTGTTCT) (Crudo et al., 2013).

1.2.4 Physiological Roles of Glucocorticoids: Blood-Glucose Regulation

The main purpose of glucocorticoid’s effect on blood-glucose regulation is to preserve plasma glucose for the brain during stress by increasing peripheral blood glucose levels (Kuo et al., 2015) (Figure 1.1). This is done by various pathways including the promotion of gluconeogenesis by GC in liver resulting in increased expression of major gluconeogenic enzymes such as phosphoenolpyruvate carboxykinase (PEPCK) and glucose-6-phosphatase (G6Pc) (Imai et al., 1990). In skeletal muscle and white adipose tissue, GCs decrease glucose uptake and utilization by antagonizing insulin response resulting in hyperglycemia and insulin resistance (Charmandari et al., 2005; Di Dalmazi et al., 2012; Kuo et al., 2013). GCs also exhibit tissue-specific effects on glycogen metabolism (Kuo et al., 2015). In the liver, GCs increase glycogen storage, whereas in skeletal muscle they are shown to inhibit insulin-stimulated glycogen synthesis (Exton et al., 1972; Ruzzin et al., 2005; Stalmans & Laloux, 1979). GCs also regulate blood glucose levels by modulating the function of pancreatic α and β cells thereby altering the secretion of glucagon and insulin (Beaudry & Riddell, 2012; Wise et al., 1973).
Figure 1.1 Blood-glucose regulation by glucocorticoids
GC production is regulated by the hypothalamic-pituitary adrenal axis. Chronic GC secretion leads to increased blood glucose via i) inhibition of insulin release from pancreatic β cells, ii) inhibition of glucose uptake in tissues, iii) the breakdown of muscle and adipose in order to provide substrates for glucose production and iv) increased glucose production via gluconeogenesis in the liver.

1.2.5 Physiological Roles of Glucocorticoids: Anti-Inflammatory Effects

Due to their ability to suppress pro-inflammatory genes and activate anti-inflammatory genes, GCs are widely used for the treatment of chronic inflammatory diseases such as rheumatoid arthritis, inflammatory bowel disease, asthma and autoimmune diseases (Barnes, 1998). GC provide anti-inflammatory effects by increasing the transcription of genes coding for anti-inflammatory proteins such as lipocortin-1, interleukin-10 (IL-10) and neutral endopeptidase (NEP), and also act by inhibiting the expression of pro-inflammatory genes (Adcock et al., 1995, 1994; Barnes & Karin, 1997). The protein complex heavily involved in regulating the immune response, NF-κB (nuclear factor kappa-light-chain-enhancer of activated B cells), has been shown to be inhibited by GR activation (Nelson, 2003). The combined actions of GCs to repress inflammatory genes and activate anti-inflammatory genes led to the widespread adoption of GCs as therapeutic agents for the treatment of inflammatory diseases.

1.2.6 Glucocorticoid Drug Properties

In 1948, the first patient with rheumatoid arthritis was treated with cortisone and subsequent administration of hydrocortisone and cortisone began in 1950-1951 (Benedek, 2011). GCs have similar molecular structures and clinical effects and their distinction is determined by
potency and relative mineralocorticoid activity (Na\(^+\)/H\(_2\)O retention) (Becker, 2013). Cortisol, prednisone and methylprednisolone have the highest mineralocorticoid activity (unlike dexamethasone, triamcinolone and betamethasone) and dexamethasone and betamethasone are known to have the longest biological half-life in humans (36-72 hrs, compared to 8-12 hrs cortisol, 12-36 hrs prednisone and methylprednisolone) (Becker, 2013). Due to its lack of mineralocorticoid affinity, dexamethasone has been widely used in the laboratory to study the selective activation of GR. Various studies have classified “short term” GC administration as 5-7 days and “chronic” GC administration has been reported to be considered a minimum of 3 weeks, but could also be months and years (Angeli et al., 2006; Becker, 2013; Bonadonna et al., 2005; Giustina et al., 1992; Wellman, 2001).

1.3 Liver X Receptors

Similar to GR, liver X receptors (LXRs) are ligand-activated transcription factors that are tightly associated with metabolism. LXRs are present in two isoforms encoded by two separate genes (Laurencikiene & Rydén, 2012). LXR\(\alpha\) (NR\(1H3\)) is highly expressed in the liver, intestine, adipose tissue, lung, macrophage, kidney and adrenal gland whereas LXR\(\beta\) (NR\(1H2\)) is ubiquitously expressed (Steffensen & Gustafsson, 2004; Bookout et al., 2006). LXRs are located in the nucleus as obligate heterodimers with the retinoid X receptor (RXR) and bind to the LXR response element (LXRE) which consists of direct repeats (DR) of the hexameric sequence 5’-AGGTCA-3’ in which the half sites are separated by four nucleotides (Quack et al., 2002; Repa & Mangelsdorf, 2000; Song et al., 1994; Willy et al., 1995). Unliganded LXR-RXR heterodimers are bound by a multi-protein complex with co-repressors such as nuclear receptor co-repressor (NCoR) and silencing mediator for retinoid or thyroid-hormone receptors (SMRT) (Chen & Evans, 1995; Hörlein et al., 1995) (Figure 1.2). Upon activation by oxidized cholesterol derivatives known as oxysterols, LXR-RXR undergoes a conformational change leading to the exchange of the co-repressor complex for a co-activator complex to regulate the transcription of target genes involved in cholesterol metabolism, gluconeogenesis, lipogenesis and inflammation (Commerford et al., 2007; Joseph et al., 2003; Mangelsdorf & Evans, 1995) (Figure 1.2). The LXR-RXR is known as a permissive heterodimer since it can be activated by ligands for either LXR (oxysterols) or RXR (9-cis-retinoic acid) or synergistically by both (Peet et al., 1998; Rühl et al., 2015).
LXR activation leads to downstream upregulation of genes involved in cholesterol metabolism in the liver (CYP7A1), lipogenesis (fatty acid synthesis) via SREBP1c and FASN, cholesterol efflux and transport by the ABC-binding cassette transporters (ABCA1/G1/G8) in the liver and macrophage and inhibition of inflammatory mediators (IL-6, IL-1β) in the macrophage.

1.3.1 Physiologic Roles of the LXRs: Anti-Inflammatory Effects

LXRs serve as important regulators of inflammatory gene expression. Gene expression analysis showed that LXR agonist treatment can limit the transcriptional upregulation of pro-inflammatory genes such as cyclooxygenase 2 (Cox2), inducible nitric oxide synthase (Nos2) and tumor necrosis factor alpha (Tnfα) mediated by NFκB and activating protein 1 (AP-1)(Castrillo et al., 2003; Ghisletti et al., 2007, 2009; Joseph et al., 2003). In terms of the isoform-dependent inflammatory responses of LXRs, Joseph et al. (2004) treated LXRα-/‐ and LXRβ-/‐ mice with Listeria monocytogenes resulting in increased susceptibility to infection due to defective macrophage function and decreased expression of macrophage survival factor AIM/Spα (Joseph et al., 2004). It was found that the increased susceptibility of LXRα/β-/‐ macrophages to respond...
to pathogen-induced apoptosis was due to the downregulation of the antiapoptotic gene \( \text{Sp}_\alpha \), the first LXR\( \alpha \) selective target gene to be described (Joseph et al., 2004). Ligand induced activation of LXR\( \beta \) was also shown to diminish the proliferative capacity of B and T cells demonstrating LXR\( \beta \)’s ability to regulate lymphocyte proliferation (Bensinger et al., 2008). Similarly, it was shown that mice lacking LXR\( \beta \) demonstrated lymphoid hyperplasia compared to LXR\( \alpha \) knockout mice (Bensinger et al., 2008). These results suggest isoform specific roles of LXR in the immune response.

1.3.2 Physiologic Roles of LXRs: Cholesterol Metabolism

LXR/RXR heterodimer functions as a sensor of cellular cholesterol concentration and mediates cholesterol efflux by inducing the transcription of key cholesterol transport proteins in order to prevent cholesterol buildup in the liver (Zhu et al., 2012). Studies using atherosclerotic mouse models (\( \text{Apoe}^{-/-} \) and \( \text{Ldlr}^{-/-} \) mice) have demonstrated that the use of LXR agonists reduces the development of atherosclerosis as well as can block the progression of existing disease (Bischoff et al., 2010; Joseph et al., 2002; Terasaka et al., 2003). This has been shown to be the result of increased expression of genes involved in cholesterol efflux (\( \text{Abca1}, \text{Abcg1} \) and \( \text{Apoe} \)), cholesterol conversion to bile acids (\( \text{Cyp7a1} \)) and cholesterol excretion via secretion into bile (\( \text{Abcg5}, \text{Abcg8} \)) (Costet et al., 2000; Repa & Mangelsdorf, 2000; Venkateswaran et al., 2000). LXR\( \alpha \) has been shown to directly regulate expression of ATP binding cassette transporters, \( \text{Abcg5} \) and \( \text{Abcg8} \), which function as heterodimers to mediate excretion of cholesterol to the bile as well as play a prominent role in limiting cholesterol absorption in the intestine (Repa et al., 2002; Wilund et al., 2004; Yu et al., 2003; Yu et al., 2014). LXR\( \alpha \) has been shown to regulate the expression of cytochrome P450 7A1 (\( \text{Cyp7a1} \)), the rate limiting enzyme in cholesterol conversion to bile acids (Peet et al., 1998) (Figure 1.3). It should be noted that the LXR binding in the \( \text{Cyp7a1} \) promoter is only observed in mice and not humans (Menke et al., 2002). Apolipoprotein E (ApoE) is secreted by macrophages in the arterial wall and, through its ability to promote lipid efflux via expansion of the HDL core with cholesterol, has been known to play a protective role against the development of atherosclerosis (Laffitte et al., 2001; Mahley, Huang, & Weisgraber, 2006). The ApoE gene is a direct target of LXR\( \alpha \) and LXR\( \beta \) in macrophages and adipose but not in the liver (Laffitte et al., 2001). Single knockout mice of LXR\( \alpha \) on a background of \( \text{Apoe}^{-/-} \) or \( \text{Ldlr}^{-/-} \) had increased atherosclerosis while LXR\( \beta \)
knockout mice on this background had no effect in the pathogenesis of the disease (Bischoff et al., 2010). In contrast, LXRβ has been shown to compensate for the loss of LXRα in mediating the anti-atherogenic response to potent synthetic LXR agonists (Bischoff et al., 2010; Joseph et al., 2004). Due to their significant role in regulating the expression of genes involved in reverse cholesterol transport, LXRs have been considered promising pharmaceutical targets for the treatment of atherosclerosis.

Figure 1.3 LXR target genes in reverse cholesterol transport
Reverse cholesterol transport begins with ABCA1 aiding in the efflux of excess cholesterol to the Apolipoprotein acceptor ApoA1 thereby creating a nascent HDL particle. ABCG1 regulates macrophage cholesterol efflux to more HDL with the purpose of incorporating more cholesterol, forming a mature molecule of HDL. HDL is transported to the liver where CYP7A1 acts as the rate limiting enzyme in the conversion of cholesterol to bile acids. ABCG5 and ABCG8 function as heterodimers and aid in cholesterol secretion via biliary duct and prevent cholesterol reabsorption in the intestine by facilitating excretion into the feces.

1.3.3 Physiologic Roles of LXRs: Fatty Acid Synthesis
LXR activation leads to an increase in the levels of long chain polyunsaturated fatty acids (PUFAs), increased hepatic triglyceride secretion in the form of very low-density lipoprotein (VLDL) particles and increased triglyceride synthesis (Bischoff et al., 2010; Okazaki et al., 2010). This is done by regulating various genes involved in fatty acid synthesis, one of them being sterol regulatory element binding protein 1c (SREBP1-c), a preferential target gene of LXRα (Horton et al., 2002). Deletion of the Srebp1-c gene in mice resulted in a 50% reduction of hepatic fatty acid synthesis and SREBP-1c was found to be the dominant transcription factor that upregulated lipogenic genes, including acetyl-coA carboxylase 1 (Acc1) and fatty acid synthase (Fasn) in the liver of patients with NAFLD (Higuchi et al., 2008; Liang et al., 2002). The gene Scd1 (stearoyl CoA desaturase-1) which encodes the enzyme that catalyzes the rate-limiting step in monounsaturated fatty acids (MUFAs) synthesis, was found to increase in wildtype and Lxrb-/- mice, but not in Lxra-/- mice, with LXR agonist T0901317 treatment (Zhang et al., 2014). In a different study, Fasn, Scd1 and Srebp1 were shown to be down-regulated in Lxra-/- mice on a low-cholesterol diet suggesting that LXRα plays a role in the basal regulation of these genes (Peet et al., 1998)(Figure 1.3). Several desaturases and fatty acid elongases including Fasn and Scd1, are also direct LXR target genes (Chu et al., 2006; Peet et al., 1998; Schultz et al., 2000). Thus, fatty liver and hypertriglyceridemia are undesired side effects associated with LXR full agonist activation.

1.4 LXR as Therapeutic Targets

1.4.1 LXR Agonists

At present, there are no approved pharmaceutical drugs targeting LXRs. Pre-clinical LXR probe drugs used for research purposes include the LXR dual-agonist GW3965 (EC₅₀ =190 nM) which was shown to have potent anti-atherogenic activity in Ldlr-/- and Apoe-/- mice (Joseph et al., 2002). When macrophages were pretreated with GW3965, there was significant reduction in nitric oxide synthase (iNOS) mRNA, an enzyme involved in the inflammatory response and atherosclerotic lesion development (Joseph et al., 2003). Even though GW3965 showed promising anti-inflammatory and anti-atherosclerotic effects, GW3965 has been shown to stimulate lipogenic gene expression in human hepatocytes as well as a modest increase in liver and plasma triglyceride levels (Grefhorst et al., 2005; Groot et al., 2005; Zhou et al., 2008).

T0901317, another non-selective LXR agonist (EC₅₀ = 50 nM), was also reported to exert strong anti-atherogenic effect in LDL receptor-deficient mice compared to vehicle (Terasaka et al., 2003). It was later reported that T0901317 is not LXR specific as it also activates pregnane X.
receptor (PXR) (Mitro et al., 2007). T0901317 also has been shown to increase hepatic expression of key enzymes in fatty acid metabolism (SREBP-1c, ACC, FAS and SCD-1) in wildtype but not in Lxra/β-/- or Lxra/-/ mice (Quinet et al., 2006; Schultz et al., 2000).

Unlike the LXR agonists previously mentioned, the agonist IMB-808, (LXRα EC50 = 150 nM, LXRβ EC50 = 530 nM) was shown to promote cholesterol efflux in RAW264.7 macrophages but not induce the expression of lipogenic genes in HepG2 cells, a common limitation in LXR agonism (Li et al., 2017). The ability to increase cholesterol efflux and reduce cholesterol levels in macrophage cell lines was maintained in IMB-808 thereby suggesting it could be a promising compound to target atherosclerosis without increased lipogenesis via LXR regulation (Li et al., 2017).

1.4.2 LXR Antagonists and Inverse Agonists

The LXR inverse agonist, SR9238 (LXRα IC50 = 214 nM, LXRβ IC50 = 43 nM), that is reported to be liver-selective, was shown to suppress hepatic steatosis in mice fed a high fat diet via suppression of genes involved in fatty acid synthesis including Srebp-1c, Fas and Scd1 (Griffett & Burris, 2013). SR9238 also reduced inflammation and fibrosis in mice with diet induced- non-alcoholic steatohepatitis (NASH) compared to control (Griffett et al., 2015). A significant concern with development of LXR inverse agonists for the treatment of fatty liver disease is that the beneficial effect of LXR-cholesterol sensing for reverse cholesterol transport may be jeopardized due to LXR antagonism.

GSK2033 was the first synthetic dual LXR antagonist (LXRα IC50 = 100 nM, LXRβ IC50 = 40 nM) to be discovered (Zuercher et al. 2010). The compound was tested against a panel of other nuclear receptors and found to be highly selective for LXRs but exhibited a high intrinsic clearance (CLint > 1 mL/min/mg protein) in rat and human liver microsomes suggesting that it would not be suitable for in vivo studies. Studies found GSK2033 suppresses Srebp-1c mRNA expression in THP-1 cells (Ignatova et al., 2013) and HepG2 cells (Zuercher et al., 2010). Griffett and Burris (2016) reported that 10 μM GSK2033 could activate a number of nuclear receptors including the glucocorticoid receptor (GR), pregnane X receptor (PXR) and farnesoid X receptor (FXR) (all showed ~3-fold activation), which are all involved in liver
metabolism. These data contradict that of Zuercher et al. (2010) that found that the \( \text{XC}_{50} \)’s for GSK2033 on a wide panel of NRs (including FXR and PXR) were > 10 \( \mu \text{M} \). Our group also tested the agonist activity of GSK2033 up to 1 \( \mu \text{M} \) on GR and FXR and found no significant difference from vehicle, in agreement with Zuercher’s data (unpublished data, Rucha Patel).

Human LXR\( \alpha \) (447 amino acids) and LXR\( \beta \) (460 amino acids) share 54% overall sequence homology and 77% sequence homology in their ligand-binding domains with only a single amino acid residue difference in the sequence lining the ligand binding pocket (Färnegårdh et al., 2003). For this reason, it is extremely challenging to rationally develop isoform selective agonists or antagonists.

1.5 Cross-talk between LXR and GR

In a study by Patel et al., 2011, it was shown that LXR\( \alpha/\beta\)-/- mice had 2-fold higher circulating GC levels yet were lean and more glucose tolerant compared to wildtype mice (Patel et al., 2011). When treated with Dex to test whether LXR\( \alpha/\beta\)-/- mice were resistant to the metabolic effects of elevated GCs, Dex-treated LXR\( \alpha/\beta\)-/- mice did not develop hyperglycemia in contrast to wildtype mice (Patel et al., 2011). Both wildtype and LXR\( \alpha/\beta\)-/- mice demonstrated significant spleen atrophy with chronic Dex treatment demonstrating that mice devoid of LXR\( \alpha/\beta \) exhibit dissociated glucocorticoid actions by being resistant to the glycemic response of Dex but sensitive to lymphoid organ atrophy (Patel et al., 2011). GR target genes involved in gluconeogenesis (Pepck, G6pc) and their transcriptional regulators (Pgc\( \text{1}\)a and Foxo1) were increased in the presence of Dex for WT mice but not LXR\( \alpha/\beta\)-/- mice (Patel et al., 2011). Using primary mouse hepatocytes, it was determined that the LXR\( \beta \) isoform was responsible for mediating Pepck gene upregulation and glucose production in response to Dex (Patel et al., 2011). In contrast, the expression of another GR target gene, Tat, was induced equally well by Dex in wildtype, LXR\( \alpha\)-/- and LXR\( \beta\)-/- cells showing that GC-sensitive gene regulation by LXR is gene selective. To address the mechanism of these promoter-specific actions of GCs, Patel et al. 2011 investigated promoter occupancy by in vivo chromatin immunoprecipitation of GR bound to the GRE of either Pepck or the Tat gene promoters. Interestingly, GR occupancy of the Pepck promoter but not the Tat promoter, was decreased in LXR\( \alpha/\beta\)-/- and LXR\( \beta\)-/- mice in
response to Dex thereby supporting the notion that LXRβ is required for selective binding of GR to the Pepck promoter.

Since the development of insulin resistance is highly correlated with the presence of a fatty liver phenotype, the next step was to determine whether insulin tolerance in the LXRα/β-/- mice after Dex treatment correlated with decreased fat accumulation in the liver. Oil red O staining demonstrated significant lipid droplets in livers of WT and LXRα-/-mice treated with Dex whereas no change was detected in the liver histology of LXRβ-/- and LXRα/β-/-mice between vehicle and Dex treatment (Patel et al., 2011). Liver TG levels were significantly increased with Dex in all genotypes except in LXRβ-/- supporting the idea that loss of LXRβ protects against fatty liver in the presence of Dex.

The anti-inflammatory effects of GCs are achieved by either direct or indirect repression of proinflammatory genes or the transactivation of anti-inflammatory genes (Patel et al., 2017). Since hepatic steatosis and hyperglycemia were differentially induced in the LXRβ-/- mice in response to Dex, it was assessed whether the anti-inflammatory response to GCs was preserved. Treatment of Dex for 14 days showed significant spleen atrophy in LXRα-/-and LXRβ-/- mice (Patel et al., 2011). Taken together, these results clearly show that once LXRβ is knocked out, mice treated with glucocorticoids demonstrate the desired effects of immunosuppression without the detrimental side effects of hyperglycemia and hepatic steatosis observed with GC-induced type 2 diabetes.

To assess whether the detrimental actions of LXRβ in the presence of GCs could be inhibited with the use of a small molecule, studies were performed with GSK2033, a potent dual LXRα/LXRβ antagonist. The induction of Pepck by Dex was inhibited by cotreatment with GSK2033 in both WT and LXRα-/- hepatocytes. Mechanistically, it was found that GSK2033 impaired Dex-induced transcription factor recruitment (GR, C/EBPβ, MED1) to the Pepck promoter (Patel et al., 2017). Pepck mRNA expression was not increased by Dex in LXRβ-/- primary hepatocytes and remained unchanged when co-treated with GSK2033 (Patel et al., 2017). Likewise, gene expression studies were performed with wildtype, LXRα-/- and LXRβ-/- primary macrophages treated with Dex ± GSK2033. Dex strongly repressed the expression of proinflammatory genes (Il1β and Il6) and this was unaltered by GSK2033 co-treatment (Patel et al., 2017). Even at the lowest dose of Dex (0.1 nM), GSK2033 did not interfere with the ability
of Dex to inhibit \( \text{Il}1\beta \) expression concluding that GC-mediated immune suppression is unaffected by LXR antagonism in mouse primary macrophages.

1.6 Rationale and Hypotheses

1.6.1 Rationale

Synthetic GCs are widely used for their immunosuppressive and anti-inflammatory roles in various diseases. However due to their ability to induce the expression of various gluconeogenic genes (\( \text{Pepck, G6pc} \)), chronic GC administration is associated with a number of side effects including hyperglycemia and glucocorticoid-induced diabetes (De Bosscher, 2010; Patel et al., 2017).

The GR and LXR are members of the nuclear receptor superfamily involved in overlapping transcriptional programs in the context of glucose and lipid metabolism (Patel et al., 2011). Our lab has shown that mice lacking LXR\( \beta \) but not LXR\( \alpha \) were resistant to Dex-induced hyperglycemia, hyperinsulinemia, and hepatic steatosis, but remained sensitive to the anti-inflammatory benefits of Dex (Patel et al., 2011). Similarly, antagonizing LXR\( \beta \) by GSK2033 in LXR\( \alpha \) deficient mice showed that the gluconeogenic and immunosuppressive actions of GR activation can be dissociated by pharmacological antagonism of LXR\( \beta \) (Patel et al., 2017). For this reason, the treatment with an LXR\( \beta \) antagonist in the presence of GC drugs could provide safer use of glucocorticoid therapy as it would lead to diminished diabetogenic side effects while preserving the anti-inflammatory actions of glucocorticoids.

Humans have both isoforms of LXRs and LXR\( \alpha \) is highly involved in basic physiological functions including the regulation of reverse cholesterol transport. For this reason, antagonism of LXR\( \alpha \) by GSK2033 could result in unwanted pathophysiology due to the modification of these pathways. Based on findings from our lab and our knowledge on the implication of LXR\( \alpha \) in the regulation of reverse cholesterol transport, the lab has collaborated with Dr. Arturo Orellana from York University in creating a novel, selective LXR\( \beta \) antagonist (LO520), and we hypothesize that the selectivity of LXR\( \beta \) antagonism will provide a means to prevent the diabetogenic side effects of GC.
1.6.2 Hypothesis

The use of the LXRβ antagonist LO520 in the presence of glucocorticoids will impede the development of the diabetogenic side effects of glucocorticoids.

1.7 Objectives

The main objective of this thesis is to characterize a pre-clinical drug lead candidate targeting LXRβ (LO520) that could be used along with GC therapy to prevent diabetogenic side effects of GC drugs.

There are four major aims of my thesis:

1. Characterize the in vitro activity of LO520 for antagonism of LXRα and LXRβ
   a) Determine the IC₅₀ against mouse and human LXRα and LXRβ in HEK293 cells
   b) Treat primary mouse hepatocytes with Dex ± LO520 to assess Pepck induction

2. Determine the pharmacokinetic (PK) properties of LO520 after intraperitoneal (i.p.) dosing
   a) Optimize the extraction efficiency of LO520 using LC-MS/MS assay
   b) Perform pilot study to titrate the LXRβ antagonist activity of LO520 in vivo against an LXR agonist (T0901317)
   c) Perform a pharmacokinetic study after i.p. dosing of LO520 to C57Bl/6 mice

3. Perform an in vivo efficacy study with LO520 in GC-induced diabetic mouse model

4. Establish the side-effect profile of LXRβ antagonism with respect to reverse cholesterol transport
   a) Measure gene expression profile of cholesterol efflux pathway in THP-1 cells ± LO520
   b) Quantify the efflux of [³H]-cholesterol from macrophages to high-density lipoproteins (HDL)
Chapter 2: Methods

2.1 Cell culture conditions

HEK293 cells were grown under aseptic conditions in 10 cm plates using high glucose DMEM (Invitrogen, Burlington, ON) with 10% FBS (Gibco-Invitrogen) and 1% penicillin/streptomycin (Sigma-Aldrich, Oakville, ON). Cells were passaged at 80% confluency every 2-3 days using 0.25% trypsin and maintained at a density of 5 x 10^5 cells/mL. THP-1 cells were grown in tissue culture flasks (75 cm^2, 250 mL, vented, polystyrene, Sarstedt, Nümbrecht, Germany) in 90% RPMI-1640 (Sigma-Aldrich), 10% FBS, 200 nM glutamine (Sigma-Aldrich), 1% penicillin-streptomycin, and medium was changed every second day by spinning down cells at 130 x g for 8 minutes and seeded at a cell density of 1.5 x 10^5 cells/mL.

2.2 Calcium-phosphate co-precipitation transfection assays

HEK293 cells were split using DMEM high glucose and 10% charcoal-stripped FBS (Cedarlane, Burlington, ON) into a white clear-bottom 96-well plate (VWR, Mississauga, ON) aiming for 80% confluency the next day (final volume in well is 100 μL). A concentration-response luciferase assay was performed in order to calculate the EC_{50} of receptors in the presence of nuclear receptor agonists including T0901317 (LXR agonist, Cayman Chemicals, Burlington, ON), dexamethasone (GR agonist, Sigma-Aldrich), or chenodeoxycholic acid (CDCA, farnesoid X receptor (FXR) agonist, Sigma-Aldrich). From there, IC_{50} experiments with either LO520 or GSK2033 were carried out using 20 ng of various receptor plasmids (GAL4-hLXRα, GAL4-hLXRβ, CMX-mLXRα, CMX-mLXRβ, GAL4-hGR, GAL4-hFXR), 20 ng β-galactosidase, 25 ng UAS-luciferase promoter or 25 ng LXRE reporter plasmid (LXREx3-tk-Luc) and 20 ng pGEM filler plasmid with a final DNA concentration of 150 ng per well (CMX-LXR plasmids were transfected along with 20 ng CMX-mouse RXRα). The morning after seeding, the cells were treated with 10 μL of a master mix containing calcium chloride in a buffered phosphate solution thereby generating a calcium-phosphate-DNA co-precipitate and left in the cell culture incubator for 6 to 8 hours. Cells were then treated with various ligands (ligand volume added was 40 μL with a total volume of 150 μL per well). The next morning, cells were harvested and
50 μL of luciferase buffer was added to each well via multichannel pipette and subsequently treated with 125 μL β-galactosidase solution. Luciferase and β-galactosidase activities were measured on a Victor 1420 Multilabel counter (Perkin Elmer, Vaughan, ON). Each transfection reaction was carried out in triplicate, and values were normalized to β-galactosidase activity and expressed as Relative Luciferase Units (RLU). Results are presented as the mean ± SD of triplicate measurements.

2.3 Drug extraction from plasma

Wildtype blank mouse plasma (50 μL) was spiked with LO520 and precipitated with five volumes of either methanol (MeOH), acetonitrile (ACN), zinc sulfate (0.2M ZnSO₄ in MeOH) or methyl tert-butyl ether (MTBE). Samples were resuspended in HPLC-grade methanol (150 μL) to yield a final concentration of 10 μM LO520 for LC/MS/MS analysis. Samples were analyzed using an Agilent 1200 HPLC and 6410 QQQ with a Zorbax C18 column (4.6 x 50 mm, 5 μm, Agilent, Mississauga, ON). The mobile phase consisted of HPLC grade water (A) and ACN (B) both containing 5 mM NH₄Ac. The flow rate was 0.6 mL/min and the following gradient was run: 35% B (0-1 min), 35-90% B (1-3 mins), 35% B (9.1-13 mins). MS parameters were as follows: gas temperature 300°C, nebulizer pressure 35 psi, drying gas (nitrogen) 10 L/min, VCap 6000V (positive), column temperature 40°C. Using MRM monitoring (in positive-ion mode) the following transitions were observed: LO520 (m/z 705→226, RT 8.2 min), GSK2033 (m/z 592→245, RT 6.3 min). Fragmentor voltage and collision energy settings are summarized in Table 2.

2.4 Animal studies

WT and LXRα knockout mice were maintained on mixed strain background and housed in temperature and light-controlled environment. All mice were male aged between 3 and 8 months. Mice were treated intraperitoneally with LO520 in 5% Tween®80, 5% DMSO and 90% of 0.9% NaCl or 5 mg/kg dexamethasone (5% ethanol, 95% sesame oil) or T0901317 (90% PBS, 5% DMSO, 5% Tween®80). Saphenous blood was collected by pricking the saphenous vein with a 25G needle. To reduce the production of endogenous glucocorticoids from stress, mice were
sacrificed within 1 minute from initial handling by decapitation. All animals were maintained according to the recommended procedures of the Faculty of Medicine and Pharmacy Animal Care Committee at the University of Toronto and the Canadian Council on Animal Care.

2.4.1 Blood plasma collection

Saphenous and trunk mouse blood was collected in Eppendorf tubes containing 5 μL of 0.5M EDTA (BioShop, Burlington, ON), gently inverted and placed on ice until centrifuged at 500 x g at 4°C for twenty minutes. Plasma was stored at -80°C.

2.4.2 Dex injection study

Wildtype mice were injected subcutaneously twice daily for five days eight hours apart with 5 mg/kg Dex. LO520 was injected interperitoneally at 40 mg/kg daily. Vehicle-treated mice were injected with an equal volume of 0.9% NaCl/ Tween®80/DMSO (90:5:5). On the sixth day, mice were decapitated within two hours of lights turning on to ensure low endogenous glucocorticoid levels.

2.4.3 Plasma analysis

Trunk blood was collected in EDTA tubes on ice and plasma was separated by centrifuging blood samples at 500 x g at 4°C. Plasma analytes were measured using commercially available colorimetric kits: Auto Glucose Reagent kit (Wako, VWR), Total Infinity Cholesterol (VWR) and Infinity Triglyceride (VWR) kits.

2.4.4 White blood cell differential

Blood (150 μL) was collected in microcuvettes coated with EDTA on ice (Sarstedt) and hematology analysis was performed using the ABAXIS: VetScan HM5 (VWR).

2.4.5 Mouse primary hepatocyte isolation
Wildtype and LXRα-null mice were anesthetized with isoflurane and the portal vein was cannulated using a 24 G angiocatheter. Mouse liver was subsequently perfused with 25 mL perfusion buffer (Invitrogen) at 4 mL/min flow rate for five minutes via peristaltic pump. The perfusion was continued using liver digestion buffer (Invitrogen) for twelve minutes at a flow rate of 3 mL/min. The liver was then removed aseptically and washed in 10 mL warm low glucose DMEM to manually remove the blood clots, gall bladder and other adherent tissues. The cells were then filtered through a 100 μm strainer and centrifuged at 50 x g for three minutes at 10°C. The supernatant was removed and cells were washed three times, checked for viability and plated at a final density of 0.5x10⁶ cells per well onto 6-well Corning Primaria plates (Fischer Scientific, Mississauga, ON) in attachment medium (William’s E medium [Invitrogen], 10% charcoal-stripped FBS, 1% penicillin-streptomycin and 10 nM insulin [Sigma-Aldrich]). After two hours, cells were washed twice with PBS and were treated overnight with M199 medium supplemented with 5% charcoal-stripped FBS, 1 nM insulin and 1% penicillin/streptomycin. The next day ligands were added to the cells in M199 medium without FBS and cells were harvested 16 h after ligand treatment for RNA extraction. A similar experiment was performed using hepatocytes from a male, 9-week-old PD-1 (programmed cell death-1) whole body knockout mouse provided by the Uetrecht lab (University of Toronto).

2.5 RNA isolation, cDNA synthesis, and real-time QPCR analysis

Total RNA was extracted from tissues using RNA STAT-60, treated with RNase-free DNase I, and reverse transcribed into cDNA with random hexamers using the High Capacity Reverse transcription system (Applied Biosystems, Mississauga, ON). Validated human or mouse primers used are shown in Table 1. Real-time QPCR reactions were performed on an ABI 7900 in 384-well plates containing 12.5 ng cDNA, 150 nM of each primer, and 5 μL 2X SYBR Green PCR in a 10 μL total volume. Relative mRNA levels were calculated using the comparative Ct method normalized to cyclophilin or 36B4 mRNA. 36B4 is also known as acidic ribosomal phosphoprotein Po (RPLPo).
2.6 THP-1 gene expression studies

Cells were plated at a density of 2.5 x 10^6 cells/well in a 6-well plate and treated for two days with RPMI-1640 supplemented with 10% charcoal-stripped FBS containing 100 nM phorbol-12-myristate-13-acetate (PMA, BioShop) in order for the cells to differentiate into macrophages. Cells were treated with various ligands in serum free medium for 16 hours until harvested for mRNA extraction.

2.7 Cholesterol efflux studies

On day 1, THP-1 cells were plated at a density of 1.5x10^6 cells/well in a 24-well plate and treated for two days with RPMI-1640 supplemented with 10% charcoal-stripped FBS containing 100 nM PMA (to initiate monocyte to macrophage differentiation) with a final volume of 1mL/well. On day 3, medium was removed, and cells were washed twice with PBS (Sigma-Aldrich). Cells were incubated with 0.5 μCi/mL [¹³H]-cholesterol (Cholesterol, [1,2-3H(N)], 1 μCi/μL, Perkin Elmer) suspended in RPMI-1640 supplemented with 10% charcoal-stripped FBS for 48 hrs in a final volume of 0.5 mL/well. On day 5, the [¹³H]-cholesterol labelling media was removed, and cells were washed twice with PBS. Equilibration medium (RPMI-1640 with 2% fatty acid free BSA (bovine serum albumin) [BioShop] with ligand) was added for 18 hrs in a final volume of 250 μL/well. On day 6, the equilibration medium was removed, and cells were washed twice with PBS. Cells were incubated with efflux medium containing 2% fatty acid free BSA in which half of the cells were treated with 50 μg/mL HDL and the other half did not receive the acceptor (in order to estimate various ligand effects on cholesterol efflux in the presence and absence of HDL) in a final volume was 125 μL/well. Samples from the supernatant and cells were collected at various time points and 6 hrs was deemed optimal. The supernatant was collected in labelled Eppendorf tubes and centrifuged at 5000 rpm to remove cell debris. The supernatant (100μL) was transferred to labelled scintillation vials pre-filled with 5 mL ScintiVerse BD Cocktail scintillation liquid (Fisher Brand, Mississauga, ON) and ran on Tri-Carb 4910TR liquid scintillation analyzer (Perkin Elmer) to quantify the radioactivity. Cells were treated with 500μL of 0.5 M NaOH and the plates rocked for 2 hours at room temperature. Rocked lysate (100 μL) was then added to 5 mL of scintillation liquid and ran on Tri-Carb 4910TR liquid scintillation
analyzer to quantify radioactive cell content. The percent cholesterol efflux was calculated as follows: \( \frac{\text{CPM}_{\text{supernatant}}}{\text{CPM}_{\text{supernatant}} + \text{CPM}_{\text{intracellular}}} \) with the CPM values adjusted to reflect the volumes assayed and represented as difference between well with the HDL acceptor and those without. The Quanta Smart program was used to measure counts per minute (CPM). Swipe tests were performed within one day after each experiment to ensure no contamination had occurred.

2.8 Statistical analyses

One-way ANOVA followed by the Student-Newman-Keuls test was used to compare more than two groups. Outliers were removed using Grubb’s test (calculated as the difference between the outlier and the mean divided by the standard deviation, value is considered an outlier if the ratio is larger than 1.96 SD). \( P<0.05 \) was considered significant. All statistical tests were performed using the software program GraphPad Prism 6.
<table>
<thead>
<tr>
<th>Gene Name</th>
<th>Abbrev.</th>
<th>Accession #</th>
<th>Forward and Reverse Primers</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ribosomal protein, large, P0</td>
<td>36b4</td>
<td>NM_007475.5</td>
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Table 2 - Multiple Reaction Monitoring (MRM) transitions of LO520, GSK2033.

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<th>Compound Name</th>
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<th>Precursor Ion</th>
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Chapter 3: Results

Our lab has previously shown that mice lacking LXRβ but not LXRα are resistant to the diabetogenic side-effects of the synthetic glucocorticoid dexamethasone (Dex) but remain sensitive to its immunosuppressive actions (Patel et al. 2011). Further investigation into the therapeutic efficacy of LXRβ antagonism was tested by dosing a dual LXRα/LXRβ antagonist (GSK2033) to LXRα knockout mice treated with Dex. Data from this experiment suggested that inhibition of LXRβ was sufficient to reduce hyperglycemia, hyperinsulinemia, and hepatic steatosis caused by Dex while preserving its immunosuppressive effects. Since these results were obtained in the LXRα-/- background, we hypothesized that the use of a selective LXRβ antagonist would allow prevention of GC-mediated side effects in a wildtype mouse. In this chapter, we show the drug characterization and proof-of-concept studies of a novel LXRβ antagonist (LO520) using various in vivo and in vitro approaches.

3.1 - LO520 is a highly selective LXRβ antagonist.

In order to confirm that LO520 acts as a LXRβ antagonist, we had to first establish the LXR isoform selectivity of LO520. Luciferase assays were conducted to quantify LXR receptor activity in the presence of LO520. We first performed a dose-response of T0901317, an LXRX agonist, to assess the EC₈₀ for activation of each LXR isoform. The EC₈₀ for GAL4-hLXRα and GAL4-hLXRβ on a UAS-Luc reporter was estimated to be 1 μM and 332 nM respectively (Figure 3.1A). Using each receptor’s approximate EC₈₀, we then assessed the inhibitory potency (IC₅₀) of LO520 against each receptor. The IC₅₀ of LO520 against GAL4-hLXRα and GAL4-hLXRβ in HEK293 cell transfection assay was estimated to be 160 μM for LXRα and 0.505 μM for LXRβ, a 320-fold difference between receptors (Figure 3.1B).

A similar study was performed using mouse receptor plasmids in which the EC₈₀s for T0901317 against CMX-mLXRα and CMX-mLXRβ on a LXREx3-tk-Luc reporter were estimated to be 316 nM and 108 nM, respectively (Figure 3.2A). With these values, the IC₅₀ of LO520 against murine LXRβ receptor was estimated to be 925 nM and the IC₅₀ was not determinable for LXRα (Figure 3.2B). The inhibitory constant (Kᵢ) for LO520 was calculated in
order to deduce the strength of the binding interaction between LO520 and the LXR isoforms. The $K_i$ was calculated using the following equation:

$$K_i = \frac{IC_{50}}{1 + \frac{[\text{Ligand}]}{K_d \text{ of ligand}}}$$

The dissociation constants, $K_d$s, for the LXR ligand T0901317 were reported to be 7 nM and 22 nM for human LXRα and LXRβ, respectively (Yue et al. 2005). Incorporating these values into the $K_i$ equation gave an inhibitory constant of 1112 nM for LXRα and 31 nM for LXRβ, a 36-fold difference between receptors. LO520 did not significantly antagonize two other nuclear receptors tested, GAL4-hGR and GAL4-hFXR, demonstrating receptor specificity (Supplemental Figure 1). We performed similar IC$_{50}$ experiments for the dual LXRα/LXRβ antagonist GSK2033 and found a 21-fold difference between murine LXR receptors (Supplemental Figure 2), compared to a 320-fold difference that we measured for LO520 (Figure 3.1B). Together these results demonstrate that LO520 is a highly selective antagonist for both mouse and human LXRβ receptors.
Figure 3.1: LO520 is a highly selective human LXRβ antagonist.

HEK293 cells were co-transfected with GAL4-hLXRα or GAL4-hLXRβ with a UAS-luciferase reporter plasmid. A) A dual LXR agonist T0901317 was dosed at the EC$_{80}$ for each reporter (1 μM for GAL4-hLXRα and 250 nM for GAL4-hLXRβ). B) The IC$_{50}$ of LO520 against LXRα and LXRβ in HEK293 cell transfection assay was estimated to be 160 μM for LXRα and 0.505 μM for LXRβ. For the LO520 dose response, results were pooled from three independent experiments (N=3/experiment) performed on different days. Data presented as Mean ± SD (N=9 for LO520, N=3 for T091317). The EC$_{50}$ and EC$_{80}$ values were determined using log(agonist) vs. response - Find ECanything function and the IC$_{50}$ values were determined using log(inhibitor) vs. normalized response - Variable slope function (Prism 6, GraphPad).
Figure 3.2: LO520 is a highly selective mouse LXRβ antagonist.

HEK293 cells were co-transfected with CMX-mLXRα or CMX-mLXRβ and CMX-mRXRα with a LXRE reporter plasmid (LXREx3-tk-Luc). **A)** A dual LXR agonist T0901317 was dosed at the EC₈₀ for each reporter (316 nM for LXRα, 108 nM for LXRβ). **B)** The estimated IC₅₀ values of LO520 against mouse LXRα and LXRβ receptors transfected into HEK293 cells. No value could be estimated for LXRα because of the weak inhibition. The EC₅₀ and EC₈₀ values were determined using log(agonist) vs. response - Find ECanything function and the IC₅₀ values were determined using log(inhibitor) vs. normalized response - Variable slope function (Prism 6, GraphPad). Results were pooled from two independent experiments (Mean ± SD, N=9) performed on different days for the LO520 dose response. Results for T0901317 dose response are from one independent experiment (Mean ± SD, N=3).

3.2 - **LO520 extraction is optimal with 5x-MeOH and its standard curve in plasma is linear.**

To ensure optimal detection of LO520 from plasma in our planned pharmacokinetic studies, we first determined the optimal extraction method of LO520 from plasma. Next, we created a standard curve of LO520 using blank plasma to allow us to interpolate the levels of LO520 after *in vivo* dosing. In order to obtain the most accurate detection of LO520 in plasma by LC/MS/MS assay, the extraction efficiency from various solvents was assessed: i) 5:1 MeOH:plasma; ii) 5:1 ACN:plasma; iii) 5:1 0.2M ZnSO₄ in MeOH:plasma; iv) 5:1 MTBE:plasma. From the four different solvents tested in mouse wildtype plasma containing LO520, methanol had the highest percentage extraction efficiency (MeOH, 89%) followed by acetonitrile (ACN, 82%), zinc sulfate (ZnSO₄, 52%) and methyl tert-butyl ether (MTBE, 47%)
(Figure 3.3A). From there, a standard curve of increasing concentrations of LO520 extracted by 5x methanol was created and had an R² value of 0.9992 demonstrating robust linearity between 25 nM and 25 μM (Figure 3.3B).

**Figure 3.3:** LO520 in plasma is most efficiently extracted with methanol and standard curve is linear.

Wildtype blank mouse plasma (50 μL) was spiked with LO520 and precipitated with five volumes of either methanol (MeOH), acetonitrile (ACN), zinc sulfate (0.2M ZnSO4 in MeOH) or methyl tert-butyl ether (MTBE). Samples were resuspended in HPLC-grade methanol (150 μL) to yield a final concentration of 10 μM LO520 for LC/MS/MS analysis. A) Extraction efficiency was compared to 10 μM LO520 prepared in pure methanol. B) Wildtype blank mouse plasma samples were spiked with GSK2033 as the internal standard (final concentration 0.33 μM) along with increasing concentrations of LO520 (25 nM to 25 μM) and precipitated with five volumes of methanol. Samples were resuspended in HPLC-methanol for LC/MS/MS analysis performed by Michael F. Saikali. Data represent the Mean ± SD, N=3.

3.3 - Pharmacokinetic profile of LO520 after intraperitoneal dosing.

In order to establish a dosing frequency of LO520, we performed a pharmacokinetic study after intraperitoneal dosing (40 mg/kg) of LO520 to wildtype mice (N=4 mice per time point). Blood was collected at 5, 15, 30 minutes, 1, 2, 4, 6 and 12 hrs and plasma LO520 levels were measured using LC/MS/MS. Samples were taken more frequently around the 1-hour time point because this was the anticipated t_max for an intraperitoneal dose. The resulting concentration vs. time curve was used to calculate LO520 pharmacokinetic parameters (Figure 3.4, Table 3). The t_max of LO520 was observed at four hours and the corresponding C_max was 238.5 μM (Figure 3.4A). The elimination rate constant (k) of LO520 was calculated to be 0.4764
hr⁻¹ and its half-life was estimated to be 1.5 hrs (Table 3). The overall exposure of drug over time, represented by the area under the curve (AUC) was estimated to be 735 μg*hr/mL with 99% of the AUC accounted for by the 12 hr time point (Table 3, Supplemental Table 1). This study demonstrated that after a single dose of 40 mg/kg, LO520 would remain above the IC₅₀ for mLXRβ (~1 μM) for at least 15 hours suggesting that this daily dose could be used for future in vivo efficacy studies.

**Figure 3.4: Pharmacokinetic profile of LO520**

Wildtype mice were injected intraperitoneally with 40 mg/kg LO520 (90% 0.9% NaCl, 5% Tween® 80, 5% DMSO) and blood was collected via saphenous vein or after decapitation at various time points ranging from five minutes to 12 hours (A). The semi-log plot was used to estimate the elimination half-life (B). LO520 was extracted from plasma and analyzed by LC/MS/MS. Concentration-time relationship data are represented as Mean ± SEM, N=4 mice/timepoint. LC/MS/MS analysis performed by Michael F. Saikali, i.p injections performed by Jia-Xu Li, saphenous blood collection performed by Cigdem Sahin.
### Table 3: Pharmacokinetic parameters of LO520 and their calculations

<table>
<thead>
<tr>
<th>PK parameter</th>
<th>Calculation</th>
<th>Value Estimates Calculated</th>
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<tbody>
<tr>
<td>Elimination phase (k)</td>
<td>[ k = -slope \left( \frac{lnC_p}{time(hr)} \right) ]</td>
<td>0.4764 hr(^{-1})</td>
</tr>
<tr>
<td>Half-life (t(_{1/2}))</td>
<td>[ t_{1/2} = \frac{ln(2)}{k} ]</td>
<td>1.5 hrs</td>
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<td>Area Under Curve (0(\rightarrow)12hrs)</td>
<td>[ AUC_{(0\rightarrow12hrs)} = \sum AUC_{0\rightarrow0.08hrs} + AUC_{0.25hrs\rightarrow0.5hrs} + AUC_{0.5hrs\rightarrow1hr} + ] [ AUC_{1hr\rightarrow2hrs} + AUC_{2hrs\rightarrow4hrs} + AUC_{4hrs\rightarrow6hrs} + ] [ AUC_{6hrs\rightarrow12hrs} ]</td>
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<td>Area Under Curve (12(\rightarrow)infinity)</td>
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<td>3.39 mL</td>
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3.4 - LO520 suppresses the induction of known LXRα target genes at 60 mg/kg in the presence of the dual LXR agonist T0901317.

A similar experiment was performed where LO520 was injected with increasing doses (20, 40, 60 mg/kg) against 5 mg/kg of the dual LXR agonist T0901317 in wildtype mice thereby titrating its antagonistic activity against known dual LXR (Abcg5, Abcg8) and LXRα preferential target genes (Cyp7a1, Srebp1c). As expected, T0901317 increased mRNA expression of known LXR target genes between 2 and 4-fold. At the highest dose of 60 mg/kg, however, we observed significant suppression of the LXRα preferential target genes, Cyp7a1 and Srebp1c (Figures 3.5A and 3.5B). These results suggest that LO520 has the capability to significantly antagonize T0901317 induced LXRα target genes when dosed at 60 mg/kg.

![Image of bar graphs showing gene expression changes](image_url)

**Figure 3.5:** LO520 can suppress the induction known LXRα target genes in the liver when dosed at 60 mg/kg.

Expression of the LXRα preferential target genes Cyp7a1 (A) and Srebp1c (B) and LXRα/LXRβ dual target genes Abcg5 (C) and Abcg8 (D) in WT mouse liver. Mice were treated with 5 mg/kg T0901317 alone or in combination with increasing concentrations of LO520. RNA was extracted, reverse transcribed, and analyzed for gene expression by real-time QPCR. Data presented as Mean ± SD, N=3. *P<0.05 by One-Way ANOVA and Newman-Keuls test. I.p injections performed by Jia-Xu Li.

3.5 - LO520 marginally suppresses Dex-induced Pepck expression in mouse primary hepatocytes.
Our lab has previously shown that LXRβ is required for the recruitment of GR to the Pepck promoter, suggesting that the absence of LXRβ (Patel et al., 2011) and/or antagonism of LXRβ (using GSK2033 in LXRα-/- mice as seen in Patel et al., 2017) would be beneficial in suppressing the gluconeogenic effects of GCs. Thus, we wanted to similarly determine the extent of Pepck induction by Dex when dosed in the presence of LO520 to establish the magnitude of gluconeogenic repression that could be expected by this drug. Due to difficulty we encountered isolating viable primary hepatocytes, we performed only two pilot experiments to date (Figure 3.6 and Supplemental Figure 3). Primary hepatocytes of one wildtype and one LXRα-/- mouse was used. As expected, Dex increased Pepck mRNA levels significantly (Figure 3.6A, 3.6B). In the presence of Dex, LO520 was able to marginally suppress Pepck upregulation, unlike GSK2033 which significantly downregulated Dex-induced Pepck expression (Figure 3.6A, 3.6B). In the second pilot study, primary hepatocytes of a male 8-week old mouse with whole-body knockout of programmed cell death -1 (PD-1) was used. PD-1 is involved in suppressing the immune response via reducing T cell activity and is not expected to be expressed in the liver or influence the gluconeogenic pathway (Van de Velde et al. 2017). Similar to the first study, Dex was able to induce Pepck expression, however this time LO520 was not shown to diminish Dex-induced Pepck upregulation (Supplemental Figure 3A). The LXRα target gene Srebp1c, which encodes a transcription factor involved in the regulation of lipogenic genes, was used as a positive control (Supplemental Figure 3B). As expected, the dual LXR agonist T0901317 was able to induce Srebp1c due to its activation of LXRα (Supplemental Figure 3B). Since GSK2033 antagonizes LXRα, we expect downregulation of Srebp1c with GSK2033 treatment, as seen in Supplemental Figure 3B. As an LXRβ antagonist, LO520 does not suppress nor induce Srebp1c via lack of LXRα modulation, which is also seen in Supplemental Figure 3B. Taken together, these results suggest that even though LO520 is LXRβ selective, it is unable to reproducibly suppress the glucocorticoid-mediated induction of Pepck.
Figure 3.6: LO520 marginally suppresses GC-induced Pepck expression
Expression of the GR target gene Pepck in wildtype (A) and LXRα-/- whole body knockout (B) mouse primary hepatocytes after 18 hours of ligand treatment. Cells were treated with LO520 alone or in combination with Dex (100 nM), or with 10 μM GSK2033 and 1 μM T0901317 as positive controls. RNA was extracted, reverse transcribed, and analyzed for gene expression by real-time qPCR. Data presented as Mean ± SD, N=3. *P<0.05 by One-Way ANOVA and Newman-Keuls test. Primary hepatocytes extracted by Dr. Carolyn L. Cummins.

3.6 - LO520 lowers plasma glucose levels in the presence of Dex.

Despite weak/conflicting in vitro evidence (primary hepatocyte data) suggesting LO520 would be able to suppress Pepck expression, we tested the efficacy of LO520 in an in vivo mouse model of glucocorticoid-induced diabetes. Mice were injected subcutaneously with 5 mg/kg Dex twice daily for five days to precipitate the development of insulin resistance and hyperglycemia. LO520 was dosed i.p. at 40 mg/kg once daily. We measured the effect of Dex ± LO520 on physiological changes including body and organ weights, plasma analytes and liver gene expression of key gluconeogenesis genes regulated by LXRs. Body weight and food intake remained the same across all treatments (Figure 3.7A and 3.7B) along with no change in liver weight (Supplemental Figure 4), demonstrating that LO520 does not modify these parameters. As expected, Dex increased plasma glucose levels (Figure 3.8A). Remarkably, even though LO520 was unable to fully suppress Dex-induced Pepck mRNA expression in mouse primary
hepatocytes (Figure 3.6, Supplemental Figure 3), LO520 significantly lowered Dex-induced plasma glucose levels (Figure 3.8A). Dex increased plasma cholesterol levels, however, LO520 was unable to attenuate the Dex-induced increase in cholesterol (Figure 3.8B). Plasma triglyceride levels remained unchanged between all treatment groups (Figure 3.8C).

Figure 3.7: LO520 and/or Dex do not change body weight or food intake.

Wildtype mice were injected subcutaneously twice daily for five days eight hours apart with 5 mg/kg Dex. LO520 was injected interperitoneally at 40 mg/kg daily. On the sixth day, mice were decapitated within two hours of the lights coming on. A) Body weight was measured at first day of injections (Day 1) and last day (Day 5). B) Food intake represented by grams of normal chow diet eaten per day. Data presented as Mean ± SEM, N=5. *P<0.05 by One-Way ANOVA and Newman-Keuls test. Body weight and food intake measured by Jia-Xu Li.
Figure 3.8: LO520 lowers plasma glucose levels in the presence of dexamethasone.

Wildtype mice were injected subcutaneously twice daily for five days eight hours apart with 5 mg/kg Dex. LO520 was injected interperitoneally at 40 mg/kg daily. On the sixth day, mice were decapitated within two hours of the lights coming on. Plasma glucose (A), cholesterol (B) and triglycerides (C) were measured using colorimetric assays by Jia-Xu Li. Data presented as Mean ± SEM, N=5. *P<0.05 by One-Way ANOVA and Newman-Keuls test.

3.7 – LO520 does not attenuate Dex-induced hepatic lipid accumulation.

Along with other glucocorticoids, Dex has been known to induce a fatty liver phenotype that is apparent by the appearance of hepatic lipid droplets. Histologic examination of mouse liver sections was performed with oil red O staining to detect neutral lipid content. An increase in lipid droplets were seen in mice treated with 5 mg/kg Dex compared to Vehicle (Figure 3.9A and 3.9B). Mice treated with LO520 alone did show any difference from Vehicle (Figure 3.9C). In the combined treatment of LO520+Dex, LO520 was unable to attenuate Dex-induced lipid accumulation in the liver (Figure 3.9D).
Figure 3.9: LO520 does not attenuate dexamethasone-induced lipid accumulation in the liver.

Wildtype mice were injected subcutaneously twice daily for five days eight hours apart with 5 mg/kg Dex. LO520 was injected interperitoneally at 40 mg/kg daily. On the sixth day, mice were decapitated within two hours at lights on. Liver sections of Vehicle (A), Dex (B), LO520 (C) and LO520+DEX (D) treated mice were stained with oil red O and examined under brightfield microscopy. Original magnification, ×20 (representative image from N = 5/treatment group).

3.8 - Mice treated with LO520 remain sensitive to the immunosuppressive effects of Dex.

Glucocorticoids are used therapeutically for their potent anti-inflammatory and immunosuppressive effects. In order to establish whether or not the anti-inflammatory effects of Dex were preserved with co-administration of LO520, spleen weight was used as a marker of immune function since lymphoid organs atrophy in response to Dex. As expected, Dex significantly decreased spleen weight and remained low when co-dosed with LO520 (Figure 3.10A). Interestingly, we observed a significant increase in spleen weight in mice that were dosed with LO520 alone. Since activation of LXRα are known to display anti-inflammatory activities, LXR antagonism could exacerbate inflammation which may explain the increased spleen weight (Figure 3.10A). Examination of the white cell type distribution among the treated
mice showed that Dex lead to extravasation of lymphocytes to the periphery and neutrophil demargination, and this effect on the immune cells was preserved even when Dex was co-treated with LO520 (Figure 3.10B). This result demonstrates that the immune system’s response to Dex is maintained in the presence of LO520.

**Figure 3.10: Mice treated with LO520 remain sensitive to the immunosuppressive effects of dexamethasone.**

Wildtype mice were injected subcutaneously twice a day for five days eight hours apart with 5 mg/kg Dex. LO520 was injected intraperitoneally at 40 mg/kg for five days in between Dex treatment. On the sixth day, mice were decapitated within 2 hours of the lights coming on. Hematology was performed using ABAXIS: VetScan HMS by Jia-Xu Li. (A) Spleen weight (g) was normalized to mouse femur length. *P<0.05 by One-Way ANOVA and Newman-Keuls test. (B) Total white blood cell type represents each column as a percentage of WBC distribution per treatment. *P<0.05 by Two-Way ANOVA and Tukey's multiple comparisons test. Data represents the Mean ± SEM, N=5/treatment.

3.9 - LO520 does not attenuate Dex-induced gluconeogenic gene expression in vivo.

Similar to results obtained with GSK2033 in LXRα-/- mice (Patel et al., 2017), LO520 was able to suppress Dex-induced hyperglycemia in vivo (Figure 3.8A). To investigate the underlying mechanism of this in vivo result we measured liver gene expression by qPCR. In agreement with the known effect of GR activation on gluconeogenesis, an 8-fold increase in *Pepck* expression was seen when mice were treated with Dex (Figure 3.11A). However, similar
to the results seen in primary hepatocytes, LO520 was unable to fully attenuate Dex-induced *Pepck* expression (Figure 3.11A). Another key gluconeogenic gene, glucose-6-phosphatase (*G6pc*), and a transcriptional regulator of the pathway, forkhead box protein O1 (*Foxo1*) were induced by Dex and there were non-significant trends towards decreases in these genes when Dex was co-treated with LO520 (Figures 3.11B and 3.11C). Another co-activator involved in the transcriptional regulation of *Pepck* and *G6pc*, peroxisome proliferative activated receptor-gamma co-activator 1 (*PGC-1α*), had no significant gene expression changes regardless of treatment, though it tended to increase with Dex (Figure 3.11D). Tyrosine aminotransferase (*Tat*), a GR gene involved in the breakdown of amino acids to form precursors for glucose, was induced by Dex and this induction was significantly decreased in the presence of LO520 (Figure 3.11E). Taken together, these data suggest that the mechanism by which GSK2033 and LO520 impact circulating glucose levels appear distinct. Further studies are required to elucidate the mechanism by which LO520 impacts glucose levels.
Figure 3.11: LO520 does not attenuate dexamethasone-induced gluconeogenic gene expression in liver.

Wildtype mice were injected subcutaneously twice daily for five days eight hours apart with 5 mg/kg Dex. LO520 was injected interperitoneally at 40 mg/kg daily. On the sixth day, mice were decapitated within two hours of the lights coming on. Gene expression of gluconeogenic genes including *Pepck* (A), *G6pc* (B), *Foxo1* (C), *Pgc1α* (D) and *Tat* (E) were quantified by real-time QPCR. Data presented as Mean ± SEM, N=3. *P*<0.05 by One-Way ANOVA and Newman-Keuls test.

3.10 - LO520 has minimal basal effect on LXRα target genes in vivo.

In order to deduce the impact of LO520 *in vivo* on known LXRα target genes in the presence of Dex, mRNA expression was assessed. Unlike our previous findings where LO520 attenuated T0901317-induced LXR target gene activation (Figure 3.5), basally, LO520 did not affect *Cyp7a1* mRNA expression (Figure 3.12A). LO520 also induced *Srebp1c* upregulation basally, which was unexpected (Figure 3.12B). Taken together, these results show LO520’s lack of basal antagonism on LXRα target genes *in vivo* that is consistent with the 40 mg/kg dose chosen.
Figure 3.12: LO520 basally has minimal effect on LXRα target genes *in vivo*.

Wildtype mice were injected subcutaneously twice daily for five days eight hours apart with 5 mg/kg Dex. LO520 was injected interperitoneally at 40 mg/kg daily. On the sixth day, mice were decapitated within two hours of the lights coming on. Gene expression of known LXRα target genes involved in bile acid, Cyp7a1 (A) and fatty acid synthesis, Srebp1c (B) was measured by real-time qPCR. Data presented as Mean ± SEM, N=3. *P<0.05 by One-Way ANOVA and Newman-Keuls test.

3.11 - LO520 basally antagonizes LXR target genes involved in reverse cholesterol transport.

Due to the involvement of LXRαs in the regulation of genes important for cholesterol efflux, a possible side effect of LO520 administration is inhibition of the reverse cholesterol transport pathway potentially leading to subsequent development of atherosclerosis. To test for this potential adverse effect, we examined the gene expression of ABCA1 and ABCG1, the ATP-binding cassette transporters responsible for the efflux of cholesterol from macrophages to HDL, in human THP-1 macrophage cells. We first performed a dose-response analysis to determine the concentration of the dual LXR agonist T0901317 that would give us the highest ABCA1 and ABCG1 mRNA induction (Figure 3.13). It was concluded that 50 nM T0901317 was the lowest dose to give us the highest ABC gene expression before reaching a plateau of mRNA expression (Figure 3.13). Next, we treated cells with 50 nM T0901317 to induce near maximal ABC target gene expression and co-dosed with increasing concentrations of LO520 (5 μM to 25 μM) to establish the potency with which LO520 would antagonize the induction of the genes by
Complete inhibition of the T0901317 induced expression of ABCA1 mRNA was observed at 10 μM LO520 (Figure 3.14). To test whether LO520 would affect the basal levels of the ABC transporter genes, THP-1 macrophages were treated with LO520 alone. At high concentrations (5 μM to 25 μM), LO520 acted as an inverse agonist by antagonizing basal expression of ABCA1 and ABCG1 mRNA (Figure 3.15). Once assessing gene expression with lower concentrations (10 nM to 500 nM) of LO520, we still see basal downregulation (Figure 3.16). The IC\textsubscript{50}s of LO520 for antagonizing the basal expression of ABCA1 and ABCG1 were estimated by plotting the percent inhibition normalized to vehicle and found to be 57.5 nM and 112.3 nM, respectively (Supplemental Figure 5). We also measured the effect of Dex alone and in combination with LO520 in THP-1 cells since the eventual goal would be to dose LO520 along with a glucocorticoid drug in humans. Alone, Dex did not have an effect on ABC transporter gene expression and did not change LO520’s ability to downregulate these genes (Figures 3.15 and 3.16). As expected, GSK2033 downregulated ABC gene expression since dual-LXR antagonism intercepts LXR’s atheroprotective effects (Figures 3.15 and 3.16). Together these data suggest that LO520 is a potent suppressor of the basal expression of ABCA1 and ABCG1 mRNA in THP-1 cells.
Figure 3.13: T0901317 induces LXR target genes involved in reverse cholesterol transport in a dose-dependent manner.

THP-1 cells were treated with 100 nM PMA for 48 hrs to initiate monocyte to macrophage differentiation. Concentrations of T0901317 from 1 nM to 100 nM were added for 16 hr to induce upregulation of LXR target genes involved in reverse cholesterol transport, ABCA1 (A) and ABCG1 (B). RNA was extracted, reverse transcribed, and analyzed for gene expression by real-time qPCR. Data represent the Mean ± SD, N=3. *P<0.05 by One-Way ANOVA and Newman-Keuls test.
Figure 3.14: LO520 downregulates the T0901317-induced activation of atheroprotective LXR target genes at all concentrations tested.

THP-1 cells were treated with 100 nM PMA for 48 hrs to initiate monocyte to macrophage differentiation. Concentrations of LO520 from 5 μM to 25 μM co-treated with 50 nM T0901317 was added for 16 hr. The dual LXR antagonist GSK2033 (10 μM) served as a positive control. RNA of LXR target genes involved in reverse cholesterol transport, ABCA1 (A) and ABCG1 (B) was extracted, reverse transcribed, and analyzed for gene expression by real-time qPCR. Data represent the Mean ± SD, N=3. *P<0.05 by One-Way ANOVA and Newman-Keuls test.
Figure 3.15: LO520 downregulates the basal expression of atheroprotective LXR target genes at high concentrations.

THP-1 cells were treated with 100 nM PMA for 48 hrs to initiate monocyte to macrophage differentiation. Concentrations of LO520 from 5 μM to 25 μM ± Dex (1 μM), or GSK2033 (10 μM) ± Dex were added for 16 hr. Gene expression of ABCA1 (A) and ABCG1 (B) was measured by real-time qPCR. Data represent the Mean ± SD, N=3. *P<0.05 by One-Way ANOVA and Newman-Keuls test.
Figure 3.16: LO520 downregulates the basal expression of atheroprotective LXR target genes at nanomolar concentrations.

THP-1 cells were treated with 100 nM PMA for 48 hrs to initiate monocyte to macrophage differentiation. Lower concentrations of LO520 from 1 nM to 500 nM ± Dex (1 μM), or GSK2033 (10 μM) ± Dex were added for 16 hr and genes involved in reverse cholesterol transport, ABCA1 (A) and ABCG1 (B) were quantified by real-time qPCR. Data represent the Mean ± SD, N=3. *P<0.05 by One-Way ANOVA and Newman-Keuls test.

3.12 - LO520 and GSK2033 suppress RCT induced by T0901317 but do not decrease basal RCT.

To test whether the changes we observed in the mRNA expression of ABCA1 and ABCG1 would result in functional differences in cholesterol efflux, we performed an in vitro reverse cholesterol transport assay. In this assay, macrophages are pre-loaded with 0.5 μCi/mL [3H]-cholesterol and incubated with compounds that either induce or inhibit ABC transporter expression. Two days after cholesterol loading, [3H]-cholesterol was removed in order to allow equilibration and start ligand treatment (T0901317, GSK2033 or LO520). After 18 hrs, cells were treated with or without HDL (50 μg/mL), which acted as the acceptor molecule for cholesterol. Supernatant was then removed to measure extracellular labeled cholesterol and lysate was collected to measure intracellular labeled cholesterol, allowing for comparison of relative levels between both. We first determined the optimal time for efflux by performing a time course analysis after addition of HDL or vehicle for 2, 4, or 8 hours (Supplemental Figure
6). Because both the 4 hr and 8 hr time points were comparable, we decided to perform all future studies using a 6 hr efflux period. Since we know that LXR activation is reported to increase reverse cholesterol transport, 1μM T0901317 was used as a positive control (Zanotti et al., 2008; Ma et al., 2014). Similarly, the potent LXR antagonist GSK2033 (10 μM) was used as we anticipated a suppressive effect on cholesterol efflux. As expected, T0901317 basally increased cholesterol efflux and GSK2033 basally decreased cholesterol efflux when compared to vehicle (Figure 3.17). When treated with T0901317, GSK2033 and LO520 were shown to downregulate T0901317-induced cholesterol efflux, however, unlike GSK2033, LO520 did not downregulate basal cholesterol efflux (Figure 3.17).

![Figure 3.17: LO520 suppresses RCT induced by T0901317 but does not decrease basal RCT.](image)

THP-1 cells were treated with 100 nM PMA for 48h to initiate monocyte to macrophage differentiation. Cells were then treated with 0.5 μCi/mL [3H]-cholesterol for 48hrs followed by ligand treatment for 18 hrs. T0901317 is used as a positive control to induce RCT. The extent of cholesterol efflux was quantified from the supernatant and cellular pools after 6 hrs exposure to ± 50 μg/mL HDL and counted in a liquid scintillation counter. Percent cholesterol efflux was calculated as follows: [supernatant/(supernatant+intracellular)] and represented as difference between HDL treated wells and wells without HDL acceptor. The data represent the Mean ± SD, N=3. *P<0.05 by One-Way ANOVA and Newman-Keuls test.

Since the conditions to grow the THP-1 cells are different when performing the RCT assay compared to the gene expression assay, we decided to examine the mRNA expression of ABCA1 and ABCG1 after treatment of cells in the RCT protocol. Cells underwent the same
protocol as those undergoing cholesterol efflux quantification. Before reaching the step for HDL treatment, cells were processed for qPCR analysis looking at ABCA1 and ABCG1. As expected, T0901317 increased expression of both genes (Figure 3.18). GSK2033 and LO520 basally suppressed mRNA expression of the ABC transporters which was also consistent with our prior mRNA data (Figures 3.15, 3.16). However, in the presence of 1 μM T0901317, while GSK2033 still strongly suppressed ABCA1 and ABCG1 expression, there was no effect of 25 μM LO520 on the induction of these genes by T0901317. Therefore, LO520’s effect on mRNA expression of ABCA1 and ABCG1 shows opposing results from the RCT study. Notably, in the efflux studies, LO520 did not basally antagonize cholesterol efflux, yet mRNA analysis shows that LO520 basally downregulates ABC genes. Additionally, the efflux study showed that LO520 suppressed T0901317-induced reverse cholesterol transport (Figure 3.17) yet LO520 did not attenuate the T0901317 upregulation on gene expression (Figure 3.18). Taken together, we can conclude that the effects of LXR antagonism on genes important in RCT do not consistently reflect expected functional outcomes due to unknown mechanisms that have yet to be examined.

Figure 3.18: LO520 and GSK2033 downregulate basal expression of atheroprotective LXR target genes.

THP-1 cells were treated with 100 nM PMA for 48h to initiate monocyte to macrophage differentiation. Cells were then treated with RPMI 1640 supplemented with 10% FBS and 1% L-glutamine for 48hrs followed by ligand treatment for 18hours. Gene expression of ABCA1 (A) and ABCG1 (B) was measured by real-time qPCR. Data represent the Mean ± SD, N=3. *P<0.05 by One-Way ANOVA and Newman-Keuls test.
Chapter 4: Discussion

In this thesis, the novel LXRβ antagonist LO520, was pharmacologically characterized for the treatment of glucocorticoid-induced diabetes. Our lab has previously shown that mice lacking LXRβ, but not LXRα, are resistant to GC-induced hyperglycemia, hyperinsulinemia, and hepatic steatosis, but remain sensitive to the anti-inflammatory benefits of GCs. This implies that therapeutically targeting LXRβ may offer a novel adjunctive therapy to limit the diabetogenic side effects associated with exogenous GC treatment, while preserving their beneficial anti-inflammatory actions. The LXRs are ligand-activated transcription factors and members of the nuclear receptor superfamily. They are involved in a multitude of pathways and play a significant role in lipid and cholesterol metabolism. The goal of this thesis was to characterize LO520 using in vitro and in vivo models in order to examine the isoform specificity of LXR antagonism and investigate whether LO520 would impact LXR’s role in maintaining cholesterol homeostasis.

In the liver, both LXR isoforms positively regulate the transcription of several cholesterol efflux transporter proteins, and thus play a central role in preventing the lipotoxicity associated with aberrant accumulation of free cholesterol within hepatocytes (Zhu et al., 2012). The antagonism of both LXRs by GSK2033 is not a practical option for the treatment of glucocorticoid-induced diabetes since without LXRα, physiological constraints of cholesterol transport might arise, such as lipotoxicity due to cholesterol accumulation within hepatocytes (Ertunck et al., 2016). With this intent, the first aim of this study was to confirm selective LXRβ isoform antagonism by LO520. It was concluded that LO520 is a highly selective LXRβ antagonist (IC50 of 500 nM). Since a low IC50 value (in the nM range) demonstrates strong binding between ligand and receptor, LO520 acts as a weak LXRα antagonist with an LXRα IC50 of 160 μM. Compared to GSK2033, which has been shown in literature to have a 10-fold difference in IC50 between receptors (Patel et al., 2017), LO520 has a greater IC50 fold difference between LXR receptors (320-fold), thereby acting as a more selective LXRβ antagonist.

The study of pharmacokinetics in preclinical drug characterization is paramount since these animal studies form the basis for predicting drug dosing, drug interactions and toxicity for
human studies. From our pharmacokinetic study in wildtype mice, the half-life of LO520 was estimated to be nine times longer than the half-life of GSK2033. The $t_{\text{max}}$ following an intraperitoneal dose was estimated to be around four hours, an unexpected delay of drug absorption into the systemic circulation. The explanation underpinning this slow absorption rate could be related to molecular size, lipid solubility, protein binding and pH of the drug, among other reasons (Chillistone and Hardman, 2017). The molecular size of LO520 is similar to that of GSK2033 however according to Griffet and Burris (2016), when 30 mg/kg GSK2033 was administered i.p, its $t_{\text{max}}$ was seen at one hour (with a $C_{\text{max}}$ value of around 250 nM). The reasoning behind LO520’s delay in absorption could be due to its higher lipophilicity compared to GSK2033. The lipophilicity of LO520 may lead to its accumulation in fatty tissues thereby delaying absorption into the systemic circulation. Alternatively, LO520 could be entering the lymphatic system before reaching the circulatory system and cause a slower rate of absorption.

Using perfused liver and primary mouse hepatocytes, it has been demonstrated that LXRβ plays a central role in driving GC-induced hyperglycemia by recruiting the GR to the PEPCK promoter (Patel et al., 2011). Therefore, we tested whether LO520 would have an effect on hepatic Pepck expression via two experimental models: 1) mice injected with 40 mg/kg LO520 and 5 mg/kg Dex (*in vivo*) and, 2) primary mouse hepatocytes treated with 25 μM LO520 and 100 nM Dex (*in vitro*). As expected, Dex increased Pepck mRNA expression in both studies, however, LO520 was ineffective at reversing this induction (Figure 3.6 and Supplemental Figure 3). Previous studies from our lab have shown that LXRβ−/− mice are protected from GC-induced hyperglycemia after 14 days of Dex treatment (Patel et al., 2011). When testing the effects of GR-mediated Pepck expression *in vitro*, Patel et al. 2017 found that Dex treatment increased Pepck expression in wildtype and LXRα−/− primary hepatocytes and that the induction of Pepck by Dex was inhibited once hepatocytes were co-treated with the dual LXR antagonist GSK2033 (Patel et al., 2017). In LXRβ−/− primary hepatocytes, Dex did not increase Pepck expression demonstrating that LXRβ is essential for mediating the activating effects of GCs on the Pepck promoter. Since we have demonstrated that LO520 is a highly selective LXRβ antagonist, we anticipated this compound to be effective at suppressing the Dex-mediated induction of Pepck. Dex was shown to induce other gluconeogenic genes (*Foxo1, G6pc, Tat*) in primary hepatocytes, yet similar to Pepck, LO520 did not significantly modulate their gene expression (with the
exception of Tat that did decrease, Figure 3.11). It is widely known that gene expression does not always correlate to protein expression and physiological function. An example of this was observed when we measured plasma glucose levels from the in vivo Dex-induced diabetic mouse model study (Figure 3.8). Since the primary hepatocyte and in vivo study showed LO520 to not significantly attenuate Dex-induced Pepck mRNA expression, we did not expect plasma glucose to be decreased once Dex-treated mice were injected with LO520. Remarkably, LO520 lowered Dex-induced plasma glucose in wildtype mice (Figure 3.8), prompting us to consider the possibility that LO520 might be working through a different molecular pathway, unlike GSK2033 and knockout mice which show similar trends in LXRβ antagonism and gluconeogenic gene suppression (Patel et al. 2011, Patel et al., 2017). LO520 was injected intraperitoneally 17 hours before mice were sacrificed. It is possible that depending on mRNA turnover rates, we were not able to see changes in Pepck gene expression because the compound was already cleared from the system. The time of the sacrifice (within two hours after lights on) was considered the ideal time to harvest blood since endogenous cortisol is lowest in the early morning (Chung et al. 2011). The variable timepoints of LO520 administration in vitro and in vivo would likely have an effect on the opposing Pepck mRNA and protein levels seen due to variable protein turnover.

Glucocorticoids have been found to inhibit various steps of the insulin signaling pathway in muscle, bone, liver and adipose tissue which contribute to insulin resistance and glucose intolerance. In skeletal muscle and adipose tissue, GCs antagonize insulin signaling by decreasing transcription of insulin receptor substrate -1 (IRS-1), while in the liver GCs decrease IRS-2 activating phosphorylation (Almon et al., 2005; Bazuine et al., 2004; Burén et al., 2008; Saad et al., 1993). It is interesting to note, however, that glucocorticoids and insulin play opposite roles in gluconeogenesis. While glucocorticoids induce gluconeogenesis via induction of Pepck and G6pc, insulin suppresses gluconeogenesis via transcriptional inhibition of these genes, however regulation is not through the interaction with GR (Hall et al., 2007; Zinker et al., 2007). From this, we can hypothesize that LO520 could play a role in the modulation of insulin by either increasing insulin secretion from the pancreas or enhancing hepatic insulin sensitivity leading to repression of gluconeogenesis despite the presence of Dex. Similarly, the hormone glucagon, which opposes the action of insulin, is known to stimulate gluconeogenesis in fasted
state (Exton, 1979). LO520 could work to inhibit gluconeogenesis via inhibition of glucagon or increasing glucagon sensitivity in hepatocytes, leading to lower plasma glucose levels in the presence of Dex. This could be caused by LO520 acting on the pancreas where insulin and glucagon is secreted. LO520 would either increase peripheral insulin levels by promoting the release and/or production of insulin through beta cells or by inhibiting glucagon production via modulation of alpha cells of the pancreas.

The LXRαs are known to play a central role in cholesterol metabolism by acting as sterol sensors yet their isoforms have been revealed to have separate physiological roles in lipogenesis. Oral administration of the dual LXR agonist T0901317 was shown to increase lipogenic gene expression along with increased plasma triglyceride and phospholipid levels (Schultz et al., 2000). It was found that T0901317 activates the lipogenic transcription factor Srebp1c through an LXR response element (LXRE) within its promoter. Srebp-1c, in turn goes on to activate the transcription of genes involved in fatty acid synthesis including Acc, Fas and Scd-1 (Schultz et al., 2000). When knockout mice were fed a standard rodent diet, LXRα−/− and LXRα/β−/− mice showed reduced liver Srebp-1 mRNA, while no change was seen in mice lacking LXRβ (Peet et al., 1998). The loss of LXRα has also been shown to increase plasma LDL cholesterol since LXRα−/− mice were unable to induce Cyp7a1, the gene encoding the rate-limiting enzyme in bile acid synthesis in the presence of a high cholesterol diet (Peet et al., 1998). It should be noted that unlike LXRα−/− mice, LXRβ−/− mice fed a high cholesterol diet do not show a modified lipid phenotype (Schultz et al., 2000). It has been hypothesized that in LXRβ−/− mice, LXRα compensates for the lipid phenotype whereas lack of LXRα cannot be compensated by LXRβ (Peet et al., 1998; Schultz et al., 2000). With this information in mind, we acknowledged the importance of preserving LXRα expression and tested to see the effects of LO520 in the presence of T0901317 in vivo, looking at primarily LXRα genes: Cyp7a1 and Srebp1c. By inducing these genes with the LXR dual agonist, we could then quantify the extent of possible antagonism by LO520 on LXRα target genes. At lower concentrations (20 mg/kg and 40 mg/kg), LO520 did not downregulate T0901317-induced gene expression of Cyp7a1 and Srebp1c. At 60 mg/kg however, LO520 antagonized T0901317-induced LXRα target gene expression demonstrating that at higher doses, LO520 has an effect on LXRα. In the absence of T0910317, 40 mg/kg of LO520 has minimal effect on these genes basally (Figure 3.12). In vivo, LO520 was seen to
basally induce *Srebp1c* which was unexpected due to its lack of LXRα regulation seen in our primary hepatocyte study (Supplementary Figure 3) and its antagonistic ability at 60 mg/kg in the presence of T0901317 (Figure 3.5). The potential reason for this unexpected increase is still unknown.

Glucocorticoids are widely used for their immunosuppressive and anti-inflammatory effects, therefore a main concern of ours was that the therapeutic effect of Dex (immunosuppression) would remain preserved in the presence of LO520. We tested the effects of LO520 on Dex-induced anti-inflammation by analyzing two physiological indicators of inflammation: spleen weight and white blood cell type distribution. Dexamethasone has been known to induce T cell immunosuppression in the spleen as well as other peripheral lymphoid organs via the inhibition of genes encoding cytokines (*Il-1,2* and *6, Tnfa*) and side effect of chronic glucocorticoid treatment is lymphoid organ atrophy (McKay and Cidlowski, 2003). Previous studies done by our lab have shown that both wildtype and LXRα/β-/- mice exhibited significant spleen atrophy with chronic Dex treatment (Patel et al., 2011). Complementary to those results, our studies showed spleen weight of Dex-treated mice to decrease significantly, along with Dex treated mice co-treated with LO520, demonstrating the immunosuppressive effects of Dex were preserved in the presence of LO520. LO520-treatment alone also increased spleen weight, which was expected since it was found that LXRβ knockout mice demonstrate lymphoid hyperplasia compared to LXRα knockout mice (Bensinger et al, 2008). Another indicator of immune response is white blood cell type distribution. Neutrophils are known as the primary defense for the innate immune system and are distributed equally between the circulating blood vessels and in the capillaries found primarily in the lung (Saffar et al., 2011). In the presence of glucocorticoids, neutrophil demargination occurs, in which neutrophils will detach from the vascular wall thereby entering the circulatory system (Fay et al., 2016). Glucocorticoid therapy leads to an increase in neutrophils due to its suppression of pro-apoptotic molecules (Saffar et al., 2011). Glucocorticoids have also shown that treatment lead to lymphocyte redistribution from the circulatory system towards peripheral lymphoid organs (Kovacs, 2014). Similar to this, our studies have found that dexamethasone significantly lowered lymphocyte content in Dex-treated mice compared to vehicle, and LO520 did not modify this change (Figure 3.10) suggesting that LO520 does not alter Dex-mediated modulation of white
blood cell distribution. For this reason, we can conclude that based on these two physiological indications of immune response, LO520’s actions are independent of dexamethasone and can be used in conjunction for the treatment of anti-inflammatory conditions.

The LXRαs are highly involved in reverse cholesterol transport via regulation of relevant genes in the liver, macrophages and intestine (Lo Sasso et al., 2010). LXR agonists have been shown to induce RCT via induction of LXR target genes such as Abca1, Abcg1, Abcg5, Abcg8, and ApoE (Laffitte et al., 2001; Repa et al., 2000, 2002; Venkateswaran et al., 2000). Abca1, which is highly expressed in the liver and macrophages is involved in the transfer of cellular cholesterol and phospholipids to lipid-poor apolipoprotein A-I (ApoA-I) to form nascent HDL particles (Bortnick et al., 2000; Remaley et al., 2001). Abcg1, another LXR target gene has been suggested to be involved in the efflux of cholesterol thereby creating mature HDL (Klucken et al., 2000; Wang et al., 2004). For this reason, we investigated whether LXRβ antagonism by LO520 would increase susceptibility to atherosclerosis via inhibition of RCT. From our gene expression studies in human macrophages (THP-1), LO520 basally downregulates ABCA1 and ABCG1 mRNA expression (Figures 3.15 and 3.16). We were surprised to see that LO520 was extremely potent at decreasing ABC gene expression in macrophages. This effect could be a result of differential expression of LXR co-regulators in macrophages leading to robust changes in LXR target gene expression. The ability of GSK2033 to basally downregulate ABCA1 and ABCG1 was expected since LXR antagonism downregulates LXRα target genes however we did not expect LXRβ antagonism by LO520 to have the same inhibition as the dual LXR antagonist with respect to ABC gene expression. We expected LXRα to compensate for the LXRβ antagonism as previously described in by Peet et al (1998) where when LXRβ−/− mice were fed a high cholesterol diet, LXRα was considered to compensate for the loss of LXRβ and prevent a lipid accumulation phenotype. We quantified cholesterol efflux using the RCT tritiated-cholesterol efflux to macrophages assay and found that cells treated with LO520 did not affect basal RCT efflux yet cells treated with GSK2033 did (Figure 3.17). This is understandable due to GSK2033’s potent dual LXR antagonism which is also seen by GSK2033’s ability to basally suppress ABC gene expression (Figure 3.18). We did not expect LO520 to also basally suppress ABC gene expression once qPCR analysis was performed on cells that would have been quantified for cholesterol efflux (Figure 3.18). LO520 downregulating genes involved in
cholesterol efflux but not having an effect on the physiological output of cholesterol efflux might be due to the time of LO520 treatment which leads to varied mRNA and protein expression. We also cannot exclude other genes involved in reverse cholesterol transport (ApoA1, ApoE) that might be differentially modulated by LO520 and GSK2033.
Chapter 5: Future Studies, Limitations, Summary and Significance

5.1 Limitations and Future Studies

There are numerous limitations associated with the interpretation of these results. It is important to note certain constraints of LO520 that have come up when interpreting receptor selectivity. Even though LO520 demonstrates dramatic selectivity (320-fold) between LXR receptors based on comparison of IC$_{50}$ values, its binding affinity (K$_i$) demonstrates only a 36-fold difference between receptors. Low K$_i$ values indicate high binding affinity and even though the K$_i$ value of LXR$\alpha$ is high (1.1\(\mu\)M), the 36-fold difference between receptors may suggest possible LXR$\alpha$ modulation by LO520. In the future, it would be of interest to take into account the binding affinity of the ligand and the LXRs in order to gain a more accurate representation of receptor selectivity.

Certain results of the pharmacokinetic study are estimates (i.e., clearance, volume of distribution, elimination rate) because our study was performed i.p. and since the peak absorption appeared much later than we anticipated, only three data points were available in the elimination phase. Thus, further PK studies need to be performed in order to confirm these parameters including dosing LO520 at a higher/lower dose in order to see a higher/lower concentration to determine whether it demonstrates linear kinetics. An intravenous study would provide complementary data to ensure accurate PK parameters and would allow for the calculation of bioavailability (F), volume distribution (Vd) and clearance (CL). The unexpected delay in drug absorption (t$_{max}$ at 4 hours) could be due to LO520 being sequestered in the lymphatic system or fatty tissue before entering into the circulatory system. In order to test this hypothesis, it would be beneficial to measure the concentration of LO520 in adipose tissue and/or lymph nodes of the mice treated in the pharmacokinetic study. If the highest concentration of LO520 is seen before the four-hour time point, then we could hypothesize LO520 has increased tendency to absorb into adipose before reaching circulation. Protein binding could also be a factor in the pharmacokinetic profile of LO520. Drugs that are minimally protein bound (mostly unbound) penetrate tissue better than
those bound (Scheife, 1989). In order to test whether protein binding could be a factor in the delayed absorption seen for LO520, it would be of interest to elucidate the plasma protein binding ($K_{puu}$) of LO520 using the Rapid Equilibrium Dialysis (RED) device (Thermo Fisher).

Since the mRNA and functional output of cells treated with LO520 show contradictory results, Western blots looking at genes of interest (i.e., Pepck, ABC genes) would help clarify the influence LO520 on protein expression. Further studies looking at insulin levels (insulin and glucose tolerance test, measuring glucagon in plasma) in the presence of LO520 can help investigate the effect of LO520 on insulin sensitivity and levels. Similarly, a time course of LO520 on Dex-induced Pepck mRNA expression and/or protein levels (i.e., Western blot) would help elucidate the possible relationship between time of LO520 administration and protein turnover rate. This could shed light on the contradictory results from the primary hepatocytes and in vivo functional results. Experiments looking at post-translational modifiers, including protein turnover and chromatin modifications (ChIP) could be performed in order to elucidate other possible molecular modulations by LO520. We can conclude that the effects of LXR antagonism on genes important in RCT do not consistently reflect expected functional outcomes due to unknown mechanisms that have yet to be examined, including the effects of LO520 on ApoE and ApoAI, which are also involved in reverse cholesterol transport and are LXR target genes.

5.2 Summary

To date, we have identified the first LXRβ selective antagonist, LO520, for the treatment of glucocorticoid-induced diabetes. LO520 shows highly selective LXRβ antagonism for mouse and human LXR receptors, an increased half-life (1.5 hrs) compared to its analog, the dual LXR antagonist GSK2033 (10 minutes) and does not significantly antagonize other nuclear receptors (FXR, GR). In terms of proof of concept, LO520 attenuates the Dex-induced hyperglycemia and does not modify Dex-mediated anti-inflammation which was concluded by decreased spleen weight and a shift in white blood cell distribution that matches that of Dex-treatment alone. A possible side effect of LXR antagonism is the modulation of reverse cholesterol transport due to LXRs robust role in this physiological pathway. LO520 antagonized gene expression of LXR target genes ABCA1 and ABCG1 yet did not basally modify cholesterol efflux in a functional
study. Further studies elucidating its role in the presence of dexamethasone need to be performed to better understand the impact that LO520 may have on cholesterol efflux in this setting. Future studies in an atherosclerotic mouse model (Apoe-/- or Ldlr-/-) could be performed to establish the in vivo relevance of our findings.

5.3 Significance

Diabetes, recently considered a global epidemic affecting half a billion people worldwide, is characterized by elevated blood glucose levels (hyperglycemia) caused by insulin resistance or defective insulin secretion. A known cause of hyperglycemia is the long-term use of glucocorticoid drugs which are commonly used to treat chronic inflammation. Studies in our lab found an unexpected novel crosstalk between GR and LXRβ in the regulation of glucose metabolism and anti-inflammation by GCs. It was found that LXRβ promotes glucocorticoid-induced hyperglycemia and hepatosteatosis, and that LXRβ antagonism attenuates these GC-induced side effects. LO520, which to date is the first LXRβ selective antagonist, serves as a lead candidate for the treatment of glucocorticoid-induced diabetes since it showed promising LXRβ selectivity demonstrated by lowering plasma glucose in the presence of dexamethasone and did not affect dexamethasone’s anti-inflammatory effects. LO520 serves as a positive initial drug for cotreatment with glucocorticoids and further studies elucidating its mechanism of action could help identify its potential in lowering the prevalence of GC-induced diabetes.
References


Supplemental Figure 1: LO520 antagonism is LXR selective.
HEK293 cells were co-transfected with GAL4-hFXR and GAL4-hGR with a UAS-luciferase reporter plasmid. 

A) Cells were dosed with increasing concentrations of LO520 along with the EC\textsubscript{80} of the FXR agonist CDCA (50 µM) and values were normalized to vehicle (CDCA alone).

B) Cells were dosed with increasing concentrations of LO520 along with the EC\textsubscript{80} of the GR agonist Dex (100 nM) and values were normalized to vehicle (Dex alone). Results were pooled from triplicate wells of one independent experiment (Mean ± SD, N=3).
Supplemental Figure 2: GSK2033 antagonism is LXR selective.
HEK293 cells were co-transfected either GAL4-hFXR, GAL4-hGR or CMX-mLXR with either UAS-luciferase reporter plasmid or LXRE full length plasmid. 

A) Cells were dosed with increasing concentrations of GSK2033 along with the EC\textsubscript{80} of the FXR agonist CDCA (50 μM) and values were normalized to vehicle (CDCA alone). 

B) Cells were dosed with increasing concentrations of GSK2033 along with the EC\textsubscript{80} of the GR agonist Dex (100 nM) and values were normalized to Dex alone. 

C) Cells were dosed with increasing concentrations of GSK2033 along with the EC\textsubscript{80} of the LXR agonist T0901317 (316 nM for LXRα, 108 nM for LXRβ) and values were normalized to vehicle (T0901317 alone). 

(D) Chemical structure of GSK2033. Data presented as Mean ± SD (N=3) and determined from log(inhibitor) vs. response – Variable slope (four parameters) function (Prism, GraphPad).
Supplemental Figure 3: LO520 does not suppress GC-induced Pepck expression in primary hepatocytes from PD-1 mice.

Expression of the GR target gene Pepck (A) and the LXRα target gene Srebp1c (B) in PD-1 (programmed cell death-1) whole body knockout mouse primary hepatocytes after 18 hours of ligand treatment. Cells were treated with LO520 alone or in combination with Dex (100 nM), or with 10 μM GSK2033 and 1 μM T091317 as positive controls. RNA was extracted, reverse transcribed, and analyzed for gene expression by real-time qPCR. Data presented as Mean ± SD, N=3. *P<0.05 by One-Way ANOVA and Newman-Keuls test. “$” represents significantly different from all other treatments. Primary hepatocytes extracted from Allison Jee of Utrech lab.
Supplemental Figure 4: LO520 and Dex do not change organ weights
Wildtype mice were injected subcutaneously twice daily for 5 days with 5 mg/kg dexamethasone. LO520 was injected intraperitoneally at 40 mg/kg for five days. On the sixth day, mice were decapitated within two hours of lights on. Organ weights of epididymal white adipose tissue, eWAT (A), inguinal white adipose tissue, iWAT (B), mesenteric white adipose tissue, mWAT (C) and liver weight (D) were normalized to femur length. Data presented as Mean ± SEM, N=5. *P<0.05 by One-Way ANOVA and Newman-Keuls test.

Supplemental Figure 5: LO520 antagonizes ABC transporter gene expression
THP-1 cells were treated with 100 nM PMA for 48 hrs to initiate monocyte to macrophage differentiation. Concentrations of LO520 from 1 nM to 25 μM were added for 16 hr. Gene expression of ABCA1 and ABCG1 was determined by real-time qPCR. Data was normalized to vehicle (absence of LO520) and represent the Mean ± SD, N=3. *P<0.05 by One-Way ANOVA and Newman-Keuls test.
Supplemental Figure 6: Time course of reverse cholesterol transport (RCT) from THP1 macrophages pre-loaded with [3H]-cholesterol.

THP-1 cells were treated with 100 nM PMA for 48h to initiate monocyte to macrophage differentiation. Cells were then treated with 0.5 μCi/mL [3H]-cholesterol for 48 hrs followed by ligand treatment: 50 nM T0901317, 10 μM GSK2033 and/or T0901317+GSK2033 for 18 hrs. T0901317 is used as a positive control to induce RCT. The extent of cholesterol efflux was quantified from the supernatant and cellular pools after either 2 hr (A), 4 hr (B) or 8 hr (C) exposure to ± 50 μg/mL HDL and counted in a liquid scintillation counter. Percent cholesterol efflux was calculated as follows: [supernatant/(supernatant+intracellular)] and represented as difference between wells treated with and without HDL acceptor. The data represent the Mean ± SD, N=3. *P<0.05 by Two-Way ANOVA and Tukey's multiple comparisons test.
Supplemental Table 1: Concentration vs. time data for LO520 after 40 mg/kg i.p. dose

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