Impact of Diabetes on Drug Disposition
Mechanisms in Pregnancy

by

Gregory John Anger

A thesis submitted in conformity with the requirements
for the degree of Doctor of Philosophy

Department of Pharmaceutical Sciences
University of Toronto

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2011

Abstract

Over 220 million people worldwide are diagnosed with diabetes and rising prevalence is reported in nearly all surveyed populations. Accordingly, the percentage of pregnancies affected by pre-existing type 1 or 2 diabetes or by diabetes that develops during pregnancy, called gestational diabetes mellitus (GDM), is also on the rise. Today, approximately 8% of all pregnancies are complicated by diabetes. Diabetes alters drug disposition mechanisms in non-pregnant subjects but the impact of diabetes on drug disposition in pregnancy has not been properly evaluated. Atypical drug disposition in pregnancy has implications for maternal and fetal health. Because liver tissue from pregnant women is not readily available, this thesis investigated drug disposition mechanisms primarily in a rat model of experimental GDM. This model consisted of administering streptozotocin, a diabetogenic toxin, to pregnant rats on gestational day 6. One key finding was that elevated circulating lipids in GDM rats competed with drugs (e.g., glyburide and saquinavir) for plasma protein binding so as to increase free drug concentrations. Another key finding was that important hepatic drug efflux transporters (e.g., Mdr1a/b) and metabolic enzymes (e.g., Cyp3a2 and Ugt1a1) were upregulated in GDM as a consequence of, most likely, enhanced nuclear receptor activity (e.g., pregnane X receptor upregulation). Upregulation of hepatic drug efflux transporters and metabolic enzymes, coupled with larger unbound drug fractions, would be expected to increase the hepatic clearance of many drugs. Consistent with this, in GDM, maternal and fetal exposure to the Mdr1 and Cyp3a2 substrate lopinavir was substantially lower than controls post-administration and data supporting enhanced lopinavir metabolite formation were obtained. Placental drug efflux
transporters were also examined in this lopinavir study. Elevated placental Mdr1b and Bcrp expression was observed in GDM, which was associated with decreased fetal exposure to lopinavir (even after correcting for maternal unbound concentrations). Taken together, this thesis demonstrates that experimental GDM can significantly impact drug disposition by altering key drug disposition mechanisms. If confirmed in humans, this drug-disease interaction would need to be considered when atypical therapeutic outcomes occur in diabetic pregnancies. Data from experiments with human placentas, obtained from pregnancies complicated by insulin-managed diabetes, is included/discussed.
For Sarah and our families.
Acknowledgments

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Table of Contents

Acknowledgments .............................................................................................................................. v
Table of Contents ............................................................................................................................. vii
List of Tables ...................................................................................................................................... xi
List of Figures ...................................................................................................................................... xii
List of Appendix Figures .................................................................................................................. xiii
List of Equations ............................................................................................................................... xiv
List of Abbreviations ........................................................................................................................ xv
List of Symbols ................................................................................................................................. xvii
Chapter 1. Introduction ..................................................................................................................... 18
  1.1 Diabetic pregnancy .................................................................................................................... 19
    1.1.1 Classification and pathogenesis of diabetic pregnancy (in relation to other forms of diabetes mellitus) ................................................................. 19
    1.1.2 Epidemiology .................................................................................................................... 23
    1.1.3 Management of diabetic pregnancy .................................................................................. 24
    1.1.4 Short- and long-term consequences of mismanagement .................................................. 24
  1.2 Drug disposition mechanisms ..................................................................................................... 26
    1.2.1 Plasma protein binding .................................................................................................... 27
    1.2.2 Drug transporters ............................................................................................................ 29
    1.2.3 Drug metabolizing enzymes .......................................................................................... 37
    1.2.4 Regulation of drug transporters and metabolic enzymes ................................................. 39
    1.2.5 Pharmacokinetic parameters in this thesis ...................................................................... 43
  1.3 Rationale .................................................................................................................................... 46
  1.4 Streptozotocin-induced diabetic pregnancy ................................................................................ 49
  1.5 General hypotheses .................................................................................................................... 54
  1.6 Research objectives .................................................................................................................... 55
    1.6.1 Objective 1: characterize hepatic drug efflux transporter expression in non-pregnant, female rats with streptozotocin-induced T1DM......................................................... 55
    1.6.2 Objective 2: characterize plasma protein binding capabilities and hepatic drug efflux transporter and metabolic enzyme expression in a rat model of GDM .......... 55
    1.6.3 Objective 3: determine if maternal and fetal exposure to lopinavir is altered in a rat model of GDM .................................................................................................................... 55
    1.6.4 Objective 4: characterize placental drug efflux transporter expression in human pregnancies complicated by diabetes ................................................................. 56
Chapter 2. Impact of Acute Streptozotocin-Induced Diabetes on ABC Transporter Expression in Rats .......................................................... 57

2.1 Abstract .......................................................................................................................... 58
2.2 Introduction ..................................................................................................................... 59
2.3 Methods .......................................................................................................................... 61
  2.3.1 Animals ..................................................................................................................... 61
  2.3.2 Animal treatments/monitoring and tissue collection ................................................. 61
  2.3.3 IL-6, TNF-α and CRP ELISAs ............................................................................... 62
  2.3.4 ALT and GGT assays ............................................................................................ 62
  2.3.5 Urinalysis ................................................................................................................ 62
  2.3.6 Accumulation experiments ..................................................................................... 62
  2.3.7 PCR ........................................................................................................................ 63
  2.3.8 Glutathione assays ............................................................................................... 63
  2.3.9 Statistics ................................................................................................................ 64
2.4 Results and discussion .................................................................................................... 65
  2.4.1 Characteristics of streptozotocin-injected rats consistent with unmanaged T1DM ... 65
  2.4.2 Alteration of hepatic ABC transporter expression in T1DM .................................. 68
  2.4.3 Endogenous glutathione disposition was altered in T1DM ..................................... 71
  2.4.4 Preservation of renal ABC transporter expression in T1DM .................................. 73
  2.4.5 Potential mechanisms for the alteration of hepatic ABC transporter expression in T1DM ...................................................................................................................... 75
2.5 Appendix: supplementary figures/data .......................................................................... 78

Chapter 3. Impact of Hyperlipidemia on Plasma Protein Binding and Hepatic Drug Transporter and Metabolic Enzyme Regulation in a Rat Model of Gestational Diabetes .... 79

3.1 Abstract .......................................................................................................................... 80
3.2 Introduction ..................................................................................................................... 81
3.3 Methods .......................................................................................................................... 83
  3.3.1 Animals ..................................................................................................................... 83
  3.3.2 Animal treatments/monitoring and tissue collection ................................................. 83
  3.3.3 Blood chemistry ...................................................................................................... 84
  3.3.4 Protein binding assays ........................................................................................... 84
  3.3.5 Proteomics: sample preparation and isobaric mass tagging .................................. 85
  3.3.6 Proteomics: LC-MS/MS ......................................................................................... 85
  3.3.7 Proteomics: database processing and analysis ....................................................... 86
  3.3.8 Characterization of hepatic Mdr1 expression .......................................................... 87
### 3.3.9 Statistics .......................................................................................................................... 88

### 3.4 Results .................................................................................................................................. 89

#### 3.4.1 STZ-injected pregnant rats exhibited a variety of characteristics that were consistent with unmanaged, or poorly managed, GDM ........................................................................... 89

#### 3.4.2 Decreased plasma protein binding was observed in GDM.................................................. 91

#### 3.4.3 Evidence for hyperlipidemia as a mechanism for decreased plasma protein binding in GDM ......................................................................................................................... 91

#### 3.4.4 Alterations in the expression of hepatic membrane proteins in GDM were largely linked to lipid metabolism...................................................................................................................... 93

#### 3.4.5 Evidence for nuclear receptor activation contributing to drug transporter and metabolic enzyme upregulation ........................................................................................................... 101

### 3.5 Discussion ............................................................................................................................ 104

### 3.6 Appendix: supplementary figures/data .................................................................................. 108

---

**Chapter 4. Mechanisms of Reduced Maternal and Fetal Lopinavir Exposure in a Rat Model of Gestational Diabetes ................................................................. 112**

#### 4.1 Abstract ............................................................................................................................ 113

#### 4.2 Introduction ....................................................................................................................... 114

#### 4.3 Methods ............................................................................................................................ 117

##### 4.3.1 Pharmaceutical and chemical products ...................................................................... 117

##### 4.3.2 Animals ....................................................................................................................... 117

##### 4.3.3 Animal treatments/monitoring and tissue collection .................................................. 117

##### 4.3.4 Sampling of fetal compartments for time course determination .................................. 118

##### 4.3.5 Determination of lopinavir and lopinavir metabolite concentrations ................................ 118

##### 4.3.6 ATPase activation assay ............................................................................................. 120

##### 4.3.7 Blood chemistry ........................................................................................................ 120

##### 4.3.8 PCR ............................................................................................................................ 121

##### 4.3.9 Western blotting ......................................................................................................... 121

##### 4.3.10 Lopinavir protein binding assay .................................................................................. 122

##### 4.3.11 Statistics .................................................................................................................. 123

#### 4.4 Results ............................................................................................................................... 124

##### 4.4.1 Characterization of STZ-induced GDM ....................................................................... 124

##### 4.4.2 Maternal and fetal lopinavir exposure at 45 min .......................................................... 126

##### 4.4.3 Influence of fetal uterine horn position on fetal lopinavir exposure ................................ 129

##### 4.4.4 Time course of fetal lopinavir exposure in GDM ......................................................... 129

##### 4.4.5 Drug transporter and Cyp3a2 expression in GDM ....................................................... 132

##### 4.4.6 Lopinavir metabolite formation in maternal liver at 45 min ......................................... 135
Chapter 5. Placental ABC Efflux Transporter Expression in Pregnancies Complicated by Insulin-Managed Diabetes ................................................................. 148

5.1 Abstract ........................................................................................................149
5.2 Introduction ....................................................................................................150
5.3 Methods ..........................................................................................................151
  5.3.1 Sample acquisition ......................................................................................151
  5.3.2 Western blotting ........................................................................................151
  5.3.3 Statistics ......................................................................................................152
5.4 Results and discussion ....................................................................................154
5.5 Appendix: power analysis .............................................................................158

Chapter 6. Discussion ............................................................................................ 159

6.1 Examining hepatic drug transporters in non-pregnant rats with streptozotocin-induced T1DM: proof of concept .........................................................160
6.2 Model performance ........................................................................................161
6.3 Drug plasma/serum protein binding in a rat model of diabetic pregnancy ..........165
6.4 Expression of hepatic drug transporters and metabolic enzymes in a rat model of diabetic pregnancy .................................................................169
6.5 Expression of placental drug efflux transporters in diabetic pregnancy ..........174
6.6 Non-routine methods ......................................................................................176
  6.6.1 The relative quantitation of drug transporters and metabolic enzymes in complex, iTRAQ-labeled membrane protein mixtures ......................................176
  6.6.2 A surgical method for use in studies of rat fetal pharmacokinetics ..............179
6.7 Future studies ..................................................................................................183
  6.7.1 Studies that expand the scope of my work with STZ-induced GDM ..........183
  6.7.2 Studies that test some of my mechanistic claims .........................................184
  6.7.3 Studies that determine if the effects described in this thesis translate into human pregnancies that are complicated by diabetes .................................185
6.8 Conclusions .....................................................................................................188

References ............................................................................................................. 189
List of Tables

Table 2.1. The effect of STZ-injection on various physiological and biochemical characteristics.....66

Table 3.1. The effect of STZ-induced GDM, with and without insulin treatment, on various physiological and biochemical characteristics .................................................................90

Table 3.2. Identified proteins related to lipid metabolism in STZ-induced GDM.........................96

Table 3.3. Identified proteins related to lipid metabolism in insulin-treated STZ-induced GDM ....98

Table 3.4. Comparisons of proteins that were altered in iTRAQ experiments to “Tox Lists” in the Ingenuity Knowledge Base .........................................................................................................................102

Table 4.1. The effect of STZ-induced GDM, with and without insulin treatment, on various physiological and biochemical characteristics .................................................................125

Table 4.2. Pharmacokinetic parameters derived from concentration-versus-time curves for fetal lopinavir exposure following maternal administration (10 mg/kg; intravenous) .........................131

Table 5.1. Inclusion/exclusion criteria .....................................................................................153

Table 5.2. Clinical data ........................................................................................................156

Table 6.1. Evidence for decreased protein binding of drugs in STZ-induced GDM...............168

Table 6.2. Evidence for altered hepatic drug transporter and metabolic enzyme expression in STZ-induced GDM .................................................................................................172
List of Figures

**Figure 1.1.** Localization of selected human transport proteins in a variety of tissues ..........................32

**Figure 1.2.** Streptozotocin .....................................................................................................................53

**Figure 2.1.** Accumulation of an Mdr1 and Mrp2 substrate in IL-6-treated rat hepatocytes ..........67

**Figure 2.2.** Hepatic expression of apical (A) and basolateral (B) ABC transporters in diabetic and control rats as determined by qRT-PCR ......................................................................................70

**Figure 2.3.** Disposition of glutathione in the liver (A), plasma (B) and urine (C) of diabetic and control rats ........................................................................................................................................72

**Figure 2.4.** Renal expression of ABC transporters in diabetic and control rats as determined by qRT-PCR ........................................................................................................................................74

**Figure 2.5.** Scatter plots depicting the association between plasma CRP concentration and mRNA expression for significantly altered transporters ..........................................................................................77

**Figure 3.1.** Plasma protein binding characteristics ..................................................................................92

**Figure 3.2.** Volcano plots depicting the magnitude and statistical significance of hepatic protein alterations in iTRAQ experiments ..................................................................................................................................95

**Figure 3.3.** Lipid metabolism network in the GDM group .......................................................................99

**Figure 3.4.** Lipid metabolism network in the insulin-treated group .....................................................100

**Figure 3.5.** Hepatic Mdr1 expression ......................................................................................................103

**Figure 4.1.** The dominant Cyp3a-mediated metabolic pathway for lopinavir ........................................116

**Figure 4.2.** Maternal and fetal lopinavir concentrations 45 min after lopinavir administration ......127

**Figure 4.3.** Tissue/unbound maternal serum lopinavir concentration ratios ........................................128

**Figure 5.1.** Placental ABC efflux transporter expression in pregnancies complicated by insulin-managed diabetes ......................................................................................................................................157

**Figure 6.1.** Summary of direct and indirect interactions between PPARα and PXR in the presence of intracellular starvation and their implications for the regulation of proteins related to hepatic energy homeostasis as well as drug transport and metabolism ..................................................................................173
List of Appendix Figures

Appendix 2.1. Time course of STZ-induced hyperglycemia and weight loss.................................78

Appendix 3.1. Work flow for iTRAQ experiments........................................................................108

Appendix 3.2. Time course of STZ-induced hyperglycemia and weight gain in pregnancy........109

Appendix 3.3. Photographs demonstrating overt hyperlipidemia in experimental GDM (A) and the effect of delipidation on a hyperlipidemic sample (B).................................................................110

Appendix 3.4. Lipemic indices (A) and plasma C-reactive protein concentrations (B) in Anger et al., 2009 (Chapter 2) and Anger and Piquette-Miller, 2010 (Chapter 3).........................................................111

Appendix 4.1. Cartoon illustration of fetal positioning within the pregnant rat at approximately GD20.................................................................................................................................142

Appendix 4.2. Lopinavir and lopinavir metabolite chromatography............................................143

Appendix 4.3. Placental size in experimental GDM ......................................................................144

Appendix 4.4. Mdr1a/b, Mrp2 and Bcrp expression in placentas from a group of fetuses for which sex was determined...........................................................................................................145

Appendix 4.5. Lopinavir and lopinavir metabolite concentrations in maternal liver without normalization..........................................................................................................................147
List of Equations

Equation 1.1. Hepatic clearance ................................................................. 43

Equation 1.2. Fetal:maternal free drug concentration ratio ........................................ 45
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>AAG</td>
<td>alpha 1-acid glycoprotein</td>
</tr>
<tr>
<td>ABC</td>
<td>ATP-binding cassette</td>
</tr>
<tr>
<td>AGE</td>
<td>advanced glycation endproduct</td>
</tr>
<tr>
<td>ALT</td>
<td>alanine aminotransferase</td>
</tr>
<tr>
<td>ANOVA</td>
<td>analysis of variance</td>
</tr>
<tr>
<td>APR</td>
<td>acute phase response</td>
</tr>
<tr>
<td>AUC</td>
<td>area under concentration versus time curve</td>
</tr>
<tr>
<td>Bcrp/BCRP</td>
<td>rodent/human breast cancer resistance protein</td>
</tr>
<tr>
<td>BDL</td>
<td>bile duct ligation</td>
</tr>
<tr>
<td>CAR</td>
<td>constitutive androstane receptor</td>
</tr>
<tr>
<td>CRP</td>
<td>C-reactive protein</td>
</tr>
<tr>
<td>CV</td>
<td>coefficient of variation</td>
</tr>
<tr>
<td>Cyp/CYP</td>
<td>rodent/human cytochrome P450 enzyme</td>
</tr>
<tr>
<td>ELISA</td>
<td>enzyme-linked immunosorbent assay</td>
</tr>
<tr>
<td>ESI</td>
<td>electrospray ionization</td>
</tr>
<tr>
<td>FXR</td>
<td>farnesoid X receptor</td>
</tr>
<tr>
<td>FFA</td>
<td>free fatty acid</td>
</tr>
<tr>
<td>GD</td>
<td>gestational day</td>
</tr>
<tr>
<td>GDM</td>
<td>gestational diabetes mellitus</td>
</tr>
<tr>
<td>GGT</td>
<td>gamma-glutamyl transpeptidase</td>
</tr>
<tr>
<td>GSH</td>
<td>reduced glutathione</td>
</tr>
<tr>
<td>GSSG</td>
<td>glutathione disulfide</td>
</tr>
<tr>
<td>HAART</td>
<td>highly active antiretroviral therapy</td>
</tr>
<tr>
<td>HDL</td>
<td>high-density lipoprotein</td>
</tr>
<tr>
<td>HIV</td>
<td>human immunodeficiency virus</td>
</tr>
<tr>
<td>HNF1</td>
<td>hepatic nuclear factor 1</td>
</tr>
<tr>
<td>HPLC</td>
<td>high performance liquid chromatography</td>
</tr>
<tr>
<td>IKB</td>
<td>Ingenuity Knowledge Base</td>
</tr>
<tr>
<td>IL</td>
<td>interleukin</td>
</tr>
<tr>
<td>iTRAQ</td>
<td>isobaric tags for relative and absolute quantitation</td>
</tr>
<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>LPS</td>
<td>lipopolysaccharide</td>
</tr>
<tr>
<td>LPV</td>
<td>lopinavir</td>
</tr>
<tr>
<td>LXR</td>
<td>liver X receptor</td>
</tr>
<tr>
<td>MRM</td>
<td>multiple reaction monitoring</td>
</tr>
<tr>
<td>Mrp/MRP</td>
<td>rodent/human multidrug resistance-associated protein</td>
</tr>
<tr>
<td>MS</td>
<td>mass spectrometry</td>
</tr>
<tr>
<td>Mdr1/MDR1</td>
<td>rodent/human multidrug resistance protein 1</td>
</tr>
<tr>
<td>Mdr2/MDR2</td>
<td>rodent/human multidrug resistance protein 2</td>
</tr>
<tr>
<td>Nr2</td>
<td>nuclear factor erythroid 2-related factor 2</td>
</tr>
<tr>
<td>NF-κB</td>
<td>nuclear factor kappa-light-chain-enhancer of activated B cells</td>
</tr>
<tr>
<td>Oatp/OATP</td>
<td>rodent/human organic anion transport protein</td>
</tr>
<tr>
<td>OGTT</td>
<td>oral glucose tolerance test</td>
</tr>
<tr>
<td>PKC</td>
<td>protein kinase C</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
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</tr>
<tr>
<td>PPAR</td>
<td>peroxisome proliferator-activated receptor</td>
</tr>
<tr>
<td>PXR</td>
<td>pregnane X receptor</td>
</tr>
<tr>
<td>qRT-PCR</td>
<td>quantitative reverse transcriptase-polymerase chain reaction</td>
</tr>
<tr>
<td>RAGE</td>
<td>advanced glycation endproduct receptor</td>
</tr>
<tr>
<td>ROS</td>
<td>radical oxygen species</td>
</tr>
<tr>
<td>RTV</td>
<td>ritonavir</td>
</tr>
<tr>
<td>RXR</td>
<td>retinoic acid receptor</td>
</tr>
<tr>
<td>SRM</td>
<td>selected reaction monitoring</td>
</tr>
<tr>
<td>SLC</td>
<td>solute carrier</td>
</tr>
<tr>
<td>STZ</td>
<td>streptozotocin</td>
</tr>
<tr>
<td>SCX</td>
<td>strong cation exchange</td>
</tr>
<tr>
<td>T1DM</td>
<td>type 1 diabetes mellitus</td>
</tr>
<tr>
<td>T2DM</td>
<td>type 2 diabetes mellitus</td>
</tr>
<tr>
<td>TGSH</td>
<td>total glutathione</td>
</tr>
<tr>
<td>TR</td>
<td>thyroid hormone receptor</td>
</tr>
<tr>
<td>TNF-α</td>
<td>tumor necrosis factor-alpha.</td>
</tr>
<tr>
<td>Ugt/UGT</td>
<td>rodent/human UDP-glucuronosyltransferase</td>
</tr>
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## List of Symbols

<table>
<thead>
<tr>
<th>Symbol</th>
<th>Definition</th>
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<tbody>
<tr>
<td>AUC&lt;sub&gt;0-60&lt;/sub&gt;</td>
<td>area under the concentration-time curve from zero to 60 min</td>
</tr>
<tr>
<td>C&lt;sub&gt;filtered&lt;/sub&gt;</td>
<td>concentration in plasma/serum ultrafiltrate</td>
</tr>
<tr>
<td>C&lt;sub&gt;max&lt;/sub&gt;</td>
<td>maximum plasma/serum drug concentration during a dosing interval</td>
</tr>
<tr>
<td>C&lt;sub&gt;unfiltered&lt;/sub&gt;</td>
<td>concentration in unfiltered plasma/serum</td>
</tr>
<tr>
<td>CL&lt;sub&gt;F&lt;/sub&gt;</td>
<td>overall clearance from the fetal circulation</td>
</tr>
<tr>
<td>CL&lt;sub&gt;FM&lt;/sub&gt;</td>
<td>clearance from the fetal circulation into the maternal circulation</td>
</tr>
<tr>
<td>CL&lt;sub&gt;intrinsic&lt;/sub&gt;</td>
<td>intrinsic clearance</td>
</tr>
<tr>
<td>CL&lt;sub&gt;liver&lt;/sub&gt;</td>
<td>liver/hepatic clearance</td>
</tr>
<tr>
<td>CL&lt;sub&gt;MIF&lt;/sub&gt;</td>
<td>clearance from the maternal circulation into the fetal circulation</td>
</tr>
<tr>
<td>f&lt;sub&gt;u&lt;/sub&gt;</td>
<td>fraction unbound in plasma/serum</td>
</tr>
<tr>
<td>Q&lt;sub&gt;liver&lt;/sub&gt;</td>
<td>liver/hepatic blood flow</td>
</tr>
<tr>
<td>T&lt;sub&gt;max&lt;/sub&gt;</td>
<td>time to maximum plasma/serum drug concentration during a dosing interval</td>
</tr>
<tr>
<td>% unbound&lt;sub&gt;f&lt;/sub&gt;</td>
<td>percentage of drug unbound in fetal circulation</td>
</tr>
<tr>
<td>% unbound&lt;sub&gt;M&lt;/sub&gt;</td>
<td>percentage of drug unbound in maternal circulation</td>
</tr>
</tbody>
</table>
Chapter 1. Introduction
This thesis describes a series of studies that examine the impact of diabetes on drug disposition mechanisms in pregnancy.

1.1 Diabetic pregnancy

Diabetes mellitus is the term given to a group of metabolic diseases that are principally characterized by hyperglycemia, resulting from defective insulin secretion, insulin action or both (American Diabetes Association, 2011a). Insulin is a hormone, produced by the pancreas, that plays an essential role in energy homeostasis within the body. One hundred years ago, very little was known about diabetes let alone diabetic pregnancy. The average life expectancy of a woman with what would now be considered type 1 diabetes mellitus (T1DM, see below) was very short and sterility was an issue for those surviving to sexual maturity. Women with what would now be considered type 2 diabetes mellitus (T2DM, see below) and women who developed diabetes during pregnancy, called gestational diabetes mellitus (GDM, see below), were at major risk of death during pregnancy or in the postpartum period. Fortunately, the prognosis for these women was forever changed for the better when Banting, Best, Collip and MacLeod discovered insulin at the University of Toronto in 1922. To borrow the words of Dr. Priscilla White, “…diabetes [was] no longer a contraindication to pregnancy” (White, 1928). Exogenous insulin injections allowed women with pre-existing diabetes to overcome sterility, survive pregnancy/carry their fetuses to term and survive in the postpartum period. Survival rates for pregnant women with T2DM and women who developed GDM also rose sharply. In the years that followed, the concepts of fluctuating maternal insulin sensitivity/requirements across gestation and the dependence of fetal health on maternal maintenance of euglycemia arose and matured. The field of diabetes and pregnancy has advanced considerably since the discovery of insulin. In most high-income countries, detection tools and screening protocols as well as effective therapeutic management strategies are now in place and undergoing constant refinement. It is in this period of refinement, when the focus of research is shifted somewhat from mere preservation of maternal life to the optimization of both maternal and fetal outcomes, that the research described in this thesis was conducted.

1.1.1 Classification and pathogenesis of diabetic pregnancy (in relation to other forms of diabetes mellitus)

There are many diabetes classification systems available but none have received as much attention or have as much global influence as that of the American Diabetes Association (ADA). The World Health Organization (WHO) develops its own diabetes classification system in cooperation with the ADA and many countries have as their classification system a variant of
ADA’s. For simplicities sake, I will focus only on this organization’s classification system. The ADA’s diabetes classification system, in its most recent form, is based on four broad etiopathogenic categories, including: 1) T1DM, 2) T2DM, 3) other specific types of diabetes and 4) GDM.

T1DM, formerly referred to as insulin-dependent diabetes mellitus (IDDM) or juvenile-onset diabetes, is the result of autoimmune destruction of β-cells within the pancreas and accounts for 5-10% of overt diabetes (American Diabetes Association, 2011a). The role of the pancreas in diabetes was first discovered when the organ was removed from dogs, which resulted in permanent hyperglycemia (von Mering and Minkowski, 1889). The pancreas is an organ that serves both endocrine and exocrine functions. Exocrine cells make up approximately 98% of cells within the pancreas, with endocrine cells dispersed throughout them in small erratic clusters called the islets of Langerhans. Islets contain four types of cells, including the insulin producing β-cells. In response to glucose stimulation, which occurs with the consumption of carbohydrates, β-cells release insulin in a pulsatile manner (Hellman et al., 2009). Insulin then causes the uptake of glucose by cells in the liver, muscles and adipose tissue. In the liver and muscle, glucose is stored as glycogen while it is stored in adipose tissue as fat. Autoantibodies targeting β-cell antigens, such as insulin, glutamic acid decarboxylase and a protein tyrosine phosphatase molecule called IA-2 are found in most individuals with T1DM and can be used to distinguish T1DM from other forms of diabetes (Verge et al., 1996; Leslie et al., 1999). Destruction of β-cells in individuals with T1DM is highly variable but tends to occur rapidly in infants and children and slowly in adults (American Diabetes Association, 2011a). Individuals with T1DM are dependent on exogenous insulin for lifelong survival.

When researchers discovered that defective insulin secretion was responsible for T1DM, it was theorized that all diabetics must have low insulin levels; however, this was discredited once sensitive insulin detection methods were developed. Researchers soon discovered that diabetic patients that were not completely dependent on insulin for survival often had normal or high plasma insulin concentrations. These patients had what would now be considered T2DM. T2DM, formerly referred to as non-insulin-dependent diabetes mellitus (NIDDM) or adult-onset diabetes, refers to individuals with peripheral insulin resistance and some degree of insulin secretion deficiency. T2DM accounts for 90-95% of overt diabetes (American Diabetes Association, 2011a). The cause of this form of diabetes is unknown but autoimmune destruction of β-cells does not occur and patients in this category do not have any of the specific types of diabetes listed below. Obesity is very strongly associated with T2DM and this is speculated to be the result of obesity-induced resistin secretion (Steppan et al., 2001; Sheng et al., 2008) and/or obesity-induced systemic inflammation (Hotamisligil
et al., 1995). Aging is also associated with T2DM (Reviewed in Davidson, 1979). In the earlier stages, T2DM often goes undiagnosed because symptoms develop gradually over time. Individuals who exhibit blood glucose concentrations that are too high to be considered normal but too low to be considered overt diabetes are typically diagnosed as having impaired fasting glucose, impaired glucose tolerance or, simply, prediabetes (depending on the tests performed). Many of these individuals have T2DM but are in a protracted presymptomatic phase. The 5-year risk of developing overt diabetes for prediabetic individuals in one study was found to be 25-50% (Zhang et al., 2010).

The ADA’s diabetes classification category for other specific types of diabetes encompasses eight etiopathogenic sub-categories of comparatively rare and specific forms of diabetes, including: A) genetic defects of β-cell function; B) genetic defects of insulin action; C) diseases of the exocrine pancreas, such as pancreatitis; D) endocrinopathies, such as Cushing’s syndrome; E) drug or chemical induced diabetes, such as diabetes induced by glucocorticoid use; F) infections, such as congenital rubella; G) uncommon forms of immune-mediated diabetes, such as diabetes resulting from anti-insulin receptor autoantibodies; H) other genetic syndromes sometimes associated with diabetes, such as Down syndrome (For a complete list of other specific types of diabetes, see American Diabetes Association, 2011a).

The ADA’s current diagnostic criteria for diabetes in any of the above ADA diabetes classifications (i.e., diabetes that is not GDM) involves meeting at least one of four criteria (American Diabetes Association, 2011a). First, an HbA1c percentage ≥ 6.5%. In proportion to their concentration in the blood, glucose molecules are able to react with normal hemoglobin (HbA0) in red blood cells to produce HbA1c. HbA1c concentrations are a reflection of average blood glucose concentrations over the previous 3-4 months, which is the approximate lifespan of red blood cells (Rahbar et al., 1969). For example, HbA1c concentration data for 1,439 patients in the landmark Diabetes Control and Complications Trial strongly correlated with data obtained from their self-monitoring of blood glucose (Rohlfing et al., 2002). Second, a fasting plasma glucose concentration ≥ 7 mmol/L. Fasting is defined as no caloric intake for eight hrs. Third, a plasma glucose concentration ≥ 11.1 mmol/L after consuming a glucose load that is equivalent to 75 g of anhydrous glucose dissolved in water (a.k.a., an oral glucose tolerance test/OGTT). Measurements are taken 2 hrs after consuming the glucose load. Fourth, in patients exhibiting the classic symptoms of diabetes, a casual blood glucose concentration ≥ 11.1 mmol/L.

The final category in the ADA’s diabetes classification system, GDM, has undergone a significant recent revision with respect to definition. Upon initiation of my doctoral research in
2006, the ADA and most other diabetes classification systems defined GDM as any degree of glucose intolerance with onset or first recognition during pregnancy (American Diabetes Association, 2004). This definition had been in place since the first ADA classification system was inherited from the National Diabetes Data Group (NDDG) of the United State’s National Institutes of Health (NIH) in 1979. The problem with this long-standing definition is that it makes for a highly heterogeneous category because it makes no distinction between GDM that resolves with birth and GDM that is later found to be overt diabetes. This should be kept in mind when pre-2011 studies of GDM are referenced, which is the case not just for this thesis but for most of the studies currently available in the literature. In its most recent form, the ADA’s diabetes classification system defines GDM as it has in the past but now recommends that, “…high-risk women found to have diabetes at their initial prenatal visit, using standard criteria, receive a diagnosis of overt, not gestational, diabetes” (American Diabetes Association, 2011a).

If a case of diabetes is categorized as GDM under the ADA’s most recent diabetes classification system, it is now assumed that the underlying pathology is maternal insulin receptor insensitivity/insulin resistance brought on by the metabolic demands of pregnancy. Insulin receptor insensitivity and exagerated postprandial insulin response is a well-documented phenomenon in normal pregnancies (Burt, 1956; Spellacy and Goetz, 1963; Kühl, 1975; Buchanan et al., 1990). Early in pregnancy, rising estrogen and progesterone increase the release of maternal insulin and this actually results in mild hypoglycemia. As pregnancy progresses, though, insulin receptor insensitivity occurs due to a combination of chronic insulin receptor activation and the effects of rising human chorionic somatomammotropin, cortisol, prolactin, estrogen and progesterone (Reviewed in Yogev et al., 2003). The hypothesized evolutionary drive behind this state of insulin resistance is the direction of maternal glucose to the developing fetus. For most pregnant women, increased insulin requirements are easily met with increased insulin secretion. For some women, however, this is not the case and diabetes ensues for the remainder of gestation. Thus, the pathogenesis of GDM resembles T2DM because a combination of insulin resistance and insulin secretion deficiency is at play. Not surprisingly, then, women with prediabetes, the precursor for T2DM, are at an increased risk of developing GDM and many women who develop GDM in pregnancy will receive a diagnosis of overt diabetes, primarily T2DM, later in life (Reviewed in Kim et al., 2002; Damm, 2009). The traditional risk factors for GDM are also risk factors for T2DM: high maternal weight and age as well as previous GDM and/or a family history of diabetes (American Diabetes Association, 2004).

The ADA’s current guidelines for the screening and diagnosis of GDM consists of administering a 75-g OGTT at 24-28 weeks gestation in women who are not already diagnosed with
overt diabetes. This is done in the fasted state and plasma glucose is measured both pre- and post-administration. A diagnosis of GDM is made if at least one of the following plasma glucose concentrations are met or exceeded: a fasting concentration of 5.1 mmol/L, a 1 hr concentration of 10 mmol/L and a 2 hrs concentration of 8.5 mmol/L (American Diabetes Association, 2011a).

1.1.2 Epidemiology

Population growth, aging, urbanization, obesity and physical inactivity are presently fueling a global diabetes epidemic. Over 220 million people worldwide are diagnosed with diabetes and rising prevalence, for both T1DM and T2DM, is reported in nearly all surveyed populations (Onkamo et al., 1999; World Health Organization, 2011). In 2004, the WHO predicted that the global diabetes prevalence in adults would reach 6.4% by 2030 (Wild et al., 2004). This prediction represents an increase in prevalence of 60% globally and 65% in high-income countries like Canada from 1995 to 2030 (Wild et al., 2004); however, increasing rates of obesity were not taken into consideration in the WHO study and it is, therefore, believed to be an underestimate. In Canada’s largest province, Ontario, health care is government funded, as is the case in all other Canadian provinces, which results in nearly all 12 million residents being represented in epidemiological studies that tap into patient record databases. This means that the Ontario Diabetes Database is an excellent tool with which to assess the WHO’s prevalence estimates. In a recent study of Ontario residents, the age-adjusted and sex-adjusted diabetes prevalence in adults was found to have increased an alarming 69% from 1995 to 2005 (Lipscombe and Hux, 2007). According to this study, 8.8% of Ontarians were diagnosed with diabetes as of 2005 (Lipscombe and Hux, 2007). Thus, Ontario exceeded WHO estimates for 35 years in just 10 years. Similar trends are present in other areas of the world.

In accordance with the rise in overt diabetes, the percentage of pregnancies affected by pre-existing T1DM, T2DM and GDM is also on the rise. GDM is on the rise in this respect because, as stated above, overt diabetes is often detected for the first time in pregnancy and has historically factored directly into GDM statistics. The abovementioned Ontario Diabetes Database study noted that the greatest increases in prevalence occurred in men and women between the ages of 20 and 49 (Lipscombe and Hux, 2007), which is when the majority of women in Ontario bear children (Statistics Canada, 2011). Studies published prior to the ADA’s most recent diabetes classification system place the average number of pregnancies affected by GDM at approximately 8%, ranging from 1-14% in accordance with the prevalence of overt diabetes in the specific populations studied (O’Sullivan et al., 1973; Benjamin et al., 1993; Magee et al., 1993; Murphy et al., 1993; Janghorbani et al., 2006; Hossein-Nezhad et al., 2007).
1.1.3 Management of diabetic pregnancy

The treatment goal for GDM is the maintenance of normal blood glucose concentrations (i.e., euglycemia) across gestation and in the postpartum period. For pregnancies affected by pre-existing diabetes, euglycemia prior to conception is also a goal. The first-line approach to treating GDM is medical nutrition therapy (MNT). MNT is tailored to meet the specific blood glucose goals of individual patients. In the event that GDM persists after MNT is initiated, exogenous insulin injections are typically self-administered as a combination of short and intermediate acting forms. Supplementary insulin tables are used to adjust dosages to meet the specific blood glucose goals of individual patients across gestation. Statistics for “mismanagement” of hyperglycemia via the self-administration of insulin are limited but the percentage of patients with GDM who do not achieve their specific blood glucose goals is between 20-30% (Langer et al., 2000; Langer et al., 2005b). In recent years, the use of oral hypoglycemic drugs, such as glyburide, in pregnancy has garnered a great deal of interest but insulin remains the current standard of care (American Diabetes Association, 2011b). Management of pregnancy that is complicated by overt diabetes follows a path that is similar to the management of GDM.

1.1.4 Short- and long-term consequences of mismanagement

Serious short- and long-term maternal and fetal consequences are associated with unmanaged, or poorly managed, GDM. Throughout gestation, pregnant women with GDM can experience polyuria, polydipsia and hyperphagia (a.k.a., the classical triad of diabetes symptoms). Polyuria is caused by osmotic diuresis, which occurs at high blood glucose concentrations. Polydipsia occurs in response to the large volumes of fluid lost through polyuria. Hyperphagia is, simply put, the body’s response to the intracellular starvation that occurs in the absence of proper insulin signaling. Pregnant women with GDM can also experience hyperlipidemia (Knopp et al., 1977; Knopp et al., 1986; Koukkou et al., 1996; Couch et al., 1998) and reduced weight gain (Cunningham et al., 2001).

In the prenatal period, there is increased production of fetal insulin, which leads to increased storage of excess glucose as fetal adipose tissue and the development of macrosomia (Silverman et al., 1995; Silverman et al., 1998). Macrosomic fetuses are at increased risk of birth trauma (Casey et al., 1997). Stillbirth and intrapartum death are more common in unmanaged GDM than in managed GDM (Crowther et al., 2005; Langer et al., 2005a; Yang et al., 2006) but the frequency in managed GDM is still greater than in uncomplicated pregnancy (Langer et al., 2005a). In the postnatal period,
neonates affected by GDM are at increased risk of hypoglycemia, hypocalcemia, jaundice, polycythemia and respiratory depression (Martin et al., 1987; Hunter et al., 1993).

The long-term consequences of unmanaged, or poorly managed, GDM revolve primarily around the risk of future metabolic disease. Women who develop GDM in pregnancy are more likely to develop GDM in subsequent pregnancies or, as mentioned above, overt diabetes later in life (Reviewed in Kim et al., 2002; Damm, 2009). Children affected by GDM are at increased risk of obesity and diabetes either in childhood or as adults (Pettitt et al., 1983; Martin et al., 1985; Pettitt et al., 1988; Silverman et al., 1995; Silverman et al., 1998).

All of these short- and long-term consequences are believed to be directly related to the degree of maternal hyperglycemia (i.e., the severity of maternal disease across gestation). Very little attention has been paid to possible consequences in the domain of altered drug disposition mechanisms. At present, drug-disease interactions have not been investigated/identified in GDM.
1.2 Drug disposition mechanisms

In a very general sense, a drug can be defined as a substance that, when introduced into the body, brings about a change in biological function by way of its chemical actions (Katzung, 2004). Drug disposition is a term for an administered drug’s absorption, distribution, metabolism and excretion/elimination, commonly abbreviated to ADME. An in-depth description of the drug disposition mechanisms present within the mammalian body is the domain of textbooks and well beyond the scope of this thesis; however, in this section, I provide a few orienting paragraphs that present the basics of ADME. I then introduce the specific drug disposition mechanisms that were the focus of my doctoral research.

Unless administered directly to their site(s) of action, drugs must gain access to systemic circulation, commonly referred to as the central compartment, before being distributed throughout the body. For orally administered drugs, this entails being absorbed in the digestive tract. Thus, the amount of drug reaching the central compartment for an orally administered drug is the amount ingested minus the amount lost during absorption and associated processes. The amount that reaches the central compartment describes an orally administered drug’s bioavailability and this is affected by such factors as drug solubility, stability and ability to pass, without metabolism, through the intestinal wall. Drugs leaving the intestine flow first through the liver via the portal vein, where they are subjected to what is called first-pass clearance prior to reaching the central compartment. First-pass clearance can dramatically reduce a drug’s bioavailability. For drugs that have low oral bioavailability, alternate routes of administration can be used. These include parenteral injection (i.e., intravenous or intramuscular), dermal absorption and inhalation. Since intravenous injection involves delivering a drug directly to the central compartment, bioavailability via this route is 100%.

Once a drug reaches the central compartment, a fraction of the drug will become involved in reversible binding interactions with plasma proteins. Once binding reaches a state of equilibrium in the central compartment, the balance between unbound and bound drug remains constant. Bound drug will not be able to distribute out of the central compartment because drug-protein complexes are too large to pass through cell membranes (see section 1.2.1). Plasma protein binding is the primary determinant of a drug’s apparent volume of distribution, which is the quotient of the amount of drug in the body (i.e., the administered amount) and the amount of drug retained in the central compartment (i.e., in blood plasma). Because drug distributes out of the central compartment, apparent volume of distribution can be much larger than the actual volume of the central compartment when plasma protein binding is low. Drug may also be retained outside the
central compartment via binding to tissue proteins. Extensive tissue protein-binding increases apparent volume of distribution.

As drugs distribute throughout the body, they reach both their site(s) of action (e.g., drug receptors) and elimination. The latter consists of metabolism and/or excretion in organs like the kidney and liver. Drug metabolism is presented in section 1.2.3 and excretion, mediated in these organs by drug transport proteins, is presented in section 1.2.2.

It should be noted that pregnancy has a significant impact on ADME. For example, the absorption of orally administered drugs is altered because rising progesterone levels delay gastric emptying and reduce intestinal motility (Parry et al., 1970). Hormonal changes are also associated with alterations in the expression of drug metabolizing enzymes. I have reviewed the effects of pregnancy on ADME elsewhere (Reviewed in Anger et al., 2011, a book chapter).

1.2.1 Plasma protein binding

Once a drug reaches the central compartment, as stated above, it immediately begins to reversibly interact with proteins that are either present in the blood or accessed by the blood (e.g., tissue membranes). These interactions consist of binding and releasing and typically occur on a scale measured in milliseconds. For some drugs, protein interactions occupy a high percentage of the total drug concentration. These drugs are said to be highly protein bound. Many clinically important drugs are highly protein bound. For example, many of the human HIV protease inhibitors are over 98% protein bound (Holladay et al., 2001; Aweeka et al., 2010). For other drugs, protein interactions occupy a low percentage of the total drug concentration. These drugs are said to exhibit low or moderate protein binding. Of particular importance to drug disposition are the interactions between drugs and three primary drug-binding proteins: albumin, alpha 1-acid glycoprotein (AAG) and plasma lipoproteins.

Albumin is the most abundant protein present in blood plasma. It is a basic protein composed of three homologous α-helical domains, which contain ten helices that are divided into anti-parallel six-helix and four-helix sub-domains. The primary ligands for albumin are fatty acids (Gordon and Cherkes, 1956; Gordon and Cherkes, 1958; White and Engel, 1958) and crystallographic experiments have identified seven fatty acid binding sites that are asymetrically distributed across all three α-helical domains (Curry et al., 1998; Bhattacharya et al., 2000; Petitpas et al., 2001; Simard et al., 2005). In addition to fatty acids, albumin is noted for its ability to bind an incredibly diverse range of ligands. These include thyroid hormones, unconjugated bilirubin and most acidic drugs that exhibit plasma protein binding (Sjöholm et al., 1979; Kragh-Hansen, 1988).
Two drug-binding sites have been identified in albumin’s sub-domains, which exhibit overlap with fatty acid binding sites (He and Carter, 1992; Carter and Ho, 1994). Albumin is capable of transporting multiple ligands at once.

AAG, also known as orosomucoid, is another important drug-binding protein in blood plasma. It is an acidic protein with poorly elucidated endogenous functions (Reviewed in Hochepied et al., 2003). The crystal structure of unglycosylated human AAG has been published, which indicates AAG has a broadly defined drug binding site (Schönfeld et al., 2008). In its capacity as a drug-binding protein, AAG is believed to bind several hundred basic and acidic drugs. In this way, AAG complements albumin so that most drugs, be they acidic, neutral or basic, will exhibit some degree of protein binding in blood plasma.

A lipoprotein is a biochemical assembly in the blood that consists of a hydrophobic lipid core, containing cholesterol and triglycerides, surrounded by a hydrophilic phospholipid monolayer and apoproteins. Lipids are, by definition, insoluble in water so their transport in blood plasma depends on the formation of these assemblies. Lipoproteins are classified according to their density, which is based on the relative amounts of protein and lipids in the assembly. While it is said that lipoproteins bind drugs, this is a misnomer because very lipophilic drugs simply dissolve into the lipid monolayer (as opposed to binding to specific sites, as with albumin and AAG).

Only the fraction of an administered drug that is not engaged in protein binding is able to distribute throughout the body, exert pharmacological effects and, in the end, be metabolized and/or eliminated. As is described in section 1.2.2, a drug-protein complex is far too large to cross cellular membranes so only unbound drug distributes throughout the body. Once an administered drug dose fully distributes, the unbound drug concentrations observed in extracellular water are roughly equivalent to the unbound drug concentration in serum water. This equilibrated concentration will exert the drug’s effects on therapeutic targets but will also be subjected to elimination processes when in the liver and kidneys. By regulating exposure to elimination processes, plasma protein binding plays a major role in determining a drug’s half-life because the bound drug fraction acts as a reservoir, which delays distribution to sites of elimination (half-life is defined as the amount of time it takes for half of the drug dose to be eliminated). Tissue protein-binding also provides a reservoir – drugs that bind to tissue proteins generally have longer half-lives.

The plasma protein binding of drugs can be altered in three ways. First, the concentration of binding protein can change. For example, certain diseases and physiological states are associated with decreased hepatic synthesis of albumin (Ghoneim and Pandya, 1975; Dean et al., 1980; Leopold et al., 1985; Moshage et al., 1987; Townsend, 1990; Viani et al., 1992; Mattock et al., 2001; Ruot et
...al., 2002) and increased hepatic synthesis of AAG (Kjeldsen et al., 1995; Lacki et al., 1995; Kjeldsen et al., 1997). Second, the concentration of substances that inhibit drug binding or compete with drugs for binding sites can change. This can occur either through the upregulation/downregulation of endogenous substances, like hormones or various lipids, or through the consumption of exogenous substances such as drugs. This is the underlying principle for displacement drug-drug interactions, wherein the protein binding of one drug entity decreases because it is displacedblocked from binding sites by another drug entity (perhaps with greater affinity for the binding protein). Finally, the structure of binding proteins can change in such a way that drug-binding affinity is altered. One such modification is glycosylation, which can occur to albumin and AAG in the presence of persistantly high blood glucose concentrations (Wörner et al., 1992; Koyama et al., 1997; Barnaby et al., 2011).

1.2.2 Drug transporters

Drugs, as well as other xenobiotics and endobiotics, encounter a variety of epithelial and endothelial barriers as they make their way to their site(s) of action and elimination. To circumvent these barriers, they are required to cross cell membranes. For example, a drug being absorbed in the small intestine must first cross the apical membrane of the intestinal epithelial cell and then cross its basolateral membrane to gain access to the central compartment. Drugs cross cell membranes mainly via passive diffusion, facilitated/carrier-mediated diffusion, endocytosis/exocytosis and active transport. These processes occur in both an inward (uptake) and outward (efflux) direction. The permeation process that a drug uses is dictated largely by its physicochemical characteristics.

Passive diffusion involves the movement of drugs down their concentration gradient. For hydrophilic drugs, passive diffusion is highly dependent on molecular size. Aqueous channels in cell membranes are 8-10 Å wide and can accommodate only molecules of a molecular size less than 150-200 Da (Seeman and Kalant, 1998). Caffeine and ephedrine are examples of drugs that cross membranes via this process (Seeman and Kalant, 1998). The passive diffusion of lipid-soluble drugs, on the other hand, is much less dependent on molecular size than on octanol/water partition coefficient (up to about 700 Da). A drug’s oil/water partition coefficient describes the force with which it is driven into the cell membrane, where it can accumulate and eventually transfer into the cytoplasm. Since the majority of drugs are greater than 200 Da in size (250-450 Da) and are lipid-soluble, passive diffusion is the principle mechanism of drug permeation (Seeman and Kalant, 1998).

Facilitated/carrier-mediated diffusion is a process that allows drugs that are hydrophilic but too large to utilize aqueous channels to cross cell membranes. Carrier proteins that are embedded in
the cell membrane are able to form temporary complexes with these drugs, permit diffusion across the membrane and then dissociate at the intracellular portion of the membrane. Carrier proteins exhibit ligand-specificity and this process, as with passive diffusion, involves the movement of drugs down their concentration gradients.

Endocytosis involves processes that allow large drugs, 1000 Da or greater, to cross cell membranes. These processes involve the cell membrane “pinching” together to form an intracellular membrane-bound compartment, called an endosome, that contains extracellular material. Extracellular material can include large drugs that are released into the cytoplasm when endosomes are engulfed by lysosomes. Unlike passive and facilitated/carrier-mediated diffusion, endocytosis can move drugs against their concentration gradients. Also unlike these other processes, endocytosis requires energy (Silverstein et al., 1977). Endocytosis is almost always coupled to exocytosis, the opposite process. For example, insulin, at almost 6000 Da, utilizes endocytosis and exocytosis to cross cell membranes of the blood-brain barrier (Duffy and Pardridge, 1987).

Active transport is a process that, like endocytosis and exocytosis, utilizes energy to move drugs against their concentration gradients. Active transport is executed by families of transport proteins that play very important roles in the uptake and efflux of drugs within the body. This is particularly true in polarized cells, such as those found in intestinal epithelia (Fig. 1.1A), kidney proximal tubules (Fig. 1.1B), blood-brain barrier (Fig. 1.1C), hepatocytes (Fig. 1.1D) and placenta (Fig. 1.1E). Active transport proteins are subdivided according to the source of energy they utilize.

Primary active transport proteins are directly coupled to the hydrolysis of adenosine triphosphate (ATP) and this type of active transport protein is exemplified by the ATP-binding cassette (ABC) superfamily of transporters. In humans, there are 48 ABC transporters, in seven distinct subfamilies (ABCA-ABCG), that share a common sequence and organization of their ATP-binding domains (Dean et al., 2001). These transporters consist of one to three transmembrane domains (TMDs) and one or two nucleotide-binding domains (NBDs). ABC transporter TMDs are diverse and typically consist of α-helices that contain substrate-binding sites capable of conferring substrate specificity to the transporter. ABC transporter NBDs are highly conserved and are the sites of ATP binding and hydrolysis. Transporters with one TMD and one NBD are referred to as half transporters and must dimerize to function (Reviewed in Mo and Zhang, 2009). ABC transporters make possible the unidirectional efflux of their substrates across the cell membrane. As efflux transporters, many serve important excretory roles in clearance organs (e.g., kidney proximal tubules and hepatocytes) as well as barrier roles in other tissues and organs (e.g., counteracting other
permeation processes in the intestinal epithelia, blood-brain barrier and placenta). ABC transporter substrates are numerous and structurally diverse, ranging from lipids, steroid hormones and bile acids to large polypeptides and countless clinically important drugs and drug conjugates. Key members of this family include multidrug resistance protein 1 (MDR1), the multidrug resistance-associated proteins (MRPs) and breast cancer resistance protein (BCRP).

Secondary active transport proteins transport one solute against its concentration gradient by utilizing the electrochemical potential of a second solute being transported down its concentration gradient. When both solutes are traveling in the same direction, the transporter is said to be a symporter, cotransporter or coupled transporter. When the solutes are traveling in opposite directions, the transporter is said to be an antiporter or exchanger. This type of active transport is exemplified by many members of the solute carrier (SLC) superfamily of transporters (as presented below, some SLC members are not active). The Human Genome Organization (HUGO) Gene Nomenclature Committee (HGNC) Database currently has entries for 378 SLC superfamily members, in 51 families, which makes it one of the largest families of transmembrane proteins (HUGO Gene Nomenclature Committee, 2011). SLC transporters are capable of bidirectional transport of their substrates across the cell membrane but, with respect to drug disposition, they are most frequently associated with intracellular drug uptake. As uptake transporters, they serve important roles in the uptake of drugs for elimination by clearance organs (e.g., kidney proximal tubules and hepatocytes) but oppose many of the ABC transporters’ barrier roles in other tissues and organs (e.g., intestinal epithelia, blood-brain barrier and placenta). The range of SLC transporter substrates is as large and diverse as that of the ABC transporters. Key members of this family that transport substrates via secondary active transport include the organic anion transporting polypeptides (OATPs) and organic anion transporters (OATs).

In my introduction to ABC and SLC superfamilies, which follows, and throughout this thesis, I utilize capitalized letters for the abbreviated names of human genes and proteins (e.g., MDR1 or OATP1A1) but only capitalize the first letter of abbreviated names for rodent genes and proteins (e.g., Mdr1 or Oatp1a1). When referring to a gene or protein in general terms, without reference to a specific species, it is treated as human when the abbreviated name is provided. Also, the recently standardized human gene name nomenclature is periodically used or referenced but transporter genes and proteins are typically referred to by the names commonly used in this field (e.g., MDR1 instead of ABCB1 or OATP1A1 instead of SLCO1A1). These same naming conventions are used when referring to drug metabolizing enzymes (e.g., UGT1A1 for humans and Ugt1a1 for rodents).
Figure 1.1. Localization of selected human transport proteins in a variety of tissues. Influx transporters are coloured in yellow while efflux transporters are coloured in blue. A, The apical (luminal) membranes of intestinal epithelial cells contain members of the organic anion transporting polypeptide (OATP) family, peptide transporter 1 (PEPT1; SLC15A1), ileal apical sodium/bile acid cotransporter (ASBT; SLC10A2), multidrug resistance 1 protein (MDR1 or P-glycoprotein/P-gp; ABCB1), breast cancer resistance protein (BCRP; ABCG2) and multidrug resistance protein 2 (MRP2; ABCC2). The basolateral membranes of these cells contain organic cation transporter 1 (OCT1; SLC22A1) and MRP3 (ABCC3). B, The apical (luminal) membranes of renal proximal tubule epithelial cells contain organic anion transporter 4 (OAT4; SLC22A11), organic cation/ergothioneine transporter (OCTN2; SLC22A5), urate transporter 1 (URAT1; SLC22A12), PEPT1, PEPT2 (SLC15A2), MDR1, BCRP, MRP2, MRP4 (ABCC4), multidrug and toxin extrusion protein 1 (MATE1; SLC47A1) and MATE2-K (SLC47A2). The basolateral membranes of these cells contain OATP4C1 (SLCO4C1), OCT2 (SLC22A2), OAT1 (SLC22A6), OAT2 (SLC22A7) and OAT3 (SLC22A8). C, The apical (luminal) membranes of brain capillary endothelial cells contain a number of transport proteins that contribute to the function of the blood-brain barrier, including OATP1A2 (SLCO1A2), OATP2B1 (SLCO2B1), MDR1, BCRP, MRP2, MRP4 and MRP5 (ABCC5). The basolateral membranes of these cells contain MRP4. D, The basolateral (sinusoidal) membranes of hepatocytes contain OCT1, OCT2, OAT7 (SLC22A9), OATP1B1, OATP1B3 (SLCO1B3), OATP2B1, sodium/taurocholate cotransporting peptide (NTCP; SLC10A1), MRP1 (ABCC1), MRP3 and MRP4. The apical (canalicular) membranes of these cells contain efflux transporters that transport substrates into the bile canaliculi, including MDR1, multidrug resistance 3 protein (MDR3; ABCB4), BCRP, MRP2, MATE1 and bile salt export pump (BSEP; ABCB11). E, The syncytiotrophoblast (S) layer of the placenta is comprised of multinucleated (N) cells that contain a number of transport proteins that contribute to the function of the placental barrier, including OATP4A1 (SLCO4A1), MDR1, BCRP and MRP2. The basolateral membranes of these cells contain OCT1, OATP2B1, MRP1 and MRP3. The apical (luminal) membranes of the fetal capillary endothelial cells, located within the adjacent mesenchyme (M), contain MRP1 and MRP3. All bracketed gene names refer to human gene names. The localization of transporters to specific membranes and tissues, as presented in this figure and discussed in this thesis, is based on currently available evidence. The expression of transporters with bolded names and extra thick borders in panels D and E were quantified in Chapter 3 and/or 4. Panels A-D were adapted from The International Transporter Consortium, 2010.
1.2.2.1 Multidrug resistance protein 1 (MDR1; ABCB1)

MDR1, also commonly referred to as P-glycoprotein/P-gp, was originally identified because of its ability to confer multidrug resistance to Chinese hamster ovary (CHO) cells and is the most extensively studied ABC transporter (Juliano and Ling, 1976). Structurally, MDR1 is a full ABC transporter with two TMDs, consisting of six α-helices each, and two NBDs. In rodents, MDR1 is encoded by the Mdr1a and Mdr1b genes. MDR1’s substrates are characteristically neutral or cationic compounds with a bulky structure and are known to include steroid hormones (e.g., progesterone), antineoplastics, antibiotics, antiretrovirals (e.g., all of the HIV protease inhibitors), immunosuppresants and calcium channel blockers (Saeki et al., 1993; Booth et al., 1998; Kim et al., 1998; Schuetz et al., 1998; Smit et al., 1998b; Su et al., 1998; Song et al., 1999; Wang et al., 2000; Reviewed in Cascorbi, 2011). In normal human tissues, MDR1 is primarily expressed in liver, kidney, intestine, blood-brain barrier, blood-testis barrier and placenta (Reviewed in Cascorbi, 2011). In the liver, MDR1 is located in the apical (canalicular) membranes of hepatocytes where it effluxes its substrates into the bile canaliculi for elimination via the intestinal tract (i.e., biliary excretion). This is a major route of elimination for many drugs. Smit and colleagues demonstrated that the cumulative biliary excretion of several cationic compounds was reduced in Mdr1a gene knockout mice (Smit et al., 1998a; Smit et al., 1998c). In the placenta, MDR1 is located in the maternal-facing apical membrane of the syncytiotrophoblast layer and its important barrier function in this tissue has been highlighted by studies employing Mdr1a/b gene knockout mice. Lankas and colleagues mated mice that carried a mutation causing a lack of placental Mdr1 and examined the teratogenic effects of avermectin treatment on their litters (Lankas et al., 1998). Pups were 100% susceptible to cleft palate if they were homozygous for the mutation, heterozygous mice were somewhat less sensitive and wild type mice were unaffected (Lankas et al., 1998). This finding was then replicated using targeted Mdr1a/b gene knockout mice and potent Mdr1 inhibitors, namely PSC833 and GG918 (Schinkel et al., 1997; Smit et al., 1999). In this latter study, it was shown that accumulation of radiolabeled digoxin, paclitaxel and saquinavir was dramatically increased in fetuses from Mdr1a/b gene knockout mice and mice treated with Mdr1 inhibitors.

1.2.2.2 Multidrug resistance-associated proteins (MRPs; ABCCs)

Nine MRPs have been identified throughout the body, referred to as MRP1-9. The HGNC has assigned the gene names ABCC1-6 to MRP1-6 and ABCC10-12 to MRP7-9. Of particular importance to the studies described in this thesis are the first four MRPs. Structurally, MRP1-3 are full ABC transporters with three TMDs, totalling 17 α-helices combined, and two NBDs. MRP4,
however, is considered a “short MRP” because its structure more closely resembles MDR1. MRP1-3 substrates are characteristically amphiphilic with at least one negative net charge. MRP1 substrates are known to include glutathione, glucuronide and sulfate conjugates, antineoplastics and antiretrovirals. In normal human tissues, MRP1 is ubiquitously expressed but at fairly low levels (Reviewed in Borst et al., 2000). MRP2 substrates are known to include glutathione, glucuronide and sulfate conjugates, antineoplastics, antiretrovirals and statins. In normal human tissues, MRP2 is primarily expressed in the liver, kidney, intestine and placenta (Reviewed in Borst et al., 2000; St-Pierre et al., 2000). MRP3 substrates are known to include glutathione, glucuronide and sulfate conjugates (with glucuronide conjugates being the better MRP3 substrates), antineoplastics and bile salts. In normal human tissues, MRP3 is primarily expressed in the liver, kidney, intestine and placenta (Reviewed in Borst et al., 2000; St-Pierre et al., 2000). MRP4 substrates are characteristically nucleotide analogues and are known to include glucuronide conjugates, cyclic nucleotides and antiretrovirals. In normal human tissues, MRP4 is ubiquitously expressed (Reviewed in Borst et al., 2000). In the liver, MRP1, MRP3 and MRP4 are located in the basolateral membranes of hepatocytes where they efflux their substrates into the central compartment. In contrast, MRP2 is located in the apical membranes of hepatocytes where it effluxes its substrates into the bile canaliculi for elimination via the intestinal tract. Studies have consistently shown a compensatory upregulation of MRP1, MRP3 and MRP4 in the liver, as well as kidney, when MRP2-mediated hepatobiliary excretion is compromised (Soroka et al., 2001; Tanaka et al., 2002; Kuroda et al., 2004; Chen et al., 2005; Chu et al., 2006; Vlaming et al., 2006). For example, Mrp2 gene knockout mice have been shown to exhibit elevated Mrp3 and Mrp4 expression (Chen et al., 2005; Chu et al., 2006; Vlaming et al., 2006). For some substrates, compensation of this nature is believed to reduce intrahepatic accumulation and lead to increased renal elimination (Tanaka et al., 2002; Chu et al., 2006; Villanueva et al., 2008). In the placenta, MRP1 and MRP3 are located in the fetal-facing basolateral membrane of the syncytiotrophoblast layer, where they promote the transfer of their substrates into the fetal compartment. MRP1 and MRP3 are also located in the apical (luminal) membranes of the fetal capillary endothelial cells where they again promote the transfer of their substrates into the fetal compartment. MRP2, in contrast, is located in the maternal-facing apical membrane of the syncytiotrophoblast layer where it antagonizes the actions of MRP1 and MRP3.

1.2.2.3 Breast cancer resistance protein (BCRP; ABCG2)

BCRP is a relatively recent addition to the ABC transporter superfamily. BCRP’s activity was first observed in human breast carcinoma cell lines that had grown resistant to mitoxantrone,
doxorubicin and daunorubicin but not via MDR1 or MRP upregulation (Chen et al., 1990; Nakagawa et al., 1992; Lee et al., 1997). Around the same time, the transporter was isolated in breast cancer by Doyle et al and called BCRP (Doyle et al., 1998), in the placenta by Allikmets et al and called ABCP (Allikmets et al., 1998) and in colon cancer by Miyake et al and called MXR (Miyake et al., 1999). The HGNC designated it BCRP and assigned it the gene name ABCG2. Structurally, BCRP differs from MDR1 and the MRPs in that it is a half transporter that must homodimerize to function as a transporter (Ozvegy et al., 2001). BCRP’s single TMD consists of six α-helices (Wang et al., 2008). BCRP’s substrates are known to include steroid hormones (e.g., progesterone and estradiol), cholesterol, glucuronide and sulfate conjugates, antineoplastics, antibiotics, antiretrovirals, statins, natural health products and dietary carcinogens (Janvilisri et al., 2003; Merino et al., 2005; Pavek et al., 2005; van Herwaarden et al., 2006; Zamek-Gliszczynski et al., 2006; Ando et al., 2007). In normal human tissues, BCRP is primarily expressed in liver, kidney, intestine, blood-brain barrier, lung and placenta (Maliepaard et al., 2001). In the liver, BCRP is located in the apical membranes of hepatocytes where it effluxes its substrates into the bile canaliculi for elimination via the intestinal tract. In the placenta, BCRP is located in the maternal-facing apical membrane of the syncytiotrophoblast layer and its important barrier function in this tissue has been highlighted by studies employing BCRP inhibitors. For example, relative to controls, pregnant Mdr1a/b gene knockout mice treated with the Mdr1/Bcrp-specific inhibitor GF120918 exhibited twice the fetal penetration of the Mdr1/Bcrp substrate topotecan (Jonker et al., 2000). Placental BCRP is also partly responsible for the limited fetal exposure that occurs when women use the oral hypoglycemic agent glyburide in pregnancy (Gedeon et al., 2008).

1.2.2.4 Other efflux transporters

Multidrug resistance protein 3 (MDR3) and the bile salt export pump (BSEP) are ABC efflux transporters that exhibit specific substrate preferences. MDR3 is related to MDR1 in both structure and regulation but is believed to only transport phospholipids. In contrast to MDR1’s expression throughout the body’s major organ systems, functional MDR3 protein is only found in the apical membranes of hepatocytes (Smit et al., 1994). BSEP is also largely restricted to the apical membranes of hepatocytes and is believed to only transport bile salts. While both MDR3 and BSEP to not directly contribute to drug disposition, they are susceptible to inhibition and this can affect other transporters by inducing intrahepatic cholestasis (Kullak-Ublick et al., 2000). Additional transporters that are believed to play important roles in the cellular efflux of drugs appear in figure
1.1 and are reviewed elsewhere (Reviewed in Koepsell et al., 2007; International Transporter Consortium et al., 2010; Reviewed in Klaassen and Aleksunes, 2010).

1.2.2.5 Uptake transporters

The OATPs transport amphiphilic organic compounds via anion exchange. OATPs consist of 12 transmembrane domains/helices and a large extracellular domain (Noé et al., 1997). They are encoded by SLCO genes but not every human OATP has a rodent ortholog and vice versa. There are also some species differences in OATP localization. For example, Oatp4a1 is a placenta-specific transporter in the mouse but OATP4A1 can be found in many human tissues (Cheng et al., 2005). Known OATP transporter substrates include bile acids, glucuronide and sulfate conjugates, antineoplastics and statins. OAT and OCT transporters also consist of 12 transmembrane domains/helices (Burckhardt and Wolff, 2000). The OATs transport amphiphilic organic anions using sodium and dicarboxylate gradients that are established by the sodium-dicarboxylate co-transporter and the sodium-potassium ATPase. Unlike OATPs and OATs, the OCTs simply mediate facilitated diffusion. Transport, therefore, occurs down the concentration gradient of substrates (Reviewed in Koepsell and Endou, 2004). OCT substrates are usually less than 500 Da in size and hydrophilic organic cations. Antihistamines (e.g., cimetidine and ranitidine) and the oral hypoglycemic agent metformin are examples of OCT substrates (Kimura et al., 2005). OATPs, OATs and OCTs are expressed in a variety of normal human tissues, although OATs and OCTs are strongly associated with the uptake of drugs by kidney proximal tubules (Fig. 1.1). Additional transporters that are believed to play important roles in the cellular uptake of drugs appear in figure 1.1 and are reviewed elsewhere (Reviewed in Koepsell et al., 2007; International Transporter Consortium et al., 2010; Reviewed in Klaassen and Aleksunes, 2010).

1.2.3 Drug metabolizing enzymes

For lipophilic drugs to be successfully removed from the body, they usually must be rendered polar, or more polar, by way of biotransformation/biochemical modification. The biotransformation of drugs within the body’s tissues is referred to as drug metabolism and it is executed by highly specialized families of metabolic enzymes. These enzymes are broadly classed as either phase I or phase II enzymes. Phase I metabolic enzymes typically convert a drug (the parent) to a more polar molecule (a metabolite) by introducing or unmasking a functional group (e.g., -OH, -NH₂, -SH, etc.) by way of oxidation, reduction or hydrolysis. The products of phase I metabolism can be active but are most often inactive (with prodrugs, the metabolite is more active than the parent). In some instances, the products of phase I metabolism are sufficiently polar to be removed
rapidly from the body. If they are not, though, they may be subjected to subsequent conjugative biotransformation. This conjugative biotransformation, called phase II metabolism, combines drugs or their metabolites with endogenous substrates such as glucuronic acid, glutathione, sulfate, acetic acid or amino acids to produce highly polar conjugates. Phase I metabolism need not precede phase II metabolism when a drug already contains suitable functional groups. While metabolic capabilities have been detected in pretty much every tissue, most drug metabolism occurs in the liver and intestine. For some drugs, metabolism in the kidney is also important. In many instances, metabolites are much better substrates for ABC and SLC transporters. Indeed, transport is often referred to as phase III metabolism.

1.2.3.1 Phase I enzymes (cytochrome P450s)

The cytochrome P450 (CYP) superfamily is the most notable superfamily of phase I enzymes. CYPs are moderately sized (approximately 50 kDa), membrane-bound proteins that activate molecular oxygen, in the presence of co-factors like cytochrome P450 reductase, to add oxygen to substrates, remove hydrogen atoms from substrates or simply remove electrons from substrates (they are mono-oxygenases). Substrate specificity for a given CYP is conferred by the specific oxidative mechanism employed and the specific three-dimensional structure of its substrate-binding pocket, which determines substrate orientation in relation to the reactive oxygen molecule. There are 57 functional CYP enzymes in humans but considerably more in rodent species (HUGO Gene Nomenclature Committee, 2011). CYPs are responsible for the metabolism of approximately 75% of the top 200 prescription drugs in the United States (Williams et al., 2004). Moreover, just five CYPs are responsible for 95% of this metabolism: CYP3A4 (approximately 50%), CYP2C9, CYP2C19, CYP2D6 and CYP1A2 (Williams et al., 2004). CYP3A4 is considered the most important CYP by virtue of the massive number of drugs it metabolizes. CYP3A4 also accounts for roughly 30% of total hepatic CYP content and 40% of total intestinal CYP content (Davis and Rodrigues, 2009). Metabolism in the liver facilitates excretion whereas intestinal metabolism reduces bioavailability. CYP3A4’s substrates include, but are most certainly not limited to, antineoplastics, antibiotics, antiretrovirals (e.g., all of the HIV protease inhibitors), immunosuppressants, statins, calcium channel blockers and several recreational drugs. In rats, Cyp3a2, not CYP3A4, is the dominant constitutive Cyp3a isoform (Nelson et al., 1993) and has been shown to metabolize prototypical human CYP3A4 substrates like verapamil (Tracy et al., 1999; Choi and Burm, 2008; Hanada et al., 2008). CYPs are also involved in the metabolic pathways of endobiotics. For example, several CYP4As are involved in the hydroxylation of fatty acids.
1.2.3.2 Phase II enzymes

Phase II enzymes, as touched upon above, add hydrophilic groups to their substrates in the presence of a co-factor. The co-factor provides this hydrophilic group and generally defines the enzyme family. There are three particularly important families of phase II enzymes which render their substrates more polar via conjugative biotransformation. First, the uridine 5’-diphospho (UDP)-glucuronosyltransferases (UGTs) conduct glucuronidation reactions in aliphatic alcohols and phenols. UGT-mediated metabolism is the second most common clearance mechanism of the top 200 prescription drugs in the United States after CYP-mediated metabolism, although this only equates to about 10% (Williams et al., 2004). UGTs tend to share substrates, differing only in relative affinities, but UGT1A1 exhibits noteworthy substrate diversity. Second, the glutathione S-transferases (GSTs) catalyze the reaction between tripeptide glutathione and reactive electrophilic sites of, most frequently, the products of phase I metabolism. GSTs merely increase the rate of this reaction because it can occur in their absence. Third, the sulfotransferases (SULTs) catalyze the transfer of sulfonate from 3’-phosphoadenosine-5’-phosphosulfate to susceptible substrates. Additional families of phase II enzymes are involved in acetylation or methylation reactions, but these reactions do not necessarily produce more polar metabolites so these enzyme families are more often associated with the termination of pharmacological activity.

1.2.4 Regulation of drug transporters and metabolic enzymes

The human, rat and mouse genomes contain genes for 48, 47 and 49 nuclear receptors, respectively (Zhang et al., 2004b). Most ligand-activated nuclear receptors heterodimerize with the 9-cis retinoic acid receptor (RXR) and directly interact with their DNA response elements to control the transcription of a wide array of target genes. As such, these nuclear receptors are ligand-activated transcription factors. Nuclear receptors contribute to processes involved in reproduction, development, energy homeostasis, response to inflammation and xenobiotic detoxification/drug disposition. With respect to their roles in energy homeostasis, many nuclear receptors act as sensors for aberrant intracellular levels of endobiotics, such as lipids and glucose, and are able to coordinate adaptive responses by way of their transcriptional activity. In this way, nuclear receptor activity/inactivity has been associated with metabolic disease. Nuclear receptors are also associated with inflammatory disease as several are suppressed by inflammation and exposure to pro-inflammatory cytokines (Beigneux et al., 2000; Pascussi et al., 2000; Beigneux et al., 2002; Kim et al., 2003; Fang et al., 2004; Kim et al., 2007). With respect to their roles in drug disposition, nuclear receptors have been found to act as transcription factors for all of the abovementioned drug
transporters and metabolic enzymes and, not surprisingly, share ligands/substrates with them (Reviewed in Omiecinski et al., 2011; Reviewed in Tirona, 2011). As such, they are perfectly positioned to link drug exposure to the transcription of genes encoding appropriate drug transporters and metabolic enzymes. This is particularly true of the pregnane X receptor (PXR) and constitutive androstane receptor (CAR), which exhibit a great deal of ligand promiscuity. Peroxisome proliferator-activated receptors (PPARs), liver X receptors (LXR) and the farnesoid X receptor (FXR) are also linked to the regulation of drug transporters and metabolic enzymes. All of these nuclear receptors, to some extent, exhibit crosstalk with each other’s signalling pathways. Many of these nuclear receptors also regulate each other’s expression; sometimes directly.

1.2.4.1 Pregnane X receptor (PXR; NR1I2)

PXR is primarily expressed in the liver, kidney, intestine and blood-brain barrier (Kliewer et al., 1998; Bauer et al., 2004; Lamba et al., 2004b). Owing to its large and flexible ligand-binding pocket, PXR is activated by a more diverse array of drug compounds than any other nuclear receptor (Watkins et al., 2001). The most robust PXR drug ligands are those that are also established CYP3A inducers, such as dexamethasone, phenobarbital and spironolactone (Lehmann et al., 1998; Shi et al., 2010). Other notable ligands include: antiretrovirals, hyperforin (St. John’s wort), nicotine, omeprazole, paclitaxel, phenytoin and many others (Tirona, 2011). Bile acids, bilirubin and steroid hormones (e.g., progesterone) are endogenous PXR ligands (Timsit and Negishi, 2007). Pregnenolone-16α-carbonitrile is frequently used experimentally to achieve PXR activation in rodents but this ligand is not as efficacious in humans, just as rifampin is an established PXR activator in humans but is not as efficacious in rodents (Xie et al., 2000; Tirona et al., 2004). This illustrates that there are species differences in the ability of ligands to activate PXR. PXR activation has been implicated in increased transcription of many drug transporters, such as MDR1, MRP2, MRP3, BCRP, OATP1A1 and OATP1B1 (Geick et al., 2001; Kast et al., 2002; Teng et al., 2003; Jigorel et al., 2006; Miki et al., 2006; Meyer zu Schwabedissen et al., 2008; Jiang et al., 2009). PXR has also been implicated in the transcription of many drug metabolizing enzymes, such as CYP2As, CYP2Bs, CYP2Cs, CYP3As, UGT1As and SULTs (Goodwin et al., 1999; Pascussi et al., 1999; Goodwin et al., 2001; Chen et al., 2004; Itoh et al., 2006; Alnouti and Klaassen, 2008). Recent studies have shown that, in addition to regulating drug transporters and metabolic enzymes, PXR plays an important role in lipid and glucose metabolism (Reviewed in Wada et al., 2009).
1.2.4.2 Constitutive androstane receptor (CAR; NR1I3)

CAR is primarily expressed in the liver, kidney, intestine and blood-brain barrier (Choi et al., 1997; Lamba et al., 2004a; Burk et al., 2005). As its name suggests, CAR is constitutively active in the absence of ligand but is regulated by agonists and inverse agonists. The CAR/RXR complex binds to the phenobarbital response element – so named because the prototypical CAR activator is phenobarbital. CAR and PXR have many xenobiotic and endobiotic ligands in common. Consistent with this, there is also a great deal of overlap in the drug transporters and metabolic enzymes that CAR and PXR modulate. For example, CAR is implicated in the transcription of MDR1, MRP2, MRP3 and BCRP (Geick et al., 2001; Kast et al., 2002; Assem et al., 2004; Jigorel et al., 2006). One of the regulatory properties that differentiates CAR from PXR is the preferential induction of CYP2B6 over CYP3A4, even though the inducers of both these genes are similar (Faucette et al., 2007). As with PXR, CAR has been shown to play an important role in lipid and glucose metabolism (Reviewed in Wada et al., 2009).

1.2.4.3 Peroxisome proliferator-activated receptors (PPARs; NR1C1-3)

There are three PPAR isoforms: PPARα (NR1C1), PPARδ (NR1C2) and PPARγ (NR1C3). PPARα is found in the liver, kidney, intestine and heart and regulates a number of genes that are critically involved in fatty acid oxidation (Reviewed in Braissant et al., 1996; Lemberger et al., 1996; Lee et al., 2003; Bünger et al., 2007). PPARγ is found primarily in adipose tissue and regulates a number of genes that are involved in adipocyte differentiation and the maintenance of peripheral insulin sensitivity (Reviewed in Chawla et al., 1994; Braissant et al., 1996; Lemberger et al., 1996; Lee et al., 2003). Comparitively less is known about PPARδ, which is ubiquitously expressed, but it has recently been ascribed roles in glucose metabolism and insulin sensitivity in muscle (Braissant et al., 1996; Lee et al., 2006). Free fatty acids and eicosanoids are endogenous PPAR ligands (Kliewer et al., 1997). Hypolipidemic drugs such as fibrates (PPARα agonist), thiazolidinediones (PPARγ agonist) and aleglitazar (balanced PPARα/PPARγ agonist) are potent PPAR ligands (Kliewer et al., 1997; Willson and Wahli, 1997; Bénardeau et al., 2009). One study that examined the effects of PPARα activation on the expression of hepatic drug transporters in mice found upregulation in Mdr1, Mrp3, Mrp4 and Bcrp (Moffit et al., 2006). The PPARs also regulate a handful of UGTs and SULTs (Reviewed in Runge-Morris and Kocarek, 2009). Interestingly, a functional PPARα binding site has been identified within the proximal promoter of the PXR gene (Aouabdi et al., 2006).
1.2.4.4 Liver X receptors (LXRs; NR1H3/2)

There are two LXR isoforms: LXRα (NR1H3) and LXRβ (NR1H2). LXRα is primarily expressed in the liver, kidney, intestine, spleen, adipose tissue and lung whereas LXRβ is ubiquitously expressed (Apfel et al., 1994; Willy et al., 1995). Oxidized cholesterol derivatives, called oxysterols, are the established endogenous ligands for LXR (Janowski et al., 1996; Lehmann et al., 1997; Janowski et al., 1999). A role for the LXRs in glucose sensing has been proposed but remains controversial (Lazar and Willson, 2007; Mitro et al., 2007; Denechaud et al., 2008). As sensors of sterols, the LXRs play an important role in triggering adaptive responses to high intracellular cholesterol levels. For example, LXR-mediated upregulation of the cholesterol transporters ABCG5 and ABCG8 promotes the hepatobiliary excretion of cholesterol and inhibits its intestinal absorption (Repa et al., 2002; Yu et al., 2003). LXR-mediated upregulation of 7-α-hydroxylase (CYP7A1), the rate-limiting enzyme in the hepatic synthesis of bile acids from cholesterol, is another adaptive response (Lehmann et al., 1997). While these effects are beneficial, non-specific activation of LXR increases the transcription of genes for lipogenic proteins and lipogenic transcription factors (i.e., the sterol regulatory element binding protein-1/SREBP-1) and this leads to increased hepatic triglyceride synthesis, hypertriglyceridemia and hepatic steatosis (Zhou et al., 2008). Studies employing specific and non-specific LXR ligands have ascribed the lipogenic effects to the LXRα isoform (Miao et al., 2004; Lund et al., 2006). While the study of LXR’s drug transporter and metabolic enzyme target genes appears to be in its infancy, there have been links to members of the OATP family. For example, OATP1B1 mRNA was induced in human hepatoma-derived cells and primary human hepatocytes following incubation with the LXRα ligand TO901317 (Meyer zu Schwabedissen et al., 2010).

1.2.4.5 Farnesoid X receptor (FXR; NR1H4)

FXR is predominantly expressed in the liver, kidney and intestine (Houten et al., 2007) and plays an important role in triggering adaptive responses to high intracellular bile acid levels (Parks et al., 1999). For example, FXR indirectly suppresses the transcription of CYP7A1 by upregulating a protein that interferes with its transcription (Lu et al., 2000). FXR is also involved in the regulation of genes that promote β-oxidation and triglyceride clearance as well as the suppression of lipogenic gene transcription (Watanabe et al., 2004; Zhang et al., 2004a). Thus, the actions of FXR and LXR are diametrically opposed. Studies examining FXR’s drug transporter and metabolic enzyme target genes suggest that it regulates MRP2, BSEP, OATP1B1 and OATP1B3 (Ohtsuka et al., 2006; Meyer
zu Schwabedissen et al., 2010) as well as CYP3A4, SULT2A1 and various UGTs (Kast et al., 2002; Plass et al., 2002; Gnerre et al., 2004; Miyata et al., 2006; Kaeding et al., 2008).

1.2.5 Pharmacokinetic parameters in this thesis

Pharmacokinetics is a field within pharmacology that employs a variety of metrics, or parameters, to describe the time course of drug concentrations in the body as well as the relationship between drug dosage and drug concentration at sites within the body. The alterations to drug disposition mechanisms that are presented in this thesis are periodically discussed in terms of the pharmacokinetic parameters that characterize their actions. What follows is a simplified background for hepatic clearance, which is a parameter that characterizes the efficiency of drug elimination through the activity of hepatic drug transporters and metabolic enzymes, and the fetal:maternal free drug concentration ratio, which is a parameter that characterizes the extent of fetal drug exposure relative to that of the mother.

1.2.5.1 Hepatic clearance

Equation 1.1, adapted from Wilkinson and Shand, can be used to calculate hepatic clearance as a function of blood flow, protein binding and intrinsic clearance capacity (Wilkinson and Shand, 1975). This equation is based on a simple, well-stirred venous equilibrium model and could be applied to any clearance organ.

$$\text{CL}_{\text{liver}} = \frac{Q_{\text{liver}} \cdot f_u \cdot \text{CL}_{\text{intrinsic}}}{Q_{\text{liver}} + f_u \cdot \text{CL}_{\text{intrinsic}}}$$

**Equation 1.1. Hepatic clearance.** Where $\text{CL}_{\text{liver}}$ = hepatic clearance, $Q_{\text{liver}}$ = blood flow to the liver, $f_u$ = fraction of drug unbound and $\text{CL}_{\text{intrinsic}}$ = intrinsic clearance.

As this equation indicates, there are three values that can influence hepatic clearance: $Q_{\text{liver}}$, $f_u$ and $\text{CL}_{\text{intrinsic}}$. $Q_{\text{liver}}$ determines the rate at which drug is presented to the liver. The $f_u$ value determines what fraction of drug that is presented to the liver is not bound to protein and, therefore, available for clearance. $\text{CL}_{\text{intrinsic}}$ is the intrinsic ability of the liver to remove the drug and is determined by transport and metabolic processes. The extraction ratio for a given drug is defined as the fraction of the drug entering the liver that is irreversibly extracted in one pass of blood and is itself determined using these same three values in equation 1.1. The extraction ratio can be used to predict the extent to which alterations in $Q_{\text{liver}}$, $f_u$ and $\text{CL}_{\text{intrinsic}}$ will influence $\text{CL}_{\text{liver}}$. An extraction ratio of 0 indicates no extraction while a ratio of 1 indicates total extraction in a single pass. Examples of drugs with low extraction ratios (~0.3 or lower) in humans include: antipyrine,
diazepam, glyburide, phenytoin, theophylline and warfarin. Examples of drugs with high extraction ratios (~0.7 or higher) in humans include: lignocaine, morphine, propranolol, verapamil and the protease inhibitors saquinavir and lopinavir (lopinavir only when given without ritonavir).

Variation in $Q_{\text{liver}}$ has a greater effect on the clearance of high extraction ratio drugs than it does on low extraction ratio drugs. High extraction ratio drugs could theoretically be removed at a rate that is much higher than blood flow (i.e., $Q_{\text{liver}} << f_u \times CL_{\text{intrinsic}}$). Since drugs cannot be cleared faster than they are presented to an eliminating organ, high extraction ratio drugs are said to exhibit blood flow limited hepatic clearance (i.e., $CL_{\text{liver}} \equiv Q_{\text{liver}}$). Low extraction ratio drugs, on the other hand, remove drugs at a rate that is far below the rate of its presentation (i.e., $Q_{\text{liver}} >> f_u \times CL_{\text{intrinsic}}$). Low extraction ratio drugs are, therefore, said to exhibit capacity limited hepatic clearance since they are insensitive to changes in blood flow (i.e., $CL_{\text{liver}} \equiv f_u \times CL_{\text{intrinsic}}$).

The clinical significance of protein binding is an intensely debated issue (Sansom and Evans, 1995; Benet and Hoener, 2002; Schmidt et al., 2010). For the purposes of this thesis, I am taking the generalized position that variation in $f_u$ has a greater effect on the clearance of low extraction ratio drugs than it does on high extraction ratio drugs. For low extraction ratio drugs, an increase in $f_u$ will increase the opportunities for drug transporters and metabolic enzymes to act upon the drug (i.e., $f_u \times CL_{\text{intrinsic}}$) and lead to an increase in $CL_{\text{liver}}$. For high extraction ratio drugs, clearance mechanisms are already so efficient that increasing opportunities for interaction will be of little consequence.

Variation in $CL_{\text{intrinsic}}$ also has a greater effect on the clearance of low extraction ratio drugs than it does on high extraction ratio drugs because, as described above, hepatic clearance is generally capacity limited with the former and blood flow limited with the latter. For low extraction ratio drugs, even slight changes in $CL_{\text{intrinsic}}$ will produce corresponding changes in $CL_{\text{liver}}$. For high extraction ratio drugs, $f_u \times CL_{\text{intrinsic}}$ would have to be reduced to below $Q_{\text{liver}}$ to significantly affect $CL_{\text{liver}}$ (such a scenario occurs with advanced liver disease).

The influence of $Q_{\text{liver}}$, $f_u$ and $CL_{\text{intrinsic}}$ on $CL_{\text{liver}}$ has been presented here using extraction ratio extremes (i.e., low versus high). It is important to note that between these extremes are a number of drugs with intermediate extraction ratios (~0.3-0.7) in humans, for which these three parameters are important determinants of $CL_{\text{liver}}$. Examples of drugs with intermediate extraction ratios in humans include: cyclosporine, midazolam, nortriptyline, paracetamol and quinidine. It is also important to note that it is not uncommon for more than one of these parameters to be altered. In most scenarios where perturbations to $f_u$ are believed to be associated with a clinically meaningful
alteration in hepatic clearance, for example, a change in drug transporter and/or metabolic enzyme activity is also suspected.

1.2.5.2 Fetal:maternal free drug concentration ratio

Equation 1.2 is a modified form of the Henderson-Hasselbach equation, adapted from Garland, that can be used to estimate the fetal:maternal free drug concentration ratio (F/M) by correcting for differences in maternal and fetal protein binding and materno-fetal/feto-maternal transfer mechanisms (Garland, 1998).

$$\frac{F}{M} = \frac{\% \text{unbound}_M \cdot \text{CL}_{MF}}{\% \text{unbound}_F \cdot \text{CL}_{FM} \cdot \text{CL}_F}$$

Equation 1.2. Fetal:maternal free drug concentration ratio. Where % unbound$_M$ = percentage of drug unbound in maternal circulation, % unbound$_F$ = percentage of drug unbound in fetal circulation, CL$_{MF}$ = clearance from the maternal circulation into the fetal circulation, CL$_{FM}$ = clearance from the fetal circulation into the maternal circulation and CL$_F$ = overall clearance within the fetal compartment.

The percentage of drug unbound in maternal circulation and CL$_{MF}$ (the numerators) are values that can influence the transfer of drugs in the materno-fetal direction. Drug transporters in the placental syncytiotrophoblast layer play a major role in determining CL$_{MF}$. For example, the uptake transporter OATP4A1 in the apical membrane and the efflux transporters MRP1, MRP3 and MRP5 in the basolateral membrane contribute to the CL$_{MF}$ of their substrates (Fig. 1.1E). Drug transporters in fetal capillary endothelial cells, located in mesenchyme that is adjacent to the syncytiotrophoblast layer, also contribute to CL$_{MF}$ (e.g., MRP1, MRP3 and MRP5; Fig. 1.1E).

The percentage of drug unbound in fetal circulation, CL$_{FM}$ and CL$_F$ (the denominators) are values that can influence the transfer of drugs in the feto-maternal direction. Drug transporters in the placental syncytiotrophoblast layer also play a major role in determining CL$_{FM}$. For example, the efflux transporters MDR1, MRP2 and BCRP in the apical membrane and the uptake transporters OCT1, MCT1 and OATP2B1 in the basolateral membrane contribute to the CL$_{FM}$ of their substrates (Fig. 1.1E). CL$_F$ is determined by fetal drug metabolism/elimination.

As equation 1.2 indicates, the fetal:maternal free drug concentration ratio represents the balance between transfer in the materno-fetal and feto-maternal directions.
1.3 Rationale

There is an association between inflammation and altered drug disposition that research conducted in the laboratory of my supervisor, Dr. Micheline Piquette-Miller, helped establish. In this section, the nature of this association and data supporting the assertion that GDM is a systemic inflammatory disease are presented.

Inflammation is the body’s defensive response to harmful stimuli, such as pathogen exposure or tissue damage, and includes local and systemic effects that are executed by the innate arm of the immune system. The local effects of inflammation are initiated by the movement of plasma and leukocytes from the blood compartment into the affected site(s). As the inflammatory response propagates and matures, monocytes differentiate into macrophages. The systemic effects of inflammation, collectively referred to as the acute phase response (APR), are initiated when these macrophages “ramp-up” their release of pro-inflammatory cytokines into systemic circulation. Systemic pro-inflammatory cytokines, primarily interleukin-6 (IL-6) and tumor necrosis factor-α (TNF-α), stimulate the synthesis of acute phase proteins in the liver (Ramadori and Christ, 1999). These proteins include C-reactive protein (CRP), AAG, serum amyloid A and other proteins that are associated with the fever, increased blood pressure, somnolence, lethargy and decreased appetite that accompany systemic inflammation. While stimulation of acute phase proteins is generally beneficial, the process leading to it can affect drug disposition when the harmful stimuli elicits a particularly severe response or persists over time (as in chronic inflammation).

For example, increased synthesis of the aforementioned acute phase proteins in the liver can have an impact on the plasma protein binding of drugs. Increased AAG is observed in patients with diseases that are associated with inflammation, such as those with inflammatory bowel disease (Kjeldsen et al., 1995; Kjeldsen et al., 1997) or rheumatoid arthritis (Lacki et al., 1995), which can translate into increased plasma protein binding of basic drugs. One study comparing ex vivo carbamazepine protein binding in serum from patients with various inflammatory diseases to serum from healthy controls found that plasma protein binding was enhanced in the disease state (Baruzzi et al., 1986). In this study, carbamazepine $f_{10}$ values were inversely correlated with serum AAG concentrations (Baruzzi et al., 1986). Not all alterations involve increased synthesis. Decreased synthesis of albumin has been observed in inflammation and this can translate into decreased plasma protein binding of acidic drugs (Moshage et al., 1987; Ruot et al., 2002).

One of the first clinical examples of inflammation-induced changes in human drug response to appear in the literature was a 1978 report that described decreased theophylline clearance during
confirmed upper-respiratory tract infections (Chang et al., 1978). This observation was attributed not to plasma protein binding but to a downregulation of the CYPs responsible for theophylline metabolism (Renton, 1978; Renton, 2001). In the decades that followed, numerous studies utilized human and rodent cell lines to demonstrate CYP suppression in response to pro-inflammatory cytokines such as IL-1β, IL-6 and TNF-α (Reviewed in Renton, 2001; Reviewed in Morgan et al., 2008). Consequently, it is widely accepted that inflammation is capable of downregulating CYPs, particularly within the liver, and that this effect is mediated by pro-inflammatory cytokines.

More recent evidence suggests that a relationship similar to that between inflammation and metabolism exists between inflammation and drug transporters. By injecting rats with endotoxins known to produce systemic inflammation, my supervisor and her colleagues demonstrated, for the first time, that inflammation could decrease Mdr1 expression and activity in isolated hepatocytes (Piquette-Miller et al., 1998). Her research group later found that incubating cultured hepatocytes with the pro-inflammatory cytokines IL-1β (for 3 days) or IL-6 (for 24 hours) also decreased Mdr1 expression and activity (Sukhai et al., 2000; Sukhai et al., 2001). In addition to replicating these Mdr1 findings, subsequent studies in her laboratory and the laboratory of others have demonstrated similar inflammation-mediated changes in hepatic Mrp2, Mrp3, Bsep and several Oatps (Tang et al., 2000; Hartmann et al., 2002; Hidemura et al., 2003; Cherrington et al., 2004; Siewert et al., 2004; Teng and Piquette-Miller, 2005). While similar results have been obtained in the intestine, kidney and CNS (Ando et al., 2001; Goralski et al., 2003; Kalitsky-Szirtes et al., 2004), comparatively little is known about inflammation-mediated changes to drug transporter expression in the placenta or maternal and fetal tissues in pregnancy. One in vitro study observed decreased MDR1 and BCRP expression in human primary term placental trophoblasts following incubation with pro-inflammatory cytokines (Evseenko et al., 2007). From the laboratory of my supervisor, Wang and colleagues observed a significant downregulation of placental Mdr1a/b mRNA in pregnant rats injected with LPS versus saline (Wang et al., 2005). Fetal tissues from LPS-injected rats also accumulated 3.5 times more \[^{99m}Tc\]-sestamibi, a radiopharmaceutical agent and Mdr1 substrate (Wang et al., 2005). This same group found that Mdr1, Mrp1-3, Bcrp and several Oatps were downregulated in placental samples from LPS-injected pregnant rats (Petrovic et al., 2008).

As mentioned in section 1.2.4, many nuclear receptors are suppressed via the activation of cytokine signalling pathways. For example, the expression and/or activity of PPARs, PXR, CAR, LXRs, FXR and RXR is downregulated in livers obtained from rodent models of inflammation as well as in primary human hepatocyte cultures treated with pro-inflammatory cytokines (Beigneux et
Nuclear receptors are the putative link between inflammation and the expression of drug transporters and metabolic enzymes.

My rationale for examining the impact of diabetes on selected drug disposition mechanisms in pregnancy stem primarily from the findings of several studies that suggest diabetes is an inflammatory disease. First, both IL-6 and CRP correlate with blood glucose concentrations in patients with T2DM (Rodriguez-Moran and Guerrero-Romero, 1999; Leinonen et al., 2003; Rodriguez-Moran and Guerrero-Romero, 2003). Second, lowering blood glucose via standard treatment decreases plasma CRP and IL-6 concentrations (Arnalich et al., 2000). Third, in vitro experiments with human peripheral blood monocytes have demonstrated that exposure to high glucose medium stimulates the release of IL-6 and TNF-α (Morohoshi et al., 1995; Morohoshi et al., 1996). Finally, induction of hyperglycemia in healthy volunteers while simultaneously blocking insulin secretion significantly increases plasma IL-6, TNF-α and IL-18 after just five hrs (Esposito et al., 2002). This latter study suggests that hyperglycemia does not have to be chronic to elicit a systemic inflammatory response.

GDM, as with diabetes in the non-pregnant state, is believed to be associated with a systemic inflammatory response. In human GDM, increases in plasma concentrations of the pro-inflammatory cytokines IL-6 and TNF-α have been reported (Kirwan et al., 2002; Winkler et al., 2002; Atègbo et al., 2006; McLachlan et al., 2006). Evidence for the inflammatory marker CRP is less conclusive. As compared to controls, there have been reports of both elevated plasma CRP (Qiu et al., 2004) and unaltered plasma CRP (Retnakaran et al., 2003; McLachlan et al., 2006) in GDM. The diabetes-induced systemic inflammatory response is likely promoted by a corresponding placental inflammatory response. For example, studies have shown that cultured placental explants from GDM pregnancies release significantly more TNF-α in response to increased media glucose concentrations than those from uncomplicated pregnancies (Coughlan et al., 2001). Studies have also shown that placentas from women with GDM not only contain more TNF-α but exhibit significant upregulation in genes associated with pro-inflammatory pathways (Radaelli et al., 2003). Thus, the literature supports the assertion that GDM is a systemic inflammatory disease and this makes developing an understanding of the consequences of this for drug disposition mechanisms an important goal.
1.4 Streptozotocin-induced diabetic pregnancy

At present, drug-disease interactions have not been investigated/identified in GDM so it is not surprising that very little attention has been directed toward the impact of GDM on the specific mechanisms that determine drug disposition. For ethical reasons, drug disposition studies are difficult to conduct in human pregnancy and when they are, they are not typically conducted in a way that allows for the specific contribution of an obstetrical complication to be identified (Reviewed in Anger and Piquette-Miller, 2008). For example, healthy controls are understandably not included. This means that researchers must rely on within-subject comparisons made in the postpartum period, which does not isolate the effects of obstetrical complications from the effects of pregnancy itself. Studies of specific drug disposition mechanisms are even more difficult to conduct in human pregnancy than drug disposition studies. In fact, most of the objectives outlined at the end of this chapter would be more or less impossible to accomplish with human volunteers at this point in time. For example, liver tissue from pregnant women is not readily available, which is a critical hurdle when attempting to study the effects of obstetrical complications on hepatic drug transporters and metabolic enzymes. Thus, a rodent model of GDM was extensively utilized in the research described in this thesis because data generated in rodents can be used to justify more difficult studies in human patients or volunteers.

For the most part, rodent models of diabetes are either cytotoxin-induced or genetically predetermined. Alloxan and streptozotocin (STZ) are examples of cytotoxins that can be used to induce T1DM because they target insulin production in the pancreas. BioBreeding (BB) rats and Non-Obese Diabetic (NOD) mice are examples of animals with genetically predetermined T1DM while Goto-Kakizaki (GK) rats, Kasukabe-K (KK) mice, Lepr\(^{db/db}\) mice and Lepr\(^{fa/fa}\) rats (a.k.a., Zucker rats) are examples of animals with genetically predetermined T2DM. An in-depth description of the experimental models of diabetes that are currently available, including the specific types of human diabetes that they most closely emulate, is beyond the scope of this thesis but can be found elsewhere (Reviewed in McNeill, 1999; Shafrir and Desoye, 2003).

Unfortunately, there is no “gold standard” rodent model of human GDM. Most rodent models of GDM simply involve adapting an established model of human T1DM or T2DM to the pregnant state. Given the fertility issues that plague many of the genetically predetermined models, such as complete infertility in homozygous KK mice, Lepr\(^{db/db}\) mice and Lepr\(^{fa/fa}\) rats, most studies of diabetic pregnancy in rodents have utilized a cytotoxin. For my studies, I ultimately decided to use...
STZ to model GDM in rats. Rats were chosen over mice because their larger size is more conducive to drug disposition and fetal pharmacokinetic studies.

To date, the vast majority of studies that examine drug disposition in animals with diabetes have employed either alloxan or STZ. Alloxan is a cyclic urea analog that exhibits β-cell toxicity and was the first diabetogenic agent used to produce T1DM in laboratory animals (Shaw-Dunn et al., 1943). STZ [N-(Methylnitrosocarbamoyl)-α-D-glucosamine] is a broad-spectrum antibiotic that was discovered by Upjohn Laboratories in Streptomyces achromogenes var. streptozoticus (Vavra et al., 1959). STZ’s diabetogenic properties were first described by Upjohn but were ascribed to β-cell toxicity in dogs and rats by Rakieten and colleagues (Rakieten et al., 1963). While both alloxan and STZ induce diabetes by targeting pancreatic β-cells, alloxan was quickly replaced by STZ in research because of STZ’s greater selectivity (Junod et al., 1969), lower mortality (Hoftiezer and Carpenter, 1973) and longer half-life within the body (Schein et al., 1973). STZ injection is by far the most widely utilized method of inducing diabetes in laboratory animals.

The chemical structure of STZ consists of a glucose molecule and a highly reactive nitrosourea side chain (Fig. 1.2). STZ’s glucose moiety has been found to direct STZ to glucose transporter 2 (GLUT2) receptors, which are highly expressed in the cell membranes of β-cells (Schnedl et al., 1994). The nitrosourea side chain is responsible for the intracellular effects that ultimately lead to β-cell death but the exact nature of these effects are a subject of debate (Reviewed in Rodrigues et al., 1999). The basic, most supported, theory is that, once inside the cell, the nitrosourea side chain decomposes to form carbonium ions. Carbonium ions induce DNA breaks via the alkylation of DNA bases, which triggers cell repair mechanisms (Uchigata et al., 1982). In particular, nuclear poly(ADP-ribose) synthetase is activated and this leads to the depletion of cellular pyridine nucleotides like nicotinamide adenine dinucleotide (NAD+). The absence of NAD+ within β-cells halts NAD+-dependent energy metabolism and posttranslational protein modifications (Uchigata et al., 1982). This causes cell death in a short period of time. The fact that coadministering nicotinamide and STZ reduces the latter’s diabetogenic effects supports this theory (Wilson et al., 1984). Evidence suggests that free radicals and nitric oxide are also involved (Gandy et al., 1982; Kwon et al., 1994).

STZ must be administered parenterally: intravenously, intraperitoneally or subcutaneously. Although the validity of the practice has been called into question (Axler, 1982), most researchers prepare STZ in citrate buffer adjusted to pH 4.5 because of purported stability issues. STZ is also typically injected shortly after preparation. Reported dosages for rodents vary greatly in the literature.
but an early, seminal study of the effects of intravenous STZ administration in rats established that there are no effects below 25 mg/kg and profound hyperglycemia at or above 100 mg/kg (Junod et al., 1969). Stable hyperglycemia develops within 24 to 48 hrs of administration (Junod et al., 1969).

Two approaches can be adopted when using STZ to model GDM in rats. In the first approach, which is the approach I adopted, pregnant dams are injected with STZ, after the early organogenesis phase of gestation, and GDM is established once they become hyperglycemic. STZ-induced GDM usually occurs within two days of injection and persists through to parturition. STZ-induced GDM is the most common approach to using STZ to model GDM. The second approach involves using the offspring of dams with STZ-induced GDM. The offspring of dams with STZ-induced GDM are exposed to aberrant metabolic programming in utero as a result of STZ-induced intrauterine growth restriction (IUGR). STZ-induced IUGR modifies the intrauterine environment of rodent species in such a way as to provoke fetal adaptations that can lead to the development of T2DM in adulthood or GDM in pregnancy (Boloker et al., 2002). The nature of fetal adaptations to this environment often depend on the severity of maternal hyperglycemia but fetal insulin overproduction and insensitivity are common features (Oh et al., 1988). One criticism of using STZ-induced GDM over STZ-induced IUGR revolves around the type of human diabetes they most closely emulate. As described, GDM is a problem of insulin insensitivity (similar to T2DM) and is not necessarily associated with decreased insulin production (T1DM). As such, relative to STZ-induced GDM, the pathophysiology observed in rats that experienced in utero STZ-induced IUGR more closely resembles the clinical presentation of GDM. However, STZ-induced GDM has the significant advantages of being more successful in achieving overt GDM, less variable in severity, more stable across gestation, cheaper and much more extensively characterized (Herrera et al., 1985; Oh et al., 1988; Aerts et al., 1997; Plagemann et al., 1998; Plagemann et al., 1999; Merzouk et al., 2000; Menezes et al., 2001; Merzouk et al., 2001). Moreover, STZ-induced IUGR’s real benefit comes from its ability to model the effects of GDM on the physiology of offspring rather than the physiology of mother and fetus across gestation.

To the best of my knowledge, other than the studies described later in this thesis, there is but one published account of an experimental rodent model of diabetic pregnancy being employed for the purpose of examining drug disposition. Mulay and Varma conducted a study where maternal and fetal exposure to a maternally administered dose of [³H]-dexamethasone was determined in pregnant rats rendered diabetic on the first day of gestation via STZ injection (Mulay and Varma, 1984). By inducing diabetes so close to conception, it would be difficult to argue that this is not a model of pregnancy complicated by pre-existing T1DM. On gestational day 20 (GD20), these researchers
found that both maternal and fetal exposure to dexamethasone was lower in the diabetic group than in the pregnant vehicle control group (3 hrs post-administration). The ratio of drug concentration in fetal serum and tissues to maternal serum were equivalent in the diabetic and control groups (e.g., both groups had fetal:maternal serum drug concentration ratios of 0.2), suggesting fetal exposure was a reflection of maternal exposure. Diabetes did not significantly influence the plasma protein binding of [\textsuperscript{3}H]-dexamethasone in this study. Interestingly, these researchers obtained evidence that fetuses in the GDM group excreted approximately twice as much [\textsuperscript{3}H]-dexamethasone into amniotic fluid than fetuses in the vehicle control group. This suggests that a greater amount of [\textsuperscript{3}H]-dexamethasone was eliminated by fetuses in the GDM group because maternally administered drugs reach the amniotic fluid by way of materno-fetal transfer and then fetal urination (Seeds, 1981).

Dexamethasone is renally excreted. Greater fetal elimination without a corresponding impact on the fetal:maternal serum concentration indicates that there was greater placental transfer of [\textsuperscript{3}H]-dexamethasone, at some point in time, that was compensated for by fetal renal elimination. Mdr1 is believed to limit the passage of dexamethasone across the placenta (Parry and Zhang, 2007), which could possibly have been affected in the GDM group but was not assessed (in 1984, Mdr1 was not widely studied in non-tumorous tissues). The expression of hepatic drug transporters or metabolic enzymes was also not assessed.
Figure 1.2. Streptozotocin. A, The chemical structure of streptozotocin, which consists of a glucose molecule and a highly reactive nitrosourea side chain. B, D-glucose is presented for comparison.
1.5 General hypotheses

Upon initiation of my doctoral research, my primary general hypothesis was that STZ-induced GDM would cause changes in drug disposition mechanisms, particularly in plasma/serum protein binding and drug efflux transporter/metabolic enzyme expression in the liver and placenta, that would ultimately contribute to altered maternal and fetal drug exposure. My secondary general hypothesis was that observed changes in drug efflux transporter expression would be linked to pro-inflammatory cytokines.
1.6 Research objectives

1.6.1 Objective 1: characterize hepatic drug efflux transporter expression in non-pregnant, female rats with streptozotocin-induced T1DM

My first research objective was to determine whether experimental T1DM in non-pregnant rats resulted in the downregulation of drug efflux transporters, as has been reported in animal models of inflammation, and thereby provide preliminary support for future studies involving pregnant rats. To do this, mRNA levels of key drug efflux transporters were examined in non-pregnant, female rats rendered diabetic via subcutaneous STZ injection (total duration of diabetes: ~7 days). Because hepatic drug efflux transporters play an important role in the maternal disposition of drugs and has been a primary focus of previous literature examining inflammation-mediated changes, this study was restricted to the liver (although a few renal transporters were examined as well). Plasma IL-6, TNF-α and CRP concentrations were assayed and statistically examined to determine if they correlated with transporter expression. This study is described in Chapter 2.

1.6.2 Objective 2: characterize plasma protein binding capabilities and hepatic drug efflux transporter and metabolic enzyme expression in a rat model of GDM

My second research objective involved establishing an animal model of GDM and then determining if plasma protein binding capabilities and hepatic drug efflux transporter and metabolic enzyme expression were altered in it. As described in section 1.4, the rat model of GDM that I eventually employed was STZ-induced GDM. Pregnant rats were rendered diabetic via subcutaneous STZ injection on GD6 (total duration of diabetes: ~13-14 days). An insulin-treated control group, absent in my study of STZ-induced T1DM, was included. To determine plasma protein binding capabilities, ex vivo assays were performed with plasma samples. To determine hepatic drug efflux transporter and metabolic enzyme expression, a proteomics approach was adopted. Proteomic data was supplemented with Mdr1 quantitation via qRT-PCR and western blotting. Plasma CRP concentrations were assayed to determine if inflammation was involved. This study is described in Chapter 3.

1.6.3 Objective 3: determine if maternal and fetal exposure to lopinavir is altered in a rat model of GDM

My third research objective was to determine if maternal and fetal exposure to a maternally administered drug was altered in STZ-induced GDM. Lopinavir (LPV) is the preferred HIV protease inhibitor in HIV-positive pregnant women but it is unknown if GDM affects its
disposition, even though GDM occurs more frequently in HIV-positive women. LPV is a suitable substrate with which to assess the effects of altered drug disposition mechanisms because it is highly protein bound, a substrate for drug efflux transporters and extensively metabolized by the liver. LPV concentrations were measured in maternal and fetal samples at two time points (45 and 60 min) and a fetal time course was generated using a novel serial sampling protocol to provide fetal pharmacokinetic data (e.g., \( \text{AUC}_{0-60} \)). An insulin-treated control group was again included. Protein binding was measured by ultrafiltration. The expression of relevant transporters, like Mdr1, and Cyp3a2, which metabolizes LPV in rodents, was assessed in maternal liver via qRT-PCR and western blotting. The expression of relevant transporters was also assessed in placenta via qRT-PCR alone. This study is described in Chapter 4.

1.6.4 Objective 4: characterize placental drug efflux transporter expression in human pregnancies complicated by diabetes

My final research objective was to address the issue of clinical relevance by determining whether protein levels of key placental drug transporters were altered in human pregnancies that were complicated by diabetes. To do this, placental samples from pregnancies complicated by T1DM or MNT-resistant GDM, as well as control samples from healthy pregnancies, were obtained from the Research Centre for Women’s and Infants’ Health (RCWIH) BioBank program of Mount Sinai Hospital. MDR1, MRP2 and BCRP expression was then measured via western blotting. All patients with samples included in this study received insulin therapy to manage their hyperglycemia, which, as described in section 1.1.3, is the standard of care for both T1DM and MNT-resistant GDM. On the basis of available clinical data, I describe both diabetic groups as being properly managed with respect to their diabetes. Recruitment opened in April 2010 and applied retroactively to the entire BioBank sample catalogue. Recruitment closed in January 2011. A total of 35 placental samples were analyzed. This study is described in Chapter 5.
Chapter 2. Impact of Acute Streptozotocin-Induced Diabetes on ABC Transporter Expression in Rats

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2.1 Abstract

Hepatic ABC efflux transporters control the cellular uptake (in basolateral membranes) and excretion (in apical membranes) of many substrates. Since type 1 diabetes mellitus (T1DM) is associated with altered hepatobiliary excretion of many endogenous and exogenous substances, we examined key hepatic ABC transporters and levels of the endogenous substrate glutathione in rats with acute streptozotocin-induced T1DM. Renal transporters and inflammatory markers were also examined. Mdr1, Mrp1-4 and Bcrp were measured using qRT-PCR. Glutathione was measured in liver tissue, plasma and urine. Inflammatory markers, including C-reactive protein (CRP), were measured in plasma via ELISA. In diabetic rats, Mdr1a, Mrp2 and Bcrp (apical) were decreased while Mrp4 (basolateral) was increased. Mdr1a and Mrp2 inversely correlated with plasma CRP. Diabetic and control rats exhibited similar hepatic glutathione concentrations but levels in diabetic plasma were lower. When normalized to urinary output, diabetic rats excreted 6.7-fold more glutathione in urine than controls. Renal transporter levels were normal in diabetic rats. Results show apical transporters involved in hepatobiliary excretion are downregulated in T1DM, possibly through an inflammation-mediated process. Findings suggest there may be a vectorial shift from hepatic to renal excretion for some substrates in T1DM.
2.2 Introduction

Several ATP-binding cassette (ABC) efflux transporters are highly expressed in the liver where they counteract hepatic uptake (in basolateral/sinusoidal membranes) and make possible the biliary excretion (in apical/canalicular membranes) of various endogenous and exogenous substances. Key hepatic ABC transporters include Mdr1 (P-glycoprotein/P-gp, encoded in rodents by the Mdr1a and Mdr1b genes), Mrp1-4 (multidrug resistance-associated proteins 1-4/Mrp1-4) and Bcrp (breast cancer resistance protein). Mdr1, Mrp2 and Bcrp are located in apical membranes while Mrp1, Mrp3 and Mrp4 are located in basolateral membranes. Expression of Mrp1, Mrp3 and Mrp4 is low under normal physiological conditions but studies have consistently shown a compensatory upregulation in the liver, as well as kidney, when apical hepatobiliary excretion is compromised (Soroka et al., 2001; Tanaka et al., 2002; Kuroda et al., 2004; Chen et al., 2005; Chu et al., 2006; Vlaming et al., 2006). For example, Mrp2 gene knockout mice have been shown to exhibit elevated Mrp3 and Mrp4 expression (Chen et al., 2005; Chu et al., 2006; Vlaming et al., 2006). For some substrates, compensation of this nature is believed to reduce intrahepatic accumulation and lead to increased renal elimination (Tanaka et al., 2002; Chu et al., 2006; Villanueva et al., 2008). A growing body of literature indicates that the expression of ABC transporters is altered in pathological states associated with inflammation (Piquette-Miller et al., 1998; Tang et al., 2000; Hartmann et al., 2001; Hartmann et al., 2002; Cherrington et al., 2004; Siewert et al., 2004; Hartmann et al., 2005; Teng and Piquette-Miller, 2005; Wang et al., 2005).

It has been previously demonstrated that inflammation induced by endotoxin and turpentine injection decreases the expression and activity of Mdr1 in rodent liver (Piquette-Miller et al., 1998; Hartmann et al., 2001; Achira et al., 2002; Hartmann et al., 2005). Decreased expression and activity of Mdr1 has also been reported in vitro in interleukin-1β (IL-1β) and interleukin-6 (IL-6) treated primary rat hepatocyte cultures (Sukhai et al., 2000; Sukhai et al., 2001). Numerous in vivo and in vitro studies have not only confirmed inflammation-mediated changes to the expression and function of Mdr1 but extended these findings to members of the Mrp subfamily, such as Mrp1-3 (Tang et al., 2000; Hartmann et al., 2002; Hidemura et al., 2003; Cherrington et al., 2004; Siewert et al., 2004; Hartmann et al., 2005; Teng and Piquette-Miller, 2005; Wang et al., 2005). For instance, a pronounced reduction in the hepatic expression of Mrp2 and decreased hepatobiliary excretion of doxorubicin has been reported in Shiga-like toxin II treated rats (Hidemura et al., 2003). In many of these studies, pro-inflammatory cytokines, particularly IL-6, have been implicated as principle mediators of inflammation-induced changes in transporter expression.
Diabetes mellitus is a condition that has been linked to altered levels of hepatic bile constituents such as bile salts, phospholipids and cholesterol in both humans and experimental animals (Bennion and Grundy, 1977; Akiyoshi et al., 1986; Andersen et al., 1987; Villanueva et al., 1990; Stone et al., 1997). It is possible that inflammatory-mediated changes in hepatic ABC transporters play a role in these alterations. Diabetes has been linked to increased levels of systemic inflammatory mediators such as pro-inflammatory cytokines and APR proteins (Reviewed in Pickup and Crook, 1998). Both IL-6 and CRP correlate with blood glucose (Rodriguez-Moran and Guerrero-Romero, 1999; Leinonen et al., 2003). Lowering blood glucose via standard treatment regimens results in decreased levels of these proteins (Arnalich et al., 2000). Experiments with human peripheral blood monocytes have demonstrated that high glucose stimulates the in vitro release of IL-6 and tumor necrosis factor-α (TNF-α) (Morohoshi et al., 1995; Morohoshi et al., 1996). Moreover, acute induction of hyperglycemia in healthy volunteers has been shown to increase levels of IL-6, TNF-α and interleukin-18 (IL-18) (Esposito et al., 2002). Since the production of pro-inflammatory cytokines is governed largely by the transcription factor nuclear factor-kappa B (NF-κB), it is not surprising that studies have documented the activation of this protein in both type 1 (Ho and Bray, 1999; Eldor et al., 2006) and type 2 (Yuan et al., 2001; Lehrke et al., 2004; Chen, 2005) diabetes mellitus.

To date, little is known about the effects of acute T1DM on the expression of several hepatic ABC transporters that have roles not just in the excretion of endogenous bile constituents but also in the excretion of a multitude of clinically important drugs. To this end, we examined the expression of Mdr1 (encoded in rodents by Mdr1a and Mdr1b), Mrp1-4 and Bcrp in streptozotocin (STZ)-induced T1DM. We also examined the functional consequences of T1DM on the disposition of total glutathione (TGSH) and glutathione disulfide (GSSG) and investigated the potential role of inflammatory mediators in changes observed in hepatic transporters. TGSH (and its components: reduced glutathione, or GSH, and GSSG) is transported primarily by Mrp2 at the apical membrane (Elferink et al., 1989) and by Mrp4 at the basolateral membrane (Lai and Tan, 2002). While several studies have examined the effects of acute (~7 days) STZ-induced T1DM on the pharmacokinetics of various drugs (Kim et al., 2005; Bae et al., 2006; Kim et al., 2006; Choi et al., 2008), few have determined whether altered expression of hepatic ABC transporters could be a contributing factor. Our findings could have important implications for the disposition of drugs, specifically those eliminated via hepatobiliary excretion, in both experimentally induced and clinical T1DM.
2.3 Methods

2.3.1 Animals

Female Sprague-Dawley rats (220-280 g) were purchased from Charles River Laboratories, Inc. (Senneville, QC) and housed in a temperature controlled facility on a 12:12 hr light-dark cycle. Rats were given free access to water and standard chow (Harlan Teklad Global Diet 2018). Experiments were approved by the Office of Research Ethics at the University of Toronto and performed in accordance with Canadian Council on Animal Care guidelines.

2.3.2 Animal treatments/monitoring and tissue collection

T1DM was induced by way of a single subcutaneous injection of streptozotocin (STZ; Sigma-Aldrich, Oakville, ON) in 0.1 M citrate buffer (pH 4.5) at a dosage of 45 mg/kg. Vehicle control animals received a single subcutaneous injection of citrate buffer. Blood glucose and body weight were measured prior to respective injections (day 0) and on days 1, 2, 3, 6 and 9. Blood glucose was measured in all animals at noon, in a non-fasted state, using a commercially available glucometer (Freestyle Freedom Meter, Abbott Laboratories, Alameda, CA) with blood obtained via tail-prick. Blood glucose values between 10-14 mmol/L were considered mild hyperglycemia while values greater than 14 mmol/L were considered T1DM. Following measurement of blood glucose and body weight on day 9, a subgroup of control and diabetic animals (G1; n = 7/treatment group) were sacrificed for the collection of blood (cardiac puncture), liver and kidney. Blood was immediately spun to isolate plasma. Plasma aliquots were immediately snap-frozen and stored at -80 °C until use. Samples from this first group were used to examine hepatic and renal transporter expression, hepatic and plasma glutathione (GSH) levels and plasma IL-6, TNF-α and CRP levels. A second group of animals (G2; n = 5/treatment group) was used for conducting general urinalysis and to determine GSH concentrations in urine as well as plasma GGT and ALT concentrations. G2 animals were housed in metabolic cages for 24 hrs, starting on day 8, to collect urine and feces. The first 1-2 ml of urine produced on day 8 was divided into two aliquotes and either placed on ice and transported to Vita-Tech Laboratories (Markham, ON) for urinalysis or used to determine GSH concentrations. Following measurement of blood glucose and body weight on day 9, G2 animals were sacrificed for blood collection (cardiac puncture). G2 whole blood was immediately placed on ice and transported to Vita-Tech Laboratories for analysis. There were no significant differences between blood glucose and body weight values obtained in G1 and G2 rats (data not shown).
2.3.3 IL-6, TNF-α and CRP ELISAs

To determine the concentrations of IL-6, TNF-α and CRP in plasma samples, commercial ELISA kits were utilized from R&D Systems, Inc. (Minneapolis, MN), Abcam, Inc. (Cambridge, MA) and BD Biosciences (Mississauga, ON), respectively. ELISA kits were used according to their manufacturer’s instructions. In our hands, the detection limits for these ELISA kits with plasma samples were 31.25 pg/ml for IL-6, 12.5 pg/ml for TNF-α and 4.2 ng/ml for CRP.

2.3.4 ALT and GGT assays

Alanine aminotransferase (ALT) and gamma-glutamyl transpeptidase (GGT) were measured in plasma by Vita-Tech Laboratories using an autoanalyzer and commercially available reagents (Roche Diagnostics, Laval, QC).

2.3.5 Urinalysis

Urinalysis was conducted with the first 1-2 ml of urine produced by G2 animals on day 8 while housed in metabolic cages. The first 1-2 ml of urine was used to minimize degradation as this volume could be collected from all animals within 2-3 hrs. Urinalysis was performed by Vita-Tech Laboratories using dipstick-based kits (Chemstrips, Roche Diagnostics) and included the following tests: specific gravity, pH, glucose, ketones, protein, bilirubin and urobilinogen. Specific gravity and pH results are quantititative. All other results are presented as negative (below detection limits), trace or positive.

2.3.6 Accumulation experiments

Rat hepatocytes were isolated in the laboratory of Dr. Peter J. O’Brien (University of Toronto) by way of the collagenase perfusion method as previously described (Moldeus et al., 1978). For accumulation experiments, isolated hepatocytes were first washed in a series of low-speed centrifugations (10-60 g) and resuspensions in Liebovitz’s L15 medium (Sigma-Aldrich) containing 2% BSA and 10% FBS (pH 7.6). Hepatocyte number and viability in the final suspension was assessed using the trypan blue exclusion method and a hemocytometer. Cells were then resuspended in seeding/incubation medium and plated in 24-well plates at a density of approximately 20,000 cells per cm² and allowed to adhere overnight. Seeding/incubation medium consisted of Williams’ E medium (Sigma-Aldrich) modified to contain 10% FBS, 2 mM glutamine, 100 IU/ml penicillin and 10 mg/ml streptomycin. Once adhered, cells were washed with PBS and medium was replaced with seeding/incubation medium containing various concentrations of recombinant rat IL-6 (0-200 pg/ml; R&D Systems). After 24 hrs of IL-6 treatment, cells were washed three times with PBS and
medium was replaced with seeding/incubation medium containing 3 µM saquinavir (Roche Laboratories, Nutley, NJ) spiked with [3H]-saquinavir (American Radiolabeled Chemicals, Inc., St. Louis, MO). Following a 30 min incubation in this medium, cells were washed three times with PBS, lysed with NaOH and subjected to liquid scintillation counting in a Beckman Coulter LS 6500 (Beckman Coulter Canada, Inc., Mississauga, ON). Saquinavir accumulation was extrapolated from a standard generated with the same saquinavir-containing medium used for accumulation. Saquinavir accumulation was normalized to the protein content of lysates from each well using the Bradford assay method of protein determination and a BSA standard (Bradford, 1976). MTT assays were employed to examine the potential toxicities of both saquinavir and IL-6 exposure. For MTT assays, rat hepatocytes were prepared and treated with IL-6 as they were for accumulation experiments but in 96-well plates. Following IL-6 treatment, cells were incubated for three hrs with MTT (Sigma-Aldrich) in seeding/incubation medium. Cells were solubilized and the extent of MTT reduction to formazan was quantified by spectrophotometry (570 nm).

2.3.7 PCR

Mdr1a/b, Mrp1-4 and Bcrp mRNA levels were examined in liver and kidney. Total RNA was extracted from tissue using the TRIZOL method (Invitrogen, Carlsbad, CA) and then reverse transcribed to cDNA via the First Strand cDNA Synthesis Kit (Fermentas, Burlington, ON), according to manufacturer’s instructions. The mRNA levels of ABC transporter genes were each determined by quantitative reverse transcriptase-polymerase chain reaction (qRT-PCR) using LightCycler technology with SYBR Green I fluorescence detection (Roche Diagnostics). PCR oligonucleotides were synthesized at The Hospital for Sick Children (DNA Synthesis Centre, Toronto, ON) and their sequences have been previously published (Denk et al., 2004; Petrovic et al., 2008). All mRNA levels were normalized to 18S rRNA and expressed as a gene/18S ratio.

2.3.8 Glutathione assays

TGSH and GSSG were measured in liver, plasma and urine using a commercial glutathione assay kit according to the manufacturer’s instructions (Sigma-Aldrich). These assays were based on the glutathione recycling method of Tietze (Tietze, 1969), as modified by Griffith (Griffith, 1980). Values in liver are presented as concentrations per amount of tissue examined (µmol/g) while values in plasma are presented as concentrations (µM). TGSH concentrations in urine were normalized to urinary output (ml/kg/day) and are presented as µg/kg/day.
2.3.9 Statistics

Data was analyzed using Prism 4 for Macintosh (GraphPad Software, Inc., San Diego, CA). For comparisons of blood glucose and body weight, two-way ANOVAs followed by Bonferroni post-tests were performed. These same tests were employed to examine any potential differences between the G1 and G2 cohorts with respect to blood glucose and body weight. For comparisons of hepatic and renal ABC transporter mRNA expression, t-tests were performed. T-tests were also performed for CRP, ALT and GGT levels as well as water intake, urinary output, feed intake and fecal output values. Non-parametric Spearman correlation was used to assess associations between CRP concentrations and hepatic ABC transporter mRNA expression. Levels of significance for statistical analyses were set at or below $\alpha = 0.05$, indicated as follows: *, $p < 0.05$, **, $p < 0.01$ and ***, $p < 0.001$. Results are presented as mean ± SEM.
2.4 Results and discussion

2.4.1 Characteristics of streptozotocin-injected rats consistent with unmanaged T1DM

Induction of T1DM in STZ-injected rats was determined on the basis of blood glucose and body weight. The time course of STZ-induced hyperglycemia and weight loss was constructed from data obtained pre-injection (baseline) and on days 1, 2, 3, 6 and 9 and compared to vehicle controls (Appendix fig. 2.1). Ten of the 12 STZ-injected rats developed mild hyperglycemia (10-14 mmol/L) within 24 hrs and then T1DM (> 14 mmol/L) within two days. Two of the 12 STZ-injected rats were excluded from all analyses because they did not develop mild hyperglycemia until two days post-injection and T1DM until day 3. All animals considered diabetic in this study were, therefore, diabetic for at least 7 days. Analysis of mean blood glucose values for days 3, 6 and 9 revealed elevated blood glucose in STZ-injected rats as compared to vehicle control rats (Table 2.1). Differences in blood glucose concentration were statistically significant at every time point except for baseline (p < 0.001). Analysis of body weight indicated that while control rats gained weight across the study duration in an age-appropriate manner, STZ-injected rats exhibited a modest decrease in body weight (Table 2.1).

In addition to blood glucose and body weight alterations, STZ-injected rats exhibited other physiological and biochemical characteristics consistent with unmanaged T1DM. STZ-injected rats exhibited significant polydipsia and polyuria on day 8. While in metabolic cages, STZ-injected rats consumed six times as much water and produced nearly ten times as much urine relative to controls (Table 2.1). Moreover, urinalysis revealed the presence of glucose exclusively in the urine of STZ-injected rats (Table 2.1). Rats also exhibited significant hyperphagia with an associated increase in fecal output (Table 2.1). Biomarkers associated with diabetes, including alanine aminotransferase (ALT), gamma-glutamyl transpeptidase (GGT) and the inflammatory cytokines/markers IL-6, TNF-α and CRP, were also examined in plasma samples from STZ-injected and vehicle control rats (Table 2.1). Results indicated that plasma ALT concentrations were significantly elevated (p < 0.01) in diabetic rats while plasma GGT concentrations were below detection limits (Table 2.1). Results indicated that plasma ALT concentrations were significantly elevated (p < 0.01) in diabetic rats while plasma GGT concentrations were below detection limits (Table 2.1).

IL-6 and TNF-α results were inconclusive due to detection limitations. In control animals, both IL-6 and TNF-α were below detection limits. In diabetic animals, IL-6 levels were detectable in only three animals while TNF-α levels were below detection limits in all animals. CRP was detectable in all animals and was found to be significantly higher (p < 0.01) in diabetic animals relative to controls (Table 2.1). The highest three CRP values corresponded with the three diabetic
animals for which IL-6 levels were detectable: values being 62.6, 112.8 and 125 pg/ml. Experiments were conducted with primary rat hepatocyte cultures to determine if IL-6 values in this range could sufficiently cause alterations in the accumulation of the ABC transporter (Mdr1 and Mrp2) substrate saquinavir. IL-6 was found to be non-toxic, via MTT assay, at a range of 1.5-200 pg/ml and saquinavir accumulation was found to be temperature dependent and saturable as well as linear at the 30 min time point selected for experiments. Exposure to medium containing IL-6 at a concentration of 100 and 200 pg/ml produced a statistically significant increase in the intracellular accumulation of [3H]-saquinavir and a trend toward increased accumulation was observed with 50 pg/ml (Fig. 2.1).

Table 2.1. The effect of STZ-injection on various physiological and biochemical characteristics.

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Vehicle</th>
<th>STZ</th>
<th>Fold-difference</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blood chemistry</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Blood glucose (mM)</td>
<td>7.1 ± 0.3</td>
<td>22.2 ± 0.4</td>
<td>3.1</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>CRP (pg/ml)</td>
<td>267.2 ± 16.7</td>
<td>373.6 ± 25.7</td>
<td>1.4</td>
<td>&lt; 0.01</td>
</tr>
<tr>
<td>ALT (IU/L)</td>
<td>35 ± 1.8</td>
<td>99.6 ± 16.4</td>
<td>2.8</td>
<td>&lt; 0.01</td>
</tr>
<tr>
<td>GGT (IU/L)</td>
<td>&lt; 3 IU/L</td>
<td>&lt; 3 IU/L</td>
<td>n.a.</td>
<td>n.a.</td>
</tr>
<tr>
<td>Metabolic cage measurements</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Water intake (ml/kg/day)</td>
<td>85.9 ± 17.1</td>
<td>520.8 ± 102.9</td>
<td>6.1</td>
<td>&lt; 0.01</td>
</tr>
<tr>
<td>Urinary output (ml/kg/day)</td>
<td>47.1 ± 10.1</td>
<td>458.5 ± 100.6</td>
<td>9.7</td>
<td>&lt; 0.01</td>
</tr>
<tr>
<td>Feed intake (g/kg/day)</td>
<td>37.6 ± 7.4</td>
<td>118.3 ± 16.5</td>
<td>3.1</td>
<td>&lt; 0.01</td>
</tr>
<tr>
<td>Fecal output (g/kg/day)</td>
<td>9.8 ± 2.4</td>
<td>38.4 ± 6.9</td>
<td>3.9</td>
<td>&lt; 0.01</td>
</tr>
<tr>
<td>Urinalysis</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Specific gravity</td>
<td>1.014 ± 0.002</td>
<td>1.044 ± 0.003</td>
<td>1.03</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>pH</td>
<td>8.75 ± 0.25</td>
<td>7.0 ± 0.32</td>
<td>0.8</td>
<td>&lt; 0.01</td>
</tr>
<tr>
<td>Glucose</td>
<td>Negative</td>
<td>Positive</td>
<td>n.a.</td>
<td>n.a.</td>
</tr>
<tr>
<td>Ketones</td>
<td>Negative</td>
<td>Negative</td>
<td>n.a.</td>
<td>n.a.</td>
</tr>
<tr>
<td>Protein</td>
<td>Negative</td>
<td>Negative</td>
<td>n.a.</td>
<td>n.a.</td>
</tr>
<tr>
<td>Bilirubin</td>
<td>Negative</td>
<td>Negative</td>
<td>n.a.</td>
<td>n.a.</td>
</tr>
<tr>
<td>Urobilinogen</td>
<td>Normal</td>
<td>Normal</td>
<td>n.a.</td>
<td>n.a.</td>
</tr>
<tr>
<td>Other</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Body weight (% baseline)</td>
<td>103 ± 1</td>
<td>94 ± 1</td>
<td>0.9</td>
<td>&lt; 0.001</td>
</tr>
</tbody>
</table>
Figure 2.1. Accumulation of an Mdr1 and Mrp2 substrate in IL-6-treated rat hepatocytes. Rat hepatocytes were pretreated with various concentrations of recombinant rat IL-6 for 24 hrs and then exposed to culture medium containing 3 µM radiolabeled saquinavir for 30 min. Rat hepatocytes pretreated with IL-6 at concentrations greater than or equal to 100 pg/ml accumulated significantly more saquinavir. Accumulation was normalized to protein content. Values are the result of quadruplicate experiments and are reported as mean ± SEM. *, p < 0.05; ***, p < 0.001.
2.4.2 Alteration of hepatic ABC transporter expression in T1DM

To determine the effects of T1DM on hepatic ABC transporter mRNA, qRT-PCR was performed with primers targeting Mdr1a/b, Mrp1-4 and Bcrp. Relative to controls, Mdr1a, Mrp2 and Bcrp mRNA levels were significantly downregulated in the livers of diabetic rats while Mrp4 mRNA was significantly upregulated (Fig. 2.2). In diabetic rats, Mdr1a was 40 ± 10.9% of control values, Mrp2 was 6.8 ± 2.3% (p < 0.01) and Bcrp was 37.5 ± 12.6% (p < 0.05) while Mrp4 was 358.5 ± 116.7% (p < 0.01). There was also a trend toward increased Mrp3 expression. There was no significant alteration in Mdr1b or Mrp1 expression.

Our findings demonstrate that STZ-induced T1DM alters the expression of key hepatic ABC transporters after approximately seven days of T1DM. Interestingly, downregulated transporters (Mdr1a, Mrp2 and Bcrp) were localized within apical membranes of hepatocytes where they are known to facilitate the excretion of substrates into bile. In contrast, upregulated transporters (Mrp3 and Mrp4) were localized within basolateral membranes of hepatocytes where they are known to efflux substrates out of the hepatocyte and into systemic circulation.

Mdr1, which is encoded by the Mdr1a and Mdr1b genes in rodents, is responsible for the hepatobiliary excretion of an incredibly diverse array of drug substrates including vinca alkaloid and anthracycline anticancer drugs (Booth et al., 1998; Smit et al., 1998b; Song et al., 1999), immunosuppressive agents (Saeki et al., 1993), macrolide antibiotics (Schuetz et al., 1998; Wang et al., 2000) and most HIV protease inhibitors (Kim et al., 1998; Su et al., 1998). In the present study, hepatic Mdr1a was significantly downregulated in diabetic animals whereas hepatic Mdr1b was unaltered. In contrast, Brady et al (2002) and Kemeyama et al (2008) observed a significant increase in Mdr1b mRNA. The discrepancy between these studies and the present study could be due to differences in the dosage and route of administration employed, the time points at which transporters were examined and even quantification methodology (e.g., qRT-PCR vs. conventional RT-PCR). The expression of Mdr1a and Mdr1b during STZ-induced T1DM has also been examined in the brain; however, results in this organ also conflict. Relative to controls, Liu and colleagues (2006) found decreased levels of Mdr1 protein in cortical samples from rat brains isolated five weeks after STZ administration (Liu et al., 2006). Additionally, these rats exhibited significantly higher brain-to-plasma concentration ratios (Kp values) for the Mdr1 substrate Rhodamine 123 and vincristine after i.v. administration (Liu et al., 2006). In contrast, Maeng et al (2007) found induction of both Mdr1a and Mdr1b mRNA in the blood-brain barrier and a corresponding decrease in the Kp value for intravenously administered cyclosporine A in diabetic versus control rats after four
weeks (Maeng et al., 2007b). From this discussion it should become clear that interexperimental variability in STZ dosage as well as the time point(s) at which transporters are assessed limit direct comparisons to some extent. That being said, our findings are entirely consistent with much of the literature concerning inflammation-mediated downregulation of hepatic Mdr1a (Sukhai et al., 2000; Hartmann et al., 2005; Wang et al., 2005).

Mrp1-4 are responsible for the transport of conjugated and unconjugated endogenous substrates such as bilirubin (Jedlitschky et al., 1996; Hirohashi et al., 2000; Tanaka et al., 2002), GSH and GSSG (Jedlitschky et al., 1996; Keppler et al., 1996; Leier et al., 1996; Rius et al., 2003). Exogenous substrates for Mrp1-4 include several HIV protease inhibitors (Huisman et al., 2002), nonconjugated organic anion drugs like methotrexate (Chen et al., 2003) and many others. With respect to the Mrp subfamily of transporters, Mrp2 expression was found to be downregulated (approximately 7% of control values) while a trend toward increased expression in Mrp3 and significantly increased expression of Mrp4. Consistent with our findings, an 80% reduction in hepatic Mrp2 protein and decreased elimination of GSH conjugates in STZ-treated rats has been previously reported (Lu et al., 1997; van Waarde et al., 2002). The impact of STZ-induced T1DM on the expression of Mrp1, Mrp3 and Mrp4 has not been previously reported.

The present study also examined hepatic Bcrp expression. Compared to controls, Bcrp mRNA expression was significantly decreased in diabetic rats. Relative to Mdr1 and Mrp1-4, much less is known about the regulation of hepatic Bcrp and our study represents the first investigation into its hepatic expression in STZ-induced T1DM. Bcrp has been implicated in the hepatobiliary excretion of sulfate conjugates and drugs such as topotecan and nitrofurantoin (Jonker et al., 2000; Suzuki et al., 2003; Merino et al., 2005). Consistent with our findings, Liu et al (2007) found decreased levels of Bcrp protein in cerebral cortex samples from rats rendered diabetic via STZ for five and eight weeks (Liu et al., 2007). Associated increases in Kp values were seen in diabetic rats after i.v. administration of prazosin and cimetidine (Liu et al., 2007). Decreased Bcrp has recently been observed in the bile duct ligation (BDL) model of cholestasis (Villanueva et al., 2008).

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Figure 2.2. Hepatic expression of apical (A) and basolateral (B) ABC transporters in diabetic and control rats as determined by qRT-PCR. Values represent ratios of transporter mRNA to 18S, normalized to control levels (100%), and are presented as mean ± SEM of 5-7 rats. *, p < 0.05; **, p < 0.01.
2.4.3 Endogenous glutathione disposition was altered in T1DM

TGSH and GSSG were examined in diabetic and control animals as these represent well-characterized endogenous substrates for transporter-mediated hepatobiliary excretion. Hepatic levels of these GSH forms were similar in diabetic and control animals at 6.4 ± 0.8 µmol/g vs. 5.6 ± 0.2 µmol/g (p > 0.05) for TGSH and 0.07 ± 0.01 µmol/g vs. 0.07 ± 0.01 µmol/g (p > 0.05) for GSSG, respectively (Fig. 2.3A). In contrast, relative to controls, plasma concentrations of these GSH forms were lower in diabetics at 1.9 ± 0.3 µM vs. 2.8 ± 0.5 µM (p > 0.05) for TGSH and 0.3 ± 0.1 µM vs. 0.5 ± 0.1 µM (p < 0.05) for GSSG, respectively (Fig. 2.3B). Because an increase in the ratio of TGSH to GSSG is indicative of oxidative stress, TGSH/GSSG ratios were evaluated. In the liver, the TGSH/GSSG ratio was 93.6 ± 9.8 in diabetics and 91.6 ± 11.6 in controls (p > 0.05). In plasma, the TGSG/GSSG ratio was 9.4 ± 3.6 in diabetics and 5.3 ± 0.4 in controls (p > 0.05). Analyses of urine TGSH content revealed comparable concentrations; however, when normalized to urinary output, the total amount of TGSH excreted in urine on day 8 was 6.7-fold greater in diabetics (p < 0.01; Fig. 2.3C). In urine, levels of GSSG were below detection limits.

Our results indicate that hepatic concentrations of TGSH and GSSG were maintained in diabetic rats at levels comparable to controls. These substrates are transported primarily by Mrp2 at the apical membrane (Elferink et al., 1989) and by Mrp4 at the basolateral membrane (Lai and Tan, 2002). In fact, virtually no glutathione is observed in the bile of mutant TR- rats, which lack Mrp2 (Elferink et al., 1989). The finding of comparable TGSH and GSSG in diabetic and control rats could be explained by the absence of Mrp2 causing compensatory alterations in Mrp4 expression. Elevated Mrp3 and Mrp4 expression in the basolateral membrane serves to remove substrates from the hepatocyte and prevent toxicity associated with accumulation. A similar increase in Mrp3 has been observed in Eisai hyperbilirubinuria rats, which accumulate bilirubin glucuronide due to an Mrp2 deficiency (Kuroda et al., 2004). These models are not completely comparable to the present study; however, it is worth noting that many cholestatic animal models, as with STZ-induced diabetes in the present study, are associated with inflammation. Increased hepatic and systemic levels of TNF-α and IL-6 are observed in rats after 28 days of BDL (Fernandez-Martinez et al., 2006). Although observed increases in hepatic Mrp3 were not statistically significant, it is possible that these differences might become significant at later stages of T1DM.
Figure 2.3. Disposition of glutathione in the liver (A), plasma (B) and urine (C) of diabetic and control rats. Values represent µmol/g for liver (A), µM for plasma (B) and µg/kg/day for urine (C) but are presented in this figure as percentages of corresponding control levels. Filled bars and open bars depict data from the diabetic (n = 5) and control (n = 5) groups, respectively. *, p < 0.05; **, p < 0.01.
2.4.4 Preservation of renal ABC transporter expression in T1DM

To determine the renal mRNA expression of transporters that were found to be altered in the liver by STZ-induced T1DM, qRT-PCR was performed with primers targeting Mdr1a, Mrp2, Mrp4 and Bcrp. A general trend toward increased expression was observed (Fig. 2.4).

Renal transporter expression results, when coupled with plasma TGSH and GSSG results, suggest renal compensation may occur in T1DM. Despite T1DM associated changes in the expression of several hepatic ABC transporters, we did not detect a statistically significant impact of T1DM on the expression of these transporters in the kidney of diabetic rats. In the nephron’s proximal tubule, all of these transporters, including Mrp4, are localized within the luminal membrane of renal epithelial cells where they efflux substrates into the urine (Thiebaut et al., 1987; Schaub et al., 1997; van Aubel et al., 2002; Schinkel and Jonker, 2003; Huls et al., 2008). As such, preservation of these transporters in diabetic rats could provide an alternate route for substrates normally excreted via these transporters’ activity in the liver. With respect to GSSG, the absence of hepatocyte accumulation despite decreased apical transporter expression and the decreased presence of GSSG in plasma (51% less) indicate that this substrate is being removed via an extrahepatic route of elimination that is likely renal. Further experiments are required as renal clearance rates of GSSG were not determined. Renal compensation has been reported in several studies where ABC transporter-mediated hepatobiliary excretion is impaired (Muraoka et al., 1995; Smit et al., 1998c; Hartmann et al., 2005; Chu et al., 2006; Villanueva et al., 2008). For example, we have previously demonstrated that lipopolysaccharide (LPS)-induced inflammation decreases hepatic Mdr1 and the percentage of intravenous doxorubicin excreted in bile but increases renal Mdr1 and dramatically increases the percentage excreted in urine (Hartmann et al., 2005).

It is important to note that osmotic diuresis-based renal compensation is expected to compound transporter-based renal compensation in T1DM. Osmotic diuresis occurs when hyperglycemia is severe enough to saturate glucose transporters in the proximal tubule (i.e., glycosuria) so as to increase osmotic pressure and therefore enhance proximal tubule water loss. We observed significantly higher urinary output (9.7-fold) and positive urine glucose tests in diabetic rats, which are indicative of osmotic diuresis. For drugs that are reabsorbed in the kidney by passive mechanisms/along a concentration gradient, osmotic diuresis increases renal elimination. Consistent with this, TGSH concentrations were comparable in diabetic and control urine; however, elevated urinary output in diabetics resulted in the total amount excreted in their urine being 6.7-fold greater. Moreover, plasma TGSH concentrations in diabetic plasma were somewhat lower in diabetic rats.
TGSH is comprised primarily of reduced GSH (> 90%) which, in addition to active Mrp subfamily transport, can be excreted by the liver and kidney via passive mechanisms/along a concentration gradient (Lauterburg et al., 1984). A clinical example of osmotic diuresis-based renal compensation is the relationship between hyperglycemia and plasma lithium levels (Cyr et al., 2002).

Figure 2.4. Renal expression of ABC transporters in diabetic and control rats as determined by qRT-PCR. Values represent ratios of transporter mRNA to 18S, normalized to control levels (100%), and are presented as mean ± SEM of 5-7 rats.
2.4.5 Potential mechanisms for the alteration of hepatic ABC transporter expression in T1DM

It is felt that chemical models of diabetes that destroy insulin-producing pancreatic β-cells best represent T1DM. In the present study, we utilized the streptozotocin (STZ) T1DM model. STZ is a DNA alkylating agent that selectively targets pancreatic β-cells and STZ-treated rats represent the most widely used and well-characterized animal model of T1DM. One potential criticism of this model is the possibility that effects stem from hepatotoxicity and are not a consequence of diabetes. Oxidative stress and resultant lipid peroxidation is observed in a variety of organs, including the liver, after STZ injection (Baştar et al., 1998). Although not statistically significant, in the present study, the mean TGSH:GSSG ratio in diabetic rat plasma was elevated at 177% of control values. As mentioned, an increase in the ratio of TGSH to GSSG suggests oxidative stress is present. However, oxidative stress can be a consequence of unmanaged hyperglycemia and has been associated with the clinical presentation of diabetes (Reviewed in Ceriello, 2006). Elevated plasma ALT levels, which can be indicative of liver pathology, have also been reported with STZ injection and were observed in the present study. However, studies have demonstrated that the development of type 2 diabetes can be predicted by plasma ALT levels (Ohlson et al., 1988; Vozarova et al., 2002; Sattar et al., 2004) and elevations are a prominent feature of fulminant type 1 diabetes (Hanafusa and Imagawa, 2007). Moreover, ALT levels can be normalized in STZ-treated rats that receive insulin treatment (Yamatani et al., 1994). This suggests that elevations in hepatotoxicity indices such as oxidative stress and ALT may be the result of diabetes and not simply the result of STZ exposure.

A plausible explanation for the altered expression of hepatic ABC transporters observed in this study involves the activity of pro-inflammatory mediators. As depicted in figure 2.5, levels of the pro-inflammatory marker CRP, which were found to be significantly elevated in diabetic rats, were inversely correlated with Mdr1a and Mrp2 expression (r = -0.71, p < 0.05 and r = -0.78, p < 0.01, respectively). A similar trend was present for CRP and Bcrp. Correlations between Mdr1b, Mrp1, Mrp3 and Mrp4 and CRP were not significant. As mentioned, it is known that hyperglycemia is associated with increased production of the inflammatory cytokines/markers IL-6, TNF-α and CRP (Morohoshi et al., 1995; Morohoshi et al., 1996; Pickup and Crook, 1998; Rodriguez-Moran and Guerrero-Romero, 1999; Arnalich et al., 2000; Esposito et al., 2002; Leinonen et al., 2003). Also as mentioned, it has been previously demonstrated that inflammation decreases the expression and activity of various ABC transporters in the liver (Piquette-Miller et al., 1998; Sukhai et al., 2000; Tang et al., 2000; Sukhai et al., 2001; Hartmann et al., 2002; Cherrington et al., 2004; Siewert et al., 2004;
Hartmann et al., 2005; Teng and Piquette-Miller, 2005; Wang et al., 2005). CRP, which is a well-established marker for inflammation, was significantly higher in diabetic animals relative to controls. Significant inverse correlations were observed between the 18S ratios of Mdr1a and Mrp2 and plasma CRP concentrations. The synthesis of CRP, an acute phase protein, is induced primarily by the activity of IL-6 and can therefore be used as a surrogate marker of IL-6 (Ganapathi et al., 1988; Ganapathi et al., 1991). Indeed, the highest three CRP values corresponded with the three diabetic animals for which IL-6 levels were present at detectable concentrations. Accumulation experiments conducted with IL-6-treated rat hepatocytes revealed that IL-6 values obtained in diabetic animals were in a range capable of causing increased substrate accumulation. Studies demonstrating increased accumulation/decreased efflux in IL-6-treated hepatocytes typically utilize significantly higher IL-6 concentrations (i.e., 5-10 ng/ml) (Sukhai et al., 2000; Lee and Piquette-Miller, 2001; Sukhai et al., 2001; Hartmann et al., 2002; Lee and Piquette-Miller, 2003). Thus, the present study demonstrates that more moderate increases in pro-inflammatory mediators, increases likely observed in T1DM, are capable of affecting active transport. It is believed that pro-inflammatory cytokines exert their effect by modifying the expression and/or function of transporter transcription factors (Reviewed in Morgan et al., 2008). This mechanism of cytokine action is consistent with the present study’s demonstration of altered transporter expression at the level of mRNA. The relationship between plasma CRP concentration and Mdr1a and Mrp2 expression is suggestive of a role for inflammation in transporter downregulation in T1DM.

In conclusion, our findings demonstrate that STZ-induced T1DM alters the mRNA expression of key ABC transporters in the liver. In diabetic rats, Mdr1a, Mrp2 and Bcrp, which are localized within the apical membranes of hepatocytes, were downregulated. In contrast, Mrp3 and Mrp4, which are localized within the basolateral membranes of hepatocytes, were upregulated. Diabetic renal transporter levels were normal. We propose that upregulation of basolateral hepatic transporters and the preservation of normal renal transporters provide possible modes of compensation for this effect whereby some substrates, such as glutathione, can be shunted toward the renal route of elimination. Our results also provide preliminary support for an inflammation-based mechanism of ABC transporter downregulation as the inflammatory marker CRP was inversely correlated with alterations. Diabetes-induced alterations to hepatobiliary excretion could represent an important source of unrecognized pharmacokinetic variability in diabetic patients. Based on our findings, this is expected to be of particular relevance in situations where drug properties make renal compensation unlikely because accumulation could occur. Future studies that
further elucidate the effects of diabetes, a highly prevalent disease, on transport processes involved in hepatic drug excretion could potentially lead to important dosage recommendations.

Figure 2.5. Scatter plots depicting the association between plasma CRP concentration and mRNA expression for significantly altered transporters. Correlational analysis was performed with plasma CRP and hepatic transporter mRNA data. Values for CRP are presented as µg/ml while values for mRNA expression represent ratios of transporter mRNA to 18S and are presented as raw values. Spearman r and statistical significance values are presented on individual scatter plots.
Appendix 2.1. Time course of STZ-induced hyperglycemia (A) and weight loss (B). Blood glucose and body weight were monitored for nine days in rats injected with STZ (45 mg/kg) or vehicle control. Baseline measurements (day 0) were made prior to injection. Values represent the mean ± SD of 5–7 rats. ***, p < 0.001. Statistical test employed: two-way ANOVA followed by Bonferroni post-tests.
Chapter 3. Impact of Hyperlipidemia on Plasma Protein Binding and Hepatic Drug Transporter and Metabolic Enzyme Regulation in a Rat Model of Gestational Diabetes

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3.1 Abstract

It is currently unknown whether gestational diabetes (GDM), a prevalent obstetrical complication, compounds the changes in drug disposition that occur naturally in pregnancy. Hyperlipidemia occurs in GDM. Using a rat model of GDM, we determined if excess lipids compete with drugs for plasma protein binding. Since lipids activate nuclear receptors that regulate drug transporters and metabolic enzymes, we used proteome analysis to determine if hyperlipidemia indirectly leads to the dysregulation of these proteins in the liver. GDM was induced on gestational day 6 (GD6) via streptozotocin injection. Controls received either vehicle alone or streptozotocin with subsequent insulin treatment. Liver and plasma were collected on GD20. Glyburide and saquinavir protein binding was determined by ultrafiltration and an established solvent method was used for plasma delipidation. Proteomics analysis was performed using iTRAQ methodology with membrane-enriched hepatic protein samples. Relative to controls, GDM rat plasma contained more cholesterol and triglycerides. Plasma protein binding of glyburide and saquinavir was decreased in GDM. Delipidation normalized protein binding in GDM plasma. Proteins linked to lipid metabolism were strongly affected in the GDM proteomics dataset, with pro- and anti-hyperlipidemic changes observed, and formed networks that implicated several nuclear receptors. Upregulation of drug transporters and metabolic enzymes was observed (e.g., Mdr1/2, Cyp2a1, Cyp2b9 and Cyp2d3). In this study, GDM-induced hyperlipidemia decreased protein binding and was associated with drug transporter and metabolic enzyme upregulation in the liver. Both of these findings could change drug disposition in affected pregnancies, compounding changes associated with pregnancy itself.
3.2 Introduction

Gestational diabetes (GDM) is an obstetrical complication that affects up to 8% of all pregnancies in North America (Lawrence et al., 2008). At present, little is known about how the mechanisms that determine drug disposition differ in women with this condition relative to women with uncomplicated pregnancies. While it has been established that pregnancy itself decreases the plasma protein binding of drugs (Perucca et al., 1981; Notarianni, 1990; Tsen et al., 1999), there have been few attempts to determine if maternal diseases like GDM alter this parameter further. Given the difficulty associated with dosage determination in pregnancy, the potential compounding effects of maternal disease on protein binding could represent a largely overlooked source of variability.

Protein binding strongly influences a drug’s distribution and/or clearance. With distribution, placental transfer is limited to unbound drugs and so altered protein binding in pregnancy can significantly impact the materno-fetal transfer of drugs (Nanovskaya et al., 2006a). With clearance, the removal of drugs by the liver and kidneys, processes that depend upon drug transporters and metabolic enzymes, often varies with plasma protein binding since uptake by these organs is also limited to unbound drugs. Atypical clearance has fetal implications in the form of changing the absolute amount of drug that is available for placental transfer and maternal implications in the form of diminished therapeutic effect (when increased) or toxicity (when decreased).

Protein binding in GDM is of interest not just because there is a paucity of information concerned with it but because there are reports of changes to this parameter in other forms of diabetes (Ruiz-Cabello and Erill, 1984; Trovik et al., 1992; Arredondo et al., 1999). For example, it has been demonstrated that the plasma protein binding of itraconazole in patients with type 1 and type 2 diabetes is significantly less than in healthy volunteers (Arredondo et al., 1999). Some studies have implicated increased glycosylation of abundant plasma proteins such as albumin and alpha 1-acid glycoprotein (AAG) (Wörner et al., 1992; Koyama et al., 1997) but reports on this topic are conflicting (Olsen et al., 1995).

Hyperlipidemia is a common comorbidity among patients with type 1 or 2 diabetes (Ginsberg, 1996; Reviewed in Betteridge, 2000; Krentz, 2003; Harris et al., 2005) and represents an alternative explanation for altered protein binding in diabetes (Ruiz-Cabello and Erill, 1984; de la Fuente et al., 2002). In a study utilizing patients with type 1 diabetes, a significant positive correlation between elevated plasma lipids, such as cholesterol and triglycerides, and the urinary excretion of albumin was observed (Mattock et al., 2001). In addition to increasing the urinary excretion of this important plasma protein, it is known that lipids can interact directly with proteins to alter their
capacity for drug binding via competition or allosteric modulation. Even small increases in free fatty acids (FFAs) displace chlorophenoxyisobutyrate from human albumin (Spector et al., 1973). Along these same lines, the binding of propranolol to human AAG can be significantly increased if AAG-bound lipids are first removed (Chauvelot-Moachon et al., 1988). In pregnancy, diabetes exacerbates the natural rise in plasma lipids that occurs across pregnancy (Knopp et al., 1977; Knopp et al., 1986; Koukkou et al., 1996; Couch et al., 1998; Reviewed in Basaran, 2009).

In this study, plasma lipids and protein binding were examined in the streptozotocin (STZ) rat model of GDM. Similar to the clinical scenario, STZ-induced diabetes is associated with hyperlipidemia (Wasan et al., 1998) and our initial experiments with non-pregnant rats have demonstrated correlations between plasma lipids and the unbound percentage of both acidic and basic drugs following STZ injection (unpublished data). We hypothesized that excess plasma lipids would compete with drugs for protein binding in rats with STZ-induced GDM.

In addition to assessing plasma lipids and protein binding, insight into the effects of hyperlipidemia on hepatic proteins linked to drug transport and metabolism was sought using a global proteomics approach (8-plex iTRAQ). The regulation of drug transporters and metabolic enzymes in the liver is controlled largely by nuclear receptors, such as the farnesoid X receptor (FXR) and pregnane X receptor (PXR), which are themselves activated by various plasma lipids (Handschin and Meyer, 2005). Dysregulation of hepatic drug transporters and metabolic enzymes would be expected to exacerbate any variability in drug disposition introduced by altered plasma protein binding. We hypothesized that hyperlipidemia in STZ-induced GDM would indirectly result in hepatic drug transporter and metabolic enzyme dysregulation.

Our findings advance our understanding of the impact that GDM-induced hyperlipidemia can have on plasma protein binding and the expression of drug transporters and metabolic enzymes in the liver.
3.3 Methods

3.3.1 Animals

Timed pregnant Sprague-Dawley rats (207-260 g on gestational day 6) were purchased directly from Charles River Laboratories, Inc. (Senneville, QC) and housed individually in a temperature controlled facility on a 12:12 hr light-dark cycle. Rats were given free access to water and standard chow (Harlan Teklad Global Diet 2018). Experiments were approved by the Office of Research Ethics at the University of Toronto and performed in accordance with Canadian Council on Animal Care guidelines.

3.3.2 Animal treatments/monitoring and tissue collection

Rats were randomly assigned to one of three groups (n = 5/group): non-treated diabetics (referred to as the, “GDM group”), vehicle controls (referred to as the, “vehicle group”) and insulin-treated diabetics (referred to as the, “insulin-treated group”). Animals in the vehicle and insulin-treated groups are collectively referred to as controls. On gestational day 6 (GD6), diabetes was induced by way of a single subcutaneous injection of streptozotocin (STZ, PubChem Compound ID 5300, Sigma-Aldrich, Oakville, ON) in 0.1 M citrate buffer (pH 4.5) at a dosage of 45 mg/kg. Vehicle control animals received a single subcutaneous injection of citrate buffer on GD6. For all animals, body weight was measured prior to respective injections (GD6) and then daily until sacrifice. For animals in the GDM and vehicle groups, blood glucose was measured prior to respective injections (GD6) and on gestational days 7, 8, 9, 11, 13, 15, 18 and 20. For animals in the insulin-treated group, blood glucose was measured prior to STZ injection (GD6) and then daily until sacrifice in order to determine daily insulin dosage requirements. Blood glucose was measured at noon, in a non-fasted state, using a commercially available glucometer (Freestyle Freedom Meter, Abbott Laboratories, Alameda, CA) with blood obtained via tail-prick. When values exceeded the range of this glucometer, a commercially available enzymatic glucose assay was employed according to the manufacturer’s instructions (Autokit Glucose, Wako Chemicals USA, Inc., Richmond, VA). Mild hyperglycemia was defined as 10-14 mM and diabetes was defined as > 14 mM. For diabetic rats receiving insulin treatment, treatment was initiated once diabetes was confirmed and consisted of an initial subcutaneous injection of 8-10 U/kg Humulin-L (human biosynthetic intermediate-acting insulin, Lilly, Toronto, ON) and subsequent daily injections as required. For all animals, water intake, urinary output, feed intake and fecal output were monitored in metabolic cages on GD18. Following measurement of blood glucose and body weight on GD20, animals were sacrificed to collect maternal blood (cardiac puncture) and liver. Aliquots of whole blood were transferred to
heparin- and thrombin-coated BD Vacutainer tubes (BD Canada, Mississauga, ON) and spun to collect plasma and serum, respectively. Samples were snap-frozen and stored at -80 °C until use.

### 3.3.3 Blood chemistry

Determination of total cholesterol, high-density lipoprotein (HDL)/low-density lipoprotein (LDL) cholesterol, triglyceride, FFA and albumin concentrations in serum was outsourced to the Banting and Best Diabetes Centre (Faculty of Medicine, University of Toronto). Total cholesterol, HDL/LDL cholesterol, triglyceride and albumin concentrations were analyzed using an autoanalyzer and commercially available reagents (Roche Diagnostics, Laval, QC) while FFA was analyzed using a commercially available ELISA kit (Wako Chemicals USA, Inc.). Determination of alanine transaminase (ALT) concentrations in plasma was outsourced to Vita-Tech Laboratories (Markham, ON), where an autoanalyzer and commercially available reagents (Roche Diagnostics) were employed. C-reactive protein (CRP) and AAG concentrations were determined in-house using commercially available, rat-specific ELISA kits (BD Biosciences, San Diego, CA and GenWay, San Diego, CA, respectively), according to manufacturer’s instructions.

### 3.3.4 Protein binding assays

Glyburide (PubChem Compound ID 3488) and saquinavir (PubChem Compound ID 60787) protein binding in rat plasma was determined ex vivo by ultrafiltration using the Centrifree micropartition system (Millipore Canada, Ltd., Etobicoke, ON). Glyburide is an acidic drug that binds to albumin in plasma while saquinavir is a basic drug that binds to AAG in plasma. Aliquots of [³H]-glyburide (Perkin Elmer, Boston, MA) or [³H]-saquinavir (American Radiolabeled Chemicals, Inc., St. Louis, MO), dissolved in ethanol, were placed in microcentrifuge tubes and dried under nitrogen gas. Dried [³H]-glyburide or [³H]-saquinavir was then reconstituted with drug-free plasma from each of this study’s three groups (n = 5/group) to obtain a final glyburide or saquinavir concentration of 30 ng/ml or 0.5 µg/ml, respectively, in a final volume of 200 µl. Samples were then incubated at 36 °C for 1 hr with gentle agitation every 20 min. At 1 hr, 150 µl aliquots were transferred to ultrafiltration tubes and centrifuged at 1000 g for 30 min. Following centrifugation, aliquots (25 µl) of filtered and unfiltered sample were supplemented with 4 ml of Pico-Fluor 40 (Perkin Elmer) and subjected to scintillation counting. Percent unbound in maternal circulation (% unbound_M) was determined using the following equation: % unbound = \((C_{filtered}/C_{unfiltered}) \times 100\).

Preliminary experiments demonstrated constant glyburide and saquinavir protein binding across a wide range of concentrations. The final concentrations selected for plasma protein binding
experiments reflect values within therapeutic ranges. In order to directly determine the capacity for plasma lipids to alter protein binding, total lipid content was removed from drug-free non-treated diabetic, insulin-treated diabetic and vehicle control plasma samples (n = 5/group) using the butanol:diisopropyl ether extraction method of Cham and Knowles (Cham and Knowles, 1976). This delipidation procedure removes 100% of plasma lipids without impacting the concentrations of other plasma constituents (Cham and Knowles, 1976). Delipidated samples were then subjected to the glyburide protein binding procedure described in this methods section. Reported values are the result of triplicate experiments and are reported as mean ± SD.

### 3.3.5 Proteomics: sample preparation and isobaric mass tagging

A proteomics approach (8-plex iTRAQ) was used to characterize the expression of hepatic proteins involved in lipid metabolism and/or drug transport and metabolism (Appendix fig. 3.1). Our experimental design consisted of two experiments: a first experiment comparing four non-treated diabetic samples to three vehicle control samples and a second experiment comparing four insulin-treated diabetic samples to three vehicle control samples. In order to eliminate the contribution of blood proteins in these experiments, a proteomics-specific cohort of rats was randomly assigned to the treatments described above (n = 3-4/group, see Animal treatments/monitoring and tissue collection) but subjected, on GD20, to hepatic saline perfusion prior to liver collection (Mehta et al., 2009). Protein homogenates, obtained from perfused livers, underwent a membrane protein enrichment procedure as previously described (Molloy, 2008). For isobaric mass tagging, an 8-plex iTRAQ kit from Applied Biosystems (Scoresby, Victoria, Australia) was used according to the manufacturer's instructions. iTRAQ-labeled samples were combined (one combination per experiment) and then dried by SpeedyVac prior to storage at -20 °C to await clean-up and fractionation.

### 3.3.6 Proteomics: LC-MS/MS

Sample clean-up and fractionation was performed by strong cation exchange (SCX) chromatography on an Agilent Technologies (Forest Hill, Victoria, Australia) 1100 quaternary HPLC pump with a PolySulfoethyl A column (2.1 mm × 200 mm, 5 µm particle size, 300 Å pore) from PolyLC, Inc. (Columbia, MA). Two buffers were used for SCX: A and B. Buffer A was 5 mM phosphate and 25% acetonitrile (pH 2.7) and buffer B was 5 mM phosphate, 350 mM potassium chloride and 25% acetonitrile (pH 2.7). Combined, iTRAQ-labeled samples were resuspended in 14 ml of buffer A. This solution was filtered and 7 ml were then loaded onto the SCX column. After sample loading and washing with buffer A, the concentration of buffer B was increased from 10% to
45% in 70 min and then increased to 100% for 10 min at 300 µl/min. For each experiment, a total of 23 SCX fractions were collected and dried by SpeedyVac prior to storage at -20 °C to await mass spectrometry (MS).

Reverse phase nanoLC electrospray ionization (ESI) tandem mass spectrometry (MS/MS) was conducted on a QStar Elite MS/MS system (Applied Biosystems). SCX fractions were resuspended in 60 µl of a loading/desalting solution consisting of 0.1% trifluoroacetic acid and 2% acetonitrile. Forty µl of this solution were loaded on a reverse phase peptide Cap Trap (Michrom Bioresources, Inc., Auburn, CA) desalted with 0.1% formic acid and 2% acetonitrile at 8 µl/min for 13 min. The trap was then switched online with a ProteoCol C18 trap cartridge (150 µm × 100 mm, 3 µm particle size, 300 Å pore) from SGE Analytical Science (Ringwood, Victoria, Australia). Channel 1 contained desalting solution, channel 2A contained 0.1% formic acid and channel 2B contained 0.1% formic acid and 90% acetonitrile. The concentration of solution in channel 2 was increased from 5% to 95% in 95 min in three linear gradient steps to elute peptides. After elution, the column was cleaned by increasing the concentration of channel 2B from 0% to 100% for 20 min followed by equilibration with 95% channel 1 and 5% channel 2B for 7 min prior to injection of the next SCX fraction.

Reverse phase nanoLC eluent was subjected to positive ion nanoflow electrospray analysis in an information dependant acquisition mode (Applied Biosystems). In this mode, a time-of-flight MS survey scan was acquired (m/z 370-1600, 0.5 s) with the three most intense multiply charged ions (counts > 50) in the survey scan sequentially subjected to MS/MS analysis. Spectra were accumulated for 2 s in the range of m/z 100-1600 with a modified Q2 transition setting favouring low mass ions so that iTRAQ ion (113.1, 114.1, 115.1, 116.1, 117.1, 118.1, 119.1 and 121.1) intensities were enhanced.

3.3.7 Proteomics: database processing and analysis

Experimental nanoLC ESI MS/MS data were submitted to ProteinPilot (Applied Biosystems, version 2.0) for database processing. The Paragon Algorithm was used in thorough ID search effort with “biological modifications” selected in the “ID focus” box. Software correction factors, provided in the iTRAQ kit, were entered in the iTRAQ Isotope Correction Factors table. The detected protein threshold (Unused ProtScore) was set as larger than 1.3, which corresponds to an identification confidence of 95%. The Unused ProtScore estimates the confidence associated with identifications and uses only peptide evidence that is not better explained by a higher-ranking
protein. Significantly altered proteins (see section 3.3.9), upregulated or downregulated greater than 10%, were imported into Ingenuity Pathway Analysis (IPA) software (Ingenuity Systems, Inc., http://www.ingenuity.com). IPA software was used to identify protein networks and to generate lists of proteins associated with specific molecular and cellular functions. IPA software was also used to graphically illustrate the molecular relationships between proteins on our lists and proteins stored in the Ingenuity Knowledge Base (IKB). In these figures, proteins are represented as nodes and the biological relationships between nodes are represented as connecting lines; lines are supported by at least 1 reference from IKB, which is based on the literature or canonical information stored in the IKB. IPA software was then used to compare all altered proteins in our experiments to “Tox Lists” in the IKB. “Tox Lists” are lists of proteins that are known to be involved in particular aspects of toxicity and our focus was on lists that addressed nuclear receptor activation.

3.3.8 Characterization of hepatic Mdr1 expression

In rodents, two genes encode the Mdr1 protein: Mdr1a and Mdr1b. In this study, Mdr1a and Mdr1b mRNA levels as well as Mdr1 protein levels were determined in liver. For mRNA levels, total RNA was extracted from tissue using the TRIZOL method (Invitrogen, Carlsbad, CA) and then reverse transcribed to cDNA via the First Strand cDNA Synthesis Kit (Fermentas, Burlington, ON), according to the manufacturer’s instructions. mRNA levels were determined by quantitative reverse transcriptase-polymerase chain reaction (qRT-PCR) using LightCycler technology with SYBR Green I fluorescence detection (Roche Diagnostics). PCR oligonucleotides were synthesized at The Hospital for Sick Children (DNA Synthesis Centre, Toronto, ON) and their sequences have been previously published (Petrovic et al., 2008). Expression levels were normalized to 18S rRNA expression using the efficiency-corrected ΔCt method and presented as a gene/18S ratio.

For Mdr1 protein levels, tissue samples were first homogenized in RIPA buffer, containing freshly added PMSF (0.5 mM) and 1X protease inhibitor cocktail (Sigma-Aldrich), using a motorized pestle. Homogenates were then incubated at 4 °C for 2 hrs on a rocker and subsequently centrifuged at 18,000 g for 20 min. For each sample, the supernatant was isolated and subjected to a Bradford assay to determine total protein concentration. Samples containing 60 µg of protein in Laemmli sample buffer were heated at 36 °C for 20 min and then separated via 10% SDS-PAGE and transferred to PVDF membranes (Bio-Rad Laboratories Canada, Ltd., Mississauga, ON). Membranes were blocked in 5% fat-free milk powder at 4 °C overnight on a rocker and incubated with a mixture of monoclonal anti-Mdr1 (C219, 1:500, 1 mg/ml, Abcam, Inc., Cambridge, MA) and
anti-β-actin (AC-15, 1:20,000, 2 mg/ml, Sigma-Aldrich) antibodies at room temperature for 3 hrs. After a series of washes, membranes were incubated with a peroxidase-labeled secondary antibody for 1 hr at room temperature (goat anti-mouse at 1:3,000 from Jackson ImmunoResearch Laboratories, Inc., West Grove, PA). Immunoreactive Mdr1 and β-actin proteins were simultaneously detected in membranes using ECL Plus (Amersham Biosciences, Baie d’Urfé, QC) and scanned using an Alpha Innotech FluorChem imaging system (San Diego, CA). Using AlphaEaseFC (version 6.0) software, also from Alpha Innotech, the optical density (OD) of each band was determined. Mdr1 ODs were normalized to β-actin.

3.3.9 Statistics

Data were analyzed using Prism 4 for Macintosh (GraphPad Software, Inc., San Diego, CA). For comparisons of blood glucose and body weight, two-way ANOVAs followed by Bonferroni post-tests were performed. These same tests were employed to examine any potential differences between this study’s two cohorts, with respect to blood glucose and body weight. Protein binding of drugs (% unbound), plasma concentrations (e.g., individual lipids, albumin, etc.), hepatic Mdr1 expression data and metabolic cage variables were analyzed using one-way ANOVAs followed by Bonferroni post-tests. Linear regression was used to determine the relationship between the protein binding of glyburide and saquinavir. Paired t-tests were used to statistically evaluate the effects of plasma delipidation on the protein binding of glyburide. For proteomics data, ProteinPilot was used to generate control-normalized “fold changes” for each sample’s identified proteins. By standardizing to controls, the mean of control values was made to represent 1 ± SD while non-control values were made, consequently, to distribute around this value. Statistically significant deviations from control values were identified using t-tests. Levels of significance for statistical analyses were set at or below \( \alpha = 0.05 \), indicated as follows: *#/*, \( p < 0.05 \), **/##, \( p < 0.01 \) and ***/###, \( p < 0.001 \). All results are presented as mean ± SD.
3.4 Results

3.4.1 STZ-injected pregnant rats exhibited a variety of characteristics that were consistent with unmanaged, or poorly managed, GDM

Induction of GDM in STZ-injected rats was determined on the basis of their blood glucose concentrations as measured on GD7-9, 11, 13, 15, 18 and 20 (Appendix fig. 3.2A). All of the STZ-injected rats developed mild hyperglycemia within 24 h and then developed GDM (> 14 mM) by GD9. All animals in the GDM group were, therefore, diabetic for 12 to 13 days since sacrifice occurred on GD20. For the insulin-treated group, insulin treatment was initiated when blood glucose concentrations first exceeded 14 mM and was efficacious within hrs. Blood glucose concentrations in the GDM group, relative to controls, were higher at every time point after GD9 (p < 0.001; Table 3.1). Blood glucose concentrations in the insulin-treated group fluctuated but were different from values obtained in the vehicle group only on GD13.

As expected during gestation, all animals gained weight across the study duration; however, animals with GDM were lighter from GD8 until sacrifice (p < 0.01-0.001; Table 3.1; Appendix fig. 3.2B). On GD20, the body weight of animals in the GDM group was 136 ± 7% of baseline values versus the 150 ± 8% increase observed in the vehicle and insulin-treated groups (p < 0.01).

Animals in the GDM group exhibited other physiological and biochemical characteristics consistent with unmanaged, or poorly managed, GDM (Table 3.1). Overt hyperlipidemia was evident in the GDM group (Appendix fig. 3.3A) as plasma cholesterol (total) and triglyceride concentrations were profoundly elevated. FFA concentrations were also elevated but this finding was not statistically significant (p = 0.06). Also, animals in the GDM group exhibited obvious polydipsia and polyuria on GD18. While in metabolic cages, relative to the vehicle and insulin-treated groups, animals in the GDM group exhibited a 5.2 and 4.2-fold increase in water consumption, respectively, and a 8.6- and 4.6-fold increase in urination, respectively. Animals in the GDM group also exhibited hyperphagia with an associated increase in fecal output. Relative to controls, animals in the GDM group consumed over twice as much food and produced nearly five times as much feces. Plasma from animals in the GDM group contained higher levels of AAG and lower levels of albumin, as compared to the vehicle group. Albumin and AAG concentrations in the insulin-treated group were slightly elevated but this finding was not statistically significant. As compared to the vehicle and insulin-treated groups, ALT was found to be elevated in the GDM group while CRP values were found to be similar (p > 0.05).
Table 3.1. The effect of STZ-induced GDM, with and without insulin treatment, on various physiological and biochemical characteristics. All results are presented as mean ± SD of 5 rats/group.

* Significantly different from vehicle controls. **, Significantly different from GDM. */#, p < 0.05; **/#, p < 0.01; ***/##, p < 0.001.

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<tr>
<th>Characteristic</th>
<th>Vehicle</th>
<th>GDM</th>
<th>Insulin-treated</th>
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<tr>
<td>Blood glucose and body weight</td>
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<td></td>
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<td>Blood glucose (mM, GD6)</td>
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<td>6.6 ± 0.9</td>
<td>6.5 ± 1.8</td>
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<td>Blood glucose (mM, GD11)</td>
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<td>24.7 ± 3***</td>
<td>8.4 ± 3.2#####</td>
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<td>26 ± 2.6###</td>
<td>5.7 ± 1.9######</td>
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<td>Body weight (g, GD6)</td>
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<td>242 ± 28</td>
<td>246 ± 22</td>
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<td>Body weight (% baseline, GD11)</td>
<td>114 ± 2</td>
<td>104 ± 2###</td>
<td>112 ± 2#####</td>
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<tr>
<td>Body weight (% baseline, GD18)</td>
<td>138 ± 7</td>
<td>124 ± 5###**</td>
<td>136 ± 8######</td>
</tr>
<tr>
<td>Body weight (% baseline, GD20)</td>
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<td>Total cholesterol (mM)</td>
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<td>5 ± 1.4**</td>
<td>2.2 ± 0.6#</td>
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<td>HDL cholesterol (mM)</td>
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<td>0.7 ± 0.2</td>
<td>1.2 ± 0.1##</td>
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<td>Triglycerides (mM)</td>
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<td>19.15 ± 9.08*</td>
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<td>0.8 ± 0.1</td>
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<td>AAG (µg/ml)</td>
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<td>Albumin (g/L)</td>
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<td>21.8 ± 5.3**</td>
<td>39 ± 0.1###</td>
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<td>ALT (I.U./L)</td>
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<td>104.8 ± 20.4**</td>
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<td>CRP (µg/ml)</td>
<td>318.2 ± 33.5</td>
<td>305.2 ± 45.3</td>
<td>296.2 ± 58.1</td>
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<td>Metabolic cage (GD18)</td>
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<td>Water intake (ml/kg/day)</td>
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<td>800 ± 201**</td>
<td>189 ± 133#####</td>
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<td>Urinary output (ml/kg/day)</td>
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<td>671 ± 214**</td>
<td>148 ± 85#</td>
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<td>Feed intake (g/kg/day)</td>
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<td>Fecal output (g/kg/day)</td>
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<td>20.9 ± 0.2#</td>
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<td>Urinalysis (GD18)</td>
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<td>Total protein output (mg/kg/day)</td>
<td>11.3 ± 6.5</td>
<td>35.2 ± 43.4</td>
<td>8.2 ± 1.1</td>
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3.4.2 Decreased plasma protein binding was observed in GDM

The plasma protein binding of glyburide, an acidic drug that binds to albumin, and saquinavir, a basic drug that binds to AAG, was examined. Unbound glyburide was found to be 13.4 ± 2.6% in plasma from animals with GDM, which was higher than controls: 5.2 ± 2.6% in the vehicle group and 3.8 ± 0.9% in the insulin-treated group (p < 0.0001; Fig. 3.1A). For saquinavir, 12.2 ± 1.1% was unbound in plasma from animals with GDM, which was again higher than controls: 9.7 ± 1.2% in the vehicle group and 8.9 ± 1.1% in the insulin-treated group (p < 0.01; Fig. 3.1B). While the unbound percentages in the insulin-treated group were lower than those in the vehicle group, the magnitude of these changes were quite modest. Linear regression revealed a positive relationship between the plasma protein binding of glyburide and saquinavir (r² = 0.85; p < 0.0001; Fig. 3.1C).

3.4.3 Evidence for hyperlipidemia as a mechanism for decreased plasma protein binding in GDM

To determine the contribution of plasma lipids to the altered protein binding observed in the GDM group, plasma samples were delipidated prior to analysis of plasma protein binding capacity (Appendix fig. 3.3B). As illustrated in figure 3.1D, delipidation of plasma from the GDM group dramatically reduced the percentage of unbound glyburide from 13.4 ± 2.6% to 6.1 ± 0.3% (p < 0.01). Post-delipidation protein binding in the GDM group was not statistically different from post-delipidation protein binding in the vehicle group (p > 0.05). Delipidation had no effect on the protein binding of glyburide in the vehicle or insulin-treated groups (p > 0.05).
Figure 3.1. Plasma protein binding characteristics. The plasma protein binding of glyburide/GLIB (A) and saquinavir/SQV (B) was determined in the GDM and control groups and expressed as percent unbound. C, Linear regression was used to determine the relationship between the binding of glyburide and saquinavir. D, Plasma samples were delipidated and then subjected to the glyburide protein binding procedure. All results are presented as mean ± SD of 5 rats/group. *, Significantly different from vehicle controls. †, Significantly different from GDM. *∕†, p < 0.05; **∕††, p < 0.01; ***∕†††, p < 0.001.
3.4.4 Alterations in the expression of hepatic membrane proteins in GDM were largely linked to lipid metabolism

Proteomic analysis consisted of two iTRAQ experiments: experiment 1 compared the GDM and vehicle groups and experiment 2 compared the insulin-treated and vehicle groups. Experiment 1 detected 625 hepatic proteins, 142 of which were significantly altered (Fig. 3.2A), and experiment 2 detected 607, 125 of which were significantly altered (Fig. 3.2B). Comparison of the two iTRAQ datasets revealed a total of 476 proteins that were common: meaning that 149 proteins were detected only in the GDM versus vehicle comparison and 131 proteins were detected only in the insulin-treated versus vehicle comparison. The raw data associated with the proteomics component of this study may be downloaded from the ProteomeCommons.org Tranche network using the following hash:
T4stJnbow+8wF2ifikOiHs3zEw5QOaxzU6fUUL98P2mZ8603m4dj2YU/EXV/kB4kGcvw3DJ3TR mpV0xSp/bfzggyNlgoAAAAAAAAPRw==.

Members of the ATP-binding cassette (ABC) and solute carrier organic anion (SLC) transporters, cytochrome P450 (CYP) enzymes and UDP-glucuronosyltransferases (UGTs) were present in both datasets; however, owing probably to the extensive sharing of peptide sequences, we positively identified only a modest fraction of drug transporters and metabolic enzymes known to be present in rat liver.

Altered lipid metabolism appeared as a function in two of the top five molecular networks that were identified in GDM by IPA software. Of the 142 proteins altered in GDM, 22.5% (32) were identified as having a molecular/cellular function in lipid metabolism (Table 3.2). Of these proteins, 72% (23) were normalized by insulin treatment, 22% (7) lacked comparison data in the insulin-treatment group and 6% (2) were similarly altered.

A review of the literature concerning the proteins listed in table 3.2 indicated that alterations in the GDM group were both pro- and anti-hyperlipidemic. For the purposes of presenting and discussing results, alterations were considered pro-hyperlipidemic if they would be expected to increase the hepatic synthesis of lipids or promote their retention in the blood compartment. Conversely, alterations were considered anti-hyperlipidemic if they would be expected to decrease the hepatic synthesis of lipids or promote their retention in non-blood compartments (e.g., within hepatocytes). The upregulation of Fdft1 and Cyp51a1 in GDM are examples of pro-hyperlipidemic alterations. Examples of anti-hyperlipidemic alterations in GDM include the downregulation of Acaca, Acat1 and Fasn and the upregulation of Acadl. The expression of lipid transporting proteins
was also altered in GDM. Cd36 was downregulated while Abcb4 (Mdr2) and Slcs 27a2 and 27a5 were upregulated.

Interestingly, lipid metabolism did not appear as a function in any of the major molecular networks that were identified in the insulin-treated group. Despite this, alterations in 14 proteins involved in lipid metabolism were found in the insulin-treated group’s proteomics dataset (Table 3.3). As with GDM, alterations in the insulin-treated group were both pro- and anti-hyperlipidemic. The downregulation of Abcd3, Abcg2 and Cd36 in this group are examples of pro-hyperlipidemic alterations. Examples of anti-hyperlipidemic alterations following insulin treatment include the finding that Ebp and Soat2 were downregulated and the finding that Ldlr was upregulated.

Several transporters and metabolic enzymes were upregulated in GDM, including: Abcb4, Cyp4a11, Cyp4a14 and Cyp51a1 as well as Ugt1a1. In the insulin-treated group, Abcd3, Abcg2 and Cyp2b6 were downregulated while Cyp4f8 and Xdh were upregulated. Organic anion transport protein (Oatp) 1a1 was downregulated in both groups.

To investigate the relationships specifically between proteins involved in lipid metabolism, all altered proteins associated with this function in experiment 1 and 2 were isolated and individually subjected to IPA analysis. Figures 3.3 and 3.4 depict molecular networks for these proteins, illustrating the connections between proteins quantified in our experiments as well as a myriad of proteins implicated on the basis of IKB data. In both figures 3.3 and 3.4, one feature that stands out is the presence of network “hubs” that are comprised of nuclear receptors (these appear in blue).
Figure 3.2. Volcano plots depicting the magnitude and statistical significance of hepatic protein alterations in iTRAQ experiments. Negative log p-values for all 625 proteins identified in the GDM versus vehicle group comparison (A) and all 607 proteins identified in the insulin-treated versus vehicle group comparison (B) were plotted against log fold change. Horizontal lines correspond with $\alpha = 0.05$, meaning all proteins found above these lines are statistically different from controls. Vertical lines correspond with the 0.1-fold/10% upregulation and downregulation limits.
Table 3.2. Identified proteins related to lipid metabolism in STZ-induced GDM. Control-normalized “fold changes” were generated for proteins linked to lipid metabolism. Differences of less than 10% were disregarded and statistically significant deviations from control values were identified using t-tests. *, p < 0.05; **, p < 0.01; ***, p < 0.001. NC: no change. Comparison values in the insulin-treated group were designated as NC when they were either not statistically different from those of controls or differed from controls by less than 10%.

<table>
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<th>UniProt</th>
<th>Symbol</th>
<th>Entrez gene name</th>
<th>STZ Coverage (%)</th>
<th>Fold change</th>
<th>STZ + Insulin Coverage (%)</th>
<th>Fold change</th>
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<td>Q08201</td>
<td>Abcb4</td>
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Table 3.3. Identified proteins related to lipid metabolism in insulin-treated STZ-induced GDM. Control-normalized “fold changes” were generated for proteins linked to lipid metabolism. Differences of less than 10% were disregarded and statistically significant deviations from control values were identified using t-tests. *, p < 0.05; **, p < 0.01; ***, p < 0.001. NC: no change. Comparison values in the GDM group were designated as NC when they were either not statistically different from those of controls or differed from controls by less than 10%.

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<tr>
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<th>STZ Coverage (%)</th>
<th>Fold change</th>
<th>STZ + Insulin Coverage (%)</th>
<th>Fold change</th>
</tr>
</thead>
<tbody>
<tr>
<td>P16970</td>
<td>Abcd3</td>
<td>ATP-binding cassette, subfamily D, member 3</td>
<td>56.9</td>
<td>NC</td>
<td>61.6</td>
<td>1.13*</td>
</tr>
<tr>
<td>Q80W57</td>
<td>Abcg2</td>
<td>ATP-binding cassette, subfamily G, member 2</td>
<td>-</td>
<td>-</td>
<td>33.8</td>
<td>1.16*</td>
</tr>
<tr>
<td>Q07969</td>
<td>Cd36</td>
<td>CD36 molecule (thrombospondin receptor)</td>
<td>41.1</td>
<td>1.25**</td>
<td>31.8</td>
<td>1.19*</td>
</tr>
<tr>
<td>P705000</td>
<td>Cdipt</td>
<td>CDP-diacylglycerol—inositol 3-phosphatidyltransferase</td>
<td>-</td>
<td>-</td>
<td>49.8</td>
<td>1.19*</td>
</tr>
<tr>
<td>P04167</td>
<td>Cyp2b6</td>
<td>Cytochrome P450, family 2, subfamily B, polypeptide 6</td>
<td>51.3</td>
<td>NC</td>
<td>40.3</td>
<td>1.11*</td>
</tr>
<tr>
<td>P51869</td>
<td>Cyp4f2</td>
<td>Cytochrome P450, family 4, subfamily F, polypeptide 2</td>
<td>56.3</td>
<td>NC</td>
<td>47.3</td>
<td>1.14**</td>
</tr>
<tr>
<td>Q9JJ46</td>
<td>Ebp</td>
<td>Emopamil binding protein (sterol isomerase)</td>
<td>-</td>
<td>-</td>
<td>27.8</td>
<td>1.25**</td>
</tr>
<tr>
<td>P35952</td>
<td>Ldlr</td>
<td>Low density lipoprotein receptor</td>
<td>21.6</td>
<td>NC</td>
<td>22</td>
<td>1.23*</td>
</tr>
<tr>
<td>Q6AY20</td>
<td>M6pr</td>
<td>Mannose-6-phosphate receptor (cation dependent)</td>
<td>53.2</td>
<td>NC</td>
<td>41.4</td>
<td>1.19*</td>
</tr>
<tr>
<td>P46720</td>
<td>Oatp1a1</td>
<td>Solute carrier organic anion transporter family, member 1A1</td>
<td>27.2</td>
<td>1.14*</td>
<td>28.4</td>
<td>1.17*</td>
</tr>
<tr>
<td>Q6RUUV5</td>
<td>Rac1</td>
<td>Ras-related C3 botulinum toxin substrate 1 (rho family, small GTP binding protein Rac1)</td>
<td>50</td>
<td>NC</td>
<td>24</td>
<td>1.27*</td>
</tr>
<tr>
<td>Q9R0C9</td>
<td>Sigma1</td>
<td>Sigma non-opioid intracellular receptor 1</td>
<td>-</td>
<td>-</td>
<td>48</td>
<td>1.17**</td>
</tr>
<tr>
<td>Q7TQM4</td>
<td>Soat2</td>
<td>Sterol O-acyltransferase 2 (ACAT2)</td>
<td>37.4</td>
<td>NC</td>
<td>28.8</td>
<td>1.2*</td>
</tr>
<tr>
<td>P22985</td>
<td>Xdh</td>
<td>Xanthine dehydrogenase</td>
<td>27.1</td>
<td>NC</td>
<td>23.1</td>
<td>1.14**</td>
</tr>
</tbody>
</table>
Figure 3.3. Lipid metabolism network in the GDM group. IPA software was used to graphically illustrate the molecular relationships between altered proteins that were linked to lipid metabolism and proteins stored in the IKB. Proteins are represented as nodes and the biological relationships between nodes are represented as connecting lines. Proteins are labeled with their Entrez gene symbols. Upregulated proteins are red, downregulated proteins are green, nuclear receptors are blue and proteins inserted by IPA software are white.
Figure 3.4. Lipid metabolism network in the insulin-treated group. IPA software was used to graphically illustrate the molecular relationships between altered proteins that were linked to lipid metabolism and proteins stored in the IKB. Proteins are represented as nodes and the biological relationships between nodes are represented as connecting lines. Proteins are labeled with their Entrez gene symbols. Upregulated proteins are red, downregulated proteins are green, nuclear receptors are blue and proteins inserted by IPA software are white.
3.4.5 Evidence for nuclear receptor activation contributing to drug transporter and metabolic enzyme upregulation

The networks depicted in figures 3.3 and 3.4 highlight the involvement of nuclear receptors with roles in lipid metabolism as well as in the regulation of drug transporters and metabolic enzymes. These networks implicated involvement of FXR, liver X receptor (LXR), nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB), peroxisome proliferator-activated receptors (PPARs) and thyroid hormone receptor (TR) in GDM-mediated alterations. On the other hand, network analysis implicated hepatic nuclear factor 1 alpha and beta (HNF1A/HNF1B), NF-κB and PPARγ in alterations that were observed in the insulin-treated group. Given the apparent activity of these nuclear receptors, at least within the subset of our data concerned with lipid metabolism, we decided to compare all altered proteins observed in both iTRAQ experiments to nuclear receptor activation “Tox Lists” in the IKB (Table 3.4). At present, “Tox Lists” are available for the activation of FXR, LXR, PPARs and PXR as well as the constitutive androstane receptor (CAR) and nuclear factor erythroid 2-related factor 2 (Nrf2). With the exception of CAR, relative to animals in the insulin-treated group, animals in the GDM group consistently exhibited a higher number of alterations to proteins associated with the activation of these nuclear receptors (22 unique proteins versus 10 unique proteins). As predicted, many of the specific proteins that overlapped with nuclear receptor activation “Tox Lists” were transporters, CYPs or other proteins of relevance to drug transport and metabolism. It is important to note that the majority of proteins presented in table 3.4 were identified in both iTRAQ experiments.

Table 3.4 also includes data from two additional lists: 1) CYPs with xenobiotic substrates and 2) xenobiotic metabolism. Including these additional lists revealed a handful of differentially expressed proteins that did not appear in lipid metabolism networks or the nuclear receptor activation “Tox Lists.” For the GDM group, the additional lists identified Cyp2a1, Cyp2b9 and Cyp2d3 as xenobiotic metabolizing enzymes. All three proteins were upregulated in GDM. For the insulin-treated group, the additional lists identified Aldh1l1, Gsta4, Gstm1 and Ugt1a4 as xenobiotic metabolizing enzymes. Ugt1a4 was downregulated while Aldh1l1, Gsta4 and Gstm1 were upregulated.

Due to our evidence suggesting activation of FXR and PXR via the upregulation of their target genes (e.g., Mdr2), the FXR/PXR “target gene” Mdr1 was examined at the level of mRNA and protein. Mdr1 was absent from both iTRAQ datasets and this supplementary analysis allowed us to further test our hypothesis that hyperlipidemia would have indirect effects on drug transporters.
As figure 3.5 illustrates, Mdr1b/18S standard ratios were higher in GDM at 0.26 ± 0.15 as compared to 0.01 ± 0.004 in the vehicle group and 0.03 ± 0.02 in the insulin-treated group (p < 0.01).

Mdr1/β-actin standard ratios were also higher in GDM at 0.41 ± 0.1 as compared to 0.23 ± 0.07 in the vehicle group and 0.22 ± 0.06 in the insulin-treated group (p < 0.01). All groups had comparable Mdr1a/18S standard ratios (p > 0.05).

Table 3.4. Comparisons of proteins that were altered in iTRAQ experiments to “Tox Lists” in the Ingenuity Knowledge Base. Datasets were examined by IPA software for overlap with “Tox List” proteins. “Tox Lists” are lists of proteins that are known to be involved in particular aspects of toxicity and our focus was on lists that addressed nuclear receptor activation and xenobiotic metabolism. Bolded entries were not common to the datasets of both experiments.

<table>
<thead>
<tr>
<th>“Tox List” ID (Total number of list members)</th>
<th>STZ</th>
<th>STZ + Insulin</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Up-regulated</td>
<td>Down-regulated</td>
</tr>
<tr>
<td>CAR/RXR activation (28)</td>
<td>Ugt1a1</td>
<td>-</td>
</tr>
<tr>
<td>FXR/RXR activation (66)</td>
<td>Abcb4, Apoa1, G6pc, S1c27a5</td>
<td>Fasn</td>
</tr>
<tr>
<td>LXR/RXR activation (55)</td>
<td>Apoa1</td>
<td>Acaca, Cd36, Fasn</td>
</tr>
<tr>
<td>Nrf2 activation (148)</td>
<td>Cyp2a1, Cyp4a11, Cyp4a14</td>
<td>Hmox1</td>
</tr>
<tr>
<td>PPARα/RXR activation (118)</td>
<td>Acadl, Apoa1</td>
<td>Cd36, Fasn</td>
</tr>
<tr>
<td>PXR/RXR activation (57)</td>
<td>Aldh1a7, G6pc, Hmgcs2, Ugt1a1</td>
<td>-</td>
</tr>
<tr>
<td>Cytochrome P450 panel (rat), substrate is a xenobiotic (23)</td>
<td>Cyp2a1, Cyp2d3</td>
<td>-</td>
</tr>
<tr>
<td>Xenobiotic metabolism (89)</td>
<td>Cyp2a1, Cyp2b9, Cyp2d3, Cyp51a1, Ugt1a1</td>
<td>-</td>
</tr>
</tbody>
</table>
Figure 3.5. Hepatic Mdr1 expression. Hepatic Mdr1 was examined at the level of mRNA via qRT-PCR and at the level of protein via immunoblotting in the GDM and control groups. For mRNA, both genes encoding rodent Mdr1 were examined: Mdr1a and Mdr1b. All results are presented as mean ± SD of 5 rats/group. *, Significantly different from vehicle controls. #, Significantly different from GDM. **, p < 0.05; ***, p < 0.01.
3.5 Discussion

In this study, we employed the STZ rat model of GDM to determine if plasma lipids compete with drugs for protein binding in this disease. We also used this model to examine whether GDM is associated with alterations in hepatic drug transporters and metabolic enzymes.

With respect to plasma protein binding, we first determined the concentrations of two important plasma proteins: albumin and AAG. In humans, glyburide is 98% bound to albumin (Olsen et al., 1995) and was 95% protein bound in this study's vehicle group. In humans, saquinavir is 98% bound to AAG (Holladay et al., 2001) and was 90% protein bound in this study’s vehicle group. In GDM, albumin concentrations were decreased and AAG levels were increased. This is consistent with studies involving patients with uncontrolled clinical diabetes (Mattock et al., 2001).

If plasma protein concentrations were the sole determinant of protein binding then one would expect, based on albumin and AAG levels, decreased binding of glyburide and increased binding of saquinavir in GDM. However, in this study, the protein binding of both glyburide and saquinavir was lower in GDM than in the control groups. Moreover, as illustrated in figure 3.1C, the extent of one drug’s protein binding was found to be highly predictive of the other’s. This is indicative of a contribution from a factor other than absolute plasma protein content.

To test whether plasma lipids were, as hypothesized, competing with drugs for binding, we repeated our glyburide protein binding experiments with delipidated plasma samples. If plasma protein concentrations were the sole determinant of protein binding then one would also expect that delipidation would have no effect on protein binding; however, this was not the case in this study. Delipidation completely normalized the protein binding of glyburide in GDM yet did not affect protein binding in either the vehicle or insulin-treated groups. It has previously been reported that the binding capacity of human albumin for glyburide was halved by protein glycosylation, a process which can occur in GDM (Koyama et al., 1997). If glycosylation was preventing glyburide protein binding in our study, however, the plasma protein binding of glyburide should not have been completely normalized by delipidation alone. Moreover, delipidation did not affect glyburide protein binding in controls. Taken together, our results provide strong evidence in support of hyperlipidemia contributing directly to decreased plasma protein binding of drugs in GDM.

In our proteomics experiments, many alterations in GDM occurred in proteins that are linked to lipid metabolism. While studies have associated the etiology of hyperlipidemia in STZ-induced diabetes with decreased insulin signaling and altered fat absorption, the contribution of hepatic proteins has remained largely unexplored.
Based on our findings in GDM, there appear to be a number of pro- and anti-hyperlipidemic responses in the liver and some of these changes could greatly influence plasma lipids. One particularly interesting pro-hyperlipidemic response was the upregulation of Fdft1 and Cyp51a1 in GDM, both of which are proteins that play critical roles in the conversion of acetyl-CoA to cholesterol. In fact, Fdft1 is a potential therapeutic target for hyperlipidemia and an Fdft1 inhibitor, lapaquistat/TAK-475, has been shown efficacious in preclinical and clinical studies (Nishimoto et al., 2003; Piper et al., 2006). Many of the pro-hyperlipidemic responses we observed are likely the result of excess acetyl-CoA being shuttled to alternative routes of utilization. It is possible that, in GDM, enhanced hepatic lipid production is preferential to excess blood glucose.

With respect to interesting anti-hyperlipidemic hepatic responses in GDM, Acaca, Acat1 and Fasn were downregulated and Acadl was upregulated. Acaca catalyzes the carboxylation of acetyl-CoA to produce malonyl-CoA, a substrate for the biosynthesis of fatty acids. Acat1 catalyzes the esterification of cholesterol, which is subsequently incorporated into LDL and secreted. Fasn’s main function is to catalyze the synthesis of acetyl-CoA and malonyl-CoA into long-chain saturated fatty acids. Decreased expression of Acaca, Acat1 and Fasn in GDM would, therefore, be expected to decrease the synthesis and secretion of lipids by the liver. Acadl catalyzes the initial step in mitochondrial fatty acid oxidation, whereby fatty acids are broken down to generate acetyl-CoA. This increased expression of Acadl in GDM would be expected to increase fatty acid utilization. While interesting, it is important to note that these anti-hyperlipidemic hepatic responses, as well as those involving lipid transporting proteins, did not correct the hyperlipidemia present in our model of GDM. Thus, the regulation of proteins linked to lipid metabolism is pathologically disrupted in GDM. Studies to determine the relative contribution of the pro- and anti-hyperlipidemic alterations described here are warranted.

An indirect effect of dysregulation in lipid metabolism regulatory pathways, and one for which this study provides support, is an activation of nuclear receptor networks which have roles in the regulation of hepatic drug transporters and metabolic enzymes. Studies have demonstrated that several nuclear receptors have lipids as natural ligands (Handschin and Meyer, 2005). Activation of several of these nuclear receptors is consistent with our observation that many of the drug transporters and metabolizing enzymes that we identified were upregulated in GDM. Activation of FXR, for example, is consistent with the upregulation of Abcb4 (Mdr2) observed in GDM. FXR also regulates, with PXR, Mdr1 (Stedman et al., 2006) and upregulation of this important drug transporter was observed in GDM. Another example is the activation of CAR and PXR, which is consistent with the upregulation of Ugt1a1 and Cyp2a1 observed in GDM. These findings
complement a body of literature demonstrating increased drug metabolizing capacities in experimental animals and humans with diabetes (Barnett et al., 1990; Song et al., 1990). Interestingly, Oatp1a1, which can be downregulated in response to CAR, Nrf2 or PPARα activation (Cheng et al., 2005; Maher et al., 2006), was downregulated in the GDM and insulin-treated groups.

The finding that several ABC transporters were upregulated in GDM is contrary to our previous report in non-pregnant, female rats with acute STZ-induced T1DM, which demonstrated that the hepatic expression of Mdr1a, Mrp2 and Bcrp mRNA was downregulated (Anger et al., 2009). In that study, rats were diabetic for half as long as the rats in this study and had not yet developed overt hyperlipidemia, as measured on a lipemia index (Appendix fig. 3.4A). Moreover, in our previous report we provided preliminary evidence that inflammation plays a role in the observed downregulation but inflammatory responses can be blunted during late pregnancy (Aguilar-Valles et al., 2007), which is supported by the fact that plasma CRP was not elevated in GDM (Appendix fig. 3.4B). We speculate that the differences between STZ-induced GDM and acute STZ-induced T1DM stem from an absence of hyperlipidemia as well as the presence of inflammation in the latter. It would be interesting to compare the results obtained in our GDM model with those of a model of pregnancy complicated by maternal type 1 diabetes, wherein the total duration of maternal diabetes could be extended even further (to encompass the entire course of gestation).

Our proteomics findings in the insulin-treated group deserve further comment. First, insulin treatment did not normalize the expression of all proteins that were altered in GDM. Some of the changes in the insulin-treated group could be due to our insulin treatment protocol. Daily injections of long-acting insulin lead to oscillating blood glucose concentrations with the potential for brief periods of hyperglycemia pre-injection and hypoglycemia post-injection. It is possible that this was sufficient to stimulate some of the observed changes, both those shared with the GDM group and those unique to the insulin-treated group. While hyperlipidemia is unlikely to have been transiently induced by these brief periods of abnormal blood glucose, some of the nuclear receptors discussed here are also capable of sensing glucose. LXR is an example (Mitro et al., 2007).

Second, insulin treatment itself is likely to have affected the expression of hepatic proteins as there were proteins that were altered in the insulin-treated group that were not altered in GDM. Pro-hyperlipidemic alterations in the insulin-treated group that did not occur in GDM included the downregulation of Abed3. Abed3 is involved in the transport of lipids and downregulation of this protein would be expected to prevent the movement of plasma lipids to hepatic routes of metabolism or elimination. Insulin-mediated anti-hyperlipidemic alterations that did not occur in
GDM included the downregulation of Soat2 and the upregulation of Ldlr. Soat2 produces intracellular cholesterol esters from long-chain fatty acyl-CoA and cholesterol. As noted with Acat1, esterified cholesterol in the liver is subsequently incorporated into LDL and secreted. Ldlr expression and activity is a major determinant of plasma cholesterol content, by binding to LDL and transporting it across membranes, and its upregulation in the insulin-treatment group would be expected to play an anti-hyperlipidemic role. An explanation for these alterations not being observed in the GDM group could be that some of these proteins respond to insulin levels and are, consequently, sensitive to the exogenous administration of insulin. Ldlr is such a protein (Wade et al., 1989). Returning to plasma protein binding in our model of GDM, albumin production is also stimulated by insulin and this could explain the slightly elevated albumin concentrations and slightly increased protein binding observed in insulin-treated rats (Lloyd et al., 1987).

In conclusion, our findings demonstrate that hyperlipidemia in GDM contributes directly to decreased protein binding of drugs and indirectly to drug transporter and metabolic enzyme upregulation. Our findings advance our understanding of the impact that GDM-induced hyperlipidemia can have on these specific drug disposition mechanisms. As mentioned, protein binding strongly influences a drug’s distribution and/or clearance. With distribution, placental transfer of drugs and the uptake of drugs by the liver and kidneys could significantly increase. Elevated hepatic drug transporters and metabolizing enzymes in GDM would be expected to compound changes in clearance that relate to elevated uptake of drugs by the liver and kidneys. If confirmed in clinical populations, effects reported in this study would need to be considered when atypical therapeutic outcomes are observed in pregnancies complicated by GDM.
Appendix 3.1. Work flow for iTRAQ experiments. 1, On GD20, livers were extracted from animals treated as per this study’s groups (n = 3-4/group). 2, Protein was extracted and membrane protein content was enriched. 3 and 4, Membrane-enriched samples were given iTRAQ labels. 5, Two mixtures were generated from labeled samples: one containing a sample from each of the GDM and vehicle group samples and a second containing a sample from each of the insulin-treated and vehicle group samples. 6-8, Mixtures were processed according to the procedures described in 4.3 Methods.
Appendix 3.2. Time course of STZ-induced hyperglycemia (A) and weight gain (B) in pregnancy.
Blood glucose and body weight were monitored from GD6 to GD20 in pregnant rats injected with STZ (45 mg/kg), with or without subsequent insulin treatment, or vehicle control. Baseline measurements were made prior to injection on GD6. Values represent the mean ± SD of 9 rats/group. *, p < 0.05; **, p < 0.01; ***, p < 0.001. Statistical test employed: two-way ANOVA followed by Bonferroni post-tests.
Appendix 3.3. Photographs demonstrating overt hyperlipidemia in experimental GDM (A) and the effect of delipidation on a hyperlipidemic sample (B). Photographs depict representative samples. VEH, vehicle group; GDM, GDM group; INS, insulin-treatment group.
Appendix 3.4. Lipemic indices (A) and plasma C-reactive protein concentrations (B) in Anger et al., 2009 (Chapter 2) and Anger and Piquette-Miller, 2010 (Chapter 3). Lipemic index, an indicator of plasma lipid content, and C-reactive protein concentration, an indicator of inflammation, was determined in each plasma sample from both Anger et al., 2009 (white bars) and Anger and Piquette-Miller, 2010 (gray bars). A, Lipemic indices were determined by Vita-Tech Laboratories via turbidimetry. A value of 0 indicates no turbidity, whereas 1 indicates slightly turbid (hazy), 2 indicates moderately turbid (milky) and 3 indicates markedly turbid (creamy). B, C-reactive protein concentrations were determined by ELISA. The ELISA kit and method employed with it were the same for both studies. Additionally, the same C-reactive protein calibrator lot was used for both studies. Detailed information regarding methods can be found in the methods sections of either paper. All results are presented as mean ± SD *. Significantly different from indicated, bracketed bars (A) or significantly different from non-pregnant vehicle controls (B). * p < 0.01. Statistical test employed: one-way ANOVA followed by Bonferroni post-tests.
Chapter 4. Mechanisms of Reduced Maternal and Fetal Lopinavir Exposure in a Rat Model of Gestational Diabetes

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4.1 Abstract

Lopinavir is the preferred HIV protease inhibitor in pregnancy but it is unknown if gestational diabetes mellitus (GDM) affects its disposition. Hepatic protein expression and plasma protein binding is altered in rodent models of GDM. Because lopinavir is influenced by hepatic transporters and metabolic enzymes and is highly protein bound, it was hypothesized that streptozotocin-induced GDM would alter its disposition. Maternal and fetal tissues were collected from GDM rats and controls 45 min after lopinavir injection. In another cohort, fetuses were serially extracted 5-60 min after injection. Lopinavir was quantified using LC-MS/MS. Expression of relevant transporters, like Mdr1, and Cyp3a2, which metabolizes lopinavir in rodents, was measured in maternal liver via qRT-PCR and western blot. Expression of relevant transporters was also measured in placenta via qRT-PCR. Protein binding was determined by ultrafiltration. Relative to controls, we observed dramatically reduced maternal and fetal lopinavir exposure in GDM. As compared to controls, maternal hepatic Mdr1 and Cyp3a2 were upregulated and protein binding was reduced in the GDM group. Increased Mdr1 and Cyp3a2-mediated hepatobiliary clearance, coupled with a larger unbound lopinavir fraction, is likely to have facilitated hepatic elimination, thereby decreasing maternal and fetal exposure. Not surprisingly, upregulation of Mdr1 and Cyp3a2’s transcriptional regulator, pregnane X receptor, was demonstrated in maternal liver via western blot. Upregulation of Mdr1 in placentas isolated from the GDM group likely also contributed to decreased fetal exposure to lopinavir. This study provides preclinical support for an as yet unreported drug-disease (LPV-GDM) interaction.
4.2 Introduction

Antiretroviral drugs are administered to human immunodeficiency virus (HIV)-positive pregnant women to prevent both maternal disease progression and vertical HIV transmission. Lopinavir (LPV) co-formulated with low-dose ritonavir (RTV) is currently the preferred HIV protease inhibitor combination for pregnant women because of superior efficacy and extensive patient experience in pregnancy (http://aidsinfo.nih.gov). As such, the majority of HIV-positive pregnant women in North America now utilize LPV/RTV as part of their highly active antiretroviral therapy (HAART) regimens. For LPV/RTV to be effective, optimal LPV plasma concentrations must be maintained throughout gestation (Boffito et al., 2002; Masquelier et al., 2002).

Variation in LPV exposure has been observed with standard dosing (Guiard-Schmid et al., 2003) and is likely caused by individual variability in protein binding and drug transporter and metabolic enzyme expression. LPV is a substrate for the multidrug resistance protein 1 (MDR1) drug efflux transporter (Woodahl et al., 2005; Agarwal et al., 2007) and is extensively metabolized by cytochrome P450 3A4 (CYP3A4) (van Waterschoot et al., 2010). In the liver, MDR1 facilitates LPV’s hepatobiliary excretion while CYP3A4 converts it to increasingly hydrophilic metabolites (Fig. 4.1). Large intersubject differences in the basal expression and activity of CYP3A4 and MDR1 have been reported (Westlind-Johnsson et al., 2003; Meier et al., 2006). In the placenta, MDR1 plays a barrier role by actively transporting substrates back into the systemic circulation. LPV is also highly (>99%) bound to blood proteins, including albumin and α1-acid glycoprotein (AAG), so even small changes in binding result in large changes in unbound drug fractions. As only unbound drugs traverse cell membranes, variation in LPV protein binding translates into deviations in the with which LPV passes into maternal and fetal tissues.

Individual variability in these mechanisms can be introduced by a variety of factors, including disease. Reports of drug disposition being sensitive to disease have appeared in the literature for nearly 40 years (Reviewed in Morgan et al., 2008); however, only in the last decade have the effects of disease during pregnancy, including gestational diabetes mellitus (GDM), been investigated (Wang et al., 2005; Petrovic et al., 2008; Anger and Piquette-Miller, 2010). Studies indicate that the prevalence of GDM in HIV-positive pregnant women far exceeds that of HIV-negative pregnant women (Watts et al., 2004; Kourtis et al., 2006; Martí et al., 2007; González-Tomé et al., 2008) but, to the best of our knowledge, it is unknown if this has an effect on the disposition of HAART drugs. In rats with streptozotocin (STZ)-induced GDM, we have recently observed that the plasma protein binding of the protease inhibitor saquinavir decreases because of displacement by
elevated lipids while the expression of hepatic Mdr1 and a variety of cytochrome P450 and UDP-glucuronosyltransferase enzymes increases (Anger and Piquette-Miller, 2010). Also, altered disposition has been observed for a variety of drugs in both non-pregnant humans with diabetes (Reviewed in Gwilt et al., 1991) and non-pregnant rats with STZ-induced diabetes (Reviewed in Lee et al., 2010).

In this study, maternal and fetal LPV exposure was examined in rats with STZ-induced GDM and related to protein binding and hepatic and placental drug transporter/metabolic enzyme expression data. In terms of transporters, the expression of Mdr1 (encoded in rodents by the Mdr1a and Mdr1b genes), multidrug resistance-associated protein 2 (Mrp2) and the breast cancer resistance protein (Bcrp) was determined in maternal liver and placenta. Mrp2 and Bcrp were included because of documented interactions with LPV and/or other antiretroviral drugs. In terms of metabolic enzymes, only Cyp3a2 was examined. Whereas CYP3A4 is the dominant constitutive CYP3A subfamily isoform in humans, Cyp3a2 is the dominant constitutive Cyp3a subfamily isoform in rats (Nelson et al., 1993) and has been shown to metabolize prototypical human CYP3A4 substrates like verapamil (Tracy et al., 1999; Choi and Burm, 2008; Hanada et al., 2008). Given our previous findings with this model (Anger and Piquette-Miller, 2010), we hypothesized that maternal LPV exposure would be reduced. Our hypothesis regarding fetal LPV exposure was dependent on whether protein binding or active feto-maternal transport dominated LPV transfer (and whether placental Mdr1 was upregulated). Decreased protein binding could increase fetal LPV exposure by facilitating materno-fetal placental transfer but placental Mdr1 could limit fetal exposure by actively transporting LPV in a feto-maternal direction. Since the quantitative contribution of these processes could change with time, we utilized a novel fetal sampling procedure to determine the time course of fetal exposure. Due to its role in Mdr1, Mrp2, Bcrp and Cyp3a2 transcription (Lehmann et al., 1998; Reviewed in Tirona, 2011), pregnane X receptor (PXR) expression in maternal liver was also determined.

Our findings further advance our understanding of the impact that GDM can have on drug disposition mechanisms and provide preclinical support for an as yet unreported drug-disease (LPV-GDM) interaction. Effects associated with GDM would compound the drug disposition effects that have been associated with pregnancy itself.
Figure 4.1. The dominant Cyp3a-mediated metabolic pathway for lopinavir. LPV (A) is metabolized to M3/M4 (B; 4-hydroxy-LPV) by Cyp3a and M3/M4 is subsequently metabolized to M1 (C; 4-oxo-LPV), also by Cyp3a. Carbon-4 is the location for these metabolic reactions, which is indicated by an asterisk in A. LPV is a substrate for MDR1. The M3/M4 and M1 LPV metabolites are likely substrates for MDR1.
4.3 Methods

4.3.1 Pharmaceutical and chemical products

LPV and RTV were purchased from USP (PubChem Compound IDs 92727 and 392622, Rockville, MD). Lopinavir metabolites M1 and M3/M4 were purchased from Toronto Research Chemicals, Inc. (North York, ON). Insulin glargine was purchased from a commercial pharmacy in Toronto, ON (Sanofi-Aventis Canada, Inc., Laval, QC). Ethanol and methanol were purchased from Commercial Alcohols (Brampton, ON) and Caledon (Georgetown, ON), respectively. Unless otherwise noted, all other chemical products, including streptozotocin (PubChem Compound ID 5300), were purchased from Sigma-Aldrich (Oakville, ON). For intravenous administration, LPV was dissolved in 4:3:3 (v/v/v) ethanol:propylene glycol:5% dextrose in water (10 mg/ml).

4.3.2 Animals

Timed pregnant Sprague-Dawley rats (208–265 g on gestational day 6) were purchased directly from Charles River Laboratories, Inc. (Senneville, QC) and housed individually in a temperature controlled facility on a 12:12 hr light-dark cycle. Rats were given free access to water and standard chow (Harlan Teklad Global Diet 2018). Experiments were approved by the Office of Research Ethics at the University of Toronto and performed in accordance with Canadian Council on Animal Care guidelines.

4.3.3 Animal treatments/monitoring and tissue collection

Rats were randomly assigned to one of three groups: non-treated diabetics (the GDM group), vehicle controls (the vehicle group) and insulin-treated diabetics (the insulin-treated group). On gestational day 6 (GD6), diabetes was induced by way of a single subcutaneous injection of streptozotocin in 0.1 M citrate buffer (pH 4.5) at a dosage of 45 mg/kg. Vehicle control animals received a single subcutaneous injection of citrate buffer on GD6. For all animals, body weight was measured prior to respective injections (GD6) and then daily until sacrifice. For animals in the GDM and vehicle groups, blood glucose was measured prior to respective injections (GD6) and then daily until sacrifice. For animals in the GDM and vehicle groups, blood glucose was measured prior to respective injections (GD6) and then daily until sacrifice. For animals in the insulin-treated group, blood glucose was measured prior to STZ injection and then daily until sacrifice in order to determine daily insulin dosage requirements. Blood glucose was measured at noon, in a non-fasted state, using a typical glucometer with blood obtained via tail-prick. When values exceeded the range of this glucometer, a commercially available enzymatic glucose assay was employed according to the manufacturer’s instructions (Autokit Glucose, Wako Chemicals USA, Inc., Richmond, VA). Mild hyperglycemia was
defined as blood glucose concentration at 10 to 14 mM and diabetes was defined as blood glucose concentration at >14 mM. For diabetic rats receiving insulin treatment, treatment was initiated once diabetes was confirmed and consisted of daily subcutaneous injection of insulin glargine as required. On GD20, one cohort of animals (n = 4-5/group) received LPV (10 mg/kg; intravenous) 45 ± 1 min prior to being sacrificed to collect maternal blood (cardiac puncture) and liver as well as up to 10 fetal compartments/dam. The location of each fetal compartment within the uterine horn was noted (e.g., left versus right horn and distance, in fetus number, away from the cervical stump). Amniotic fluid was also collected from this first subset of animals. Aliquots of whole blood were transferred to thrombin-coated BD Vacutainer tubes (BD Canada, Mississauga, ON) and spun to collect serum. Samples were snap-frozen and stored at -80 °C until use.

4.3.4 Sampling of fetal compartments for time course determination

On GD20, a second cohort of animals (n = 3/group) received LPV (10 mg/kg; intravenous) while anesthetized with continuous inhalation anesthesia (5% isoflurane for induction and 1.5-2% isoflurane for maintenance) and was subsequently subjected to sequential sampling of placentas and fetuses (collectively referred to as fetal compartments) over a period of one hr. Following LPV administration, a 3-4 cm midline abdominal incision was made so as to provide access to the uterine horns via the intraperitoneal cavity. Five min post-injection, one of the uterine horns was externalized and the most distal fetal unit identified (Appendix fig. 4.1 illustrates fetal positioning within the pregnant rat at approximately GD20). The maternal vasculature supplying this fetal unit’s placenta was then blocked via ligation in order to minimize both maternal and fetal blood loss. Once the placental blood supply was blocked, the fetal unit was removed through a small incision made in the uterine wall. The remaining pups, still situated within the uterine horn, were then returned to the maternal intraperitoneal cavity to await future time points. Additional fetal compartments were collected from more proximal uterine horn positions and from the unused uterine horn at 10, 15, 20, 30, 45 and 60 min post-injection. Fetal compartments were not collected if their placental blood supply was compromised during the collection of another fetal unit (e.g., if they shifted position in such a way that their blood vessels sheared). Following the collection of the 60 min fetal unit, maternal blood (cardiac puncture) and liver was collected. Throughout this procedure, maternal body temperatures were kept at approximately 37 °C using heating pads.

4.3.5 Determination of lopinavir and lopinavir metabolite concentrations

For the preparation of samples and standards, a liquid-liquid extraction procedure was adapted from Wang and colleagues (Wang et al., 2006). Briefly, serum and tissue samples were
thawed to room temperature. Tissues (maternal livers, placentas and fetuses) were homogenized in deionized water in glass tubes. For samples, 100 µl of serum or homogenate was added to tubes containing 20 ng of pre-dried internal standard (internal standard = RTV; final internal standard concentration: 200 ng/ml). For LPV standards, 100 µl of drug-free serum or homogenate was added to tubes containing both 20 ng of pre-dried internal standard and 1-100 ng of pre-dried LPV (final LPV standard concentrations: 10-1000 ng/ml). LPV metabolites M3/M4 and M1 were only measured in maternal liver because the placenta and fetus are not significant sites of LPV metabolism. For LPV metabolite standards, 100 µl of drug-free liver homogenate was added to tubes containing internal standard as well as a combination of 1-100 ng of pre-dried LPV, M3/M4 and M1 (final standard concentration for each of LPV, M3/M4 and M1: 10-1000 ng/ml). Fifty µl of 500 mM Na₂CO₃ was then added to the resulting samples and standards. After mixing by vortex for 30 s, 1.2 ml of 1:1 (v/v) hexane:ethyl acetate was added to each tube. Mixtures were then vortexed for 2 min to ensure complete extraction. The organic layer was separated from the aqueous layer by centrifugation at 21,000 g for 10 min at 4 °C. Seven hundred µl of the organic supernatant was transferred to a fresh tube and dried under nitrogen gas. Extracts were reconstituted in 200 µl of 80% methanol, vortexed for 30 s and then centrifuged at 21,000 g for 5 min at 4 °C. Aliquots of the resulting extracts were transferred to autosampler vials and stored at 4 °C until 10 µl could be injected into the liquid chromatographic (LC) system. Using concentration estimates obtained in pilot experiments, serum and homogenates were diluted with volumes of deionized water prior to processing so as to ensure that their concentrations fell within the mid-range of calibration curves.

LPV concentrations were determined using high-performance liquid chromatography-tandem mass spectrometry (LC-MS/MS). The liquid chromatographic system consisted of a CTC PAL autosampler (LEAP Technologies, Carrboro, NC) and an Agilent Technologies (Santa Clara, CA) 1100 series pump with a 50 mm × 4.6 mm, 5 µm, Lichrosorb RP-8 column (Phenomenex, Torrance, CA), controlled at room temperature with a flow rate of 700 µl/min. Separation of LPV and internal standard was accomplished using isocratic elution with 20:80 parts of 0.1% formic acid in water and 80% methanol. After peak elution, the mobile phase was changed to 100% methanol to wash the column. Following sample injections, the syringe and injector valve were each washed 3 times with methanol and 3 times with water. MS/MS was performed on an API 4000 triple quadrupole mass spectrometer equipped with a Turbo Ionspray source operating in a positive ion multiple reaction monitoring (MRM) mode (AB Sciex, Concord, ON). The MRM transitions were
m/z 629.3-447.3 (declustering potential 58 volts, collision energy 21 volts) for LPV, m/z 645.3-447.3 (declustering potential 40 volts, collision energy 23 volts) for M3/M4, m/z 643.3-447.2 (declustering potential 80 volts, collision energy 23 volts) for M1 and m/z 721.3-268.0 (declustering potential 60 volts, collision energy 27 volts) for RTV (the internal standard). Source temperature was set to 500 °C. Peak areas of LPV, LPV metabolites and the internal standard were determined using Analyst software, version 1.4.2 (AB Sciex). For quantification, calibration curves were derived for each sample type by plotting the peak area ratio (LPV or LPV metabolite peak area/internal standard peak area) of standards against their concentrations. Linear regression equations with 1/x weighting were then used to calculate sample LPV and/or LPV metabolite concentrations. Across tissues, based on signal-to-noise ratios, the lower limit of detection was < 3 ng/ml and the lower limit of quantification was < 10 ng/ml. Appendix fig. 4.2 illustrates the chromatographic separation of lopinavir, the M3/M4 lopinavir metabolites, the M1 lopinavir metabolite and internal standard/ritonavir in a representative vehicle control liver sample.

4.3.6 ATPase activation assay

Following the manufacturer’s instructions, rat Mdr1b PREDEASY ATPase kits (Solvo Biotechnology, Szeged, Hungary) were used to determine if LPV’s M3/M4 and M1 metabolites are transported by rat Mdr1b. Briefly, rat Mdr1b-expressing Sf9 membrane vesicles were incubated in ATPase assay buffer (5 mM DTT, 0.1 mM EGTA, 50 mM KCl, 10 mM MgCl₂, 40 mM MOPS-Tris [pH 7.0] and 4 mM sodium azide) containing 5 mM ATP and M3/M4 or M1, at eight concentrations ranging from 0.05-100 µM for M3/M4 and 0.14-300 µM for M1, for 10 min at 37 °C with and without 1.2 mM sodium orthovanadate. Because it is an established Mdr1 substrate, LPV was also assayed at eight concentrations ranging from 0.14-300 µM. Specific Mdr1-related ATPase activity was determined by calculating the difference of inorganic phosphate liberation measured with and without 1.2 mM sodium orthovanadate (vanadate-sensitive ATPase activity). ABC transporters like Mdr1b are inhibited by sodium orthovanadate so determining vanadate-sensitive ATPase activity distinguishes Mdr1-related ATPase activity from the activity of other ATPases that are found in membrane preparations.

4.3.7 Blood chemistry

Determination of total cholesterol, high-density lipoprotein (HDL)/low-density lipoprotein (LDL) cholesterol, triglyceride, FFA and albumin concentrations in serum was outsourced to the Banting and Best Diabetes Centre (University of Toronto). Total cholesterol, HDL/LDL
cholesterol, triglyceride and albumin concentrations were analyzed using an autoanalyzer and commercially available reagents (Roche Diagnostics, Laval, QC) while FFA was analyzed using a commercially available ELISA kit (Wako Chemicals USA, Inc.). AAG concentrations were determined in-house using a commercially available, rat-specific ELISA kit (GenWay, San Diego, CA), according to the manufacturer's instructions.

4.3.8 PCR

In this study, Mdr1a/b, Mrp2, Bcrp and Cyp3a2 mRNA levels in maternal liver (n = 4-5 dams/group) and Mdr1a/b, Mrp2 and Bcrp mRNA levels in placenta (n = 3 placentas/dam; n = 4-5 dams/group) were determined by quantitative reverse transcriptase-polymerase chain reaction (qRT-PCR). Total RNA was extracted from tissue using the TRIZOL method (Invitrogen, Carlsbad, CA) and reverse transcribed to cDNA via the First Strand cDNA Synthesis Kit (Fermentas, Burlington, ON), according to the manufacturer's instructions. qRT-PCR was then performed using LightCycler technology with SYBR Green I fluorescence detection (Roche Diagnostics). PCR oligonucleotides were synthesized at The Hospital for Sick Children (DNA Synthesis Centre, Toronto, ON) and their sequences have been previously published (Petrovic et al., 2008). Expression levels were normalized to 18S rRNA expression using the efficiency-corrected ΔCt method and presented as a gene/18S ratio.

4.3.9 Western blotting

Mdr1, Cyp3a2 and PXR.1 protein levels were examined in maternal liver via western blotting. Tissue samples were first homogenized in RIPA buffer, containing freshly added DTT (1 mM), PMSF (0.5 mM) and 1X protease inhibitor cocktail (Sigma-Aldrich), using a motorized pestle. Homogenates were then incubated on ice for 20 min and subsequently centrifuged at 18,000 g for 15 min. For each sample, the supernatant was isolated and subjected to a Bradford assay to determine total protein concentration. Samples containing 60 µg (Mdr1), 20 µg (Cyp3a2) or 50 µg (PXR.1) of protein in Laemmli sample buffer were heated at 36 °C for 20 min (Mdr1) or 95 °C for 5 min (Cyp3a2 and PXR.1) and then separated via 8% (Mdr1) or 10% (Cyp3a2 and PXR.1) SDS-polyacrylamide gel electrophoresis and transferred to polyvinylidene fluoride membranes (Bio-Rad Laboratories Canada, Ltd., Mississauga, ON). Membranes were blocked in 5% fat-free milk powder at room temperature for 1 hr and incubated with mouse anti-Mdr1 (C219, 1:500, 1 mg/ml, Abcam, Inc., Cambridge, MA), rabbit anti-Cyp3a2 (ab78279, 1:1000, whole antiserum, Abcam, Inc.) or goat anti-PXR.1 (A-20, 1:100, 0.2 mg/ml, Santa Cruz Biotechnology, Inc., Santa Cruz, CA) antibodies at
4 °C overnight on a rocker. After a series of washes, membranes were incubated with a peroxidase-labeled secondary antibody for 2 hr at room temperature (goat anti-mouse at 1:3,000 for Mdr1, goat anti-rabbit at 1:10,000 for Cyp3a2 and donkey anti-goat at 1:10,000 for PXR.1, all from Jackson ImmunoResearch Laboratories, Inc., West Grove, PA). Immunoreactive proteins were detected in membranes using ECL Plus (Amersham Biosciences, Baie d’Urfé, QC) and scanned using an Alpha Innotech FluorChem imaging system (San Diego, CA). Using AlphaEaseFC (Version 6.0) software, also from Alpha Innotech, the optical density (OD) of each band was determined. To confirm equivalent protein loading after determining Mdr1, Cyp3a2 and PXR.1 band ODs, a peroxidase-labeled anti-β-actin antibody was employed (AC-15, 1:20,000, 2 mg/ml, Sigma-Aldrich). Mdr1, Cyp3a2 and PXR.1 ODs were normalized to β-actin. To correct for variability in gel transfer and other sources of variation that affect western blot quantification, a calibrator sample was randomly selected from the vehicle group and run in each gel. Protein/β-actin ratios for each blot were converted to percentages of that blot’s calibrator sample ratio. For PXR.1 blots, 50 µg of HeLa cell nuclear extract was also run in each gel to provide a positive control (Santa Cruz Biotechnology, Inc.).

4.3.10 Lopinavir protein binding assay

LPV protein binding in rat serum was determined by ultrafiltration (UF) with Amicon Ultra 0.5 ml centrifugal filter units (MWCO 10,000; Millipore Canada, Ltd., Etobicoke, ON). Serum samples from 4-5 dams/group, all with known serum LPV concentrations, were first equilibrated for 30 min at 37 °C. Then, 500 µl of each individual sample was transferred to centrifugal filter devices and equilibrated for another 30 min at 30 °C. After equilibration, samples were centrifuged at 5,000 g for 5 min in a centrifuge maintained at 30 °C. After this initial spin, filtrates were collected and discarded (approximately 200 µl). Centrifugation was then continued with the remaining sample at 14,000 g for 15 min and filtrate samples collected. LPV was subsequently extracted from aliquots (100 µl) of filtrate samples and quantified using LC-MS/MS as described. Filtrate sample LPV concentrations were then compared to LPV concentrations in corresponding unfiltered serum samples. Percent unbound in maternal circulation (% unbound\(_M\)) was determined using the following equation: % unbound = (C\(_{\text{filtered}}\)/C\(_{\text{unfiltered}}\)) × 100. Aweeka and colleagues validated the precision and accuracy of this method and found non-specific binding of LPV to centrifugal filter units to be very low (Aweeka et al., 2010).
4.3.11 Statistics

Data were analyzed using Prism 5 for Macintosh (GraphPad Software, Inc., San Diego, CA). Concentration (e.g., lopinavir in serum and tissues, lipids, albumin, etc.), transporter expression (mRNA and protein) and protein binding (% unbound) data were analyzed using one-way ANOVAs followed by Bonferroni post-tests. To assess the effect of uterine horn position on fetal exposure, all fetal concentrations were converted to a percentage of their corresponding litter’s average concentration and then subjected to one-way ANOVAs followed by Bonferroni post-tests (this was done within and across groups). To analyze blood glucose, body weight and some aspects of fetal exposure time courses, two-way ANOVAs followed by Bonferroni post-tests were performed. Fetal LPV AUC₀⁻₆₀, C_max and T_max values are presented as geometric group means, derived directly from the concentration-versus-time curve using Prism 5’s area under curve analysis feature (trapezoidal method). Linear regression was used to determine correlations (i.e., r² values). Levels of significance for statistical analyses were set at or below α = 0.05, indicated as follows: *, p < 0.05, **, p < 0.01 and ***, p < 0.001. All results are presented as mean ± S.D.
4.4 Results

4.4.1 Characterization of STZ-induced GDM

All of the STZ-injected rats developed at least mild hyperglycemia within 24 h and then developed GDM (> 14 mM) by GD8. All animals in the GDM group were, therefore, diabetic for 13 to 14 days before sacrifice on GD20. Blood glucose concentrations in the GDM group, relative to controls, were higher at GD8, GD11 and GD20 (p < 0.001; Table 4.1). For the insulin-treated group, insulin treatment was initiated when blood glucose concentrations first exceeded 14 mM and was efficacious within hrs. Blood glucose concentrations in the insulin-treated group fluctuated but were significantly lower than values obtained in the GDM group on GD11 and GD20 (p < 0.01-0.001; Table 4.1).

As expected during gestation, all animals gained weight across the study duration; however, animals with GDM were lighter than controls from GD8 until sacrifice (p < 0.01-0.001; See Table 4.1 for GD11 and GD20 values). Animals in the vehicle group and insulin-treated group did not differ in weight across the study duration (p > 0.05).

Blood chemistry data for animals with GDM revealed additional characteristics that were consistent with unmanaged, or poorly managed, GDM (Table 4.1). Hyperlipidemia was evident in the GDM group as serum cholesterol (total), triglyceride and FFA concentrations were profoundly elevated (p < 0.01-0.001). Polydipsia was observed in the GDM group on GD16. Also, as compared to the vehicle group, serum from animals in the GDM group contained higher levels of AAG (p < 0.01) and lower levels of albumin (p < 0.001). AAG concentrations in the insulin-treated group were also elevated (p < 0.01).

A number of fetal characteristics were also altered in the GDM group (Table 4.1). First, litters were smaller in the GDM group at an average of 11 pups per dam versus 16 in the vehicle group and 13 in the insulin-treated group. Second, relative to controls, GDM fetuses were smaller while GDM placentas were larger. Consistent with this, when the weight of each fetus was divided by the weight of its placenta, the resulting ratios were found to be lower in GDM as compared to the vehicle (p < 0.01) and insulin-treated (p < 0.05) groups (Appendix fig. 4.3). Finally, glucose concentrations in amniotic fluid samples obtained from the GDM group were much higher than in samples from controls (p < 0.05).
Table 4.1. The effect of STZ-induced GDM, with and without insulin treatment, on various physiological and biochemical characteristics. All results are presented as mean ± S.D. Maternal results are from 7-8 dams/group while fetal results are from 3-6 randomly selected fetuses/7-8 dams/group (fetal body weight). Amniotic fluid data are from one pooled sample/7-8 dams/group. *, Significantly different from vehicle controls. †, Significantly different from GDM. *, p < 0.05; ***, p < 0.01; ****, p < 0.001.

<table>
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<th>Characteristic</th>
<th>Vehicle</th>
<th>GDM</th>
<th>Insulin-treated</th>
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<td><strong>Blood glucose</strong></td>
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<tr>
<td>GD6 (mM)</td>
<td>6 ± 0.9</td>
<td>6 ± 0.4</td>
<td>6.5 ± 1.3</td>
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<tr>
<td>GD11 (mM)</td>
<td>6.3 ± 1</td>
<td>23.2 ± 2.8***</td>
<td>16 ± 6.6***##</td>
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<td>GD20 (mM)</td>
<td>5.8 ± 1.1</td>
<td>26.8 ± 2.9***</td>
<td>9.3 ± 7.2###</td>
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<td><strong>Body weight</strong></td>
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<tr>
<td>GD6 (g)</td>
<td>243 ± 10</td>
<td>242 ± 21</td>
<td>244 ± 12</td>
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<tr>
<td>GD11 (% baseline)</td>
<td>116 ± 1</td>
<td>103 ± 2**</td>
<td>108 ± 5</td>
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<tr>
<td>GD20 (% baseline)</td>
<td>154 ± 6</td>
<td>136 ± 7***</td>
<td>152 ± 1###</td>
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<td><strong>Blood chemistry (GD20)</strong></td>
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<td>Total cholesterol (mM)</td>
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<td>8 ± 4.7**</td>
<td>2.6 ± 0.6###</td>
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<td>HDL cholesterol (mM)</td>
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<td>1.3 ± 0.7</td>
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<td>25.9 ± 16.6***</td>
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<td>Free fatty acids (mEq/l)</td>
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<td>AAG (µg/ml)</td>
<td>49 ± 8</td>
<td>87 ± 30**</td>
<td>86 ± 12**</td>
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<td>Albumin (g/l)</td>
<td>34 ± 4</td>
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<td>GD16 (ml/kg/day)</td>
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<td>Litter size</td>
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<td>Body weight (g)</td>
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<td>Placenta weight (g)</td>
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<td>5 ± 0.4##</td>
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<td>Amniotic glucose (mM)</td>
<td>2.6 ± 0.8</td>
<td>32.2 ± 9***</td>
<td>7.5 ± 8.7###</td>
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</table>
4.4.2 Maternal and fetal lopinavir exposure at 45 min

Relative to controls, LPV concentrations were lower in the GDM group’s serum and tissue samples 45 min after administration (Fig. 4.2). Concentrations in serum and tissue samples from the vehicle and insulin-treated groups were not statistically different from each other (p > 0.05).

Unbound LPV concentrations were also determined in 45 min maternal serum samples. Unbound LPV concentrations were 11.7 ng/ml in the GDM group as compared to 12 ng/ml and 12.8 ng/ml in the vehicle and insulin-treated groups, respectively (p > 0.05). When expressed as percentages of total serum LPV concentrations, 1.6 ± 0.8% of the drug was unbound in the GDM group as compared to 0.6 ± 0.3% and 0.6 ± 0.4% in the vehicle and insulin-treated groups, respectively (p < 0.05). LPV plasma protein binding is constant across a range of total LPV concentrations that encompasses all values observed in our study (Kumar et al., 2004).

When LPV concentrations in tissues were divided by corresponding unbound LPV concentrations in maternal serum, significantly decreased ratios were observed in GDM maternal livers, placentas and fetuses (p < 0.05; Fig. 4.3). This indicates that maternal and fetal exposure in GDM is lower than in controls even after controlling for changes in protein binding.
Figure 4.2. Maternal and fetal lopinavir concentrations 45 min after lopinavir administration. Forty-five min after LPV administration (10 mg/kg; intravenous), drug concentrations were determined in maternal serum samples (A), maternal livers (B), placentas (C) and fetuses (D) via LC-MS/MS. All results are presented as mean ± S.D. Maternal results are from 4-5 dams/group while placental and fetal results represent 4-5 maternal means/group from 5 randomly selected placentas or fetuses/dam. *, Significantly different from vehicle controls. **, Significantly different from GDM. *##*, p < 0.01; ***####*, p < 0.001.
Figure 4.3. Tissue/unbound maternal serum lopinavir concentration ratios. LPV concentrations in maternal livers (A), placenta (B) and fetuses (C) were normalized to unbound concentrations in maternal serum. Resulting ratios are presented as mean ± S.D. Maternal liver results are from 4-5 dams/group. Placental and fetal results are from 5 randomly selected placenta or fetuses/dam and there were 4-5 dams/group (n = 20-25 data points/group). *, Significantly different from vehicle controls. #, Significantly different from GDM. ***, p < 0.001.
4.4.3 Influence of fetal uterine horn position on fetal lopinavir exposure

Position within the uterine horn was noted for all fetal compartments that were sampled 45 min after administration (up to 10 positions: right horn positions 1-5 and left horn positions 1-5). After converting fetal concentrations to a percentage of their corresponding litter’s average concentration, which corrects for variation in exposure between dams, one-way ANOVA failed to detect a significant overall difference between the position means (p > 0.05). This was true across and within this study’s three groups.

4.4.4 Time course of fetal lopinavir exposure in GDM

Pups were surgically removed from dams 5, 10, 15, 20, 30, 45 and 60 min after LPV administration in order to generate a concentration-versus-time curve for fetal exposure within each dam (Fig. 4.4). While LPV concentrations did not differ significantly until the 20 min time point, the overall time course of fetal LPV exposure for each group did. From 5 to 30 min post-injection, fetal concentrations in the vehicle group rose in a manner consistent with the distribution of LPV from maternal circulation (site of administration) to the fetal compartment. From 30 to 60 min post-injection, fetal concentrations in the vehicle group began to fall in a manner consistent with the clearance of LPV from the fetal compartment. In the GDM group, there was only one apparent phase after the 5 min time point: a phase dominated by clearance. The $C_{\text{max}}$ for LPV exposure in GDM occurred at the first time point that was examined (5 min) and fetal concentrations continued to fall thereafter. The insulin-treated time course exhibited both a distribution and a clearance phase but the distribution phase lasted only 20 min. With respect to area under the concentration-versus-time curve (AUC$_{0-60}$), fetuses in the vehicle group were exposed to more LPV than fetuses in the GDM group (Table 4.2). The insulin-treated group had an AUC$_{0-60}$ that fell between values observed in the vehicle and GDM groups (Table 4.2). Maternal serum and liver concentrations were evaluated in this cohort at the 60 min time point. Serum and liver LPV concentrations in the GDM condition were lower than those of the vehicle and insulin-treated groups, which did not differ from each other (Table 4.2).
Figure 4.4. Time course of fetal exposure to lopinavir following intravenous maternal administration. Pups were surgically removed from dams 5, 10, 15, 20, 30, 45 and 60 min after administration (10 mg/kg; intravenous) in order to generate a concentration-versus-time curve for fetal LPV exposure. All results are presented as mean ± S.D. *, Significantly different from vehicle controls. #, Significantly different from GDM. */#, p < 0.05; */###, p < 0.01; */####, p < 0.001.
Table 4.2. Pharmacokinetic parameters derived from concentration-versus-time curves for fetal lopinavir exposure following maternal administration (10 mg/kg; intravenous). AUC\(_{0-60}\), C\(_{\text{max}}\) and T\(_{\text{max}}\) values as well as maternal serum and liver values are arithmetic groups means. Maternal samples were collected at 60 min. *, Significantly different from vehicle controls. #, Significantly different from GDM. *, p < 0.05; **/#, p < 0.01; ***/##, p < 0.001.

<table>
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<th>Parameter</th>
<th>Vehicle</th>
<th>GDM</th>
<th>Insulin-treated</th>
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<tr>
<td><strong>Fetal time course</strong></td>
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<tr>
<td>AUC(_{0-60}) (µg.min/g)</td>
<td>15.63 ± 1.21</td>
<td>4.76 ± 0.77***</td>
<td>9.03 ± 1.29***.##</td>
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<td>C(_{\text{max}}) (µg/g)</td>
<td>0.37 ± 0.06</td>
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<td>T(_{\text{max}}) (min)</td>
<td>27 ± 6</td>
<td>8 ± 6*</td>
<td>13 ± 8</td>
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<td><strong>Maternal samples</strong></td>
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<tr>
<td>Serum [LPV] (µg/ml)</td>
<td>2.17 ± 0.32</td>
<td>0.62 ± 0.32**</td>
<td>1.44 ± 0.38</td>
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<td>Liver [LPV] (µg/ml)</td>
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<td>9.13 ± 7.12*</td>
<td>42.76 ± 15.37#</td>
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</table>
4.4.5 Drug transporter and Cyp3a2 expression in GDM

The expression of Mdr1a/b, Mrp2, Bcrp and Cyp3a2 mRNA was determined in maternal liver and the expression of Mdr1a/b, Mrp2 and Bcrp mRNA was determined in placenta (Fig. 4.5). In maternal liver, both Mdr1b and Cyp3a2 were significantly upregulated in the GDM group. Hepatic Mdr1b/18S standard ratios were 3.55 ± 2 in GDM compared with 0.33 ± 0.41 in the vehicle group (p < 0.05) and 0.26 ± 0.16 in the insulin-treated group (p < 0.05). Hepatic Cyp3a2/18S standard ratios were 1 ± 0.35 in GDM compared with 0.43 ± 0.04 in the vehicle group (p < 0.05) and 0.55 ± 0.22 in the insulin-treated group (p > 0.05).

Significant mRNA findings in maternal liver were confirmed at the level of protein (Fig. 4.6). Hepatic Mdr1/β-actin standard ratios (% calibrator) were 149 ± 41 in GDM compared with 92 ± 6 in the vehicle group (p < 0.05) and 85 ± 6 in the insulin-treated group (p < 0.05). Hepatic Cyp3a2/β-actin standard ratios (% calibrator) were 170 ± 63 in GDM compared with 78 ± 27 in the vehicle group (p < 0.05) and 64 ± 14 in the insulin-treated group (p < 0.05). Hepatic Mdr1 and Cyp3a2 protein concentrations correlated well with corresponding Mdr1b ($r^2 = 0.9; p < 0.001$) and Cyp3a2 ($r^2 = 0.56; p < 0.01$) mRNA concentrations.

In placenta, both Mdr1b and Bcrp were significantly upregulated in the GDM group. Placental Mdr1b/18S standard ratios were 1.9 ± 0.43 in GDM compared with 0.95 ± 0.18 in the vehicle group (p < 0.01) and 1.06 ± 0.39 in the insulin-treated group (p < 0.05). Placental Bcrp/18S standard ratios were 2.49 ± 0.96 in GDM compared with 1.08 ± 0.27 in the vehicle group (p < 0.05) and 1.45 ± 0.64 in the insulin-treated group (p > 0.05). While placental Mrp2 expression was not statistically higher in the GDM group (p = 0.16), a trend toward this was observed in the data. Significant mRNA findings in placenta were not confirmed at the level of protein due to insufficient sample volume. Intraplacental correlations were observed between Mdr1a and Mdr1b, Mdr1b and Mrp2, Mdr1b and Bcrp and Mrp2 and Bcrp (p < 0.0001). The influence of fetal sex on placental transporter upregulation in GDM was investigated as part of a pilot study. The results of this investigation are presented in appendix fig. 4.4 and discussed in section 6.7.1).

With respect to the expression of all genes and proteins examined in this study, we did not find a difference between the vehicle and insulin-treated groups (p > 0.05).
Figure 4.5. Drug transporter and Cyp3a2 mRNA expression in GDM. The mRNA expression of Mdr1a/b, Mrp2, Bcrp and Cyp3a2 in maternal liver (left bars) and Mdr1a/b, Mrp2 and Bcrp in placenta (right bars) was determined by qRT-PCR. All results are presented as a percentage of the vehicle group’s mean ± S.D. Maternal liver results are from 4-5 dams/group while placental results represent 4-5 maternal means/group from 3 randomly selected fetuses/dam. *, Significantly different from vehicle controls. #, Significantly different from GDM. *#, p < 0.05; **##, p < 0.01.
Figure 4.6. Hepatic Mdr1 and Cyp3a2 protein expression in GDM. Mdr1 and Cyp3a2 protein concentrations were determined in maternal liver via western blotting. A, Representative blots showing Mdr1, Cyp3a2 and β-actin expression in samples from each group. Quantified Mdr1 (B) and Cyp3a2 (C) results are presented as mean percentages of a calibrator sample’s values ± S.D. and are from 4-5 dams/group. *, Significantly different from vehicle controls. #, Significantly different from GDM. */#, p < 0.05.
4.4.6 Lopinavir metabolite formation in maternal liver at 45 min

Absolute combined LPV and LPV metabolite concentrations (i.e., [LPV]+[M3/M4]+[M1]) were 30 ± 13 in GDM compared with 176 ± 77 in the vehicle group (p < 0.05) and 182 ± 122 in the insulin-treated group (p < 0.05). The relative contributions of LPV and its metabolites to this combined concentration in the GDM group differed from the relative contributions observed in the control groups (Appendix fig. 4.5 presents LPV and LPV metabolite concentrations, 45 min after LPV administration, without normalization). When metabolite concentrations were divided by corresponding LPV concentrations, there was a trend toward decreased M3/M4 ratios (p < 0.05 for one-way ANOVA but p > 0.05 for Bonferroni post-tests; Fig. 4.7A) and increased M1 ratios (p < 0.01 for one-way ANOVA but p > 0.05 for Bonferroni post-tests; Fig. 4.7C) in the GDM group. However, when M1, a secondary metabolite, was divided by corresponding M3/M4 concentrations, the metabolite from which it is formed, a significant increase in M1 ratios was observed in the GDM group (p < 0.001; Fig. 4.7B). Decreased M3/M4 to LPV ratios with increased M1 to M3/M4 ratios is indicative of increased M3/M4 metabolism to M1, a process mediated by Cyp3a2. There was a significant correlation between these M1 ratios and Cyp3a2 protein expression (r² = 0.35; p < 0.05).

Mdr1b ATPase activation assays were performed with the M1 and M3/M4 metabolites but the results were somewhat inconclusive. In these assays, ATPase activity briefly rose but then fell below baseline (data not shown). Mdr1 has basal ATPase activity and depression of this activity is observed with slowly transported substrates. In support of this, LPV produced a similar pattern of ATPase activity and it is an established Mdr1 substrate.
Figure 4.7. Normalized concentration ratios for lopinavir metabolites in maternal liver 45 min after lopinavir administration. Forty-five min after LPV administration (10 mg/kg; intravenous), lopinavir and lopinavir metabolite concentrations were determined in maternal liver samples via LC-MS/MS. A, Because M3/M4 is a product of LPV (i.e., a primary metabolite), concentrations of M3/M4 were normalized to LPV concentrations. B, Because M1 is a product of M3/M4 (i.e., a secondary metabolite), concentrations of M1 were normalized to M3/M4 concentrations. C, M1 concentrations were also normalized to LPV concentrations. All results are presented as mean ± S.D. of 4-5 dams/group. *, Significantly different from vehicle controls. **, Significantly different from GDM. ***, #, p < 0.01.
4.4.7 Hepatic PXR.1 protein expression in GDM

Hepatic PXR.1 protein levels were examined due to its integral role in the regulation of both Mdr1 and Cyp3a2. As figure 4.8 illustrates, the expression of hepatic PXR.1 in the GDM group was substantially higher than in control groups. Hepatic PXR.1/β-actin standard ratios (% calibrator) were 309 ± 55 in GDM compared with 75 ± 33 in the vehicle group (p < 0.001) and 96 ± 14 in the insulin-treated group (p < 0.001). There was a significant correlation between hepatic PXR.1 protein concentrations and the hepatic concentrations of both Mdr1 ($r^2 = 0.64; p < 0.001$) and Cyp3a2 ($r^2 = 0.43; p < 0.01$).

![Figure 4.8. Hepatic PXR.1 protein expression in GDM. Protein concentrations of the PXR.1 PXR isoform were determined in maternal liver via western blotting. A, Representative blots showing PXR.1 and β-actin expression in samples from 3 dams/group. HeLa cell nuclear extract was used as a positive control. B, Quantified results are presented as mean percentages of a calibrator sample’s values ± S.D. and are from 4-5 dams/group. *, Significantly different from vehicle controls. ***, Significantly different from GDM. ****#, p < 0.001.](image-url)
4.5 Discussion

Experimental GDM had a significant impact on maternal and fetal LPV exposure in this study. Total LPV concentrations in serum from dams with GDM were less than half of control values. While unbound serum concentrations were roughly equivalent in all groups, they represented a greater percentage of total serum concentrations in the GDM group. We have previously demonstrated that GDM-induced hyperlipidemia in rats, which was observed in this study (Table 4.1) and is observed clinically in GDM (Wiznitzer et al., 2009), leads to drug displacement (Anger and Piquette-Miller, 2010). Albumin concentrations in GDM were also significantly reduced, which means there were fewer LPV binding sites. As previously stated, decreased protein binding facilitates the passage of LPV into eliminating organs and the fetal compartment.

LPV is eliminated from the body primarily by hepatobiliary excretion. In a study of LPV metabolism and disposition in the rat, 69.5% of an intravenous LPV dose was recovered in bile after 24 hrs and approximately 86% of this occurred in the first 2 hrs (Kumar et al., 2004). In maternal liver, at 45 and 60 min, LPV concentrations in GDM were less than a third of control values. Moreover, when hepatic LPV concentrations in our study were normalized to unbound serum concentrations, lower values were still observed in GDM. This suggests that more rapid clearance of LPV from liver tissue occurred in the GDM group. Significantly higher hepatic Mdr1 and Cyp3a2 expression in GDM supports this. Functionally, data supporting enhanced hepatic metabolism of the M3/M4 primary metabolites to the M1 secondary metabolite were obtained in GDM livers. The human equivalent of Cyp3a2, CYP3A4, is the principle determinant of LPV pharmacokinetics in humans. In fact, LPV is co-formulated with RTV specifically for its ability to inhibit CYP3A enzymes and thereby increase LPV exposure (Abbott Laboratories Ltd., 2010). It has been proposed by van Waterschoot and colleagues, however, that hepatic Mdr1 and Cyp3a work in a coordinated fashion to eliminate LPV in mice (van Waterschoot et al., 2010). More specifically, it has been proposed that Cyp3a drastically lowers LPV concentrations so as to prevent the saturation of Mdr1-mediated hepatobiliary excretion. Agarwal and colleagues provided evidence supporting the in vitro saturability of Mdr1 by LPV when they observed transporter activity at low (0.5 µM) LPV concentrations but not at high (5-25 µM) LPV concentrations (Agarwal et al., 2007). Data from our study indicate that LPV metabolites could also contribute to Mdr1 saturation. By monitoring ATPase activity, we demonstrated that the M3/M4 and M1 metabolites interact with rat Mdr1 in a manner similar to LPV. That being said, the fact that absolute combined LPV and metabolite concentrations were much lower in GDM livers suggests that both Mdr1-mediated efflux and
Cyp3a2-mediated metabolism contributed to enhanced hepatobiliary clearance of LPV and its metabolites in GDM.

While the specific reason for the observed pharmacokinetic findings in the GDM group dams has not been fully established, it is clear that important mechanisms have been altered. In addition to the above speculation regarding hepatic elimination, it is plausible that there was a contribution from increased renal elimination. In non-pregnant individuals, approximately $10.4 \pm 2.3\%$ of a LPV dose is recovered in urine after 8 days (Abbott Laboratories Ltd., 2010). Urinary output dramatically increases in STZ-induced diabetes as a result of osmotic diuresis (Anger et al., 2009; Anger and Piquette-Miller, 2010). While urinary output was not measured in this study, water intake was nearly 5-fold higher in the GDM group than in controls (Table 4.1) and a strong positive correlation exists between urinary output and water intake (unpublished data).

To better assess fetal exposure, LPV concentrations were determined in placentas and fetuses at 45 min and in fetuses from a second cohort at multiple time points. At 45 min, LPV concentrations were significantly lower in GDM. It stands to reason that reduced fetal exposure in GDM was largely the result of reduced maternal exposure. As maternal serum LPV levels were much lower in GDM, less LPV would have been available for placental transfer; however, when placental and fetal LPV concentrations were normalized to unbound LPV concentrations in maternal serum, ratios in the GDM group were still lower than control ratios. This suggests that LPV was also being actively excluded from the fetal compartment. This is likely attributable to placental efflux and this hypothesis is supported by increased Mdr1b mRNA expression in GDM placentas.

The time courses of fetal exposure for both the vehicle and insulin-treated groups exhibited an accumulation phase whereas the GDM group’s time course did not. Accordingly, fetuses in the vehicle and insulin-treated groups were exposed to more LPV than fetuses in the GDM group. In the absence of placental transporter data, this finding would be surprising given the GDM group’s decreased albumin concentrations and decreased LPV protein binding. Studies employing the human cotyledon perfusion model have shown that albumin plays a major role in placental LPV transfer (Gavard et al., 2006; Ceccaldi et al., 2010). For example, Gavard et al demonstrated that LPV transfer was $23.6 \pm 6.9\%$ at an albumin concentration of 2 g/l, $20.7 \pm 10\%$ at 10 g/l and only $3.3 \pm 0.5\%$ at 40 g/l (Gavard et al., 2006). Gavard et al’s data predicts higher (15%) transfer in our GDM group than in our control groups (6-7%), which is not what we observed.

The role of fetal exposure to antiretrovirals in the prevention of vertical HIV transmission is not fully established but it is generally acknowledged that exposure is desirable. The concentration of
protease inhibitors in human cord blood after in utero exposure is low but varies greatly by molecule (Mirochnick et al., 2002; Chappuy et al., 2004). While the protease inhibitors RTV, indinavir and saquinavir have low cord blood/maternal blood ratios that range from 0-0.01 (Chappuy et al., 2004), LPV has been found to have a comparatively high ratio of 0.2 ± 0.13 (Stek et al., 2006). Importantly, Gavard et al demonstrated in the human cotyledon perfusion model that this ratio leads to fetal compartment concentrations that exceed the 50% HIV inhibitory concentration for LPV under normal physiological conditions (Gavard et al., 2006; Abbott Laboratories Ltd., 2010). The fact that fetal exposure was reduced in STZ-induced GDM raises questions about GDM’s potential impact on vertical HIV transmission risk and could warrant clinical investigation.

Induction of Mdr1 and Cyp3a has been observed in tissues from male rats with STZ-induced diabetes (Maeng et al., 2007a; Kameyama et al., 2008; Hasegawa et al., 2010) but reports in female rats with STZ-induced diabetes or STZ-induced GDM are limited (Mulay and Varma, 1984; Anger et al., 2009; Anger and Piquette-Miller, 2010). We posit that disruptions to lipid and glucose homeostasis underlie the alterations to drug transporter and Cyp3a2 expression that were observed in STZ-induced GDM. Briefly, it is known that drug transporters and metabolic enzyme transcription is controlled by several nuclear receptors, like PXR, which can themselves be activated by lipids and glucose (Handschin and Meyer, 2005). We have previously demonstrated that nuclear receptor networks with roles in the regulation of hepatic drug transporters and metabolic enzymes are activated in STZ-induced GDM (Anger and Piquette-Miller, 2010). In this study, we observed a significant upregulation in the PXR.1 PXR isoform in GDM livers. PXR is Mdr1 and Cyp3a2’s primary transcriptional regulator so, not surprisingly, its hepatic protein concentrations positively correlated with hepatic Mdr1 and Cyp3a2 concentrations. It is conceivable that a similar regulatory effect may be at work in the placenta, although recent work with PXR gene knockout mice has drawn the role of placental PXR in placental transporter regulation into question (Gahir and Piquette-Miller, 2011).

While this study focused on LPV, the disposition of other drugs would likely be altered in experimental GDM. Protein binding aside, many drugs that are used today are substrates for MDR1 and/or BCRP (Chandra and Brouwer, 2004) and CYP3A4 is believed to be involved in the metabolism of approximately half of all drugs (Guengerich, 1999). Relating this to antiretroviral therapy, LPV is typically taken with other anti-HIV drugs and most of these drugs are substrates for at least one of MDR1, BCRP and CYP3A4 (Reviewed in Kis et al., 2010). For example, zidovudine/AZT and lamivudine/3TC are BCRP substrates and are often taken alongside LPV/RTV in pregnancy (Kis et al., 2010).
In conclusion, our findings demonstrate that maternal and fetal LPV exposure is reduced in a rat model of GDM. For maternal exposure, our data suggest that PXR-mediated hepatic Mdr1 and Cyp3a2 upregulation and decreased protein binding are responsible. Enhanced renal elimination in GDM may also contribute but was not directly evaluated. For fetal exposure, our data suggest that while fetal exposure is largely dictated by maternal exposure, increased placental transporter expression in GDM serves to further limit placental transfer. Using a novel sampling procedure to characterize fetal exposure, it was determined that GDM fetuses accumulated very little LPV. This study further advances our understanding of the impact that GDM can have on drug disposition mechanisms and provides preclinical support for an as yet unreported drug-disease (LPV-GDM) interaction. If confirmed in humans, the effects reported in this study would need to be considered when exposure targets are not met in GDM pregnancies.
4.6 Appendix: supplementary figures/data

Appendix 4.1. Cartoon illustration of fetal positioning within the pregnant rat at approximately GD20. A, The rat uterus consists of two uterine horns, a left horn (LH) and a right horn (RH), that extend into the abdominal cavity. In my experiments, fetuses were identified first by the uterine horn in which they implanted (LH or RH) and then by their relative order, with fetus one being the fetus closest to the cervical stump/vagina (LH1-n or RH1-n). B, Rat fetuses reside inside isolated compartments with independent access to the maternal circulation (shown in red). Since rat placentation is hemochorial, fetal trophoblasts are in direct contact with maternal blood.
Appendix 4.2. Lopinavir and lopinavir metabolite chromatography. The chromatographic separation of lopinavir (A), the M3/M4 lopinavir metabolites (B), the M1 lopinavir metabolite (C) and internal standard/ritonavir (D) in a representative vehicle control liver sample is presented.
Appendix 4.3. Placental size in experimental GDM. 

**A and B**, Placental weights in this study, presented as mean maternal means ± SD (A) and individual weights (B). 

**C and D**, Fetus:placenta weight ratios in this study, presented as mean maternal means ± SD (C) and individual ratios (D). *, Significantly different from vehicle controls. **, Significantly different from GDM. */#, p < 0.05; **/##, p < 0.01. Statistical test employed: one-way ANOVA followed by Bonferroni post-tests. 

**E**, Photograph depicting representative placentas from each group. Ruler units are in centimeters.
Appendix 4.4. Mdr1a/b, Mrp2 and Bcrp expression in placentas from a group of fetuses for which sex was determined. As part of a pilot study, transporter expression was first determined in the placentas of 15 fetuses/group as described in section 4.3. These placentas were analyzed specifically to determine the influence of fetal sex on placental transporter upregulation in GDM (i.e., their data did not contribute to the data described in 5.4 Results). The sex of each fetus was then determined. Briefly, for each sample, the fetal tail was incubated with a nucleic acid extraction solution consisting of NaOH (10 mM) and EDTA (0.1 mM), in water, for 15 min at 95 °C. Aliquots of this extraction solution were then subjected to multiplex conventional PCR with Fermentas reagents and oligonucleotides targeting rat zinc finger Y-chromosomal protein (Zfy) DNA and glyceraldehyde-3-phosphate dehydrogenase (Gapdh) mRNA. Zfy is a male-specific gene while Gapdh is a housekeeping gene that was used as an endogenous positive control. PCR oligonucleotides were synthesized at The Hospital for Sick Children and their sequences were as follows: 5'-TCCCCAGCCTCTCTCCCTCA-3' (Zfy-F), 5'-TCTTCGCCATTCTTTACAGTGCT-3' (Zfy-R), 5'-CCATCACCATCTCCAGCAGG-3' (Gapdh-F) and 5'-CCTGCTCACCACCTCTGG-3' (Gapdh-R). The following amplification conditions were used: an initial strand separation of 3 min at 94 °C, 40 cycles of 30 s at 94 °C, 30 s at 52 °C, then 60 s at 72 °C and a final elongation period of 5 min at 72 °C. Samples from adult male and female rats were analyzed alongside fetal samples to provide positive controls. PCR products were subjected to electrophoresis in agarose gels and visualized using SYBR Safe (Invitrogen; A). Using this method, the following was determined: 10 and 5 of the vehicle group fetuses were male and female, respectively; 7 and 8 of the GDM group fetuses were male and female, respectively; 7 and 8 of the insulin-treated group fetuses were male and female, respectively. Mdr1a/b (B and C), Mrp2 (D) and Bcrp (E) expression data is presented as a percentage of sex-matched vehicle group means ± S.D. * Significant different from vehicle controls. *, p < 0.05. Statistical test employed: two-way ANOVA followed by Bonferroni post-tests comparing data to the appropriate vehicle group.
Appendix 4.5. Lopinavir and lopinavir metabolite concentrations in maternal liver without normalization. Forty-five min after LPV administration, lopinavir and lopinavir metabolite concentrations were determined in maternal liver samples from the vehicle (A), GDM (B) and insulin-treated (C) groups via LC-MS/MS. Statistical tests employed: none.
Chapter 5. Placental ABC Efflux Transporter Expression in Pregnancies Complicated by Insulin-Managed Diabetes

Gregory J. Anger, Alexander M. Cressman and Micheline Piquette-Miller

Presented in this thesis as a short communication for future submission.
5.1 Abstract

To determine if placental drug efflux transporter expression is altered in pregnancies complicated by insulin-managed diabetes mellitus, we examined the expression of multidrug resistance protein 1 (MDR1), multidrug resistance-associated protein 2 (MRP2) and the breast cancer resistance protein (BCRP) via western blotting in samples obtained from affected pregnancies and compared it to expression in healthy term control samples. We illustrate that MDR1, MRP2 and BCRP expression was unaltered. Interestingly, there was a significant, positive correlation between BCRP expression and hemoglobin A1c levels, a retrospective measure of glycemic control. It is likely that placental efflux transporter function in these groups is not affected when hyperglycemia is managed; however, given the relationship between BCRP expression and hemoglobin A1c levels, it could be affected in unmanaged diabetes.
5.2 Introduction

Multidrug resistance protein 1 (MDR1), multidrug resistance-associated protein 2 (MRP2) and the breast cancer resistance protein (BCRP) are ATP-binding cassette (ABC) drug efflux transporters that are expressed in the placenta’s syncytiotrophoblast layer, where they limit the materno-fetal transfer of drugs and xenobiotics (St-Pierre et al., 2000; Evseenko et al., 2006). The importance of these transporters in determining fetal xenobiotic exposure has been established in ex vivo placental perfusion models (Mölsä et al., 2005; May et al., 2008; Pollex et al., 2008) and in mutant/knockout mice (Lankas et al., 1998; Smit et al., 1999; Zhou et al., 2007). We have previously demonstrated that placental MDR1, MRP2 and BCRP expression is elevated in rat dams with streptozotocin-induced gestational diabetes mellitus (GDM) but nearly normal in rat dams with streptozotocin-induced GDM whose blood glucose was normalized with insulin treatment (Anger and Piquette-Miller, 2011). In this study, we examined protein expression of MDR1, MRP2 and BCRP in placentas from pregnancies complicated by insulin-managed type 1 diabetes mellitus (T1DM-I) or insulin-managed GDM (GDM-I). We hypothesized that placental drug efflux transporter expression in individuals with well-managed diabetes would be equivalent to healthy term controls.
5.3 Methods

5.3.1 Sample acquisition

Thirty-five human specimens from pregnancies meeting our inclusion/exclusion criteria (Table 5.1) were obtained by the Research Centre for Women’s and Infants’ Health (RCWIH) BioBank program of Mount Sinai Hospital, in accordance with the policies of the Mount Sinai Hospital Research Ethics Board. Immediately after delivery, tissue cores through the full thickness of the placenta were obtained, avoiding chorionic plate tissue and areas with obvious evidence of thrombosis or other abnormalities. Samples were snap-frozen in liquid nitrogen and stored at -80 °C until use. Patient data was collected by the RCWIH BioBank program of Mount Sinai Hospital and made available to this study. Samples included in this study reflect RCWIH BioBank availability between April 2010 and January 2011.

5.3.2 Western blotting

Protein was isolated from samples as described elsewhere (Anger and Piquette-Miller, 2011). Samples containing 60 µg of protein in Laemmli sample buffer were separated via 8% SDS-polyacrylamide gel electrophoresis and transferred to polyvinylidene fluoride membranes (Bio-Rad Laboratories Canada, Ltd., Mississauga, ON). Membranes were blocked in skim milk and incubated with anti-MDR1 (C219, 1:500, ID Labs Biotechnology, Inc., London, ON), anti-MRP2 (M2 III-6, 1:100, Abcam, Inc., Cambridge, MA) or anti-BCRP (BXP-53, 1:50, Abcam, Inc.) antibodies. After a series of washes, membranes were incubated with a peroxidase-labeled secondary antibody (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA). Immunoreactive proteins were detected in membranes using ECL Plus (Amersham Biosciences, Baie d’Urfé, QC). The optical density (OD) of each band was determined using ImageJ for Macintosh (version 1.44o) software. To confirm equivalent protein loading, immunodetection using an anti-β-actin antibody was employed (AC-15, 1:30,000, Sigma-Aldrich) so that MDR1, MRP2 and BCRP ODs could be normalized to β-actin. To correct for gel-to-gel variability, a calibrator sample was run in each gel. Protein of interest/β-actin ratios for each blot were converted to percentages of that blot’s calibrator sample’s ratio.
5.3.3 Statistics

Data were analyzed using Prism 5 for Macintosh (GraphPad Software, Inc., San Diego, CA). Power analysis was performed using G*Power 3.1 for Macintosh (Faul et al., 2007). Levels of significance for statistical analyses were set at or below $\alpha = 0.05$, indicated as follows: *#, $p < 0.05$ and **##, $p < 0.01$. All results are presented as mean ± S.D.
Table 5.1. Inclusion/exclusion criteria.

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</tr>
<tr>
<td></td>
<td>• &gt;37 weeks + 0 days</td>
<td>• T1DM</td>
</tr>
<tr>
<td></td>
<td></td>
<td>• T2DM</td>
</tr>
<tr>
<td></td>
<td></td>
<td>• GDM (diet-managed)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Other:</td>
</tr>
<tr>
<td></td>
<td></td>
<td>• Same as term control group</td>
</tr>
</tbody>
</table>
5.4 Results and discussion

Patient data for the T1DM-I and GDM-I groups indicate that they displayed some of the known clinical characteristics of diabetic pregnancy (Table 5.2). The T1DM-I group had significantly shorter gestations ($p < 0.01$) and exhibited trends toward higher neonatal birth weight ($p = 0.14$) and larger placental volume ($p = 0.07$). In the GDM-I group, mothers had significantly higher BMIs ($p < 0.05$). Other characteristics of diabetic pregnancy were absent, though. In particular, there were no significant differences in neonatal birth weight. Given the strong correlation that exists between neonatal birth weight and maternal blood glucose concentrations, this may signify proper glycemic control in this study’s T1DM-I and GDM-I groups (Nielsen et al., 2007; Karcaaltincaba et al., 2010).

Data for a measure of glycemic control, glycosylated hemoglobin (HbA1c), was available only for the diabetic groups. In proportion to blood concentrations, glucose is able to react with normal hemoglobin (HbA0) in red blood cells to produce HbA1c. HbA1c concentrations, therefore, reflect average blood glucose concentrations over the previous 3-4 months, which is the approximate lifespan of red blood cells (Rahbar et al., 1969).

Placental MDR1, MRP2 and BCRP expression in the T1DM-I and GDM-I groups was not statistically different from controls (Fig. 5.1A-C; section 5.5 describes the results of power analysis). Normal expression in these insulin-managed groups is consistent with our observations in insulin-managed rat dams with streptozotocin-induced GDM (Anger and Piquette-Miller, 2011). Interestingly, HbA1c positively correlated with BCRP (Fig. 5.1D) but not with MDR1 and MRP2 expression. This suggests that placental BCRP could be particularly sensitive to hyperglycemia.

The overall coefficient of variation, a measure of variation about the mean, for MDR1, MRP2 and BCRP protein expression in this study was found to be 71%, 61% and 54%, respectively. This is consistent with our animal model data (Anger and Piquette-Miller, 2011) and comparable to other studies of transporter expression in human term placenta (Tanabe et al., 2001; Gil et al., 2005). Interestingly, interindividual transporter expression variation, as demonstrated in this study, appears to be small in comparison to other tissues. For example, 55-fold, 20.5-fold and 20-fold variation in human hepatic MDR1 has been observed (Schuetz et al., 1995; Owen et al., 2005; Meier et al., 2006). Similarly, there are reports of 6-fold and 3-fold variation in human intestinal MDR1 (Dürr et al., 2000; Simon et al., 2007).

There are two particularly important aspects of this study. First, it suggests that placental transporter expression in well-managed T1DM-I and a GDM-I pregnancy is not significantly different from healthy term controls. However, this may not hold true when hyperglycemia is
unmanaged. Supporting this is the observed correlation between HbA1c and BCRP. Increased levels of HbA1c were associated with increased levels of BCRP protein. Admittedly, samples from pregnancies with documented mismanagement of blood glucose were unavailable so it remains unknown if placental transporter expression is normal in T1DM-I and GDM-I because of management or because the human and rat placental response differs. Second, this study provides evidence that interindividual variability in placental transporter expression is comparatively less than that observed in other tissues. This suggests that disease-induced transporter expression changes may be comparatively easier to detect in the placenta.
Table 5.2. Clinical data. Statistical results are presented as mean ± SD. All other results are presented as [n (%)]. a, HbA1c values were measured in the 2nd and/or 3rd trimester and are presented as group means, based on 1-5 individual measurements. b, Includes drugs, vaccines and vitamin supplements. c, Wet, untrimmed weight. d, Based on measurements included in the pathology report. *, Significantly different from term controls. **, p < 0.01. N/A, not applicable or not available.

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Control (n = 14)</th>
<th>T1DM-I (n = 8)</th>
<th>GDM-I (n = 13)</th>
</tr>
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<tr>
<td>Age (years)</td>
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<td>33 ± 3</td>
<td>35 ± 4</td>
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<tr>
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<td>2 (15)</td>
</tr>
<tr>
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<td>3 (23)</td>
</tr>
<tr>
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<td>23.2 ± 4.5</td>
<td>25.9 ± 3.9</td>
<td>29 ± 6.6*</td>
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<tr>
<td>Duration of diabetes (years)</td>
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<tr>
<td>HbA1c (%)</td>
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<td>6.4 ± 0.6</td>
<td>6 ± 0.8</td>
</tr>
<tr>
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<td>4 ± 2</td>
<td>3 ± 1</td>
</tr>
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<tr>
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<tr>
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<tr>
<td>Placental weight (g)</td>
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<td>666 ± 101</td>
<td>661 ± 175</td>
</tr>
<tr>
<td>Placental volume (cm$^3$)</td>
<td>681 ± 174</td>
<td>977 ± 381</td>
<td>766 ± 283</td>
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<tr>
<td>Neonate:placenta weight ratio</td>
<td>5.9 ± 0.8</td>
<td>5.4 ± 0.9</td>
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</table>
Figure 5.1. Placental ABC efflux transporter expression in pregnancies complicated by insulin-managed diabetes. A, MDR1. B, MRP2. C, BCRP. Boxes represent quartiles enclosing 50% of the data and the whiskers at either end extend to the 25th and 75th quartiles. The median is marked by a line, the mean is marked by an “+” and outliers are marked by a “•” outside the 25th and 75th quartiles. The “Combined” group presents data from all patients (n = 33) and was not included in statistical analyses. Statistical test employed: one-way ANOVA. D, The statistical relationship between HbA1c percentage and BCRP protein expression in diabetic pregnancies is presented.
5.5 Appendix: power analysis

Using animal model-derived effect sizes of 0.78 for Mdr1 (200% mRNA increase, CV = 42%), 0.53 for Mrp2 (456% mRNA increase, CV = 120%) and 0.67 for Bcrp (230% mRNA increase, CV = 53%), a total sample size of 39 patients (13/group) would be required to achieve power ≥ 0.8 in an F test for all of the transporters examined in this study. The actual power achieved for MDR1, MRP2 and BCRP analyses in this study, based on these animal model-derived effect sizes, was 0.98, 0.77 and 0.93, respectively (group sizes were averaged for these post hoc power calculations); however, clinical effect sizes are often smaller than effect sizes in animal models. Using a more conservative effect size of 0.3 for each of the transporters, a total sample size of 111 patients (37/group) would be required to achieve power ≥ 0.8. The actual power achieved for transporter expression analyses in this study, based now on an expected effect size of 0.3, was 0.31 (group sizes were again averaged for these post hoc power calculations). Because placental transporter expression data from unmanaged, or poorly managed, diabetic patients is unavailable, the true effect size is currently unknown.
Chapter 6. Discussion
6.1 Examining hepatic drug transporters in non-pregnant rats with streptozotocin-induced T1DM: proof of concept

In the context of this thesis, my study involving non-pregnant rats was essentially a proof of concept study (see Chapter 2), the concept being: conditions that are associated with inflammation will likely also be associated with downregulation in drug transporters and metabolic enzymes. This concept provides the foundation for many of the studies currently being conducted in the laboratory of my supervisor, Dr. Micheline Piquette-Miller (Reviewed in Petrovic et al., 2007). My rationale for considering diabetes an inflammatory condition is presented in Chapter 1.

In my study involving non-pregnant rats, I induced diabetes via STZ injection and then sacrificed animals nine days later (total duration of diabetes: ~7 days). Plasma and liver tissue samples were then subjected to a battery of tests to determine: 1) if the rats were in an inflammatory state and 2) if hepatic ABC efflux transporters were downregulated. The original plan was to proceed with studies in pregnant rats, where I could study both the liver and placenta, if the results of this initial study supported the concept. A similar workflow was followed in the laboratory of my supervisor when endotoxin-induced inflammation was examined. For these studies, the ability of endotoxin-induced inflammation to downregulate Mdr1 was first examined in male and non-pregnant female rodents (Piquette-Miller et al., 1998; Hartmann et al., 2005) before moving on to pregnant rats (Wang et al., 2005; Petrovic et al., 2008).

I ultimately obtained evidence in non-pregnant rats with STZ-induced T1DM that supported the concept; however, in hindsight, I feel that I should have assessed animals at a later post-STZ injection time point (i.e., at ≥ 14 days instead of at 9 days). It is my current hypothesis that if animals were assessed at a later time point, their data would be more consistent with data obtained in my studies involving pregnant rats. More specifically, I hypothesize that inflammation-induced transporter downregulation would have subsided while the effects of hyperglycemia and hyperlipidemia would have had time to manifest (presumably leading to transporter upregulation). This belief is consistent with reports of decreased biliary excretion of bile acids 1-7 days post-STZ injection (Carnovale et al., 1987; Garcia-Marin et al., 1988) and increased biliary excretion of bile acids, digoxin, rose bengal and a handful of other chemicals 14, 28 and 35 days post-STZ injection (Watkins and Noda, 1986; Watkins and Dykstra, 1987; Watkins and Sherman, 1992). As described in Chapter 3 and 4, transporter upregulation, not downregulation, and increased drug elimination, not decreased drug elimination, was observed in my studies involving pregnant rats that were sacrificed 14 days after STZ injection.
6.2 Model performance

Throughout this thesis, the model of diabetic pregnancy that I employed has been referred to as a rat model of GDM. This is due to the fact that diabetes was initiated on GD8 in my model and the long-standing definition of GDM upon initiation of my doctoral research in 2006 was, “any degree of glucose intolerance with onset or first recognition during pregnancy” (American Diabetes Association, 2011a). I acknowledge that the pathophysiology of human GDM and STZ-induced GDM differ. Human GDM is like T2DM in that it is the result of peripheral insulin resistance and impaired hepatic glucose regulation whereas STZ-induced GDM is more like T1DM, with pancreatic insulin production being low or absent (see section 1.1.1). Nonetheless, the clinical manifestation of GDM is associated with a number of physiological and biochemical features that, together, characterize this human disease state and many of these features were present in the model that I employed. In this section, the extent to which my model of STZ-induced GDM recapitulates these human GDM features is discussed.

To begin with, maternal hyperglycemia is the defining feature of this human disease state as GDM diagnosis can be based solely on the results of blood glucose testing. Human GDM usually arises around the 24th week of pregnancy, which means the disease typically affects 60% of gestation (American Diabetes Association, 2004). Values for diagnosis, if based on casual blood glucose testing results, are typically > 11.1 mmol/L (see section 1.1.1 for alternative diagnostic criteria) but hyperglycemia can rise to much higher levels if unmanaged or poorly managed (American Diabetes Association, 2004; anecdotal physician reports/personal communications). In my model of STZ-induced GDM, diabetes was confirmed, with little variation, on GD8, which means the duration of this experimental GDM was in-line with human GDM at roughly 60% of gestation (Sprague-Dawley rat gestation: 22 days). The diagnostic criteria used in my studies was a blood glucose concentration in excess of 14 mmol/L (casual blood glucose testing) but hyperglycemia was sustained across studies at approximately 25 mmol/L.

Human GDM is also associated with the classical triad of diabetes symptoms: polyuria, polydipsia and hyperphagia. It is known that the severity of these three features is positively correlated with the severity of hyperglycemia but the classical triad is seldom quantified in human GDM. In the study described in Chapter 3, STZ-induced GDM was associated with an 8.6-fold increase in urine production, a 5.2-fold increase in water consumption and a 2.2-fold increase in feed consumption.
Maternal hyperlipidemia is another feature of the human disease state (Knopp et al., 1977; Knopp et al., 1986; Koukkou et al., 1996; Couch et al., 1998). In one study, human GDM was associated with a 2- to 4-fold increase in triglycerides and a 1.25- to 1.5-fold increase in total cholesterol (Knopp et al., 1977). In the study described in Chapter 3, STZ-induced GDM was associated with a 5.2-fold increase in triglycerides and a 2.6-fold increase in total cholesterol. In the study described in Chapter 4, STZ-induced GDM was associated with an 8.9-fold increase in triglycerides and a 4.2-fold increase in total cholesterol.

Low maternal weight gain is yet another feature of the human disease state (as mentioned in section 1.1.4). Approximately 27% of maternal weight gain in pregnancy is attributable to growing maternal fat stores (Cunningham et al., 2001) but the growth of these stores is disrupted in human GDM because adipose tissue becomes insensitive to insulin. In a prospective longitudinal study conducted by Catalano and colleagues, a significant positive correlation was observed between fat accretion and pregravid insulin sensitivity (Catalano et al., 1998). Thus, the women in their study with low pregravid insulin sensitivity (future GDM cases) had less fat accretion as compared to women with high pregravid insulin sensitivity (future uncomplicated cases). In another study by this group, total maternal weight gain in GDM was 2.4 kg less than weight gain in controls (Catalano et al., 1993). Since controls gained an average of 15 kg in this study, weight gain in patients with GDM was approximately 84% of weight gain in the controls. In my model of STZ-induced GDM, the weight gain profiles of diabetic and control dams diverged on GD8 and continued to diverge until sacrifice. On GD20, across studies, dams with STZ-induced GDM were 136% of their baseline weights whereas dams in both the vehicle and insulin-treated groups were 151% of their baseline weights. Weight gain in the GDM group was approximately 90% of weight gain in the vehicle and insulin-treated groups.

Placental and fetal enlargement are also established features of the human disease state. In human GDM, increased placental and fetal weight is well documented and the degree of placental and fetal enlargement depends on how well maternal diabetes is managed (Winick and Noble, 1967; Nummi, 1972; Spellacy et al., 1985; Laurini et al., 1987; Naeye, 1987; Clarson et al., 1989; Kucuk and Doymaz, 2009). Additionally, in this disease, placental enlargement is disproportionately greater than fetal enlargement so the fetus:placenta weight ratios that are reported for GDM are often significantly lower than ratios that are reported for uncomplicated pregnancies. In my model of STZ-induced GDM, placental enlargement was observed but fetal enlargement was not. Fetuses from dams in the GDM group were approximately 12% lighter than fetuses from the control groups (i.e., IUGR occurred). IUGR in STZ-induced GDM likely resulted from fetal insulin resistance that
was brought on by fetal hyperglycemia (Schroeder et al., 1997; Reviewed in Aerts and Van Assche, 2006; Reviewed in Fernandez-Twinn and Ozanne, 2006). As in human GDM, fetus:placenta ratios in STZ-induced GDM were significantly lower than ratios in control groups.

As is believed to be the case for human T1DM and T2DM, evidence in the literature suggests that low-grade, chronic systemic inflammation is a feature of human GDM. In human GDM, modest increases in the plasma concentrations of the inflammatory cytokines IL-6 and TNF-α have been reported (Kirwan et al., 2002; Winkler et al., 2002; Atègbo et al., 2006; McLachlan et al., 2006). Evidence for the inflammatory marker CRP is less conclusive. As compared to controls, there have been reports of both elevated plasma CRP (Qiu et al., 2004) and unaltered plasma CRP (Retnakaran et al., 2003; McLachlan et al., 2006) in GDM. Inflammation was addressed in Chapter 3 of this thesis when CRP concentrations were measured in STZ-induced GDM. My data indicated that plasma CRP concentrations in GDM were no different than concentrations in vehicle and insulin-treated controls. Also, in all groups, IL-6 and TNF-α concentrations were below detection limits in serum samples from pregnant dams (data not shown). The only evidence supporting inflammation was the specific pattern of alterations in plasma drug-binding proteins that occurred in GDM, although such alterations, on their own, are not reliable indicators of inflammation. If inflammation was a feature of my model, I was unable to detect it. Proper evaluation of this feature likely hinges on accurately determining plasma/serum concentrations of IL-6 and TNF-α (see section 6.7.2).

Finally, all of the features that characterize the human disease state are typically responsive to exogenous insulin injection and this, in itself, is another feature of human GDM. Insulin is currently the standard of care for patients with GDM who fail MNT (American Diabetes Association, 2004). In my experiments, maternal hyperglycemia, polyuria, polydipsia, hyperphagia, maternal hyperlipidemia, maternal weight gain, placental enlargement and IUGR were effectively normalized in dams with STZ-induced GDM that received regular exogenous insulin.

Five of the seven features of human GDM that I describe here were also features of STZ-induced GDM. Of these common features, the severity of maternal hyperglycemia in STZ-induced GDM is a notable difference between the clinical and experimental situations. Since the other common features have been shown to vary with the extent of maternal hyperglycemia, their severity in STZ-induced GDM is likely a reflection of maternal hyperglycemia. As such, it might be tempting to propose studies in which the severity of maternal hyperglycemia is lowered via reduced STZ dosage and/or sparingly administered insulin treatment; however, this can be problematic. First,
dams given reduced STZ doses often recover by late gestation and perform similar to controls when subjected to glucose challenge tests (Caluwaerts et al., 2003) whereas maintaining moderate hyperglycemia by way of insulin treatment in dams with severe hyperglycemia is difficult and highly variable (personal experience, data not shown). Second, using lower STZ doses and/or sparingly administered insulin treatment produces a unique disease model and is not just a less severe version of severe hyperglycemia; it could, therefore, result in the loss of some features shared by human GDM and STZ-induced GDM as well as the introduction of new disease features (Caluwaerts et al., 2003). For these reasons, I do not propose using less severe forms of STZ-induced GDM in my discussion of future studies (see section 6.7). My data should be carefully extrapolated to the clinical manifestation of GDM, focusing on cases of unmanaged, or poorly managed, GDM where the physiological and biochemical features that characterize this human disease state are pronounced.
6.3 Drug plasma/serum protein binding in a rat model of diabetic pregnancy

As Table 6.2 summarizes, my experiments provide evidence of decreased drug plasma/serum protein binding in STZ-induced GDM. This supports my primary general hypothesis. When plasma samples from the GDM and control groups were incubated with tritiated glyburide or saquinavir and then subjected to ultrafiltration, significantly higher levels of radioactivity were detected in ultrafiltrates from the GDM group. This indicates that glyburide and saquinavir protein binding was decreased in GDM. The results of these ex vivo experiments were consistent with my subsequent in vivo findings. When the serum of LPV-dosed rats from the GDM and control groups were subjected to ultrafiltration, higher concentrations of LPV were detected in ultrafiltrates from the GDM group. This indicates that LPV protein binding was also decreased in GDM.

In blood, glyburide is highly bound to albumin (Olsen et al., 1995), saquinavir is highly bound to AAG (Holladay et al., 2001) and LPV is highly bound to both (Gulati et al., 2009). Therefore, my initial attempts at explaining this phenomenon involved measuring levels of albumin and AAG in maternal plasma/serum. In two separate studies, albumin concentrations were found to be decreased in GDM while AAG concentrations were found to be increased (Table 3.1 and Table 4.1). Decreased albumin occurs in a number of clinical situations, including cirrhosis (Ghoneim and Pandya, 1975), nephrotic syndrome (Leopold et al., 1985), old age (Viani et al., 1992), pregnancy (Dean et al., 1980) and, of particular importance to this discussion, diabetes (Townsend, 1990; Mattock et al., 2001). The decrease in albumin that was observed in GDM is consistent with my protein binding data for glyburide and could partly explain my findings for LPV.

Increased AAG, as it is an acute phase protein, occurs primarily in clinical situations that are associated with inflammation. For example, increased AAG is observed in patients with inflammatory bowel disease (Kjeldsen et al., 1995; Kjeldsen et al., 1997) and in patients with rheumatoid arthritis (Lacki et al., 1995). The increase in AAG that was observed in GDM is inconsistent with my protein binding data for both saquinavir and LPV. Increased AAG would be expected to increase protein binding but the opposite occurred. This inconsistency is suggestive of a contribution from a factor, capable of influencing the protein binding of saquinavir, that is not related to absolute plasma protein content.

Unmanaged diabetes results in intracellular starvation, which triggers the liberation of fatty acids from adipocytes into the blood. Focusing on the extent to which the lipid profile was altered in plasma and serum samples from animals with GDM, obvious to the naked eye, prompted me to
question whether the protein binding of drugs could be altered in GDM via competition with circulating lipids for binding sites.

This question is not novel. One of the earliest studies documenting the crystallization of human serum albumin noted that the product contained extractable fatty acids (Kendall, 1941). Later, this was found to be because fatty acids are the primary endogenous ligands of serum albumin molecules, which transport them to the body’s tissues (Gordon and Cherkes, 1956; Gordon and Cherkes, 1958; White and Engel, 1958). Following over a decade of studies that enhanced our understanding of the interactions between albumin and fatty acids throughout the 1960’s (e.g., studies determining specific binding sites), ex vivo studies began to emerge which examined competitive albumin binding between fatty acids and other organic compounds. Some of these early studies would demonstrate that FFAs, such as oleic and palmitic acid, could displace drugs/probes such asbishydroxycoumarin, bromsulfophthalein, diphenylhydantoin, halofenate, sulfadiazine and thiopental from human and bovine albumin when their concentrations were sufficiently high (Rudman et al., 1971; Spector et al., 1973). In vivo studies conducted in the 1980’s confirmed these ex vivo findings. For example, relative to baseline data collected while in a fed condition, healthy adult subjects that were fasted for 27 hrs exhibited a 34-182% increase in FFA levels and a corresponding 9% increase in the unbound fraction of valproic acid (Bowdle et al., 1982). Valproic acid binds to albumin. Regression analysis indicated that 37% of the variation in unbound valproic acid concentrations in this study could be explained by FFA levels (Bowdle et al., 1982). The literature concerning albumin-FFA interactions clearly supports the assertion that the protein binding of drugs can be altered by circulating lipids.

Much less is currently known about AAG-FFA interactions. Given that AAG is typically associated with the binding of cations, there have been few studies examining the interaction of this plasma protein and FFAs. Nonetheless, the binding of propranolol to human AAG can be significantly increased via AAG delipidation (Chauvelot-Moachon et al., 1988).

To examine the contribution of lipids to the protein binding effects I observed in my studies, plasma samples from the GDM and control groups were delipidated, incubated with tritiated glyburide and then subjected to ultrafiltration. In contrast to findings pre-delipidation, there was no difference in the levels of radioactivity detected in post-delipidation ultrafiltrates from the GDM group and the control groups. Also, the levels of radioactivity in plasma samples from the control groups were not affected by delipidation. This supports a role for plasma lipids in the GDM group’s atypical protein binding characteristics. Plasma stocks from the majority of animals used for these studies were depleted by the end of these glyburide delipidation experiments so I was unable to
determine saquinavir plasma protein binding in the absence of plasma lipids. In the presence of plasma lipids, a positive correlation was observed between ex vivo saquinavir protein binding and ex vivo glyburide protein binding in plasma samples ($r^2 = 0.85; p < 0.0001$).

My data demonstrating decreased protein binding of both acidic and basic drugs in a rat model of diabetic pregnancy, as well as my data implicating elevated plasma lipids in this effect, is entirely consistent with the literature concerning protein binding in other forms of diabetes. Decreased plasma protein binding has been observed in type 1 and type 2 diabetes mellitus; both in animal models (McNamara et al., 1988; Nadai et al., 1990; Tanaka et al., 1993; Bae et al., 2005; Baek et al., 2006) and in clinical populations (Stafford, 1962; Ruiz-Cabello and Erill, 1984; Gatti et al., 1987; McNamara et al., 1988; Trovik et al., 1992; Wörner et al., 1992; O'Byrne et al., 1993; Arredondo et al., 1999). Moreover, in some of these studies, relationships between the protein binding of drugs and plasma FFA concentrations were established (Ruiz-Cabello and Erill, 1984; Gatti et al., 1987).

The overall significance of decreased protein binding in GDM is that drug disposition could potentially be altered as a consequence of, for example, enhanced maternal $\text{CL}_{\text{liver}}$ and enhanced placental transfer stemming from more unbound drug being available. The extent to which hepatic clearance is altered by protein binding depends on a number of factors, including the drug’s physicochemical properties and extraction ratio. Assuming their physicochemical properties are conducive to crossing into the liver, the $\text{CL}_{\text{liver}}$ of drugs would be expected to increase (provided they do not have particularly high extraction ratios). When rats in the GDM and control groups were intravenously injected with LPV, significantly less LPV was found in serum and liver samples from dams with GDM at 45 and 60 min post-administration. Unfortunately, my experimental data does not allow me to fully differentiate between the contributions of decreased protein binding, enhanced hepatic drug efflux and metabolism and possibly even enhanced hepatic blood flow; however, it is reasonable to assume that, for LPV, these processes would work in concert to increase hepatic clearance (see Equation 1.1).

The extent to which placental transfer is altered by protein binding also depends on a number of factors, including the drug’s physicochemical properties, maternal serum concentrations and whether the drug is a substrate for placental efflux transporters. Assuming their physicochemical properties are conducive to crossing the placenta, transfer would be expected to increase significantly for drugs that are present in maternal serum at sufficiently high concentrations and/or negligibly transported by efflux transporters (see Equation 1.2). When rats in the GDM and control groups were intravenously injected with LPV, fetal $\text{AUC}_{0-60}$ values were much lower in the GDM
group. Placental LPV concentrations in the GDM group were also much lower 45 min post-administration. As plasma protein binding was decreased in GDM, increased placental transfer of LPV would be expected. The fact that a decrease in placental transfer was seen is likely due to increased placental expression and function of drug efflux transporters, which oppose placental transfer (see Equation 1.2; placental transporter expression is discussed in section 6.5).

It is worth noting that the protein binding effects described in this thesis could theoretically occur not just in GDM but also in the many other clinical situations where hyperlipidemia occurs. Hyperlipidemias are divided into two types: primary (a.k.a., familial) and secondary (a.k.a., acquired). Primary hyperlipidemias are caused by specific genetic abnormalities that result in aberrant expression/activity of enzymes and receptors involved in lipid metabolism. Secondary hyperlipidemias, on the other hand, can be thought of as a consequence of another pathology. Diabetes-induced hyperlipidemia is, for example, a secondary hyperlipidemia. In addition to being caused by diabetes, secondary hyperlipidemia can be caused by certain drugs, such as diuretics and beta blockers (Lasser et al., 1984), protease inhibitors (Carr et al., 1998) and ethanol (Chait et al., 1972), or by diseases, such as hypothyroidism (O’Brien et al., 1993) and nephrotic syndrome (Radhakrishnan et al., 1993).

Table 6.1. Evidence for decreased protein binding of drugs in STZ-induced GDM. Glyburide and saquinavir data come from Chapter 3 (ex vivo experiments, in plasma) while lopinavir data comes from Chapter 4 (in vivo experiments, in serum). Data from the GDM group are significantly different (p < 0.05) than those from the vehicle and insulin-treated groups.

<table>
<thead>
<tr>
<th>Drug</th>
<th>Vehicle</th>
<th>GDM</th>
<th>Insulin-treated</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>94.8% bound</td>
<td>86.6% bound 13.4% free</td>
<td>96.2% bound 3.8% free</td>
</tr>
<tr>
<td></td>
<td>5.2% free</td>
<td>Free = 258% vehicle</td>
<td>Free = 73% vehicle</td>
</tr>
<tr>
<td>Glyburide</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>90.3% bound</td>
<td>87.8% bound 12.2% free</td>
<td>91.1% bound 8.9% free</td>
</tr>
<tr>
<td></td>
<td>9.7% free</td>
<td>Free = 126% vehicle</td>
<td>Free = 92% vehicle</td>
</tr>
<tr>
<td>Saquinavir</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>99.4% bound</td>
<td>98.4% bound 1.6% free</td>
<td>99.4% bound 0.6% free</td>
</tr>
<tr>
<td></td>
<td>0.6% free</td>
<td>Free = 267% vehicle</td>
<td>Free = 100% vehicle</td>
</tr>
</tbody>
</table>
6.4 Expression of hepatic drug transporters and metabolic enzymes in a rat model of diabetic pregnancy

Hepatic drug transporters and metabolic enzymes play an important role in determining drug disposition. For the most part, my experiments in Chapter 3 and 4 provide evidence for their upregulation, at the level of mRNA and protein, in STZ-induced GDM. This supports my primary general hypothesis. To the best of my knowledge, the studies described in these chapters are the first to examine the effects of diabetic pregnancy on the expression of hepatic drug transporters and metabolic enzymes but my results are very consistent with previous reports concerning expression in non-pregnant rats with non-acute STZ-induced T1DM. Table 6.2 provides a summary of alterations for which comparator data from the insulin-treated group is available.

With respect to transporters, hepatic Mdr1 was found to be significantly upregulated (Chapter 3 and 4). Mdr1 upregulation was normalized by insulin treatment. Hepatic Mdr2 was also found to be significantly upregulated in STZ-induced GDM, although comparator data from the insulin-treated group was not available (Chapter 3). Upregulation of hepatic Mdr1 (Kameyama et al., 2008; Zhang et al., 2011) and Mdr2 (van Waarde et al., 2002) in non-pregnant rats with non-acute STZ-induced T1DM has been previously reported. Interestingly, Oatp1a1 was found to be significantly downregulated in both STZ-induced GDM and insulin-treated STZ-induced GDM. Reports of Oatp1a1 expression in diabetes are limited but downregulation of this drug uptake transporter has been reported in the ob/ob mouse model of T2DM (Cheng et al., 2008).

With respect to metabolic enzymes, findings included the upregulation of hepatic Cyp2a1, Cyp2b9, Cyp2d3, Cyp3a2 and Ugt1a1 in STZ-induced GDM (Chapter 3 and 4). With the exception of Cyp2d3, upregulation of these enzymes was normalized by insulin treatment. In non-pregnant rats with non-acute STZ-induced T1DM, upregulation is almost exclusively reported for Cyp2a1 (Ma et al., 1989; Thummel and Schenkman, 1990; Shimojo et al., 1993), Cyp2b9 (Sakuma et al., 2001) and Cyp3a2 (Thummel and Schenkman, 1990; Shimojo et al., 1993; Chen et al., 2011; Hu et al., 2011). Cyp2d3 expression has not been reported in diabetes but increased 4-hydroxydiazepam formation following diazepam administration, a process mediated by Cyp2d3, has been observed in non-acute STZ-induced T1DM (Andrews and Griffiths, 1984). In animal models of diabetes, increased (Ahn et al., 2009), decreased (Ma et al., 1989) and unchanged (Sakuma et al., 2001) expression has been reported for other members of the Cyp2d subfamily. Ugt1a1 upregulation has been reported in BB rats, which exhibit genetically predetermined T1DM (Braun et al., 1998).
Functionally, an increase in the activity of drug transporters and metabolic enzymes in the liver would be expected to increase its CL_{intrinsic} capabilities for relevant substrates. The list of substrates that are transported and metabolized by the proteins that were altered in STZ-induced GDM is incredibly diverse and includes many clinically important drugs. Increased hepatic CL_{intrinsic} enhances CL_{\text{Liver}} for both low and high extraction ratio substrates but not to the same extent (see Equation 1.1). The greatest effect is observed with low extraction ratio drugs because drug concentrations are in excess, making their CL_{\text{Liver}} dependent entirely on CL_{intrinsic}. A lesser effect is observed with high extraction ratio drugs because CL_{intrinsic} is already quite high. For many high extraction ratio drugs, CL_{intrinsic} is higher than Q_{\text{Liver}}, so increases in CL_{intrinsic} are of no consequence.

In my LPV disposition studies, maternal exposure to this high extraction ratio drug was substantially lower in the GDM group than in the control groups. Evidence of increased LPV metabolism in the GDM group was also obtained. Because the effects in my study were so great, despite not being mentioned in Chapter 4, it is possible that Q_{\text{Liver}} was also increased. Increased hepatic blood flow in non-pregnant rats with non-acute STZ-induced T1DM has been reported (Lucas and Foy, 1977; Sato et al., 1991). Such an effect, if coupled with an increase in the amount of free drug that flows into the organ (resulting from decreased protein binding) and increased hepatic CL_{intrinsic} (resulting from increased Mdr1 and Cyp3a2 expression), could easily explain the magnitude of reduced maternal LPV exposure that I observed. Q_{\text{Liver}} was not evaluated in my LPV disposition study. Alternative explanations that should be noted include: 1) the extraction ratio of LPV could be lower in the rat, which makes CL_{intrinsic} more important and 2) decreased maternal LPV exposure in GDM could be partly attributable to increased renal elimination, as proposed in Chapter 4. These explanations are not mutually exclusive. Maternal data in my LPV disposition studies are consistent with the previously described Mulay and Varma study, in which maternal \[^3\text{H}\]-dexamethasone exposure was reduced in an STZ rodent model of diabetic pregnancy (Mulay and Varma, 1984).

As mentioned in section 6.2, plasma CRP concentrations were not elevated in GDM (Chapter 3) and concentrations of IL-6 and TNF-\(\alpha\) were below detection limits in serum samples from all groups (data not shown). Because inflammation was either not a feature of my model or was occurring at a level that I could not detect, I was not able to properly test my secondary hypothesis regarding possible relationships between pro-inflammatory cytokines and expression changes in the liver. Nonetheless, upregulation in drug transporters and metabolic enzymes runs contrary to what is currently known about the regulatory effects of pro-inflammatory cytokines (Reviewed in Petrovic et al., 2007). For example, studies conducted in my supervisor’s laboratory
have consistently demonstrated that Mdr1 is downregulated in acute inflammation (Piquette-Miller et al., 1998; Hartmann et al., 2001; Petrovic et al., 2008; Petrovic and Piquette-Miller, 2010).

In Chapter 3, I describe proteomic datasets that show hepatic proteins linked to lipid metabolism were strongly affected in GDM and formed networks that implicated several nuclear receptors in their regulation. Altered hepatic proteins and implicated nuclear receptors formed established signalling pathways for the control of drug transporter and metabolic enzyme expression but were embedded within networks involved in energy homeostasis. Thus, increased expression of drug transporters and metabolic enzymes could be a by-product of activity in molecular networks involved in energy homeostasis (i.e., lipid metabolism). This provides a way to explain hepatic effects in my model that is unrelated to inflammation. What follows is a possible explanation for upregulation of PXR and PXR targets in my model of STZ-induced GDM that employs this by-product concept. Admittedly, the explanation is simplified because the extent of crosstalk between relevant nuclear receptors is complicated and not yet fully established.

As previously mentioned, unmanaged diabetes results in intracellular starvation and this triggers the liberation of free fatty acids from adipocytes into the blood. In my model of STZ-induced GDM, I observed increased serum free fatty acid concentrations (Chapter 3 and 4). Fatty acids are ligands for the nuclear receptor PPARα and enhanced PPARα activity has been previously reported in animal models of diabetes (Kroetz et al., 1998; Asayama et al., 1999; Engels et al., 1999). Enhanced PPARα activity occurs in the presence of intracellular starvation because its targets are involved in β-oxidation, ketogenesis and gluconeogenesis (Reviewed in Burri et al., 2010). While I did not directly measure PPARα activity in my experiments, its activity was inferred in the liver on the basis of alterations in its targets. For example, Cyp4a14 was upregulated in my iTRAQ dataset for STZ-induced GDM and this protein is a commonly used indicator of PPARα activation (Corton et al., 2000; Anderson et al., 2002). In addition to these targets, a functional PPARα binding site has been identified within the proximal promoter of the PXR gene (Aouabdi et al., 2006). This binding site provides a link between PPARα and PXR. Indeed, the treatment of primary rat hepatocyte cultures with the PPARα agonist clofibrate induces PXR expression (Ma et al., 2005). PXR is best known for its role in promoting the transcription of drug transporters and metabolic enzymes but it also plays important roles in promoting the transcription of targets involved in lipogenesis and lipid accumulation. PXR can also promote lipogenesis and lipid accumulation in ways unrelated to its own transcriptional targets. For example, PXR can directly bind to insulin- and glucagon-responsive transcription factors like FoxO1, FoxA2 and CREB so as to inhibit their transcriptional activity.
FoxO1, FoxA2 and CREB are activators of genes that encode proteins involved in β-oxidation, ketogenesis and gluconeogenesis (Kodama et al., 2004; Kodama et al., 2007; Nakamura et al., 2007). Thus, PXR activation counterbalances the consequences of PPARα activation. In my model of STZ-induced GDM, upregulation of PXR and a number of its targets (Mdr1, Cyp3a2 and Ugt1a1) was observed (Chapter 3 and 4). Figure 6.1 provides a summary of direct and indirect interactions between PPARα and PXR in the presence of intracellular starvation and their implications for the regulation of proteins related to hepatic energy homeostasis as well as drug transport and metabolism.

Table 6.2. Evidence for altered hepatic drug transporter and metabolic enzyme expression in STZ-induced GDM. Methods by which evidence was obtained included qRT-PCR (PCR), western blotting (WB) and LC-MS/MS with iTRAQ labeling (iTRAQ). Data in the GDM group without a comparator in the insulin-treated groups is omitted. Changes are in comparison to vehicle group values. NC: no change. All alterations presented here are statistically significant.

<table>
<thead>
<tr>
<th>Drug transporter/ Drug metabolic enzyme</th>
<th>GDM</th>
<th>Insulin-treated</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Drug transporters</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mdr1</td>
<td>PCR: ‡</td>
<td>PCR: NC</td>
</tr>
<tr>
<td></td>
<td>WB: ‡</td>
<td>WB: NC</td>
</tr>
<tr>
<td>Mrp2</td>
<td>PCR: NC</td>
<td>PCR: NC</td>
</tr>
<tr>
<td>Bcrp</td>
<td>PCR: NC</td>
<td>PCR: NC</td>
</tr>
<tr>
<td>Oatp1a1</td>
<td>iTRAQ: ‡</td>
<td>iTRAQ: ‡</td>
</tr>
<tr>
<td><strong>Drug metabolizing enzymes</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cyp2a1</td>
<td>iTRAQ: ‡</td>
<td>iTRAQ: NC</td>
</tr>
<tr>
<td>Cyp2b9</td>
<td>iTRAQ: ‡</td>
<td>iTRAQ: NC</td>
</tr>
<tr>
<td>Cyp2d3</td>
<td>iTRAQ: ‡</td>
<td>iTRAQ: ‡</td>
</tr>
<tr>
<td>Cyp3a2</td>
<td>PCR: ‡</td>
<td>PCR: NC</td>
</tr>
<tr>
<td></td>
<td>WB: ‡</td>
<td>WB: NC</td>
</tr>
<tr>
<td>Ugt1a1</td>
<td>iTRAQ: ‡</td>
<td>iTRAQ: NC</td>
</tr>
</tbody>
</table>
Figure 6.1. Summary of direct and indirect interactions between PPARα and PXR in the presence of intracellular starvation and their implications for the regulation of proteins related to hepatic energy homeostasis as well as drug transport and metabolism. CREB, cAMP response element binding protein; DT & ME, drug transporter and metabolic enzyme; FoxA2, forkhead box protein A2; FoxO1, forkhead box protein O1; PPARα, peroxisome proliferator-activated receptor alpha; PXR, pregnane X receptor. *, Pregnancy provides an abundance of endogenous ligands that can activate PXR.
6.5 Expression of placental drug efflux transporters in diabetic pregnancy

My experiments in Chapter 4 provide evidence for elevated placental drug efflux transporter expression in STZ-induced GDM. More specifically, levels of Mdr1b and Bcrp mRNA were significantly higher in placentas from fetuses in the GDM group than in those from fetuses in the vehicle and insulin-treated groups. Placental Mdr1b and Bcrp mRNA expression in the vehicle and insulin-treated groups did not differ, demonstrating the corrective effects of insulin treatment. Indeed, in my experiments with human samples, there was a significant correlation between the extent of glycemic control in the diabetic groups, as measured by HbA1c percentage, and placental BCRP protein expression (Chapter 5). To the best of my knowledge, the studies described in Chapter 4 and 5 are the first to examine the effects of diabetic pregnancy on placental drug transporters. The results of these studies support my primary general hypothesis.

Functionally, increased placental Mdr1 and Bcrp would be expected to result in enhanced clearance of their substrates from the fetal circulation into the maternal circulation (CL_{FM}). From an alternative perspective, this would be expected to result in diminished clearance of Mdr1 and Bcrp substrates from the maternal circulation into the fetal circulation (CL_{MF}). Enhanced CL_{FM} and/or diminished CL_{MF} decreases the fetal:maternal free drug concentration ratio (see Equation 1.2). In my LPV disposition studies, in accordance with these expectations, fetuses in the GDM group were exposed to less LPV than fetuses in control groups. This was true even after normalizing fetal exposure to maternal unbound serum concentrations (% unbound_{M}), suggesting active transport might be the more dominant factor with respect to placental transfer in GDM. Fetal data in my LPV disposition studies are inconsistent with the previously described Mulay and Varma study, in which evidence was obtained that suggested fetal [³H]-dexamethasone concentrations were a direct reflection of maternal [³H]-dexamethasone concentrations (Mulay and Varma, 1984).

Placental transporter upregulation in STZ-induced GDM is in agreement with my observations in the liver and, as stated in Chapter 4, it is conceivable that a similar regulatory mechanism may be at work in both of these organs. The placenta accumulates lipids, particularly triglycerides, in humans so many nuclear receptor ligands should have been in abundance (Goldstein et al., 1985; Lindegaard et al., 2006). Placental PPARα activation could, as proposed in my discussion of expression effects in the liver, have led to PXR upregulation. PXR upregulation and activation, in turn, could have led to transporter upregulation. Indeed, the positive correlation observed between placental Mdr1b and Bcrp mRNA in STZ-induced GDM is consistent with PXR
activity because both of these transporters are PXR target genes. This speculation, however, is complicated by some of the data available in the literature.

First, placental nuclear receptors exhibit temporal fluctuations in expression because of their roles in placental development and function. This is particularly true of the PPARs. PPARα, PPARδ and PPARγ are all expressed in placental trophoblast cells after placentation and are involved in adaptive responses involving oxygen and nutrients derived from the maternal circulation (Reviewed in Barak et al., 2008). Studies have found that placental PPARα is upregulated at GD13.5 in diabetic rats (Martínez et al., 2008) but downregulated by GD21 in diabetic rats (Martínez et al., 2011) and at term in humans with GDM (Holdsworth-Carson et al., 2010). Thus, PPARα might not be involved at the time point that I examined in Chapter 4 (i.e., GD20).

Second, a recent study involving PXR gene knockout mice has drawn the role of placental PXR in placental transporter regulation into question (Gahir and Piquette-Miller, 2011). When wild-type mice in this study were treated with a PXR activator, pregnenolone-16α-carbonitrile, upregulation of PXR target genes in the liver was observed but there were no effects on the expression of targets in the placenta. This effect was not because the placenta was exposed to insufficient concentrations of the activator because upregulation of PXR target genes occurred in the livers of fetuses. It is possible that there are differences in the role that placental PXR plays in rats versus knockout mice.

Future studies that focus on the regulatory mechanisms that ultimately led to Mdr1b and Bcrp upregulation in the placenta are clearly required (see section 6.7). As was the case with the liver, I was not able to test my secondary hypothesis regarding possible relationships between pro-inflammatory cytokines and expression changes in the placenta.
6.6 Non-routine methods

At present, all of the methods used in this thesis are fairly routine in the pharmaceutical sciences with the exception of the proteomic approach utilized in Chapter 3 and the surgical method used to conduct fetal pharmacokinetic studies in Chapter 4. In this section, the advantages and disadvantages of these non-routine methods are discussed.

6.6.1 The relative quantitation of drug transporters and metabolic enzymes in complex, iTRAQ-labeled membrane protein mixtures

A proteome is the complete set of proteins in a cell, tissue or sample under study at a specific point in time (Wilkins et al., 2007). In contrast with static genomes, which are based on inherited DNA, proteomes are dynamic and vary from cell to cell and time to time in accordance with the demands of local and systemic environments. As such, the human body contains many proteomes. By comparing proteomes obtained in healthy individuals to proteomes obtained in individuals with disease, the study of proteomes, called proteomics, is able to describe the effects of disease on biological systems in great detail.

In Chapter 3, I adopted a proteomic approach involving an isobaric tag labeling strategy with eight different isobaric tags for relative and absolute quantitation (iTRAQ) that allowed me to compare liver samples from multiple non-treated or insulin-treated GDM rats to samples from vehicle controls within the same LC-MS/MS runs. With isobaric tag labeling, a unique chemical group, from a set of chemical groups with equal masses, is assigned to each unique sample in the experiment. After attaching the unique isobaric tag to each sample’s peptides (generated via the tryptic digestion of proteins), samples may be combined and simultaneously subjected to fractionation and LC-MS/MS as one “pooled” sample (i.e., sample multiplexing). During LC-MS/MS, peptides are fragmented to produce 1) sequence-specific product ions that are used to identify the peptide’s parent protein and 2) isobaric tag-derived reporter ions that are used to determine the relative contribution of each unique sample to the “pooled” sample.

The iTRAQ molecule consists of a reporter group (based on N-methylpiperazine), a mass balance group (a carbonyl group) and a peptide-reactive group (N-hydroxysuccinimide) (Ross et al., 2004; Choe et al., 2007). The 4-plex iTRAQ set consists of four tags containing reporter groups of 114.1, 115.1, 116.1 and 117.1 Da as well as corresponding balance groups ranging from 31-28 Da (final size minus the peptide-reactive group: 145.1 Da). The 8-plex iTRAQ set consists of eight tags containing reporter groups of 113.1, 114.1, 115.1, 116.1, 117.1, 118.1, 119.1 and 121.1 Da as well as corresponding balance groups ranging from 192-184 Da (final size minus the peptide-reactive group:
305.1 Da). iTRAQ molecules label peptides by covalently binding to their abundant N-termini and side chain amines.

In my study, the 8-plex iTRAQ set was employed with membrane protein-enriched samples obtained from rat livers. The primary advantage associated with my choice of proteomic approach was that it allowed me to compare eight samples in each LC-MS/MS run. Making comparisons within the same LC-MS/MS run completely eliminates the quantitative variability associated with sequential analysis of multiple peptide mixtures (Washburn et al., 2003). Reducing this form of variability (experimental error) increases the likelihood that more subtle changes in protein expression will be detected.

There are, unfortunately, two key disadvantages associated with my choice of proteomic approach that warrant discussion. The first disadvantage is the fact that iTRAQ methodology aims to identify and quantify all proteins in a sample under study but typically varies in its ability to achieve this target. In my study, I was able to identify and quantify only 625 and 607 proteins in my two experiments. These numbers are below previous reports that employ similar proteomic methods (Chick et al., 2008) and are most certainly below the number of membrane proteins believed to be expressed in rodent liver. Also, only 476 proteins were common to both experiments despite the same vehicle control samples serving as controls in both experiments. This illustrates that proteins were not consistently identified and quantified between LC-MS/MS runs. My inability to consistently detect as many proteins as others across LC-MS/MS runs is likely a function of my acquisition settings and identification criteria interacting with sample complexity.

In tandem MS, a multitude of m/z features are detected at MS1 but only a small subset is followed by data from MS2. This subset is usually automatically generated on the basis of features from the MS1 scan in a mode called information-dependent acquisition (IDA; See section 3.3). Because feature intensity is a major factor in selecting MS1 fragments for an MS2 scan, repetitive selection of high abundance peptides can occur to the detriment of low abundance peptides. The selection of high abundance peptides was an issue that prevented me from obtaining proteomics data for the placenta. When my iTRAQ experiments were replicated with membrane fractions obtained from non-perfused placentas instead of membrane fractions obtained from perfused livers, the resulting datasets were inundated with blood proteins. Low abundance proteins, in comparison to blood proteins, were scarcely detected. These experiments could not be repeated with perfused placentas because of the cost.

Peptide sharing between proteins at the point of protein identification could compound identification issues relating to IDA. At present, it is standard practice for protein identification
software to discard shared peptides and proteins with non-unique peptides so as to reduce the likelihood of identification errors. Accordingly, in my iTRAQ experiments, I used an “Unused ProtScore” of 1.3 as my cutoff point for identification. The “Unused ProtScore” is a ProteinPilot software-generated estimate of the confidence associated with protein identifications and is based only on peptide evidence that is not better explained by a higher-ranking protein (i.e., it disregards shared peptides). An Unused ProtScore of 1.3 corresponds to an identification confidence of 95%, which is a stringent and commonly employed cutoff point in proteomics research. Given the high degree of peptide sharing that occurs within drug transporter and metabolic enzyme protein families, it is reasonable to think that some proteins were not identified in my experiments because their peptides could not be confidently attributed to them.

The problem with developing an incomplete proteome, as it pertains to my study, is that I was originally interested primarily in drug transporters and metabolic enzymes but these proteins ended up being underrepresented in my datasets. Because my original interests were fairly specific, a targeted proteomic approach, involving just 10-20 carefully selected drug transporters and metabolic enzymes, may have been warranted. LC-MS/MS with selected reaction monitoring (SRM) is an example of a suitable targeted proteomic approach (Reviewed in Gallien et al., 2011).

The second disadvantage associated with my choice of proteomic approach involves iTRAQ’s underestimation of the magnitude of effects. It is now known that the dynamic range of iTRAQ is compromised in complex mixtures, like those analyzed in my experiments, because relative ratios are suppressed toward unity (Ow et al., 2009). Ow and colleagues demonstrated this effect when they mixed an 8-plex iTRAQ master mix with a 4-plex iTRAQ master mix and then observed suppression primarily in the 4-plex range (Ow et al., 2009). This disadvantage, as was the case with the previously described disadvantage, stems from sample complexity. In light of this, it is possible that the magnitude of effects described in Chapter 3 could actually be greater than what is reported. Some support for this comes from my iTRAQ data concerning glucose-6 phosphatase (G6Pase). G6Pase plays an important role in the homeostatic regulation of blood glucose and its pronounced hepatic induction in rodent models of diabetes, on the order of 2-4 fold, has been demonstrated (Segal and Washko, 1959; Massillon et al., 1996; Li et al., 1999). It is, therefore, surprising that a comparatively small induction of only 1.68-fold was observed in my comparison of non-treated GDM rats to vehicle controls (p < 0.05; Table 3.2).
6.6.2 A surgical method for use in studies of rat fetal pharmacokinetics

Due to obvious ethical constraints, detailed in vivo fetal drug biodistribution and pharmacokinetic data is essentially unobtainable in humans. Studies in humans are limited to ex vivo placental perfusion models and postnatal cord blood/neonate blood sampling protocols. Human ex vivo placental perfusion models provide insight into the likely extent to which human fetuses are exposed to maternally administered drugs (Gavard et al., 2006; Kraemer et al., 2006; Ceccaldi et al., 2008); however, the translatability of this insight is limited because placental perfusion models do not address the interplay between the placenta, fetus and amniotic fluid that exists in whole animals. Human perinatal pharmacokinetic studies that draw postnatal cord blood and/or neonate blood samples can address this interplay but only for a single postnatal time point (Acosta et al., 2001; Stek et al., 2006; Mirochnick et al., 2008; Eyal et al., 2010). As a consequence of these limitations, detailed data pertaining to fetal pharmacokinetics is often obtained from in vivo studies performed in laboratory animals.

The rat is the most frequently used laboratory animal when it comes to studies of fetal drug biodistribution and pharmacokinetics. Unfortunately, given its small size, sampling from the rat fetal compartment requires destructive measurement techniques. This means that the entire fetal compartment must be sacrificed to obtain placental or fetal drug concentrations. Generally speaking, there are two approaches to these studies.

The first approach involves maternal drug administration followed by sacrifice and the simultaneous collection of all maternal and fetal tissues of interest. Data from multiple dams, sacrificed at a common time point, can then be compared with similarly obtained data from different study groups using standard statistical tests (Mulay and Varma, 1984; Petrovic et al., 2008). This type of data is typically considered biodistribution data. To obtain pharmacokinetic parameters via this approach, data from multiple dams, sacrificed in groups at different time points, can be strung together to generate a single concentration versus time curve (Hartmann et al., 2005; Zhang et al., 2007; Zhou et al., 2007). Since only one concentration versus time curve is generated in this scenario, mathematical methods that estimate error, like Bailer’s approach, must be utilized if one wishes to compare similarly obtained pharmacokinetic parameters from different study groups using standard statistical tests (Bailer, 1988).

The second approach to studying fetal pharmacokinetics in rats involves maternal drug administration followed by the surgical removal of intact fetal compartments at serial time points (Boike et al., 1989a; Boike et al., 1989b; Clark et al., 2001; Brown et al., 2003; Alnouti et al., 2004). In
this second approach, each dam supplies a fetal compartment at each time point of interest so as to generate individual concentration versus time curves that are appropriate for standard pharmacokinetic analysis. Since a concentration versus time curve is generated for each dam, comparing the pharmacokinetic parameters of different study groups using standard statistical tests is straightforward.

Because these two approaches are associated with advantages and disadvantages, I utilized both in Chapter 4 to obtain fetal pharmacokinetic data for LPV in the rat. An initial cohort of dams (n = 4-5/group) was sacrificed 45 min after LPV administration (single time point, biodistribution only/no pharmacokinetic parameters) and a second cohort of dams (n = 3/group) was subjected to serial sampling for one hr after LPV administration (seven fetal time points/dam and a maternal time point at 60 min).

One obvious advantage to sacrificing dams and collecting all samples at a single time point is the ease with which studies can be performed. Surgical skill and equipment requirements are minimal with this approach. Because of this, collecting samples at a single time point is less time consuming for the researcher.

Another advantage is the fact that drug concentrations in maternal plasma/serum and tissues, such as liver, can be matched to drug concentrations obtained in an entire litter of fetal compartments because all samples are taken together upon sacrifice. The initial cohort of dams in my study provided me with time-matched maternal and fetal data for a single 45 min time point (Fig. 4.2). The initial cohort of dams also allowed me to use time-matched littermates to determine if fetal compartments from each of the study’s three groups had similar LPV concentrations at a given point in time (i.e., 45 min). I viewed this information as critical to the interpretation of subsequent serial sampling data because the underlying assumption of serial sampling is that drug concentrations in any one extracted fetal compartment will reflect concentrations in all fetal compartments at the time of extraction. Data from the initial cohort of dams demonstrated that fetal uterine horn position has no effect on fetal LPV exposure at 45 min. This was done by first converting all fetal concentrations (n = 5-10/dam) to a percentage of their corresponding litter’s average concentration and then performing a one-way ANOVA on resulting values after grouping them according to uterine horn position (10 positions: right 1-5 and left 1-5).

One disadvantage of sacrificing dams and collecting all samples at a single time point, at least when samples from additional groups are not collected at additional time points, is that the data provides only a snapshot into fetal exposure. Without accompanying pharmacokinetic data, this snapshot is without context: it could represent peak or plateau concentrations.
Another disadvantage of collecting samples this way is that a lot more animals are required when each animal contributes only one fetal time point. This is a disadvantage on both ethical and financial grounds and can ultimately lead to a study examining only a limited number of time points. With respect to my study, only one time point was assessed via this approach.

The advantages and disadvantages to serial sampling are basically the inverse of the advantages and disadvantages of collecting samples at a single time point of interest. In terms of advantages, a complete fetal concentration versus time curve can be generated within a single dam. This provides more than a snapshot. This also means that fewer dams are required to conduct a study of fetal pharmacokinetics because each dam provides multiple data points. To put this in perspective, the fetal concentration versus time curves that were generated for my study’s second cohort consisted of seven time points and there were three animals in each of the study’s three groups (63 data points). Consequently, what was accomplished with nine animals in my study would require at least 63 animals if each data point was derived from a single dam.

In terms of disadvantages, serial sampling studies are technically more difficult to perform. Surgical method descriptions in the literature are currently vague and do not provide enough detail to allow individuals with moderate surgical experience to perform them without essentially developing their own protocol. The surgical method that I employed in my studies had to be developed and validated specifically for my studies with the assistance of technicians from the University of Toronto’s Department of Comparative Medicine.

Another disadvantage of serial sampling is that it is generally not possible to match drug concentrations in maternal tissues, such as liver, to drug concentrations obtained in the fetal compartment because harvesting maternal tissues jeopardizes the dam’s viability and can alter fetal exposure. In the majority of published studies employing serial sampling, only blood samples are taken from the dam. In my serial sampling study, no maternal samples were taken until fetal compartment sampling was complete.

One final disadvantage of serial sampling that is worth noting is the potential for distribution volume to be lost via sampling. As mentioned in Chapter 1, a drug’s volume of distribution is the quotient of the amount of drug in the body (i.e., the administered amount) and the amount of drug retained in the central compartment (i.e., in blood plasma). For drugs that distribute extensively outside the central compartment, the removal of fetal compartments could represent lost distribution volume. Data from my study’s second cohort was used to determine $\text{AUC}_{0-60}^{\text{F}}$, $\text{C}_{\text{max}}$, and $\text{T}_{\text{max}}$ for fetal LPV exposure in all three of the study’s groups but these fetal pharmacokinetic values were derived from curves that were obtained while distribution volume was potentially being lost via
sampling. The mean weights of fetal compartments in my study were approximately 3.4 g for the vehicle group and approximately 3.2 g for the GDM and insulin-treated groups (for this discussion, fetal compartment weight = group mean placental weight + group mean fetal weight + mean amniotic fluid weight of 0.25 g). Therefore, in my fetal pharmacokinetic study, the total weight lost after removing seven fetal compartments was fairly similar across groups at approximately 22.4 g for the vehicle group and approximately 23.8 g for the GDM and insulin-treated groups. Even if the mean weight lost due to sampling is expressed as a percentage of mean maternal weight on GD20, resulting values after removing seven fetal compartments are similar across groups. To the extent that fetal compartments contribute to LPV’s volume of distribution in the pregnant rat, lost distribution volume would be expected to alter maternal pharmacokinetic parameters. This, in turn, would likely alter fetal pharmacokinetic parameters; however, in my study, it would likely do so to the same extent across groups. Thus, while distribution volume lost via sampling could have affected the accuracy of reported fetal pharmacokinetic values, it is not expected to have affected the significant differences observed between groups.

Comparisons between anesthetized and non-anesthetized rats are confounded by differences in blood flow. Anesthesia can cause a drop in blood flow that can influence the disposition of some drugs. Drug disposition can also be influenced by level of physical activity (i.e., anesthetized animals versus freely moving animals). These are considerations for both of the abovementioned approaches.
6.7 Future studies

By providing evidence for increased hepatic and placental drug transporter expression, increased hepatic drug metabolic enzyme expression and decreased plasma protein binding in a rat model of diabetic pregnancy, this thesis opens the door for future studies in which diabetic pregnancy’s effects on drug disposition mechanisms could be examined further. In this section, a series of studies are described that, if conducted, would expand the scope of my work with STZ-induced GDM, test some of my mechanistic claims and determine if the effects described in this thesis translate into human pregnancies complicated by diabetes.

6.7.1 Studies that expand the scope of my work with STZ-induced GDM

The focus of most of the expression studies described in this thesis was limited to a small number of key proteins: Mdr1, Mrp2, Bcrp and, to a lesser extent, Cyp3a2. This focus was considered acceptable because these few proteins, on their own or in combination, are involved in the transport or metabolism of a disproportionately high number of clinically important drugs (Guengerich, 1999; Chandra and Brouwer, 2004). Still, there are a multitude of additional drug transporters and metabolic enzymes that play important roles in drug disposition (Fig. 1.1). There are also a multitude of corresponding nuclear receptors that provide critical insight into the regulatory mechanisms that govern the expression of these additional drug transporters and metabolic enzymes. Future studies could expand the scope of my work by examining some of these additional drug transporters and metabolic enzymes in STZ-induced GDM, along with their corresponding transcriptional regulators (including PPARα).

The expression of these additional proteins could be examined not only in the liver and placenta but in the intestine, kidneys, blood-brain barrier and even fetal tissues as well, which is another way that future studies could expand the scope of my work. Both the intestine and kidneys play major roles in drug disposition; the intestine by governing the extent of intestinal drug absorption and metabolism and the kidneys by governing urinary drug excretion (and renal metabolism). Like the placenta, the blood-brain barrier plays a barrier role by limiting the transfer of drugs from the central compartment to the central nervous system. Expanding studies to include these additional organs/tissues would provide a more comprehensive “picture” of drug transporter and metabolic enzyme expression changes in this model. Also, it is possible that the development of fetal drug disposition mechanisms could be affected by the diabetic intrauterine environment. For this reason, it might be of interest to examine fetal tissues such as the liver.
Future studies geared toward expanding my work with STZ-induced GDM could also follow-up on the data that is presented in appendix fig. 4.4. Appendix fig. 4.4 presents preliminary data demonstrating that, relative to sex-matched controls, the expression of Mdr1b and Bcrp in male placentas from the GDM group was upregulated while the expression of these transporters in female placentas from the GDM group was not significantly altered. This suggests that male placentas could have been primarily responsible for the upregulation of placental transporters that was reported for the GDM group in Chapter 4 (i.e., the effect appears to only be present in this subgroup). There was also a trend toward transporter upregulation in female placentas from the insulin-treated group that was not apparent in male placentas from the insulin-treated group, suggesting that insulin-managed diabetes might not have the same corrective effects on female placentas as it does on male placentas. Studies of maternal asthma, pre-eclampsia and preterm delivery have shown that male and female fetuses and their placentas utilize different mechanisms when dealing with adverse prenatal conditions (Reviewed in Clifton, 2010). Perhaps male and female placentas also utilize different mechanisms when dealing with maternal diabetes. Determining fetal sex whenever data is collected from placentas in future studies could shed light on this possibility.

6.7.2 Studies that test some of my mechanistic claims

My secondary general hypothesis was that observed transporter changes would be linked to pro-inflammatory cytokines. Ultimately, I failed to obtain sufficient evidence of an inflammatory response in STZ-induced GDM and abandoned this hypothesis. This resulted not only from the absence of sufficient evidence of inflammation in my studies but also from the fact that upregulation, not downregulation, in drug transporters and metabolic enzymes was observed, which runs contrary to what is currently known about the regulatory effects of pro-inflammatory cytokines (Reviewed in Petrovic et al., 2007). Nonetheless, my claims that inflammation was not the mechanism responsible for observed changes in drug transporters and metabolic enzymes warrants future study.

My attempts to quantify the plasma concentrations of pro-inflammatory cytokines were met with detection difficulties. Due to data obtained in human diabetics and glucose-treated human monocytes, the concentrations of pro-inflammatory cytokines were never expected to be very high in STZ-induced diabetes (Morohoshi et al., 1995; Morohoshi et al., 1996; Arnalich et al., 2000; Esposito et al., 2002; Leinonen et al., 2003). Thus, to detect differences between the disease state and the control state in my experiments, a highly sensitive detection method was required. Based on previous studies in the laboratory of my supervisor that involved acute inflammation, where the differences between the disease state and control state are quite dramatic, the ELISA was utilized.
but this proved to be an inadequate approach for my work. For the vast majority of samples, across studies, the concentrations of cytokines that I examined were below detection limits. Moreover, these detection limits were generally high (certainly higher than the concentrations expected to be present in control animals). As a result of these difficulties, abundantly expressed and highly stable CRP had to be used as a surrogate marker of inflammation in Chapter 2 and 3. Unfortunately, this protein is not considered an ideal way to examine inflammation and does not provide conclusive evidence for the presence or absence of inflammation. This may be particularly true of inflammation in pregnancy as basal CRP is elevated in pregnancy to a greater extent than other inflammatory markers, suggesting the pregnant state could provide confounding regulatory factors (McLachlan et al., 2006). To provide a more rigorous characterization of STZ-induced GDM's inflammatory potential, future studies should make use of some recent advancements in cytokine quantitation that address sensitivity issues and allow for the simultaneous detection of multiple analytes. For example, multiplex assays like antibody arrays and bead-based suspension arrays are much more sensitive than ELISAs and can be used to analyze more than one analyte/sample (e.g., bead-based suspension arrays currently enable the simultaneous quantitation of up to 100 analytes/sample).

The alternative mechanism that I propose in several sections of this thesis, that excess plasma lipids lead to the activation of nuclear receptors so as to enhance drug transporter and metabolic enzyme expression, also warrants future study. Testing this mechanism in an animal model of hyperlipidemia is a logical first step because this allows the hyperlipidemia component of STZ-induced GDM to be isolated. A high-fat diet induces hypertriglyceridemia in non-pregnant, female Sprague-Dawley rats after just two weeks and does not affect blood glucose or fertility (Xue et al., 2001; Niculescu and Lupu, 2009). Based on my proposed mechanism and the data that I have to support it, I hypothesize that pregnant rats with high-fat diet-induced hypertriglyceridemia would exhibit a pattern of drug transporter and metabolic enzyme expression changes that is comparable to my observations in STZ-induced GDM. This model could also be used to further test the mechanism I propose for altered plasma/serum protein binding of drugs in STZ-induced GDM.

6.7.3 Studies that determine if the effects described in this thesis translate into human pregnancies that are complicated by diabetes

Provided researchers are interested in it at the point of delivery (i.e., at term and not at earlier stages of gestation), the entire human placenta is easily collected for study as a consequence of its ephemeral nature. For this reason, perfusion and protein expression studies are quite feasible approaches to determining if the effects described in this thesis translate into human pregnancies.
that are complicated by diabetes. Perfusion studies have been used to study the effects of diabetes on the placental transfer of nutrients, such as glucose and various lipids, by comparing transfer in placentas obtained from pregnancies complicated by diabetes to transfer in uncomplicated pregnancies (Kuhn et al., 1990; Osmond et al., 2000). Virtually the same methodology used in these studies could be employed to study the effects of diabetes on the placental transfer of drug transporter substrates, as was done in at least one small-scale study of metformin transfer (Nanovskaya et al., 2006b). Protein expression studies could be incorporated into these studies, by examining drug transporter expression in isolated lobules, or could be conducted with placental samples collected specifically for this purpose, which was done in the study described in Chapter 5. Incorporating protein expression studies into perfusion studies allows the relationship between placental drug transporter expression and the placental transfer of substrate drugs in human diabetic pregnancy to be quantified (albeit with the interpretative limitations mentioned in section 6.6.2).

Regardless of whether a perfusion, expression or combined perfusion/expression approach is taken, I believe it is important to include a measure of the quality with which diabetes was managed during pregnancy. Without such a measure, it is impossible to stratify patients according to whether their diabetes was managed, poorly managed or unmanaged while pregnant. Effects attributable to diabetic pregnancy would be diluted without the ability to stratify patients in this way because, as this thesis has shown, it is reasonable to expect dramatically diminished effects with proper management. The importance of such a measure was made clear to me while conducting the study described in Chapter 5, in which drug transporter expression was studied in human placentas obtained from pregnancies complicated by insulin-managed diabetes. While my sample sizes were too small to make patient stratification a viable option, I was able to utilize HbA1c percentage data to determine the relationship between average maternal glycemic control and placental MDR1, MRP2 and BCRP protein expression. Since there was a positive correlation between HbA1c percentage and BCRP protein expression, it stands to reason that stratification could lead to the detection of group differences that would not necessarily be detectable in heterogeneous groups.

Future studies could also attempt to determine if the effects described in this thesis translate into human pregnancies that are complicated by diabetes by actually including these individuals in clinical studies aimed at generating drug disposition information. Despite being more difficult to plan and execute than studies involving non-pregnant individuals, clinical studies of drug disposition in pregnancy regularly appear in the literature. One particularly common experimental design for these studies involves a comparison of drug disposition data obtained in pregnancy to patient-matched data obtained in the postpartum period. For example, Mirochnick and colleagues used this
design when they showed that an increased LPV/RTV dose produced desirable AUC values in pregnancy but produced AUC values in the postpartum period that were undesirably high (Mirochnick et al., 2008). This design is common because it allows researchers to focus on individuals who have already made the decision to use the drug of interest during pregnancy, such as LPV/RTV in HIV-positive individuals, but it primarily tests the effects of pregnancy itself on drug disposition and not the effects of different “types” of pregnancy. The inclusion or identification of individuals with diabetic pregnancy in these studies would make possible the comparison of drug disposition data obtained in diabetic pregnancies to data obtained in non-diabetic pregnancies.

Ethical and legal considerations will likely continue to limit the use of uncomplicated pregnancies as controls. To allow for patient stratification in these studies, I again believe the inclusion of a measure like HbA1c would be important.

While cases of completely unmanaged diabetes, regardless of type, are relatively rare in high-income countries like Canada, it is worth noting that unmanaged diabetes is a reality in many low- and middle-income countries because of inadequate access to medical care. According to the International Diabetes Federation, 80% of global expenditures relating to medical care for people with diabetes occur in high-income countries, not in the low- and middle-income countries where over 70% of people with diabetes live (International Diabetes Federation, 2006). Thus, studies attempting to determine if the effects described in this thesis translate into human GDM might be more successful if conducted in the developing world. Given the burden of the HIV/AIDS pandemic in these nations, studies might also be more meaningful if the effects of GDM on the disposition of antiretroviral drugs, like LPV, is a focus.
6.8 Conclusions

Today, approximately 8% of all pregnancies are complicated by diabetes. Diabetes alters drug disposition mechanisms in non-pregnant subjects but the impact of diabetes on drug disposition in pregnancy has not been properly evaluated. This thesis investigated drug disposition mechanisms primarily in a rat model of experimental GDM, focusing on plasma/serum drug protein binding and drug efflux transporter/metabolic enzyme expression.

My primary general hypothesis was that experimental GDM would cause changes in drug disposition mechanisms that would ultimately contribute to altered maternal and fetal drug exposure. One key finding presented in this thesis is that elevated circulating lipids in GDM rats competed with drugs (e.g., glyburide and saquinavir) for plasma protein binding so as to increase free drug concentrations. Another key finding was that important hepatic drug efflux transporters (e.g., Mdr1a/b) and metabolic enzymes (e.g., Cyp3a2 and Ugt1a1) were upregulated in GDM. Upregulation of hepatic drug efflux transporters and metabolic enzymes, coupled with increased free drug concentrations, would be expected to increase the hepatic clearance of many drugs. Consistent with this, in GDM, maternal and fetal exposure to the Mdr1 and Cyp3a2 substrate LPV was substantially lower than controls 45 and 60 min post-administration and data supporting enhanced LPV metabolite formation were obtained. Placental drug efflux transporters were also examined in this lopinavir study. Elevated placental Mdr1b and Bcrp expression was observed in GDM, which was associated with decreased fetal exposure to lopinavir (even after correcting for maternal concentrations). These findings support my primary general hypothesis.

My secondary general hypothesis was that observed changes in drug efflux transporter expression would be linked to pro-inflammatory cytokines. Because inflammation was either not a feature of my model or was occurring at a level that I could not detect, I was not able to properly test this hypothesis. Nonetheless, upregulation in drug transporters and metabolic enzymes runs contrary to what is currently known about the regulatory effects of pro-inflammatory cytokines (Reviewed in Petrovic et al., 2007). As an alternative to inflammation-mediated regulation of drug transporters and metabolic enzymes, it was proposed that alterations could be by-products of nuclear receptor activation initiated for the purpose of promoting energy homeostasis.

Taken together, this thesis demonstrates that experimental GDM can significantly impact drug disposition by altering key drug disposition mechanisms. If confirmed in humans, this drug-disease interaction would need to be considered when atypical therapeutic outcomes occur in diabetic pregnancies.
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