August 2016

The Effect of AST-120 on Hepatic Metabolism and Transport in Chronic Kidney Disease

Andrew S. Kucey
The University of Western Ontario

Supervisor
Dr. Brad Urquhart
The University of Western Ontario

Graduate Program in Physiology and Pharmacology

A thesis submitted in partial fulfillment of the requirements for the degree in Master of Science

© Andrew S. Kucey 2016

Follow this and additional works at: http://ir.lib.uwo.ca/etd
Part of the Male Urogenital Diseases Commons, and the Medicinal and Pharmaceutical Chemistry Commons

Recommended Citation
http://ir.lib.uwo.ca/etd/3990

This Dissertation/Thesis is brought to you for free and open access by Scholarship@Western. It has been accepted for inclusion in Electronic Thesis and Dissertation Repository by an authorized administrator of Scholarship@Western. For more information, please contact tadam@uwo.ca.
Abstract

Chronic kidney disease (CKD) is characterized by a progressive and irreversible decline in renal function. Patients are at high risk for adverse drug events since they are typically administered multiple medications concurrently and pharmacokinetic changes in the diseased state are relatively unexplored. Recent studies point towards molecules known as uremic toxins for playing a mechanistic role in altering the expression and function of drug metabolizing enzymes and drug transporter proteins. To further investigate this hypothesis, an adenine-induced model of CKD was used in male wistar rats. AST-120 was administered to remove uremic toxins in an attempt to recover metabolic enzyme as well as transporter protein function and activity. Animals were injected with rosuvastatin prior to sacrifice as a probe for transporter function. Impaired organic anion transportin polypeptide (OATP) and cytochrome P450 (CYP) function are important to acknowledge in a clinical setting since both of these protein superfamilies have broad substrate specificities. We have shown significant down regulation in CYP2B1 activity and expression in rats ($P<0.05$); CYP2B6 is the human ortholog and it plays a crucial role in phase one drug metabolism. If function is affected, clearance for a particular class of drug (ex. statins for OATPs or bupropion for CYP2B6) could be altered which will affect drug efficacy and toxicity.

Keywords

Chronic kidney disease, uremic toxins, cytochrome P-450, OATP, AST-120, drug metabolism, drug transport, rosuvastatin, bupropion
Co-Authorship Statement

Work from this project was aided from fellow members of the Urquhart lab. Alvin Tieu and Dr. Thomas Velenosi aided in experimental work, animal care, and training surrounding laboratory techniques. Dr. Daniel Hardy assisted specifically with the chromatin immunoprecipitation technique. Drs. Brad Urquhart and Thomas Velenosi assisted with experimental design. Dr. Brad Urquhart supervised the project to its entirety.
Acknowledgments

First and foremost I would like to thank my supervisor Dr. Urquhart for giving me the opportunity to work in his lab. It is difficult to put into word how appreciative I am of everything you have done for me and the other students under your supervision. Your dedication that was shown through countless hours of guidance and tutelage along with your unwavering enthusiasm and genuine care for your students made the most memorable graduate school experience I could have imagined. I would also like to thank the other members of the Urquhart lab, especially Tom Velenosi and Alvin Tieu. As the senior grad student in the lab, Tom, you were indispensable to the new students with your vast knowledge of laboratory techniques and basic science theory. I will be forever thankful for the support you provided me throughout my project, be it the long nights and weekends at the lab bench or the midnight hockey games, you were a great friend all the way to the end. Your keen interest in science and your innate drive to succeed will take you wherever you want to go, there are not many people out there with a work ethic like yours, never lose that fire. Maybe one day that will translate onto the ice, so keep working on that backhand. Alvin, there were many long nights in the lab and throughout our extracurricular experience in downtown London that defined my graduate school experience. You provided many new cultural experiences for me, most notably the congee adventure. I have always admired how you manage to have so much consistency in your life, probably stemming from your mantra “the last minute is the best minute”. Good luck in Ottawa, I know you will do an amazing job. I hope one day we all have a reunion at the Moonshine Flats.

My two roommates that were with me throughout the duration of grad school, Adam and Anish, thank you for the late night conversations and the animated rants that were frequently provided. You guys always supported any idea that was brought up, for better or for worse, and I would not have it any other way. Your unconditional support and penchant for turning a whimsical idea into an unforgettable experience will never be forgotten. I could not have asked for better roommates throughout the rocky ride at 1055 Richmond. One day we will revisit our stomping grounds, good luck on your PhD Anish, and stay golden Adam, you both have extremely bright futures. I would also like to thank Jon who lived with us throughout the first year of grad school, the house became a lot emptier and a lot less athletic when you moved out. Good luck in law school, I am really excited it worked out for you.

Of course I would have never had this opportunity without the support of my family. There is never enough that can be said about the boundless encouragement from my mother and father. Whether they were asking why I had not finished my thesis a year after I started or helping me plan my future, I will forever be in debt for the time and money you have invested in me. Last but not least I would like to thank my brother Daniel, sister Christine, and girlfriend Aneta for their support throughout my Masters. Aneta, although you still think that I played with rats for 2 years I am sincerely grateful for the relief you have provided me from the stress of graduate school as well as the support and encouragement you gave me through the more trying times that arose while in pursuit of this degree. Your perpetually happy demeanor is always something to look forward to. I hope your experience working towards an MSc is as enjoyable as mine was.
Table of Contents

Abstract ................................................................................................................................. i
Co-Authorship Statement ........................................................................................................ ii
Acknowledgments .................................................................................................................. iii
Table of Contents ................................................................................................................... iv
List of Tables ........................................................................................................................ viii
List of Figures ........................................................................................................................ ix
List of Appendices ................................................................................................................ xi
Abbreviations ........................................................................................................................ xii
Chapter 1 ............................................................................................................................... 1
  1 Introduction ...................................................................................................................... 1
    1.1 Chronic Kidney Disease ............................................................................................ 3
      1.1.1 Prevalence and Diagnosis .................................................................................. 3
      1.1.2 Stages ................................................................................................................ 4
      1.1.3 Treatment .......................................................................................................... 6
      1.1.4 Etiology and Comorbidities .............................................................................. 7
    1.2 Uremia ....................................................................................................................... 10
      1.2.1 Intestinal Microbiome ...................................................................................... 11
      1.2.2 Uremic Toxins .................................................................................................. 12
    1.3 Removal of Gut-Derived Uremic Toxins ................................................................. 15
      1.3.1 AST-120 ........................................................................................................... 17
    1.4 Drug Metabolism and Disposition ........................................................................... 19
      1.4.1 Metabolism ....................................................................................................... 20
      1.4.2 Transport .......................................................................................................... 23
      1.4.3 Nuclear Regulation .......................................................................................... 26
1.4.4 Altered Metabolism & Transport in Disease States.................................30
1.5 Statins.........................................................................................................33
  1.5.1 Rosuvastatin.........................................................................................33
1.6 Animal Models of CKD.............................................................................35
  1.6.1 Nephrotoxic Models............................................................................36
1.7 Objectives and Hypothesis........................................................................37
Chapter 2...........................................................................................................38
2 Methods..........................................................................................................38
  2.1 CKD Rat Model........................................................................................38
    2.1.1 Study 1 – Hepatic and Intestinal Transport (7 Weeks)......................38
    2.1.2 Study 2 – Hepatic Transport (8 Weeks)..........................................39
    2.1.3 Study 3 – Hepatic Metabolizing Enzymes (8 Weeks)......................39
  2.2 Real-Time PCR........................................................................................40
    2.2.1 Primer Validation.............................................................................40
    2.2.2 Quantification..................................................................................40
  2.3 Western Blot..............................................................................................42
    2.3.1 Microsome Isolation .......................................................................42
    2.3.2 Pierce BCA Assay...........................................................................42
    2.3.3 Gel Electrophoresis.........................................................................42
  2.4 Rosuvastatin Quantification......................................................................43
    2.4.1 Plasma Extraction............................................................................43
    2.4.2 Hepatic Extraction..........................................................................44
    2.4.3 UPLC-MS Conditions......................................................................44
  2.5 Enzyme Function Assay............................................................................45
    2.5.1 Validation......................................................................................45
    2.5.2 Hydroxybupropion Quantification..................................................45
2.6 Chromatin Immunoprecipitation ................................................................. 46
2.7 Huh7 Cell Line .................................................................................................. 47
  2.7.1 Characterization .......................................................................................... 48
  2.7.2 Uptake Transport and Uremic Toxin Incubation ......................................... 48
  2.7.3 Uremic Toxin Incubation and Transcriptional Expression ....................... 49
2.8 Statistical Analysis ........................................................................................... 49

Chapter 3 ................................................................................................................. 51
3 Results .................................................................................................................. 51
  3.1 Drug Transport in a 7-week in vivo CKD Model ............................................ 51
    3.1.1 Assessment of Kidney Damage ............................................................... 51
    3.1.2 Transporter Expression and Function ..................................................... 51
  3.2 Drug Transport in an 8-week in vivo Model of CKD ..................................... 57
    3.2.1 Assessment of Kidney Damage ............................................................... 57
    3.2.2 Transporter Expression and Function ..................................................... 57
  3.3 Drug Metabolism in an 8-week in vivo CKD Model ....................................... 61
    3.3.1 Assessment of Kidney Damage ............................................................... 61
    3.3.2 Hepatic Oatp Transcriptional Expression .............................................. 61
    3.3.3 CYP2B Transcriptional Expression ....................................................... 61
    3.3.4 CYP2B Protein Expression and Activity in CKD ................................... 65
    3.3.5 mRNA Expression of Nuclear Receptors and ChIP for CYP2B ............ 70
  3.4 Uremic Toxins as a Mechanism of Down-Regulation in vitro ....................... 73
    3.4.1 Huh7 Cell Line Validation .................................................................... 73
    3.4.2 Rosuvastatin Uptake ............................................................................. 73
    3.4.3 CYP2B6 Expression ............................................................................. 73

Chapter 4 ................................................................................................................. 78
4 Discussion ............................................................................................................. 78
4.1 Conclusions .......................................................................................................................... 79

4.1.1 Drug Transport in a 7-week Model of CKD ......................................................... 79

4.2 Drug Transport in an 8-week model .............................................................................. 80

4.3 Altered Expression and Function of CYP2B ........................................................... 81

4.4 Role of Uremic Toxins on Rosuvastatin Transport in vitro ........................................ 85

4.5 Limitations ...................................................................................................................... 86

4.6 Future Studies ............................................................................................................... 87

4.7 Overall Conclusions ...................................................................................................... 88

References ................................................................................................................................ 90

Curriculum Vitae ...................................................................................................................... 111
List of Tables

Table 1.1 - Stages and prevalence of chronic kidney disease in North America.................. 5

Table 2.1 - Primer sets for real-time PCR....................................................................... 41

Table 3.1 - Michealis-Menten parameters for $K_m$ and $V_{max}$ pertaining to the data in Figure 13. Values are presented as mean ± SEM....................................................... 69
List of Figures

Figure 1.1 - Schematic representation of gross renal anatomy (A) and the nephron (B) ........ 2

Figure 1.2 - Schematic illustration of the PBREM binding region for the transcriptional regulation of CYP2B1 ................................................................. 29

Figure 3.1 - Plasma urea (A) and creatinine (B) levels in control, CKD, control+AST-120, and CKD+AST-120 wistar rats after a 7 week study ............................................. 52

Figure 3.2 - Relative mRNA expression of hepatic transporters in a 7-week CKD model .... 53

Figure 3.3 - Relative mRNA expression of intestinal P-gp (A), Bcrp (B), Mrp2 (C), and CYP3A2 (D) in control, control+AST-120, CKD and CKD+AST-120 in wistar rats ........ 54

Figure 3.4 - Liver to plasma ratio of rosuvastatin in control, control+AST-120, CKD, and CKD+AST-120 wistar rats ........................................................................ 55

Figure 3.5 - (A) Relative liver Oatp1a4 mRNA expression in control, control+AST-120, CKD, and CKD+AST-120 in wistar rats. (B) Linear regression of control and CKD rats displaying the correlation between creatinine and Oatp1a4 ........................................ 56

Figure 3.6 - Plasma urea (A) and creatinine (B) levels in control, CKD, control+AST-120, and CKD+AST-120 wistar rats after an 8 week study ............................................. 58

Figure 3.7 - Relative mRNA expression of hepatic Oatp1a4 (A) and Bcrp (B) in an 8 week model of adenine induced CKD ............................................................... 59

Figure 3.8 - Liver to plasma ratio of rosuvastatin in control, control+AST-120, CKD, and CKD+AST-120 wistar rats ........................................................................ 60

Figure 3.9 - Plasma urea (A) and creatinine (B) levels in control, CKD, control+AST-120, and CKD+AST-120 wistar rats after an 8 week study ............................................. 62

Figure 3.10 - Relative mRNA expression of hepatic transporters in an 8-week CKD model.63
Figure 3.11 - Relative mRNA expression of CYP2B1 (A) and CYP2B2 (B) in an 8-week CKD model ................................................................. 64

Figure 3.12 - A) Relative protein expression of CYP2B from an 8-week model of CKD in wistar rats ........................................................................................................ 66

Figure 3.13 - Bupropion metabolism to OH-bupropion as a measure of CYP2B activity in rat liver microsomes from control, control+AST-120, CKD, and CKD+AST-120 animals ...... 67

Figure 3.14 - Michaelis-Menten parameters derived from figure 3.13 and displayed as $V_{\text{max}}$ (A), $K_m$ (B), and intrinsic clearance (C) ......................................................................................... 68

Figure 3.15 - Relative mRNA expression of hepatic transcription factors in an 8 week model of CKD ........................................................................................................ 71

Figure 3.16 - Binding of RNA Polymerase II to hepatic CYP2B1 (A) and CYP3A2 (B) promoters in an 8 week model of CKD ......................................................................................... 72

Figure 3.17 - Huh7 cell line characterization. Expression of mRNA is relative to human liver for each transporter ........................................................................................................ 74

Figure 3.18 - Rosuvastatin uptake in Huh7 cells after co-incubation with various uremic toxins ....................................................................................................................... 75

Figure 3.19 - Rosuvastatin uptake in Huh7 cells after pre and co-incubation with various uremic toxins ....................................................................................................................... 76

Figure 3.20 - CYP2B6 mRNA expression in Huh7 cells after a 24 hour incubation with various uremic toxins ....................................................................................................................... 77

Figure 4.1 - Proposed mechanism for the decreased mRNA expression and function of CYP2B1 in CKD ........................................................................................................ 83
List of Appendices

Appendix A - Animal Ethics Approval ................................................................. 110
## Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
</tr>
</thead>
<tbody>
<tr>
<td>ABC</td>
<td>ATP-Binding Cassette</td>
</tr>
<tr>
<td>ABCB</td>
<td>P-gp gene</td>
</tr>
<tr>
<td>ANOVA</td>
<td>Analysis of Variance</td>
</tr>
<tr>
<td>BCRP</td>
<td>Breast Cancer Resistance Protein</td>
</tr>
<tr>
<td>BEH</td>
<td>Bridged Ethylene Hybrid</td>
</tr>
<tr>
<td>BMI</td>
<td>Body Mass Index</td>
</tr>
<tr>
<td>BSEP</td>
<td>Bile Salt Export Pump</td>
</tr>
<tr>
<td>BUP</td>
<td>Bupropion</td>
</tr>
<tr>
<td>CAPD</td>
<td>Continuous Ambulatory Peritoneal Dialysis</td>
</tr>
<tr>
<td>CAR</td>
<td>Constitutive Androstane Receptor</td>
</tr>
<tr>
<td>ChIP</td>
<td>Chromatin Immunoprecipitation</td>
</tr>
<tr>
<td>CKD</td>
<td>Chronic Kidney Disease</td>
</tr>
<tr>
<td>CMPF</td>
<td>3-carboxy-4-methyl-5-propyl-2-furan-propanoic acid</td>
</tr>
<tr>
<td>CRF</td>
<td>Chronic Renal Failure</td>
</tr>
<tr>
<td>CVD</td>
<td>Cardiovascular Disease</td>
</tr>
<tr>
<td>CYP</td>
<td>Cytochrome P-450</td>
</tr>
<tr>
<td>DBD</td>
<td>DNA-Binding Domain</td>
</tr>
<tr>
<td>DKD</td>
<td>Diabetic Kidney Disease</td>
</tr>
<tr>
<td>EPPIC</td>
<td>Evaluating Prevention of Progression in CKD</td>
</tr>
<tr>
<td>ESRD</td>
<td>End-Stage Renal Disease</td>
</tr>
<tr>
<td>FXR</td>
<td>Farnesoid X Receptor</td>
</tr>
<tr>
<td>GFR</td>
<td>Glomerular Filtration Rate</td>
</tr>
<tr>
<td>GI</td>
<td>Gastrointestinal</td>
</tr>
<tr>
<td>HMG-CoA</td>
<td>3-hydroxy-3-methylglutaryl-CoA</td>
</tr>
<tr>
<td>HNF4α</td>
<td>Hepatocyte Nuclear Factor Alpha</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>HRP</td>
<td>Horseradish Peroxidase</td>
</tr>
<tr>
<td>Huh7</td>
<td>Human Hepatocarcinoma Cell Line</td>
</tr>
<tr>
<td>IgG</td>
<td>Immunoglobulin</td>
</tr>
<tr>
<td>$K_m$</td>
<td>Enzyme Affinity</td>
</tr>
<tr>
<td>LBD</td>
<td>Ligand-Binding Domain</td>
</tr>
<tr>
<td>LDL</td>
<td>Low-Density Lipoprotein</td>
</tr>
<tr>
<td>MRP</td>
<td>Multidrug Resistance-Associated Protein</td>
</tr>
<tr>
<td>NADPH</td>
<td>Nicotinamide Adenine Dinucleotide Phosphate</td>
</tr>
<tr>
<td>NF-1</td>
<td>Nuclear Factor Binding Motif 1</td>
</tr>
<tr>
<td>NIPD</td>
<td>Nocturnal Intermittent Peritoneal Dialysis</td>
</tr>
<tr>
<td>NR1</td>
<td>Nuclear Receptor Binding site 1</td>
</tr>
<tr>
<td>NR2</td>
<td>Nuclear Receptor Binding Site 2</td>
</tr>
<tr>
<td>NTCP</td>
<td>Sodium Taurochlorate Co-Transporting Polypeptide</td>
</tr>
<tr>
<td>OAT</td>
<td>Organic Anion Transporter</td>
</tr>
<tr>
<td>OATP</td>
<td>Organic Anion Transporting Polypeptide</td>
</tr>
<tr>
<td>OCT</td>
<td>Organic Cation Transporter</td>
</tr>
<tr>
<td>OH-BUP</td>
<td>Hydroxybupropion</td>
</tr>
<tr>
<td>P-gp/MDR1</td>
<td>P-glycoprotein/Multidrug Resistance Protein</td>
</tr>
<tr>
<td>PB</td>
<td>Phenobarbital</td>
</tr>
<tr>
<td>PBREM</td>
<td>Phenobarbital Responsive Enhancer Module</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase Chain Reaction</td>
</tr>
<tr>
<td>PD</td>
<td>Peritoneal Dialysis</td>
</tr>
<tr>
<td>POR</td>
<td>Cytochrome P450 Oxidoreductase</td>
</tr>
<tr>
<td>PPAR$\gamma$</td>
<td>Peroxisome Proliferator-Activated Receptor Gamma</td>
</tr>
<tr>
<td>PXR</td>
<td>Pregnane X Receptor</td>
</tr>
<tr>
<td>RLM</td>
<td>Rat Liver Microsomes</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive Oxygen Species</td>
</tr>
<tr>
<td>RXR</td>
<td>Retinoid X Receptor</td>
</tr>
<tr>
<td>SEM</td>
<td>Standard Error of the Mean</td>
</tr>
<tr>
<td>SLC</td>
<td>Solute Carrier</td>
</tr>
<tr>
<td>Term</td>
<td>Definition</td>
</tr>
<tr>
<td>--------</td>
<td>---------------------------------</td>
</tr>
<tr>
<td>SNP</td>
<td>Single Nucleotide Polymorphism</td>
</tr>
<tr>
<td>$V_{\text{max}}$</td>
<td>Maximal Enzyme Velocity</td>
</tr>
</tbody>
</table>
Chapter 1

1 Introduction

The kidneys are vital organs whose responsibilities are vast and essential to maintain homeostasis for several physiological processes. The most important functions of the kidneys are the filtration of blood, production of hormones (e.g. erythropoietin), regulation of blood pressure, clearance of toxins and xenobiotics, as well as regulating homeostasis through the body’s water and salt balance. The functional unit of the kidney is called the nephron and each adult human kidney contains approximately one million nephrons. The nephron consists of a series of tubules that filter fluid that arrives as blood at the glomerulus and eventually leaving in the collecting ducts as urine. The gross anatomical divisions of the kidney divide it into the superficial cortex (outer layer), renal medulla, and the renal pelvis. The flow of filtrate goes from superficial to internal, concentrating the fluid according to the body’s water needs. Two categories of nephron exist, and can be classified as either cortical or juxtamedullary depending on their length. Although both cortical and juxtamedullary nephrons perform similar functions, the latter have longer loops of henle, providing an apparatus for the body to produce highly concentrated urine. The components of the nephron include the glomerulus, proximal convoluted tubule, loop of henle, distal convoluted tubule, and the collecting duct (Marieb et al., 2012).

The glomerulus is a fenestrated group of capillaries supplied by the afferent arteriole and emptied by the efferent arteriole. It is located within the Bowman’s capsule and this allows solutes and fluid to be filtered out of the blood and into the renal tubules. The proximal convoluted tubule is important for its absorption and secretion functions. The reabsorption of sodium and water both occur here, as well as the near-complete reabsorption of glucose. Many drugs are secreted back in this region including methotrexate and penicillin. The loop of henle has two distinct regions comprised by the descending and ascending segments of the loop. Using a counter-current gradient it drives water reabsorption in the descending loop and ion (e.g. sodium, potassium, calcium) reabsorption in the subsequent ascending loop. The distal convoluted tubule is
Figure 1.1 - Schematic representation of gross renal anatomy (A) and the nephron (B). CRV – Cortical Radiate Vein, CRA – Cortical Radiate Artery. Images were modified from Servier Medical Art (http://www.servier.co.uk/medical-art-gallery).
an important site for calcium reabsorption and also plays a vital role in the hormone-regulated reabsorption of water. The other major tubular section for hormone inducible water reabsorption is the collecting duct. It is normally responsible for around 5% of water reabsorption but in a dehydrated state this can jump to 24% when its aquaporin channels are induced by anti-diuretic hormone (Marieb et al., 2012).

A particularly important region for the regulation of blood pressure is the juxtaglomerular apparatus. It is located in a specialized area where the afferent and efferent arterioles meet the thick ascending limb of the loop of henle. Contained within it are granular juxtaglomerular cells, which have the ability to secrete renin, a hormone that catalyzes the rate limiting step in angiotensin II formation. The renin-angiotensin mechanism initiates the release of aldosterone, which causes water retention secondary to increased sodium absorption, which will in turn increase blood volume and pressure. Macula densa cells are adjacent to this and provide chemoreceptors that monitor solute concentrations of the renal filtrate. It maintains homeostatic solute and electrolyte concentrations by signalling for renin release when concentrations fall below desired levels. There are also extraglomerular mesangial cells within this region that have contractile properties, and in conjunction to interactions with the macula densa and granular cells, they are responsible for the blood pressure regulation (Marieb et al., 2012).

1.1 Chronic Kidney Disease

1.1.1 Prevalence and Diagnosis

Chronic Kidney Disease (CKD) is a debilitating disease that affects 10-15% of Canadians (Coresh et al., 2007; Arora et al., 2013). CKD is described as a progressive decline in renal function or pathological structural changes to the kidneys that have persisted for over 3 months (National Kidney Foundation, 2002; Stevens and Levey, 2009; KDIGO, 2013). It is diagnosed using urinary albumin which is a marker of kidney damage, or estimated glomerular filtration rate (eGFR), with the latter considered the most accurate method (National Kidney Foundation, 2002; Filler et al., 2014). eGFR is a proxy for kidney function as it estimates the flow-rate of blood being filtered by the
kidney (National Kidney Foundation, 2002). Determination of eGFR can be assessed by measuring urinary creatinine clearance levels over 24 hours or by the measurement of serum creatinine and application of GFR prediction equations (National Kidney Foundation, 2002). Two equations are used in clinical practice to estimate GFR based on standardized creatinine clearance, the Modification of Diet in Renal Disease Study equation and a more recent one from the CKD-Epidemiology Collaboration which is more accurate at higher filtration rates (>60ml/min/1.73m$^2$) (Levey et al., 1999; 2009; Dias et al., 2013). Inulin and its analogue sinistrin may also be injected to assess renal function as their clearance is proportional to glomerular filtration; however they are not secreted in the nephron tubules and therefore provide the gold standard in accuracy for GFR estimation (National Kidney Foundation, 2002; Filler et al., 2014). If a diminished eGFR is present for more than 3 months, then a diagnosis of CKD is warranted (National Kidney Foundation, 2002).

### 1.1.2 Stages

Functional thresholds define the 5 different stages of CKD (Table 1.1). Approximately 10% of Canadians have stage 1 or 2 CKD while 3% are in stage 3 to 5 (Arora et al., 2013). Patients in stages 1-3 have close to normal physiology with slight abnormalities in kidney structure or urine content, increasing to moderate reduction in renal function as the disease progresses (National Kidney Foundation, 2002). At this point treatment is centered around mitigating the progression to later stages through controlling blood pressure and other risk factors such as hyperglycemia and cardiovascular disease (National Kidney Foundation, 2002). Glomerular filtration rate for these stages are >90, 60-89, and 30-59ml/min/1.73m$^2$ (1.73m$^2$ corrects for average adult body surface area) for stages 1 to 3 respectively (National Kidney Foundation, 2002). When eGFR drops below 30ml/min/1.73m$^2$ it is considered severe (stage 4) and management consists of preparing the patient for stage 5 (National Kidney Foundation, 2002). An eGFR <15ml/min/1.73m$^2$ results in the diagnosis of kidney failure and is also termed end-stage renal disease (ESRD) (National Kidney Foundation, 2002). Renal replacement therapy is required for treatment of symptoms at this point. Kidney allograft
### Table 1.1 - Stages and prevalence of chronic kidney disease in North America

<table>
<thead>
<tr>
<th>Stage</th>
<th>GFR (mL/min/1.73m²)</th>
<th>Description</th>
<th>Prevalence in North America (Canadian bolded)</th>
<th>Course of Action</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>≥90</td>
<td>Kidney damage with normal or ↑ GFR</td>
<td>9.4% (stages 1-2)&lt;sup&gt;1&lt;/sup&gt; 3.23% of Americans&lt;sup&gt;2&lt;/sup&gt;</td>
<td>Diagnosis and treatment of co-morbid conditions Slow progression</td>
</tr>
<tr>
<td>2</td>
<td>60-89</td>
<td>Kidney damage with mild ↓ GFR</td>
<td>3.7% of Americans&lt;sup&gt;2&lt;/sup&gt;</td>
<td>Estimating progression</td>
</tr>
<tr>
<td>3</td>
<td>30-59</td>
<td>Moderate ↓ GFR</td>
<td>3.1% (Stages 3-5)&lt;sup&gt;1&lt;/sup&gt; 7.85% of Americans&lt;sup&gt;2&lt;/sup&gt;</td>
<td>Evaluate and treat complications</td>
</tr>
<tr>
<td>4</td>
<td>15-29</td>
<td>Severe ↓ GFR</td>
<td>0.44% of Americans&lt;sup&gt;2&lt;/sup&gt;</td>
<td>Prepare for renal replacement</td>
</tr>
<tr>
<td>5</td>
<td>&lt;15</td>
<td>Kidney failure (ESRD)</td>
<td>0.2% of Americans&lt;sup&gt;3&lt;/sup&gt;</td>
<td>Renal replacement therapy required</td>
</tr>
</tbody>
</table>

Adapted from (Naud et al., 2012) with data from <sup>1</sup>Arora 2013, <sup>2</sup>National health and Nutrition Examination Survey (NHANES) 2007-2014, and <sup>3</sup>United States Renal Data System (USRDS 2015).
transplantation is the gold standard renal replacement method; however it is not particularly accessible due to the lack of available organs. This results in most patients being initiated on a dialysis regimen to supplement whatever residual kidney function remains.

1.1.3 Treatment

Prior to the need for renal replacement, management of CKD is centered around slowing the progression and treating comorbidities. Drugs such as angiotensin converting enzyme inhibitors and angiotensin II receptor blockers have been shown to slow the progression to ESRD while drugs such as statins and beta-blockers reduce all-cause mortality in CKD patients (Qaseem et al., 2013). Erythropoietin replacement, hypoglycemic agents, vitamin D, diuretics, and drugs to balance electrolytes such as phosphate binders are also commonly prescribed to CKD patients. Renal replacement therapy largely consists of dialysis regimens as there is a long wait for transplantation. There are two common dialysis modalities, hemodialysis and peritoneal dialysis (PD). Hemodialysis consists of connecting a catheter or fistula between an artery and a vein in an extremity to a pump that directs blood flow through a dialyzer. This filters the patient’s blood extracorporeally through a semi-permeable membrane with concomitant counter-current dialysate flow, which removes excess fluid and toxins that would normally be cleared by the kidneys. Patients typically require 3-4 hour sessions 3 times a week to account for lost kidney function. High-flux dialysis membranes are now the standard of care in treatment, they have larger pores in the semi-permeable membrane, allowing for the removal of larger ‘middle’ molecules. The composition of the dialysate is a physiological saline solution that is tailored to resupply low electrolytes and to remove molecules that are in excess (Locatelli et al., 2015). The success of dialysis treatment is largely dependent on the contents of the dialysate. Patients who undergo dialysis treatment will still experience life-threatening signs and symptoms due to the imperfect nature of current dialysis regimens, this condition is distinct from uremia and has been named residual syndrome (Depner, 2001; Dobre et al., 2013).

Peritoneal dialysis is divided into two main types, automated peritoneal and continuous ambulatory peritoneal dialysis (CAPD). CAPD involves the administration of
dialysate directly into the peritoneum and abdominal cavity via a surgically placed catheter. It requires exchange with fresh fluid 3 to 5 times per day, requiring less than 30 minutes per exchange (Tucker et al., 1991). CAPD restores some patient autonomy and may be preferred by more able-bodied CKD patients (Tucker et al., 1991). Automated peritoneal dialysis (also known as nocturnal intermittent peritoneal dialysis; NIPD) normally occurs overnight and employs a mechanized device to aid in fluid exchange (Kathuria and Twardowski, 2000). The dialysate is normally drained and delivered nocturnally (Kathuria and Twardowski, 2000). NIPD can also be supplemented into a CAPD regime if the need arises (Kathuria and Twardowski, 2000). In 2008 only 11% of Canadian ESRD patients were receiving peritoneal dialysis, while 48% were on hemodialysis and the remaining 41% had a functional kidney transplant (CORR 2011). These numbers are interesting as many studies have compared the different dialysis modalities and have found no appreciable differences in regards to quality of life and survival (Tucker et al., 1991; Mehrotra et al., 2011).

1.1.4 Etiology and Comorbidities

Incidence of CKD is increasing as the prevalence of risk factors is steadily rising globally (Coresh et al., 2007; Arora et al., 2013). In 2002 the estimated global population on dialysis was 1.1 million with an expected 7% increase annually (Jha et al., 2012). Mortality in CKD is most commonly associated with comorbidities that patients are prone to developing as opposed to the renal damage itself. Diabetes, obesity, hypertension, and cardiovascular disease (CVD) are accepted as the leading causes of CKD as well as significant comorbidities (Coresh et al., 2007). Certain toxins, immune responses, HIV, obstructions to the ureters and renal tubules such as nephroliths, and HIV associated nephropathy are also capable of initiating renal insults which can progress to CKD (Hewitson et al., 2008; Levey and Coresh, 2012; Jha et al., 2012). A vicious cycle ensues in CKD without intervention as hallmarks of CKD progression such as oxidative stress, renal fibrosis, and metabolic acidosis will result in further declines in kidney function. Anemia, uremia, and metabolic bone disease are other characteristic comorbidities of CKD (KDIGO, 2013). In our ageing population there will be a much larger burden to the healthcare system from CKD patients due to the inevitable increases
we will face and due to the rise in incidence of associated risk factors (Coresh *et al*., 2007).

### 1.1.4.1 Diabetes

Diabetes is reported to affect 6.8% of Canadians and this number climbs to 8.7% if only adults over the age of 20 are included, as risk for diabetes incidence increases with age (Public Health Agency of Canada, 2011). Diabetic kidney disease (DKD) is the leading cause of CKD and is considered a form of CKD itself by the Kidney Disease Quality Outcomes Initiative (KDQOI) (Żyłka *et al*., 2015). Hyperglycemia is necessary for the pathogenesis of DKD which makes patients with uncontrolled diabetes at increased risk to incur renal damage (Thomas, 2016). Approximately two thirds of type 2 and one third of type 1 diabetes mellitus patients will develop DKD, while diabetes affects 9% of the population worldwide (Thomas, 2016; Filla and Edwards, 2016). Microangiopathy resulting from diabetes mellitus is the causal factor for renal damage. Diabetic co-morbidities such as sustained hyperglycemia, hypertension, and hyperlipidemia will result in microangiopathy (Żyłka *et al*., 2015). Diabetic glomerulosclerosis can also lead to hypertension (Levey and Coresh, 2012).

### 1.1.4.2 Hypertension

The prevalence of hypertension varies in CKD from 30% of stage 2 patients to 50% in those with stage 4 CKD (Keith *et al*., 2004). In a retrospective study from 2012 it was estimated that 23% of Canadians have hypertension (Robitaille *et al*., 2012). This is a stark contrast to the 12% of Canadians at the start of the 20th century (Robitaille *et al*., 2012). It is also proposed to account for 13% of all deaths globally, establishing it as the leading risk factor for mortality (World Health Organization, 2009; Robitaille *et al*., 2012). Pathophysiologic renal function associated with hypertension originates from damage to the microvasculature. This can stem from high systemic blood pressure increasing the fluid load reaching the kidneys, as well as the velocity of blood passing through the renal tubules, glomerulus, and vasculature (Ravera *et al*., 2006; Levey and Coresh, 2012). Barotrauma on the renal vasculature causes mechanical stretching of glomerular capillaries, which induces a fibrotic repair mechanism, predisposing patients
to glomerular sclerosis (Ravera et al., 2006). This will further negatively impact GFR and exacerbate a patient’s condition. Tightly controlled blood pressure will help to reduce renal stress and is important for cardiovascular protection; cardiovascular disease is another leading cause of CKD (Ravera et al., 2006; Coresh et al., 2007). CKD is also one of the leading causes of hypertension and cardiovascular disease as the pathophysiology and treatment of the aforementioned conditions are highly interdependent (Tonelli et al., 2016).

1.1.4.3 Cardiovascular Disease

Much like diabetes and hypertension, cardiovascular disease (CVD) is a highly prevalent affliction globally and its incidence is rising at a rate of 8% a year (Tonelli et al., 2016). The highest risk populations for CVD are those from developing nations (Tonelli et al., 2016). CKD and ESRD patients are 5-10 fold more susceptible to developing CVD than healthy individuals (Tonelli et al., 2016). Cardiovascular disease is an umbrella term that encompasses an assortment of specific pathologic conditions such as coronary artery disease, atrial or ventricular arrhythmias, valvular defects, stroke, congestive heart failure, myocardial infarction, atherosclerosis, angina, thrombosis, cardiomyopathy, aneurysms, and thromboembolisms (Yusuf et al., 2001; Tonelli et al., 2016). CVD and CKD are highly intertwined as the risk factors typically associated with CVD such as dyslipidemia, hypertension, and advanced age are all typically present in the CKD patient demographic (Segall et al., 2014). Additionally non-traditional CVD risk factors such as anemia, proteinuria, mineral metabolism abnormalities, volume overload, oxidative stress, malnutrition, and inflammation also normally accompany CKD (Segall et al., 2014). CKD is an independent risk factor for CVD as seen by 63% of these patients developing CVD (Segall et al., 2014).

1.1.4.4 Obesity

According to Statistics Canada in 2014, approximately 54% of Canadians self-reported as having a body mass index (BMI) over 25 kg/m², a 17% rise since 2003, displaying a sharp increase in the overweight and obese conditions over the past decade. Obesity is linked with many comorbidities such as cardiovascular disease, hypertension,
type 2 diabetes, osteoarthritis, and dyslipidemia (Tsujimoto et al., 2014; Rhee et al., 2016). It is also known to be a leading cause of de novo CKD and ESRD (Munkhaugen et al., 2009; Rhee et al., 2016). Direct and indirect mechanisms have been proposed to account for obesity’s role in the onset of CKD. Indirect pathways include risk factors associated with obesity that have correlations with CKD development, such as hypertension, diabetes, and atherosclerosis (Stenvinkel et al., 2013; Rhee et al., 2016). Obesity linked mechanisms that are proposed to directly cause CKD include alterations in renal blood flow, inflammation, as well as growth factor and adipokine production (Wickman and Kramer, 2013; Stenvinkel et al., 2013; Rhee et al., 2016). A major proposed pathway follows that obesity can cause renal mesangial expansion resulting in increased metabolic demand from the kidneys, further leading to glomerular hyperfiltration, hypertrophy, and hypertension (Wickman and Kramer, 2013; Rhee et al., 2016). Increased glomerular load over a prolonged period results in glomerulosclerosis and proteinuria (Wickman and Kramer, 2013; Rhee et al., 2016). Obesity is also linked to the progression of CKD to ESRD, as increasing BMI correlates with progression to ESRD (Ahmadi et al., 2015; Rhee et al., 2016). Paradoxically in a meta-analysis from 2015 a relationship between survival of late stage non-dialysis dependent CKD patients and BMI showed that overweight and class I obesity patients had lower mortality than those who were underweight (Ahmadi et al., 2015; Rhee et al., 2016). A similar relationship was found in patients on hemodialysis as patients with a BMI between 40-45 kg/m² were shown to have a 31% lower mortality rate than patients with a BMI range from 25-27.5 kg/m² (Doshi et al., 2015; Rhee et al., 2016).

1.2 Uremia

Uremia is a term that encompasses the extensive abnormalities attributed to declining or the complete loss of renal function (Depner, 2001). As literature on the topic evolves some of the more specific symptoms previously grouped with uremia such as anemia and hypertension, have been delineated as independent conditions (Meyer and Hostetter, 2007). The current perspective on the etiology of uremia follows that decreased kidney function results in a state of intoxication due to accumulation of organic waste products that would normally be excreted in the urine (Depner, 2001; Meyer and
Early symptoms of uremia such as fatigue are difficult to allocate to a specific origin and often lead to the ailment going undetected (Meyer and Hostetter, 2007). Advanced stage CKD displays more characteristic uremic syndrome that can specifically demarcate the condition (Depner, 2001). Anorexia, pruritus, serositis, hemorrhage, reduced attention span, lethargy, fluid retention, edema, as well as biochemical and hormonal irregularities are all signs of advanced uremic syndrome (Depner, 2001). The advent of dialysis has mitigated complications associated with uremia as it makes renal replacement more accessible, dialysis is currently the only way to minimize the effects of uremia and its associated symptoms (Meyer and Hostetter, 2007). Prophylactic measures can hinder the progression of CKD to eventual ESRD and thereby reduce the effect of uremic syndrome as renal function is preserved (Meyer and Hostetter, 2007). There is currently no artificial apparatus that can compare to an actual functioning kidney and a result of this short-coming is a condition known as residual syndrome, which is experienced by dialysis patients (Depner, 2001). Large molecular weight solutes as well as smaller protein-bound molecules are poorly dialyzed, and in conjunction with tissue calcification and hormone imbalance, they are considered the putative causes for residual syndrome (Depner, 2001). These solutes are called uremic toxins and have come into prominence in the nephrology field recently. Uremic serum has been shown to impair the integrity of the tight junctions in the intestinal epithelium, allowing increased passage of these uremic toxins into the internal environment (Vaziri et al., 2012). The ensuing subsections will address the hypothesized mechanisms for their abundance as well as classifications of the commonly studied toxins.

1.2.1 Intestinal Microbiome

The microbial population of the gut is enormous, with approximately $10^{14}$ bacteria from 500-1000 different species taking up residence within it (Backhed et al., 2004; Ramezani et al., 2016). Physiologically the microbes in our gut are responsible for necessary metabolic actions that our bodies are not equipped to fulfill, including the synthesis of certain vitamins and amino acids, as well as the catabolism of certain complex polysaccharides and bile acids (Hill, 1997; Hylemon and Harder, 1998; Hooper et al., 2002; Ramezani et al., 2016). The gut appears to be a dynamic biome in which its
microbial resident demographic will change based on host diet as certain taxonomies are better adapted to metabolize different foods, which gives credence to the theory that bacterial colonies adapt to host needs (Ramezani et al., 2016).

1.2.1.1 Dysbiosis in CKD

The uremic condition leads to high levels of undigested proteins in the distal intestine, which promotes the growth of proteolytic bacteria (Sorensen, 1965; Ramezani et al., 2016). This leads to a pathologic overhaul in microbiome composition and metabolic potential which is termed dysbiosis (Ramezani et al., 2016). Patients suffering from ESRD are shown to have significant increases in relative abundance of 190 bacterial operational taxonomic units as compared to healthy controls (Vaziri, Wong, et al., 2013). It has also been shown that rats post-subtotal nephrectomy have a significant decline in overall species richness for gut microbiota (Vaziri, Wong, et al., 2013). Overall microbial diversity is shown to decrease in CKD, however there are bacterial families that are shown to excel in the disease environment and actually increase their diversity, such as the Pseudomonadaceae, which exemplifies the notion that the enteric milieu imposes selection pressures upon its occupants (Vaziri, Wong, et al., 2013). By in large aerobic bacteria outcompete anaerobic counterparts in CKD due to decreased gut motility which is secondary to decreased myoelectrical activity in enterocytes (Strid et al., 2003). Changes in excretion routes for both uric acid and oxalate combined with enteric influx of urea, long term administration of phosphate binding agents, strict dietary restrictions, and frequent use of antibiotics are the putative explanations for the observed differences in microbial flora in the CKD populous (Vaziri, Wong, et al., 2013).

1.2.2 Uremic Toxins

Metabolic differences in CKD can be attributed to changes in the gut microbiome and higher levels of undigested protein reaching the colon, which can result in higher levels of protein fermentation (SA Gibson et al., 1989; Ramezani et al., 2016). Toxic molecules derived from dietary proteins can accumulate due to the lack of renal clearance and the invigorated production of ammonia, indoles, amines, thiols, and phenols secondary to enteric dysbiosis (SA Gibson et al., 1989). There are more than 115
identified uremic toxins to date and they can been subdivided into free water-soluble low molecular weight molecules, middle molecules, and protein-bound molecules using a classification system that accounts for size and proteins binding properties (Vanholder et al., 2008; Duranton et al., 2012). Advances in techniques in the field such as untargeted metabolomics, have greatly aided investigators in the identification process and provide information on which toxins differ in concentration between control and CKD subjects (Velenosi et al., 2016).

The free water-soluble low molecular weight toxins (<500Da) comprise 46% of the currently identified uremic toxins (Duranton et al., 2012). These solutes are typically easily removed by dialysis due to their water solubility and minimal protein binding. Notable members are urea, creatinine, guanidine metabolites (e.g. guanidinosuccinic acid), and asymmetric dimethylarginine (Lisowska-Myjak, 2014). Reactive carbonyl species such as alkanals, alkenals, and 4-OH-alkenals that arise due to oxidative conditions in CKD are also included in this class of uremic toxin (Alhamdan et al., 2006). Another water-soluble uremic toxin coming into prominence is trimethylamine N-oxide (TMAO), as it has been shown to have proatherogenic properties (Koeth et al., 2013). Choline and L-carnitine are the precursors for TMAO, while their origins are derived from red meat and eggs in the diet (Moraes et al., 2015). TMAO is elevated in ESRD patients, although hemodialysis is able to clear it to a degree, it does not restore it to levels seen in controls (Bain et al., 2006; JY Choi et al., 2011).

Middle molecules account for 28% of currently identified uremic toxins and are classified by a molecular weight in excess of 500 Da. (Duranton et al., 2012; Lisowska-Myjak, 2014). Solutes over 60kDa are not typically filtered by the glomerulus and therefore are not considered uremic toxins (Duranton et al., 2012). The main constituents of this class are cytokines including IL-6, IL-8, IL-10, TNF-α, and β2-microglobulin (Duranton et al., 2012). Other members of note are adiponectin, leptin, ghrelin, endothelin, and cholecystokinin (Lisowska-Myjak, 2014). These solutes are efficiently removed by dialysis modalities that incorporate high-flux membranes (Winchester and Audia, 2006). Studies have reported correlations between lower levels of middle
molecules in dialysis patients and a decreased risk for all-cause and cardiovascular mortality (Hornberger et al., 1992; Eknoyan et al., 2002).

1.2.2.1 Protein-Bound and Gut-Derived Uremic Toxins

Protein-bound solutes account for 25% of currently identified uremic toxins, they are typically smaller molecular weight (<500Da) and many are gut-derived (Duranton et al., 2012). The protein-bound molecules are of particular interest to this study as they are poorly cleared by dialysis. Once they have breached the intestinal barrier, metabolites can be further metabolized or altered at sites such as the liver. A well-characterized protein-bound uremic toxin is indoxyl sulfate (93% bound to albumin in circulation) due to its putative effects on the cardiovascular system and drug metabolism (Niwa, 2013; Devine et al., 2014). Starting as tryptophan (derived from dietary protein) and upon exposure to intestinal bacteria containing tryptophanase, it is metabolized to indole (Niwa, 2013; Ramezani et al., 2016). Indole is then able to cross the intestinal barrier and enter portal circulation, which allows for hepatic uptake and subsequent hydroxylation followed by sulfation in the liver to indoxyl sulfate (Banoglu et al., 2001; Meijers et al., 2009; Niwa, 2013). Despite high protein binding, indoxyl sulfate is extensively cleared by renal tubular secretion in the proximal tubule cells of the kidney. In CKD, indoxyl sulfate accumulates and manifests nephrotoxic properties primarily via induction of reactive oxygen species production in both renal tubular cells and glomerular mesangial cells (Niwa, 2013). It has also been shown to have a pathophysiological role on various functions in the endothelium, heart, smooth muscle cells, intestinal cells, leukocytes, adipocytes, and hepatic metabolism and transport (Shibata et al., 2000; Sun et al., 2006; Vanholder et al., 2014). Many studies have investigated its contribution to cardiovascular disease (Niwa, 2013; Inami et al., 2014).

The metabolic pathways of both p-cresyl sulfate and hippuric acid are also well understood as they originate from the hydrophobic amino acids tyrosine and phenylalanine respectively. P-cresyl sulfate binds to albumin at the same site indoxyl sulfate does with 90% of total p-cresyl sulfate being protein-bound (De Smet et al., 1998; Meijers et al., 2009). Systemic detrimental effects related to p-cresyl sulfate have been shown in adipocytes, renal tubular cells, and leukocytes (Vanholder et al., 2014).
Elevated serum p-cresyl sulfate has also been shown to associate with higher all-cause mortality in CKD patients on hemodialysis (Wu et al., 2012). Another prominent uremic toxin is 3-carboxy-4-methyl-5-propyl-2-furanpropanoic acid (CMPF), and it is also highly protein-bound as 99% exists bound to albumin (Meijers et al., 2008). CMPF has been implicated in a plethora of pathophysiological mechanisms including thyroid dysfunction, inhibition of hepatic metabolism and mitochondrial respiration, and the production of ROS in endothelial cells (Niwa, 2012).

1.3 Removal of Gut-Derived Uremic Toxins

Gut-derived uremic toxins are typically protein bound, adding another layer of difficulty in regards to their removal as they cannot be efficiently filtered by the glomerulus. The main physiological pathway for their excretion is through tubular secretion, into the urine. Tubular secretion involves transporter proteins such as OAT1, OAT3, BCRP, and MRP4 (Poesen et al., 2013). The major reasons for the accumulation of gut-derived uremic toxins appears to be the loss of renal function, the inability of dialysis to clear the small molecules, saturation of tubular transporters, and colonic dysbiosis resulting in increased production of these solutes. There are several proposed mechanisms to reverse the pathologic retention of these molecules (Vanholder et al., 2016). Dietary restrictions are the most accessible and recommendations are in place to lower the intake of protein to reduce the accumulation of nitrogenous waste products associated with azotemia (Ritz et al., 1978). A study in rats that had undergone subtotal nephrectomy to reduce GFR were administered isocaloric diets that differed on the main caloric constituent, attempted to elucidate any benefit of reduced dietary protein in CKD. The animals receiving high protein diets had a markedly shorter lifespan than those on high fat or high carbohydrate diets, exemplifying the benefit of protein restriction in CKD (Ritz et al., 1978). Protein restriction ameliorates aspects of the uremic syndrome but it can be difficult to balance proper nutrition with optimal protein intake as uremic patients suffer from a loss of appetite and a particular aversion to protein-rich foods, making it difficult to reach daily protein intake goals, potentially resulting in malnutrition (Sirich, 2014). This makes it difficult for CKD patients to adhere to diets tailored to control protein intake.
Another option is to increase dietary fiber consumption as it decreases colonic transit time, therefore reducing the period of time the gut bacteria has to metabolize food and create protein-derived solutes (Sirich, 2014). Natural fiber is resistant to mammalian digestion and typically contains components of plant cell walls in high proportions. Synthetic fibers also exist and they have beneficial effects on gastrointestinal health, largely pertaining to gut motility (Sirich, 2014). High fiber foods may also provide more energy for the colonic bacteria which could facilitate an increase in bacterial protein synthesis and a decrease in uremic solutes as it consumes the available nitrogenous bases (Sirich, 2014). In humans with CKD treated with fiber for 5 weeks there was a 23% reduction in plasma urea in addition to a 50% increase in nitrogen excretion in the feces (Younes et al., 2006). The effect of fiber on other uremic toxins was seen in hemodialysis patients randomized to fiber supplements or corn starch; 27% and 24% reductions in indoxyl sulfate and p-cresyl sulfate respectively were observed in the patients who received the fiber supplementation (Sirich et al., 2014).

The use of probiotics and prebiotics has become a hot topic amid the notion of dietary alterations to reduce uremic toxin accumulation (Koppe et al., 2015). Probiotics are a preparation that contains viable microorganisms in sufficient number to alter the host microflora and to confer beneficial effects to the host (Schrezenmeir and de Vrese, 2001). Anaerobic bacteria such as *Lactobacillus* and *Bifidobacterium* are common in probiotic cultures (Guida et al., 2014). Prebiotics are non-digestible compounds that can support probiotic bacterial fermentation, providing a competitive advantage over pathologic bacteria in energy availability (GR Gibson et al., 2010). A convenient preparation containing both pre- and probiotics is called a synbiotic (Guida et al., 2014). Work by Guida et al in 2014 showed that synbiotics can reduce plasma p-cresol concentrations in CKD patients. A randomized clinical trial from 2016 that administered synbiotics to CKD patients showed a non-significant reduction of serum indoxyl sulfate; however, p-cresyl sulfate concentrations were significantly lowered and there was also increased growth of beneficial bacteria combined with lowered pathologic microbiota (Rossi et al., 2016).
1.3.1 AST-120

There is one other well-documented method for the removal of gut-derived uremic toxins. It involves the administration of AST-120, a spherical oral adsorbent comprised of porous microcrystalline carbon derived from petroleum pitch (Kanai et al., 1986; Niwa et al., 1991). Each particle is 0.2-0.4mm in diameter and is insoluble in both water and organic solvents (Niwa et al., 1991). AST-120 functions much like traditional activated charcoal with key differences being its fluidity in the GI tract due to its spherical shape, and its molecular weight selective properties (Niwa et al., 1991). It targets molecules between 100-10000 daltons, therefore limiting its effects on digestive enzymes. It has close to 10 fold lower affinity for endogenous enzymes such as chymotrypsin, amylase, lipase, and aminopeptidase compared to activated charcoal on a mg per g ratio of enzyme to drug (Kanai et al., 1986). AST-120 has shown an ability to lower circulating levels of uremic toxins while having only mild side effects. The only reported side effects are constipation, nausea, and diarrhea; however these ADRs occur at a similar rate in placebo treated patients (Schulman et al., 2014).

Activated charcoal has shown efficacy for alleviating pruritus in hemodialysis patients; however, AST-120 has been shown to be efficacious in this regard as well, and may be a less detrimental alternative (Niwa et al., 1991). CKD patients are on many oral medications and there have been concerns over AST-120 interfering with their absorption. Oral medications tested for this retained their desired therapeutic effect if they were consumed 30 minutes prior to AST-120 administration (Niwa et al., 1991). Since AST-120 displays a high tolerability allowing for sustained administration over a long period of time, Japan approved its use to slow the progression of CKD in 1991. Studies showed a delay in the initiation of hemodialysis and to relief of uremic symptoms (Niwa et al., 1991; Schulman et al., 2014). It was subsequently approved in both Korea (2005), the Philippines (2010) and is currently going through phase III trials in North America, Europe, and Latin America (Schulman et al., 2014). Efforts to use AST-120 in Europe and North America culminated in the Evaluating Prevention of Progression in CKD trials (EPPIC-1 & 2), which were conducted simultaneously and compiled 5840 patients across 13 countries (Schulman et al., 2015). The EPPIC trials failed to indicate a
beneficial effect for AST-120 in the progression of CKD as determined by the triple composite endpoint including either the onset of dialysis, kidney transplantation, or doubling of serum creatinine (Schulman et al., 2015). There were many limitations to this study however and it contradicted multiple clinical trials completed in Japan (Shoji et al., 2007; Hatakeyama et al., 2012).

Many studies have evaluated the reduction of serum indoxyl sulfate by AST-120, including a large clinical trial that showed a 40% reduction in moderate and severe CKD patients (Schulman et al., 2006). A metabolomics study in rats with adenine-induced CKD displays differences in prevalence for many molecules across distinctive biological matrices between control, CKD, and the aforementioned groups treated with AST-120 (Velenosi et al., 2016). AST-120 was able to significantly reduce 24 of 26 plasma metabolites that were higher in CKD than control, while lowering 50% of CKD specific metabolites in both liver and heart tissue, which had 107 and 56 metabolites identified respectively (Velenosi et al., 2016). There were 8 common metabolites significantly higher in CKD than control for plasma, liver, heart, and kidney tissues. (Velenosi et al., 2016). P-cresyl sulfate, indoxyl sulfate, hippurric acid, P-cresyl glucuronide and phenyl sulfate are all among those 8 metabolites and unsurprisingly they were all reduced by AST-120 as they have been previously identified as gut-derived toxins (Velenosi et al., 2016). The same rat model was employed by Vaziri et al in 2013 to show a restoration of the intestinal epithelium tight-junction proteins in rats that were provided AST-120. Improvement of oxidative stress markers has also been associated with AST-120 as high ROS are often attributed to uremic toxin accumulation in CKD patients (Niwa, 2013; Vaziri, Yuan, et al., 2013; Yamamoto et al., 2015). AST-120 has been shown to lower arterial pressure and serum malondialdehyde, a plasma-lipid peroxidation product in rats with CKD (Vaziri, Yuan, et al., 2013). Yamamoto et al in 2015 showed a significant reduction of oxidized albumin levels in hemodialysis patients that received AST-120. Finally, as there are many studies on the effects of indoxyl sulfate and uremic toxins on vascular disease, multiple groups have looked at AST-120 and whether it can attenuate or reverse these effects (Niwa, 2013). It has been shown to reduce carotid artery intima-media thickness, arterial stiffness, and delayed the progression of monocyte adhesion in
the endothelial cells which is characteristic of the uremic condition (Nakamura et al., 2004; Inami et al., 2014).

1.4 Drug Metabolism and Disposition

The typical CKD patient is prescribed 12 different drugs concurrently in order to manage an average of 6 comorbidities associated with the underlying disease (Manley et al., 2005). Extensive knowledge of drug pharmacokinetics is therefore of utmost importance. In order for these drugs to mediate their prescribed benefits they need to reach an appropriate concentration at their site of action in the body. A harmonious system of absorption, distribution, metabolism, and elimination govern the plasma and tissue concentration of drugs. To further illustrate this process, the pathways of an orally administered drug will be explained.

Upon ingestion of a typical orally administrated drug, it will pass through the gastrointestinal tract until it reaches the small intestine. If the drug is a weak acid then there may be some absorption in the stomach due to pH partitioning, however the majority of drug absorption will occur further down the GI tract. Reasoning for this is although rugae in the stomach increase gastric surface area, the villi and microvilli of the small intestines provide a substantially greater surface area for absorption, 3800 fold larger by some estimates (DeSesso and Jacobson, 2001). Uptake transporters can remove substrates from the intestinal lumen and translocate them across the apical intestinal epithelium. At this point enteric metabolism can occur and the drug will be left with 2 options; the first being subsequently effluxed by transporters back into the intestinal lumen for fecal excretion if it does not undergo repeated uptake, metabolism, and efflux (Nolin et al., 2008). The drug can also follow a path into the portal circulation by crossing the enteric basolateral membrane via efflux transport or passive diffusion (Nolin et al., 2008; Yeung et al., 2014). Exposure to first pass metabolism will occur through this route as passive diffusion or uptake transporters on the sinusoidal membrane of hepatocytes can internalize xenobiotics. Once in the liver drugs can be metabolized, then effluxed across the canalicular membrane and into the bile for fecal excretion. Alternatively, drugs may also move back across the basolateral membrane and into the hepatic vein and then into systemic circulation (Nolin et al., 2008). Metabolites that are
undergoing biliary elimination may be reabsorbed in the intestines through enterohepatic recirculation (Nolin et al., 2008). This typically requires the restoration of the parent drug by removal of phase II metabolic conjugations by bacterial enzymes in the intestinal lumen.

Drugs and metabolites that reach systemic circulation can be eliminated by renal excretion by filtration at the glomerulus or by transporter mediated tubular secretion. In combination with biliary excretion, these comprise the major physiological processes to remove drugs and toxins from the body. Drug dosing needs to account for renal and hepatic clearance rates in order to ensure optimal plasma concentrations while avoiding toxicity. There are clearly many factors dictating the pharmacokinetics of a drug. Metabolism and drug transport will be further expanded upon in the ensuing sections.

1.4.1 Metabolism

The metabolic processes in the body are defined by phase I and phase II reactions. These reactions occur predominantly in the liver, however the kidneys, intestine, and lungs are known to have metabolic activity as well. The primary focus of phase I metabolism is to unmask polar groups on lipophilic drugs through reduction, oxidation and hydrolysis reactions, occurring on the smooth endoplasmic reticulum of the cell. This results in deactivation or activation of a drug, depending on its properties (pro-drug or not). The majority of phase I metabolism is carried out by the Cytochrome P450 superfamily (CYP). Phase II consists of the conjugation of polar groups to lipophilic compounds or metabolites from phase I and is completed by sulfotransferases, glutathione-S-transferases, N-acetyltransferases, thiopurine methyltransferase, and UDP-glucuronosyltransferases. Phase II generally follows phase I metabolism, however it may also be solely responsible for the metabolism of certain drugs. It is estimated that 73% of currently marketed drugs require metabolism for clearance (Wienkers and Heath, 2005).

1.4.1.1 Cytochrome P450 Superfamily

Cytochrome P450 enzymes are heme-containing mono-oxygenases that are ubiquitously expressed across phylogenies and are localized to the smooth endoplasmic reticulum of cells. There are currently 57 known human CYPs, with approximately 35
isoenzymes showing clinical relevance (Martignoni, Groothuis, and de Kanter, 2006a; Guengerich, 2008). CYPs are divided into 12 different families demarcated by a number, a letter, and then another number. The first number denotes the family and signifies over 40% primary structural sequence similarity, the letter then divides them into subfamilies which are >55% identical in terms of primary structure (Martignoni, Groothuis, and de Kanter, 2006a; Guengerich, 2008). The final number in CYP nomenclature distinguishes the individual enzyme (Martignoni, Groothuis, and de Kanter, 2006a). CYP enzymes account for 75% of xenobiotic metabolism and of that, 95% is attributable to 5 enzymes (Guengerich, 2008; Schwab and Zanger, 2013). Human CYP3A4 accounts for 30% of CYP drug metabolism while 1A2, 2A6, 2B6, 2C8, 2C9, 2C11, 2D6, and 2E1 play large roles as well (Guengerich, 2008; Schwab and Zanger, 2013). The CYP3A family plays the largest role in drug metabolism and therefore is the best described in the literature for pharmacogenomic and disease-state studies.

Since the majority of marketed drugs are metabolized primarily by a small set of enzymes there is the potential for serious drug-drug as well as food-drug interactions if a target enzyme is inhibited, induced, or suffers a loss of activity. The importance of this is showcased in the classic example of felodipine bioavailability increasing with concurrent grapefruit juice consumption with the mechanism of action being enteric CYP3A4 inhibition (Bailey et al., 1998). A single 250mL serving of grapefruit juice was able to increase felodipine plasma area under the curve by 267% (Bailey et al., 1998). CYPs are also highly polymorphic, which requires another layer of caution when dosing patients with substrates. The CYP2C9 and warfarin example is very well defined in the literature and shows single nucleotide polymorphisms (SNPs) occur in 34% of patients for the 2C9 gene (Freeman et al., 2000). In addition to wild type CYP2C9*1 there are 2C9*2 and 2C9*3 SNPs (Freeman et al., 2000). The *2 and *3 SNPs result in a loss of activity in CYP2C9, which will result in extensive anti-coagulant toxicity and hemorrhage if dose adjustments are not made (Freeman et al., 2000).

1.4.1.2 CYP2B Subfamily

The CYP2B family was historically thought to account for very little of total CYP metabolism. Recent studies however estimate that CYP2B6 and 2B7 are responsible for
the metabolism of 25-30% of drugs, neutraceuticals, and herbal medicines on the market, and that 2B6 alone metabolizes 7.2% of clinically used drugs (Xie and Evans, 2001; Schwab and Zanger, 2013). CYP2B7 is expressed in the lung so the majority of attention is directed towards CYP2B6 which is expressed in the liver as well as some extrahepatic tissue (Martignoni, Groothuis, and de Kanter, 2006b). The expression of CYP2B6 is highly variable but using sensitive immunochemical detection it was shown to account for 6% of total CYP content in the liver (Stresser and Kupfer, 1999). There is very high interindividual variability reported for CYP2B6 as 25-250 fold differences have been reported; ethnicity specific differences have arisen as well, as only 30% of Japanese liver samples displayed detectable CYP2B6 expression as opposed to 85% in Caucasians (Code et al., 1997; Martignoni, Groothuis, and de Kanter, 2006b). Human CYP2B6 shares 75-76% sequence homology with rat CYP2B1 and 2B2 (Ekins and Wrighton, 1999). Rat CYP2B1 and 2B2 are isoenzymes that are difficult to differentiate as they share 97% primary structural identity and high substrate overlap (Waxman, 1988). The only notable difference between the two is CYP2B1 is considered the more catalytically active of the pair (Waxman, 1988; Pekthong et al., 2012).

1.4.1.3 Bupropion Metabolism

The metabolism of a CYP2B selective marker for activity highlights the difference between 2B1 and 2B2 (Pekthong et al., 2012). Bupropion is both a norepinephrine and dopamine reuptake inhibitor that was originally prescribed as an antidepressant but has been shown to be efficacious as a smoking cessation agent (Holm and Spencer, 2000; Stahl et al., 2004). CYP hydroxylation of this drug results in the active metabolite hydroxybupropion (OH-BUP), and this is the predominant pathway for bupropion clearance; 75% of OH-BUP generation is attributable to CYP2B1, while CYP2E1 and 2C11 account for 10.9% and 8.7% respectively (Pekthong et al., 2012). In humans CYP2B6 is also the primary metabolic enzyme responsible for bupropion’s conversion to OH-BUP and bupropion has been validated as an appropriate marker for 2B6 function as it accounts for 71% of OH-BUP formation (Hesse et al., 2000; Faucette et al., 2000). Multiple studies have reported alterations in BUP pharmacokinetics in renal impairment. A clinical study from 2007 showed a 66% reduction in the conversion of
BUP to OH-BUP in patients with CKD compared to healthy controls (Turpeinen et al., 2007). Another study showed increased plasma and brain concentrations of BUP and OH-BUP in guinea pigs that had renal impairment induced by uranyl nitrate (DeVane et al., 1986).

1.4.2 Transport

Drugs that are unable to cross the lipid bilayer of the cell require facilitation in the form of transport proteins in order to cross biological membranes. Drug transporters are integral in the absorption and excretion of xenobiotics, as well as determining the degree of penetrance into tissue (Ho and Kim, 2005). They are large, membrane bound proteins that are highly expressed at sites of drug absorption and secretion including the liver, kidney, and intestines (DeGorter et al., 2012). They are also present at sites where drug accumulation would be unfavourable such as the testes, placenta, and blood-brain-barrier (DeGorter et al., 2012). Over 400 transporters have been characterized in the human genome from the ATP-Binding Cassette (ABC) and Solute Carrier (SLC) superfamilies (Giacomini et al., 2010). These families show large interindividual differences in expression and function largely due to genetics, disease, lifestyle, and xenobiotic interactions (Ho and Kim, 2005; Giacomini et al., 2010). They can also be classified as uptake or efflux transporters.

1.4.2.1 SLC Superfamily

Members of the SLC family of transporters have >20% sequence homology and can be subdivided into Organic Cation Transporters (OCTs), Organic Anion Transporters (OATs), Organic Anion Transporting Polypeptides (OATPs), and the Sodium-Taurocholate Co-transporting Polypeptide (NTCP) (DeGorter et al., 2012). They are uptake transporters that are largely localized on the sinusoidal membrane of hepatocytes, permitting the movement of drugs from the blood into the liver. They also play a large role in uptake of substrates from the intestinal lumen into enterocytes. The SLC transporters function in a variety of ways with the most common being facilitative diffusion and secondary active transport in the form of ion coupling or ion exchange (DeGorter et al., 2012).
1.4.2.1.1 Organic Anion Transporting Polypeptide Superfamily

OATPs are very well characterized structurally and functionally, they are speculated to contain 12 transmembrane domains and drive transport in a sodium independent fashion (Hagenbuch and Meier, 2003). They harbour several highly conserved N-glycosylation domains which are imperative for proper function and membrane localization, which is dependent on the degree of glycosylation (Hagenbuch and Gui, 2008). The SLCO genes code for the respective OATP protein produced. In 2004 Hagenbuch and Meier described OATP proteins with >40% sequence homology being within the same family (OATP1-6), while >60% amino acid identity will put them in the same subfamily (OATP1A/B/C etc…). To date 52 members of the superfamily have been discovered (36 in humans), and they are organized into 6 families with 13 subfamilies (Hagenbuch and Meier, 2004). These transporters have a wide substrate specificity but some general criterion appear to be transport of amphipathic molecules, molecular weight >450Da, with high degrees of protein binding under physiological conditions (Hagenbuch and Meier, 2003). This encompasses endogenous molecules such as bile acids, thyroid hormones, as well as glucuronidated and sulfonated compounds alike (Hagenbuch and Meier, 2004). Of interest to this project, OATPs also have affinity for many xenobiotics, including the statin class of therapeutic agents and the anti-histamine fexofenadine (Hagenbuch and Meier, 2003; Ho et al., 2006; Gradhand et al., 2013). It is hypothesized that these proteins can govern bidirectional transport, depending on concentration gradients of substrates, however they are studied in large part for their role as uptake transporters (Hagenbuch and Meier, 2004). OATPs function through organic cation exchange while using bicarbonate, reduced glutathione, and glutathione conjugates as counter-ions in this process (Hagenbuch and Gui, 2008; Hagenbuch and Stieger, 2013).

OATPs are ubiquitously expressed in the body although some isoforms have tissue specific expression patterns (Hagenbuch and Stieger, 2013). Isoforms in locations such as the liver and intestine are of particular importance to this study. The role of OATPs in drug disposition is elegantly demonstrated with the diminished bioavailability of the transporter probe fexofenadine, when administered in the presence of a potential
OATP inhibitor. A study from 2005 showed a 42% reduction in the oral bioavailability of fexofenadine, potentially due to the inhibition of enteric uptake transporters by grapefruit juice, displaying the importance of OATPs in drug pharmacokinetics (Dresser et al., 2005). OATP1B1 and 1B3 (previously OATP-C and OATP8 respectively) are two proteins expressed exclusively on the sinusoidal membrane of the liver and play a large role in drug clearance (Hagenbuch and Meier, 2004). They share 80% sequence identity, are both from the OATP1B subfamily, and are of similar size, 691 and 702 amino acids long for 1B1 and 1B3 respectively (Hagenbuch and Gui, 2008). Due to their crucial role in drug disposition, there is a high potential for drug-drug interactions and adverse drug reactions (ADR) as they have affinity for such a broad spectrum of substrates (Hagenbuch and Gui, 2008).

Mutations and polymorphisms in OATPs can lead to altered function and contribute to pathological disorders (Tirona et al., 2001). There is an assortment of mendelian diseases associated with the SLC family with the severity varying from benign to life threatening (Lin et al., 2015). A mutation in the SLCO1B1 and 1B3 genes resulting in a loss of function of the respective OATP proteins has been shown to lead to rotor syndrome in humans (van de Steeg et al., 2012). It is characterized by high levels of conjugated and unconjugated hyperbilirubinemia along with coproporphyrinuria and altered bromosulfophthalein clearance (van de Steeg et al., 2012). Mutations in both the 1B1 and 1B3 genes are required to see the phenotype due to the high substrate overlap between the transporters (Lin et al., 2015).

1.4.2.2 ABC Superfamily

The ABC superfamily is responsible for efflux transport and requires energy from ATP hydrolysis to power the movement of its substrates against concentration gradients, thus a defining feature of these proteins is a highly conserved ATP-Binding motif (DeGorter et al., 2012). Constituents of this family include P-glycoprotein (P-gp/MDR1/ABCB1), breast cancer resistance protein (BCRP/ABCG2), multidrug resistance-associated protein (MRP/ABCC), and the bile salt export pump (BSEP/ABCB11) (DeGorter et al., 2012). These proteins play an important role in conferring drug resistance as they are in large part localized on the canalicular membrane of hepatocytes.
and the apical membrane of enterocytes, hepatocytes, renal proximal tubule cells, and the blood-brain-barrier endothelium (DeGorter et al., 2012). The tissue distribution of P-gp in particular shows the importance of efflux transport in detoxification pathways as it is able to move compounds into the bile, feces, urine, and away from the brain (Nolin, 2008). Other canalicular transporters of interest are BSEP and BCRP as they, along with P-gp, have broad substrate specificities and allow for the process known as enterohepatic recirculation by pumping substrates into the bile ducts (Nolin et al., 2008; Yeung et al., 2014).

1.4.3 Nuclear Regulation

The expression of different metabolic and transporter proteins can have a large effect on the therapeutic or toxicological effect of a drug. Nuclear receptors are a superfamily comprised of genes that produce transcription factors, which modulate gene expression. There are over 70 members of this superfamily, all share similar sequence identity and contain 4 similar modalities, the modulator domain, DNA-binding domain (DBD), hinge region, and ligand binding domain (LBD) (Giguère, 1999; Wang and LeCluyse, 2003). The modulator domain is located at the 5’ amino terminus and is considered the most variable of the modalities (Giguère, 1999). It contains an activation function domain-1 that allows for ligand-independent activation so long as other nuclear receptors or coactivators are present (Giguère, 1999; Urquhart et al., 2007). The DBD is the most highly conserved domain but since receptors can bind DNA as monomers, homodimers, or heterodimers there is inevitably some variation between individual receptors (Giguère, 1999). This region is aptly named as it is responsible for interaction of the receptor with target DNA sequences (Giguère, 1999). The hinge region gives the receptor flexibility between the DBD and LBD, allowing for dimerization and may pose as a docking site for repressor elements (Giguère, 1999). The LBD principally moderates ligand interactions but extends its purpose to mediate dimerization, nuclear localization, and interactions with heat shock proteins (Giguère, 1999). It also facilitates ligand-dependent-transactivation through activation function domain-2 (Giguère, 1999).

There are two perceived mechanisms of action for nuclear receptors, with the traditional one being that an inactive cytoplasmic receptor will subsequently translocate
to the nucleus once a ligand has bound to it. Many nuclear receptors heterodimerize with the retinoid X receptor (RXR) upon entering the nucleus, facilitating interactions with the promoter or enhancer regions of the target gene (Urquhart et al., 2007). Other nuclear receptors are constitutively active and do not require a ligand to facilitate DNA binding (Giguère, 1999). In the latter model, ligand binding may actually dissociate receptors from the DNA.

1.4.3.1 Orphan Nuclear Receptors

A sub-family known as orphan nuclear receptors accounts for 60% of the nuclear receptor superfamily (Giguère, 1999; Wang and LeCluyse, 2003). The orphan nomenclature originated when the first member was discovered in 1988 and all previously identified receptors had a known physiological function with an associated hormone or ligand, these had yet to be established and were instead assigned the preceding title of orphan to acknowledge this (Giguère et al., 1988). Members of this family govern the transcriptional expression of prominent metabolic enzymes and transport proteins (Wang and LeCluyse, 2003; Tirona and Kim, 2005). Exogenous and endogenous stimuli can increase or decrease nuclear receptor activity, and by virtue of this it will translate to drug metabolism and disposition proteins having inducible or repressible expression as they are the targets (Wang and LeCluyse, 2003).

1.4.3.1.1 Regulation of Metabolic Enzymes

RXR was the first orphan receptor discovered. As previously mentioned, it serves an important role for heterodimerization. These dimers can be classified as permissive or non-permissive, where permissive can be activated by RXR or its partner’s ligand, and non-permissive will only respond to the partner’s ligand (Giguère, 1999). RXR can also serve as a monomeric regulator of transcription through its ligand 9-cis-retinoic acid (Levin et al., 1992; Giguère, 1999). The pregnane X receptor (PXR) is well characterized in the literature due to its regulation of the human CYP3A subfamily. It has similar tissue expression patterns as CYP3A and is xenobiotic inducible, a necessary characteristic for a drug metabolizing enzyme regulator (Wang and LeCluyse, 2003). PXR has very little variation across species except for in the LBD, but still shares 76% sequence homology
with mouse and rat models (Wang and LeCluyse, 2003). Implications from this would be that the downstream regulatory pathways are very similar but the initiation portion may vary between species. PXR has also been shown to regulate the human CYP2B and MDR1 families, emphasizing its importance in drug metabolism and transport (Goodwin et al., 2001; Wang and LeCluyse, 2003).

Of particular importance to this study is the regulation of CYP2B6. A system of PXR, a PXR-RXR heterodimer, and the constitutive androstane receptor (CAR) regulate CYP2B6 (Goodwin et al., 2001). The activation of CYP2B6 requires interaction at the nuclear receptor binding sites (NR1 and NR2) within the distal promoter phenobarbital-responsive enhancer module (PBREM) (Czekaj, 2000; Goodwin et al., 2001). The rat homologs CYP2B1 and CYP2B2 are putatively regulated by a CAR-RXR heterodimer (Bing et al., 2014). The rat CYP2B promoter structure (Figure 1.2) follows the same map as human, as it contains the distal PBREM where CAR can bind the NR1 and NR2 sites (Shaw et al., 1996; Czekaj, 2000; Bing et al., 2014). The promoter is 2216 base pairs upstream of the transcriptional start site and contains a nuclear factor (NF-1) binding motif flanked by NR1 and NR2, all within a 51 base pair region (Shaw et al., 1996; Czekaj, 2000). There is significant sequence identity between the 2B1 and 2B2 promoters to the degree of 98-100% similarity. The transcription of 2B1 and 2B2 is phenobarbital (PB) inducible through an interaction of PB with the CAR-RXR heterodimer, causing interaction at the NR1 and NR2 sites in the PBREM (Honkakoski et al., 1998). The same phenomenon is believed to occur for CYP2B6 in humans, as CAR was shown to mediate PB inducibility in the HepG2 cell line (Sueyoshi et al., 1999).

1.4.3.1.2 Regulation of Transporter Proteins

In similar fashion to metabolic enzymes, PXR, CAR, and RXR, all play important roles in the nuclear regulation of transporter proteins (Urquhart et al., 2007). Starting with efflux transporters, P-gp is regulated by PXR as its inducer rifampin was able to upregulate it in the human intestinal goblet cell-like line, LS174T (Geick et al., 2001). Its expression may also be affected by the presence of monomeric CAR as a 2005 study showed the CAR/RXR heterodimer did not affect expression but CAR alone upregulated intestinal P-gp in vitro (Burk et al., 2005). The regulation of MRP2 was described in
Figure 1.2 - Schematic illustration of the PBREM binding region for the transcriptional regulation of CYP2B1. Images were modified from Servier Medical Art (http://www.servier.co.uk/medical-art-gallery).
2002 via the incubation of HepG2 cells with activators for CAR, PXR, and the farnesoid X receptor (FXR) causing an increase in MRP2 transcriptional expression (Kast et al., 2002). FXR has also been shown to regulate BSEP in both a mouse FXR knockout model and a human in vitro model by incubation of HepG2 cells with a FXR ligand (Sinal et al., 2000; Ananthanarayanan et al., 2001). A previously unmentioned nuclear receptor, peroxisome proliferator-activated receptor gamma (PPARγ), is able to dimerize with RXR and increase BCRP expression, shown with human dendritic cells in the presence of the PPARγ agonist rosiglitazone (Szatmari et al., 2006). The hepatocyte nuclear factor 1 alpha (HNF1α) is important for hepatic uptake of bile salts and bilirubin as it has been shown to regulate human OATP1B1 in Huh7 and HepG2 cell lines (Jung et al., 2001; Meyer zu Schwabedissen et al., 2010). Not limited to OATP1B1, OATP1B3 is also regulated by HNF1α along with a FXR/RXR heterodimer (Jung et al., 2001; 2002). Rat Ntcp, Oatp1a1 (Oatp1), 1a4 (Oatp2), and 1b2 (Oatp4) also appear to be regulated by HNF1α as a Tcf1/- mouse line was shown to have bile acid and plasma cholesterol disposition irregularities secondary to lowered mRNA of the aforementioned transporters (Shih et al., 2001). Human and mouse NTCP/ntcp differ in their regulation as HNF1α did not affect expression levels; human NTCP was later discovered to be regulated by the glucocorticoid receptor (Jung et al., 2004; Eloranta et al., 2006).

1.4.4 Altered Metabolism & Transport in Disease States

Disease states are established to have an effect on CYP expression and function. It is reasonable to assume that an organ specific disease would have an effect on its metabolic function, as is the case with hepatic cirrhosis. Although the mechanism is not always clear, it is well documented that CYP function is altered in liver disease (George et al., 1995; Frye et al., 2006; Woolsey et al., 2015). Altered metabolic function has also been seen in obesity, diabetes mellitus, congestive heart failure, and during infections (Nolin et al., 2003). This train of thought leads to the conclusion that metabolic and transporter function will be altered in renal tissues for CKD patients. Decreased renal function resulting in a lower GFR alone will lead to decreased drug exposure due to reductions in renal clearance, when combined with altered transporter and metabolic enzyme expression and function, the pharmacokinetics of drugs can be drastically altered.
Unless there has been experimental work presenting how clearance in the disease state is changed for a specific drug, the dosing adjustments will have to coincide with the known pharmacology of the drug and current creatinine clearance of the patient (Nolin, 2008).

Precautions for renally cleared drugs being administered to CKD patients are fairly well characterized, however, recent advances have shown that non-renal routes such as hepatic elimination and intestinal absorption are impaired as well (Nolin et al., 2003; Sun et al., 2004; Michaud et al., 2005; Sun et al., 2006; Naud et al., 2007; Nolin, 2008; Velenosi et al., 2012). Putative mechanisms for this are transcriptional, translational, and epigenetic alterations in metabolic enzymes and transporter proteins due to increased levels of circulating inflammatory proteins and uremic toxins (Guévin et al., 2002; Dreisbach and Lertora, 2008; Nolin et al., 2008; Velenosi et al., 2012; Zhao et al., 2013; Velenosi et al., 2014; Ladda and Goralski, 2016). Both transport and metabolism at non-renal sites are affected along with altered plasma protein and tissue binding (Nolin et al., 2008; Dreisbach and Lertora, 2008). There is a reported 40% reduction in total hepatic CYP enzymes in CKD (Leblond et al., 2000). Activity of CYP enzymes is also significantly affected as assays such as the erythromycin breath test have shown a 35% decrease in CYP activity in rat models of CKD (Leblond et al., 2000). Inflammation is a common mechanism between disease states that has been shown to affect metabolic enzyme and transporter function (George et al., 1995; Nolin et al., 2008; Morgan et al., 2008; Huang et al., 2010). Cytokines have been shown to down-regulate CYP enzymes as well as transporters such as P-gp (Morgan et al., 2008). The more important mechanism in CKD is likely to be the high circulating uremic toxins. Studies have shown reversals of the down regulation upon kidney transplant but it is not seen to the same extent in hemodialysis as the protein-bound uremic toxins will not be removed as efficiently (Nolin et al., 2006; Nolin, 2008). This was illustrated by the quantification of 4β-hydroxycholesterol (an endogenous marker of CYP3A function) in CKD patients (Suzuki et al., 2013). There was a significant rise in plasma 4β-hydroxycholesterol 90 and 180 days post-transplant compared to its formation rate prior to the transplant (Suzuki et al., 2013).
Significant reductions in the activity of rat CYP2C11, 3A1, and 3A2 secondary to lowered mRNA and protein levels of the respective isoenzymes has been reported in CKD (Leblond et al., 2001; Velenosi et al., 2012). The expression of rat CYP1A2, CYP2C6, and CYP2D have also been shown to be downregulated in CKD (Uchida et al., 1995; Guévin et al., 2002; Rege et al., 2003). Work by Velenosi et al in 2014 showed that there are epigenetic differences as well as nuclear receptor alterations potentially contributing to the altered expression and activity of CYP enzymes in CKD. Chromatin Immunoprecipitaiton (ChIP) revealed RNA polymerase II and HNF4α binding to the promoter regions of CYP2C11 and CYP3A2 was decreased by approximately 70% (Velenosi et al., 2014). They also showed a large decrease in histone 3 acetylation in the promoter regions of CYP2C11 and 3A2 (Velenosi et al., 2014). The effect of CKD on rat CYP2B1 and 2B2 expression, activity, and regulation is currently unexplored.

The altered expression and function of non-renal transporters has also been established in the literature. Specific uremic toxins were shown to decrease transport of erythromycin in rat hepatocytes, mostly affecting the OATP transporters (Sun et al., 2004). Livers from rats with CRF showed a 25% increase in P-gp protein expression, a 40% increase in Mrp2 mRNA expression, and a decrease in Oatp1a4 protein expression but no change in mRNA abundance (Naud et al., 2008). There is speculation however that Oatp1a4 function is also affected but it is currently unexplored in CKD (Sun et al., 2006; Naud et al., 2008). Intestinal efflux transporters in rats, such as P-gp, Mrp2, and Mrp3 have been shown to be down regulated while enteric Oatp1a4 and Oatp1a5 (Oatp3) are not affected in CKD (Naud et al., 2007). Indoxyl sulfate and CMPF were shown to impede P-gp’s ability to transport cyclosporin in Caco2 cells, again pointing to a uremic toxin mediated mechanism (Shibata et al., 2000). A study in 2009 incubated rat hepatocytes and enterocytes with serum from human patients with ESRD (Nolin et al., 2009). The results showed a decrease in hepatic Oatp1a4 protein expression but an increase for P-gp; enteric P-gp and CYP3A2 protein expression were both decreased while Oatp1a5 was not affected (Nolin et al., 2009). The same study also looked at the clearance of fexofenadine and found it was decreased, as it is transported by P-gp and Oatps (Nolin et al., 2009).
1.5 Statins

The statins are the highest prescribed class of drugs in the world and are used in the clinic to treat hypercholesterolemia by lowering low-density lipoprotein (LDL)-C levels. The mechanism of action is to regulate hepatic cholesterol biosynthesis through inhibition of 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA) reductase, which is the rate limiting enzyme for this process and used to reduce HMG-CoA to mevalonic acid (Nezasa et al., 2002; Knauer et al., 2010). They are noted for their efficacy in reducing the incidence of cardiovascular events (Levine et al., 1995). CKD patients are often prescribed statins as LDL-C lowering therapy is known to decrease CVD risk in stages 1-3 (National Kidney Foundation, 2002; Colhoun et al., 2004).

Statins have a low toxicity profile however there are reported cases with serious side effects. Skeletal muscle complications such as myalgia are the most common ADRs, occurring in 5-15% of patients (Sinzinger et al., 2002; Knauer et al., 2010). Instances of rhabdomyolysis have also been reported, albeit rarely, but can be life threatening through mass myocyte destruction resulting in acute renal failure (Zutt et al., 2014). Renal failure is caused by tubule obstruction and direct toxicity from myoglobin, which accumulates beyond plasma protein-binding capacity once the myocytes rupture (Zutt et al., 2014).

Some statins are excreted mostly as unmetabolized parent compounds. This emphasizes the importance of drug transport in the regulation of their clearance. OATP1B1 is responsible for a significant portion of pravastatin and pitavastatin transport as a 1B1 SNP with lower activity caused a large increase in the area under the curve for a plasma concentration-time plot in humans (Nishizato et al., 2003; Chung et al., 2005).

1.5.1 Rosuvastatin

Rosuvastatin is touted to have the highest inhibitory proficiency for HMG-CoA reductase amongst the statins from in vitro work in rat hepatocytes (McTaggart et al., 2001). It has also been shown to be more effective than atorvastatin, simvastatin, and pravastatin in terms of blood cholesterol reduction in the clinic (Olsson, 2001; JH Choi et al., 2007). The tissue distribution of rosvastatin is localized to the liver and 88.3% of the drug excreted in the bile was the parent compound, indicating a low degree of
metabolism (Nezasa et al., 2002). The excretory pathway is therefore driven by transport, and specifically hepatic transport as 98% of an 5mg/kg orally administered dose was recovered in the feces, while only 0.4% is found in the urine (Nezasa et al., 2002).

The main drug transporter for rosuvastatin is OATP1B1, accounting for 43-55% of uptake (Kitamura et al., 2008). A 2008 clinical study in the Korean population displayed the importance of OATP1B1 as the *15 SNP predisposed patients to higher plasma concentrations of rosuvastatin (JH Choi et al., 2007). Other significant OATP1B1 SNPs for rosuvastatin pharmacokinetics include *5, *9, and *18 (Ho et al., 2006). Despite the importance of OATP1B1, there are other important transporters involved in its disposition. A study in 2006 elucidated that NTCP accounts for 35% of rosuvastatin transport in humans by comparing a sodium vs sodium-independent uptake assay (Ho et al., 2006). OATP1B3, OATP2B1, and OATP1A2 all have the capacity to transport rosuvastatin and this redundancy increases the safety of the drug as a OATP1B1 loss of function will not be as significant as it is for other statins such as pravastatin and pitavastatin (Lee et al., 2005; Ho et al., 2006; Kitamura et al., 2008). Efflux transport in humans falls under the jurisdiction of BCRP, MRP2, and P-gp, as all have shown affinity for rosuvastatin (Kitamura et al., 2008).

There are species differences in the role of transporters for rosuvastatin. Uptake transport in rats is driven primarily by Oatp1a4, Oatp1a5, and Oatp1b2 (Ho et al., 2006). The largest discrepancy is that rat Ntcp does not efficiently transport rosuvastatin as it does in humans as transport was unaffected in a sodium-free buffer (Ho et al., 2006). Rat Oatp1a1, 1a4, 1a5, and 1b2 all show highly favourable interactions with rosuvastatin as a substrate (Ho et al., 2006). Canalicular transport is driven primarily by Mrp2 with Bcrp and Bsep having roles as well in sandwich cultured rat hepatocytes (Jemnitz et al., 2010). In conjunction with its limited metabolism and minimal renal clearance, rosuvastatin is a good probe substrate for hepatic OATP function (Knauer et al., 2010). The product monograph has dose reduction recommendations listed for patients with severe renal impairment, indicating potential pharmacokinetic alterations in the disease state (AstraZeneca 2012). Additionally contraindications for patients with active liver disease
are listed, insinuating that alterations to hepatic tissue may also lead to changes in rosvastatin clearance (AstraZeneca 2012).

1.6 Animal Models of CKD

Animal models are used to study chronic conditions that normally take years to develop in humans, they can accelerate pathogenesis into a matter of weeks allowing for rapid assessment of disease conditions (Hewitson et al., 2008). Animal models can be used to elucidate mechanisms underlying pathologic states and to develop therapies within a feasible timeframe due to the accelerated disease development (Furness and Harris, 1994). There are a variety of currently validated CKD models, each attempting to replicate different CKD etiologies. There are surgical, nephrotoxic, immune-mediated, and metabolic models.

There are three common surgical models to induce CKD including ureteric obstruction, sub-total nephrectomy or remnant kidney, and ischemic acute renal failure (Hewitson et al., 2008). Of the surgical models, sub-total nephrectomy is the most commonly used (Leblond et al., 2000; 2001; Hewitson et al., 2008; Velenosi et al., 2012). There is also a ligation and ablation modification of this technique. The ablation procedure involves the removal of 2/3 of one kidney (typically the left), then 5 days post-op complete ablation of the in-tact kidney is carried out, resulting in a 5/6 loss of total renal mass (Hewitson et al., 2008). The ligation version involves a uninephrectomy with subsequent ligation of 2/3 of the polar branches of the renal artery, simulating hypertensive kidney failure (Hewitson et al., 2008). Some groups do a hybrid of the ligation and ablation models to try and reduce variability between rats (Velenosi et al., 2012). Problems arising from this model revolve around post-operative inflammation and the intricate nature of the procedure leading to a large window for human error and variability between animals. Sham-laparotomies can control for some of the inflammatory irregularities however there is still an appreciable chance for human error to persist between nephrectomized animals and laparotomy controls.
1.6.1 Nephrotoxic Models

The kidney’s role in detoxification and clearance leaves it exposed to many toxins that may cause major renal damage. There are multiple currently validated nephrotoxic models including podocyte and tubular toxins, as well as tubular obstruction versions. These models typically have lower variability as they can be added to the diet of animals, causing renal damage based on consumption rates, therefore keeping renal damage proportional to weight. Larger animals will have larger kidneys, but will incur greater kidney damage due to increased appetite, theoretically controlling for size differences between animals. Folic acid or adenine supplements to food can cause CKD secondary to tubular obstruction. The adenine model of CKD was first developed in 1984 and involves long-term administration of standard rat chow supplemented with up to 0.75% adenine (Yokozawa et al., 1984; Terai et al., 2008). Renal damage occurs from the adenine metabolite, 2,8-dihydroxyadenine, precipitating and crystallizing in the proximal tubule epithelia (Hewitson et al., 2008; Ali et al., 2013). The resulting phenotype is a granular and enlarged kidney with apoptotic lesions and widespread fibrosis (Koeda et al., 1988; Tamagaki et al., 2006; Diwan et al., 2013). There are elevations in plasma urea and creatinine as well as reported azotemia (Diwan et al., 2013; Velenosi et al., 2016). The diet must be administered consistently for an extended period of time as recovery may occur after 2 weeks of treatment, after 4-6 weeks however the damage is irreversible as there is severe inflammation, fibrosis, and calcification of tubular basement membranes (Tamagaki et al., 2006; Hewitson et al., 2008). Although it reduces diversity in the degree of CKD between animals and eliminates the requirement for specialized and technical skill, there is one notable drawback to this model (Terai et al., 2008). It has been reported that there is limited food consumption within the first week of adenine administration, which makes pair-feeding a necessity for control animals (Terai et al., 2008). Both surgical and nephrotoxic animal models of CKD have been shown to decrease selected drug metabolizing enzymes compared to pair-fed control animals (Leblond et al., 2001; Velenosi et al., 2012; 2014; Feere et al., 2015).
1.7 Objectives and Hypothesis

Chronic kidney disease is a major global health burden. It can result in many comorbidities that require multiple prescriptions to manage them, leading to one of the highest pill burdens among chronic conditions. Several studies have shown altered renal and non-renal clearance of drugs in CKD, which leads to pharmacokinetic changes and commonly to severe adverse drug events. Many drugs have yet to be specified whether or not dosing regimens should be changed as it is unknown if their disposition pathway is affected by CKD. The altered clearance is a result of changes to the expression and function of metabolic enzymes and transporter proteins, both renal and non-renal. The putative mechanism for these alterations is the uremic condition that ensues in moderate and severe CKD. Specifically, gut-derived uremic toxins are proposed to cause the alterations in metabolism and transport. Better knowledge of the uremic state and how it affects drug metabolism and disposition is necessary.

The objective of this project is to elucidate whether the removal of uremic toxins can recover the expression and function of transporter proteins and metabolic enzymes. As extensively outlined in previous sections, there is reason to believe gut-derived uremic toxins alter non-renal metabolism and transport. Thus the role specific uremic toxins play in the alteration of metabolism and transport will also be explored. The first aim of the study was completed using an adenine-induced model of CKD in male Wistar rats. Administration of the drug AST-120 was used to lower gut-derived uremic toxin levels in the animals. It is hypothesized that transporter protein as well as metabolic enzyme expression and function will be altered in CKD, and AST-120 will recover expression and function back to control levels by removing the aforementioned gut-derived uremic toxins.

The second portion of this study is the in vitro aspect. The purpose of this is to determine which individual uremic toxins, if any, are responsible for alterations in transport and metabolism. The human hepatocarcinoma cell line Huh7 will be incubated with toxins and the transporter probe drug, rosuvastatin. It is hypothesized that rosuvastatin uptake will be decreased in Huh7 cells when they are incubated with uremic toxins.
Chapter 2

2 Methods

2.1 CKD Rat Model

Male Wistar rats were obtained from Charles River Laboratories (Wilmington, Massachusetts). They were allowed an acclimation period of at least 4 days while on a 12-hour light cycle and were provided with Harlan 8640 Teklad 22/5 rodent diets (Harlan Laboratories: Madison, Wisconsin) and water ad libitum. All animal care protocols and procedures followed guidelines provided by the Western University Animal Care Committee and the Canadian Council on Animal Care (Appendix A).

2.1.1 Study 1 – Hepatic and Intestinal Transport (7 Weeks)

Male Wistar rats were used as the in vivo model. After a 5-day acclimation period animals were divided into 4 groups (n=10-14 per group), which determined their diet for the duration of the 7-week study. Control and control+AST-120 groups were pair-fed to the CKD and CKD+AST-120 rats. CKD and CKD+AST-120 were provided food ad libitum throughout the 7 weeks. Control rats were fed standard rat chow (Harlan Laboratories: Madison, Wisconsin) for the entire study. CKD rats were fed standard rat chow supplemented with 0.7% adenine (Harlan Laboratories: Madison, Wisconsin) for the first 4 weeks, followed by 3 weeks of control diet. Control+AST-120 rats were fed standard rat chow for the initial 4 weeks but were changed to the same diet mixed with 8% AST-120 (provided by Kureha Corporation: Tokyo, Japan) for the remaining 3 weeks. The CKD+AST-120 group received the same diet as the CKD rats for the first 4 weeks and were provided with standard rat chow supplemented with 8% AST-120 for the final 3 weeks. On the last day of the study the rats were injected with either 5mg/kg of rosuvastatin (acquired from Toronto Research Chemicals: Toronto, Ontario) or 10mg/kg of fexofenadine intravenously (acquired from Toronto Research Chemicals: Toronto, Ontario). Sacrifice occurred 2-hours post-injection via isoflurane administration and subsequent decapitation. Liver, heart, kidney, small intestine, and pancreas were harvested, flash frozen in liquid nitrogen, and stored at -80°C. Whole blood was collected
in heparin coated tubes, centrifuged at 1300 RCF for 10 minutes to separate plasma, then the supernatant was stored at -20ºC. Serum urea and creatinine levels were determined by the London Laboratory Services Group by standard methods.

2.1.2 Study 2 – Hepatic Transport (8 Weeks)

Some differences in terms of severity of CKD were noted in the 7-week study compared to previous studies from our lab that utilized an 8-week protocol. We therefore decided to repeat the transport study with the more severe model of CKD. Wistar rats were used again as the in vivo model. The rats were divided into 4 groups (n=6-8 per group), which determined their diet for the span of the 8-week study. Control and control+AST-120 rats were pair-fed to CKD and CKD+AST-120 groups. Control rats received standard rat chow for the full 8 weeks while CKD rats were provided standard chow with 0.7% adenine for 5 weeks, and control food for the remaining 3 weeks. Control+AST-120 and CKD+AST-120 rats were provided with standard rat chow and standard rat chow with 0.7% adenine respectively until the end of week 5. They were then both switched to standard rat chow augmented with 8% AST-120 until the conclusion of the study. On the final day the rats were injected with 5mg/kg rosvastatin. Two-hours post-injection they were sacrificed through isoflurane exposure and subsequent decapitation. Whole blood, liver, heart, kidney, small intestine, and pancreatic tissues were harvested and snap frozen in liquid nitrogen, then stored at -80ºC. Whole blood was collected in heparin coated tubes, centrifuged at 1300 RCF for 10 minutes and then the plasma layer was extracted and stored at -20ºC.

2.1.3 Study 3 – Hepatic Metabolizing Enzymes (8 Weeks)

Another replicate of the rat model from study 2 was completed previously in our lab with the animals being divided into the same 4 groups (n=9-12 per group) (Velenosi et al., 2014; 2016). They did not receive injections and the same tissues were collected as described above.
2.2 Real-Time PCR

Sample preparation for qPCR was preceded by an RNA extraction from hepatic or intestinal tissue. A phenol-chloroform extraction was performed while employing guanidine isothiocyanate as a chaotropic agent by using Life Technologies’ TRizol reagent (Burlington, Ontario). RNA was quantified post-extraction using a nanodrop spectrophotometer from Thermo Scientific (West Palm Beach, Florida). Consistent concentrations were attained post-nanodrop by adding 1 µg of RNA to qScript cDNA supermix containing reverse transcriptase (Quanta Biosciences, Gaithersburg, Maryland). This mixture was subject to thermal cycling in order to synthesize cDNA.

2.2.1 Primer Validation

Primers were designed through the use of NCBI Primer BLAST. PCR amplification in conjunction with SYBR safe gel electrophoresis was completed for each potential primer set to check primer specificity. Primer efficiency was determined by serially diluting cDNA and acquiring cycle threshold (Ct) values using qPCR. Efficiency was calculated using the equation $10^{\left(-1/\text{slope}\right)}-1$, with resulting values from 80-120% deemed acceptable. Primer sets developed and validated for this project are listed in Table 2.1.

2.2.2 Quantification

The cDNA of interest was diluted 1:40 and a master mix was created for each gene of interest using a SYBR green supermix in conjunction with forward and reverse primers for the gene. Samples were pipetted in triplicate into a 384-well plate, 5 µl of cDNA and 7 µl of master mix were aliquoted to each well. An optical density lid was applied once all samples were in the plate. The plate was then put on a plate shaker for 1 minute and subsequently centrifuged at 1000 rpm for 1 minute at room temperature. A Bio-Rad thermocycler (Hercules, California) was used to complete the denaturation, annealing, and elongation steps. Relative RNA expression was determined using the $\Delta\Delta\text{Ct}$ method while using $\beta$-Actin and villin as the housekeeping genes for hepatic and enteric tissue respectively (Schmittgen and Livak, 2008). For Huh7 cell work 18s rRNA was used as the housekeeping gene. All values were relative to control groups.
Table 2.1 - Primer sets for real-time PCR

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer Sequences 5' to 3'</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Uptake</strong></td>
<td></td>
</tr>
<tr>
<td>Oatp1a1</td>
<td>FW: CATGAGGTGACTTCTCTTTG</td>
</tr>
<tr>
<td></td>
<td>RV: TTCTGCTGGTCTTTGTTTG</td>
</tr>
<tr>
<td>Oatp1a4*</td>
<td>FW: AGCTTCAAGAGCTTTACCTG</td>
</tr>
<tr>
<td></td>
<td>RV: GAATGAAAGACGGGGGACA</td>
</tr>
<tr>
<td>Oatp1a5</td>
<td>FW: CTCGAGCGACAAATGAGCTC</td>
</tr>
<tr>
<td></td>
<td>RV: ACAGTGTACTCTGACTTGCTC</td>
</tr>
<tr>
<td>Oatp1b2</td>
<td>FW: GCTTCTCAGGCAACTTCTTTG</td>
</tr>
<tr>
<td></td>
<td>RV: ACTGGGTTCATTGGCGATTTC</td>
</tr>
<tr>
<td>Ntcp</td>
<td>FW: TCAATCCAAAGCTGACAGC</td>
</tr>
<tr>
<td></td>
<td>RV: GTGGCCGAAATGACTCGG</td>
</tr>
<tr>
<td><strong>Efflux</strong></td>
<td></td>
</tr>
<tr>
<td>Bsep</td>
<td>FW: TGAAGGCAATGTTGACTCGG</td>
</tr>
<tr>
<td></td>
<td>RV: AGTGGTGGAGAACAACAGC</td>
</tr>
<tr>
<td>P-gp*</td>
<td>FW: TGTCACGATGAGCTGATG</td>
</tr>
<tr>
<td></td>
<td>RV: CCAGATATGCACAAACAGC</td>
</tr>
<tr>
<td>Berp*</td>
<td>FW: GGAGGCAAGTCTCTTGCT</td>
</tr>
<tr>
<td></td>
<td>RV: GTTTGAGGTGGCCGGTTAT</td>
</tr>
<tr>
<td><strong>Metabolic enzymes</strong></td>
<td></td>
</tr>
<tr>
<td>CYP2B1</td>
<td>FW: GCTCAAGTACCCCCTGTCC</td>
</tr>
<tr>
<td></td>
<td>RV: ATCAGTGTAGGATTTACTGCC</td>
</tr>
<tr>
<td>CYP2B2</td>
<td>FW: GTACCCCCATGTACAGAAAA</td>
</tr>
<tr>
<td></td>
<td>RV: CATCAAGGGTGTTGACTCT</td>
</tr>
<tr>
<td>CYP2B6†</td>
<td>FW: ATGGGGCAGTAAAGAAGCTGA</td>
</tr>
<tr>
<td></td>
<td>RV: AGAGGGGGGAGCGACTGAG</td>
</tr>
<tr>
<td><strong>Nuclear receptors</strong></td>
<td></td>
</tr>
<tr>
<td>RXR*</td>
<td>FW: AACCCCCCTCTAGGCGCTCAAT</td>
</tr>
<tr>
<td></td>
<td>RV: TAGTGTITGCCTGAGAGAGC</td>
</tr>
<tr>
<td>CAR*</td>
<td>FW: CCTTTTCGTTCCCTGACCA</td>
</tr>
<tr>
<td></td>
<td>RV: AGGCAAGACGTAATGCTGAGT</td>
</tr>
<tr>
<td><strong>ChIP</strong></td>
<td></td>
</tr>
<tr>
<td>CAR PBREM binding site</td>
<td>FW: GGCAGCACCCTCTCTATGAT</td>
</tr>
<tr>
<td></td>
<td>RV: GGATGACGCTTCTGGCTCC</td>
</tr>
<tr>
<td>CYP2B1 RNA Pol II binding site</td>
<td>FW: AGTCCAGTCCCCCTTTGTCT</td>
</tr>
<tr>
<td></td>
<td>RV: GGAGAGCAAGAGAGAGCAAGG</td>
</tr>
<tr>
<td><strong>Controls</strong></td>
<td></td>
</tr>
<tr>
<td>Beta-Actin*</td>
<td>FW: ACAGGCCGCCAGGGAAGAGA</td>
</tr>
<tr>
<td></td>
<td>RV: TTGGTACAAATGCGGTTCTTA</td>
</tr>
<tr>
<td>Villin*</td>
<td>FW: CTTGCTCAGCCTAGGAGACT</td>
</tr>
<tr>
<td></td>
<td>RV: GCTAGGTTGAGGACTCTTG</td>
</tr>
</tbody>
</table>

All primers are for rat genes unless marked with †

*primers that were developed by previous members of our lab
2.3 Western Blot

The western blot technique was performed to determine relative protein expression levels in order to elucidate translational activity for genes of interest.

2.3.1 Microsome Isolation

The generation of rat liver microsomes followed a protocol described previously (Velenosi et al., 2012). Rat livers were taken from -80°C storage and a portion was excised and washed with 0.9% NaCl while on ice then submerged in 1.15% KCl + 1mM ethylenediaminetetraacetic acid (EDTA). Tissue was homogenized using a T 10 Basic Ultra-TURRAX dispersing instrument from IKA Laboratory Technology (Sigma Aldrich: St. Louis, Missouri). The microsomal layer was separated using differential centrifugation with an initial spin at 9000g for 20 minutes while at 4°C. The supernatant was collected and spun at 105000g for 60 minutes and at 4°C. The ensuing microsomal pellet was suspended in 0.1M potassium phosphate buffer with 20% glycerol at pH=7.4. Samples were aliquoted and stored at -80°C.

2.3.2 Pierce BCA Assay

Protein quantification was completed using the Pierce Bicinchoninic Acid Assay kit, obtained from Fischer Scientific (Waltham, Massachusetts). The principle being that the peptide bonds in protein will reduce the Cu$^{2+}$ in copper (II) sulfate to Cu$^+$. Bicinchoninic molecules will chelate with Cu$^+$ in an alkaline environment, resulting in a purple coloured complex that has strong absorptive properties at 526 nm. The production of the purple complex is proportional to the amount of protein in the sample.

2.3.3 Gel Electrophoresis

The gels used were 10% polyacrylamide for resolving and 4% for stacking while both contained 0.1% sodium dodecyl sulfate. Ammonium persulfate and TEMED (Bio Basic Inc: Markham, Ontario) were used as polymerization agents. Samples were prepared by adding 20 µg of microsomal protein to a mixture containing the reducing agent beta-mercaptoethanol and western blot sample buffer containing 10% sodium dodecyl sulfate, 26% glycerol, and 0.5% bromophenol blue. Subsequent incubation at
80°C for 20 minutes was carried out to achieve sufficient protein denaturation. Samples were then loaded into wells in the gel and an electrical force of 150V was applied for 45 minutes or until protein migration presented with adequate separation. The gels were then put into a transfer cassette ventral to a nitrocellulose membrane and submersed in Bio-Rad transfer buffer. A current was applied at 150V for 90 minutes. The membrane was blocked with PBS + 0.15% tween (PBS-T) containing 5% skim milk powder and 0.6% bovine serum albumin for 60 minutes before being washed three times with PBS-T. Primary antibodies were applied and allowed to incubate at 4°C overnight. Primary antibodies used were a monoclonal mouse anti-rat CYP2B primary antibody from Detroit R&D Inc. (Detroit, Michigan) which was diluted 1:2000 with PBS-T containing 5% skim milk powder and a rabbit anti-rat polyclonal Oatp1a4 primary antibody from Millipore (Billerica, Massachusetts) which was diluted 1:1250 with PBS-T containing 0.6% bovine serum albumin. Membranes were washed the next day and incubated with a secondary antibody conjugated to horseradish peroxidase (Santa Cruz Biotechnology Inc: Santa Cruz, California) for 60 minutes. The blots were washed again before adding Luminata Forte western blot HRP substrate (Millipore: Billerica, Massachusetts) for chemiluminescence. The platform used to visualize the immunoblots was a Bio-Rad VersaDoc Imaging System (Hercules, California). Blots were then stripped and washed before adding a monoclonal mouse anti-beta-actin primary antibody conjugated to HRP, obtained from Sigma Aldrich and diluted 1:50000. Relative gene expression could therefore be determined to baseline protein expression using beta actin while being standardized with a loading control. Densitometry was completed using the Quantity One program from Bio-Rad (Hercules, California), values were relative to control.

2.4 Rosuvastatin Quantification

2.4.1 Plasma Extraction

Plasma was obtained after subjecting whole blood to centrifugation at 1300 RCF for 10 minutes and collecting the supernatant. The plasma was subjected to solid phase extraction via Phenomenex (Torrance, California) Strata-X polymeric reversed phase packings, this step included the addition of internal standard (D3-Rosuvastatin at 100nM) with subsequent exposure to a nitrogen evaporator at 40°C. Samples were reconstituted in
100µl of 70% water and 30% acetonitrile, then subject to quantification by a Waters Acuity I Class ultra-performance liquid chromatography (UPLC) system coupled to a XEVO G2-S quadrupole time-of-flight (QTOF) mass spectrometer (Waters Corporation: Milford, Massachusetts). The standard curve used for absolute quantification was serially diluted from 5000nM to 10nM and was extracted from human plasma in order to control for matrix effects. Quality controls were completed every 10 samples at a concentration of 125nM.

2.4.2 Hepatic Extraction

Segments of whole liver were obtained using surgical blades and these pieces were homogenized using a pestle and tube in acetonitrile containing internal standard (D3-Rosuvastatin, acquired from Toronto Research Chemicals: Toronto, Ontario). Samples were then vortexed and incubated for 20 minutes on ice. Next they were centrifuged at 14000 rpm and the supernatant was removed and diluted prior to quantification by UPLC-MS.

2.4.3 UPLC-MS Conditions

An Acuity bridged ethylene hybrid (BEH) C18 column (2.1x50mm, 1.7µm) from Waters (Milford, Massachusetts) was used with a flow rate of 0.5mL/min at 40°C with sample injection volume set to 10µl. Water with 0.01% formic acid was used as solvent A and acetonitrile with 0.01% formic acid served as solvent B. A gradient of solvent B went from 30% to 50% in 2 minutes, 50-80% from 2 to 2.1 minutes, followed by 80% B for one minute and a one-minute equilibration at 30% B. The lock-mass spray solution was 500pg/µl of leucine enkephalin (556.2771Da in positive ionization) in 50% acetonitrile with 0.1% formic acid, and the mass spectrometer was calibrated with 0.5mM sodium formate. The lock spray operated with a scan time of 0.3 seconds and at an interval of ten seconds with a mass window of 0.5Da. Electrospray ionization parameters included a capillary spray voltage of 0.5kV; desolvation gas flow of 1000L/h at 600°C; cone gas flow of 50L/h with a source temperature of 150°C; sampling cone and source offset voltages at 40V and 80V, respectively. Data was acquired using positive ionization and the instrument was run in resolution mode with a mass range from 50 to 1200Da with 0.1
scans per second.

2.5 Enzyme Function Assay

Rat liver microsomes were removed from -80°C storage and aliquoted into a 96-well plate, allowing for each sample to be completed in duplicate. The metabolism reaction mixture contained a microsomal buffer (50mM KH₂PO₄ buffer with 5mM MgCl₂ at pH=7.4), bupropion (Toronto Research Chemicals: Toronto, Ontario), rat liver microsomes, and nicotinamide adenine dinucleotide phosphate (NADPH) was added last to commence the reaction. Wells deprived of NADPH were used as negative controls. The conversion of bupropion to hydroxybupropion was used to determine CYP2B enzyme function as previously described in the literature (Pekthong et al., 2012).

2.5.1 Validation

Three preliminary validation tests were completed prior to running the experiment to its entirety. These tests varied one of three independent variables which include time, microsome concentration, and drug concentration. The appropriate time-point to stop the reaction was ascertained to be 30 minutes, while the microsomal concentration was 0.5mg/ml, and the range of concentrations for bupropion were from 10μM to 500μM.

2.5.2 Hydroxybupropion Quantification

The incubation temperature during the assay was 37°C. In order to stop the reaction at 30 minutes, ice-cold acetonitrile (containing flurazepam as an internal standard at 40ng/ml) was added at a ratio of 3:1 acetonitrile to sample. Samples were spun at 3000rpm for 5 minutes, after which the supernatant was removed and diluted 5:1 in water before being run on the UPLC-MS system for quantification. Dilution was necessary to reduce ionization suppression from the KH₂PO₄ buffer. A standard curve was generated by serially diluting concentrations from 50μM to 50nM with sample injection volumes set at 5μl. An Acuity BEH C18 column (2.1x50mm, 1.7μm) from Waters (Milford, Massachusetts) was used with a flow rate of 0.6mL/min at 40°C. Water with 0.01% formic acid was used as solvent A and acetonitrile with 0.01% formic acid served as solvent B. A gradient of solvent B started as isocratic at 20% until 1.75 minutes, it then increased to 50% from 1.75 to 2.5 minutes, 50-80% from 2.5 to 2.51
minutes, followed by 80% B for one minute, then 80-20% from 3.5 to 3.51; subsequently a 1.5-minute equilibration at 20% B took place. The lock-mass spray solution was 500pg/μL of leucine enkephalin (556.2771Da in positive ionization) in 50% acetonitrile with 0.1% formic acid, and the mass spectrometer was calibrated with 0.5 mM sodium formate. The lock spray operated with a scan time of 0.3 seconds and at an interval of ten seconds with a mass window of 0.5Da. Electrospray ionization parameters included a capillary spray voltage of 0.5 kV; desolvation gas flow of 1000L/h at 600°C; cone gas flow of 50L/h with a source temperature of 150°C; sampling cone and source offset voltages at 40V and 80V, respectively. Data was acquired using positive ionization and the instrument was run in resolution mode with a mass range from 50 to 1200Da with 0.1 scans per second. Results were plotted on a Michaelis-Menten enzyme kinetics graph to display bupropion metabolism in μM vs pmol/min/mg of CYP protein.

2.6 Chromatin Immunoprecipitation

Chromatin immunoprecipitation was performed on hepatic tissue extracted from Wistar rats according to a previously described method with subsequent modifications that are also described in the literature (Sohi et al., 2011; Velenosi et al., 2014). Tissue was homogenized in 1% formaldehyde solution that contained protease inhibitor to crosslink DNA to protein. After a 10-minute incubation at room temperature, 1M glycine in PBS was added to attenuate crosslinking. Samples were subsequently spun at 3000rpm for 5-minutes at room temperature, the supernatant was removed and 1ml of PBS was added. Samples were vortexed then spun at 3000rpm for 5 minutes, the supernatant was aspirated and 500μl of lysis buffer was added. After a 20-minute incubation at 4°C, 500μl of ChIP dilution buffer was added. Samples were then sonicated at 30 AMP for 20 one-second pulses to shear the DNA. Next they were centrifuged at 12,500rpm for 10-minutes at 4°C, after which the supernatant was diluted 1:10 with ChIP dilution buffer. The extracted chromatin was pre-cleared for 2 hours at 4°C with a protein A/G agarose and salmon sperm slurry. After centrifugation at 14000rpm for 5-minutes at 4°C the supernatant was removed and the antibody of interest was added for an overnight incubation at 4°C. Antibodies included a monoclonal mouse anti-rat RNA polymerase II (1μg per aliquot, 05-623, Millipore: Billerica, Massachusetts), polyclonal rabbit anti-rat
CAR (5µg per aliquot, sc-13065, Santa Cruz: Dallas, Texas), and anti-rat IgG from both mouse and rabbit that were procured from Millipore (12-371) and Santa Cruz (sc-2027) respectively. Aliquots to control for non-specific binding received an identical amount of non-immune IgG from the same host species as the antibody of interest. After a series of washes and spins, the chromatin was eluted and the crosslinks were reversed during a 4-hour 65ºC incubation. A phenol-chloroform-isoamyl solution was used to separate chromatin, which was precipitated using ethanol and 3M sodium acetate at -20ºC overnight. Samples were dried down the next day and reconstituted in TE buffer for real-time PCR analysis. Primer sets were designed to amplify the RNA polymerase II and CAR binding region at the CYP2B1 promoter (Table 2.1). Amplification of the RNA polymerase II binding region to the CYP3A2 promoter was used as a positive control and the primer sets were published by Velenosi et al in 2014.

2.7 Huh7 Cell Line

The human hepato-carcinoma cell line Huh7 was grown to one week post-confluence for the purposes of this work as previously described (Sivertsson et al., 2010). Cells were taken from liquid nitrogen storage and thawed in a water bath at 37ºC. They were then pipetted into a flask which was subsequently filled with 10ml of Dulbecco’s Modified Eagle’s Medium + 10% fetal bovine serum and 0.9% w/v penicillin/streptomycin, the cells were left to incubate at 37ºC with 5% CO₂. Once the cells were adhered to the flask (12-18 hours) the current media was aspirated to remove DMSO used for cell storage, new media was provided immediately. Media was changed every 2 days until the cells reached confluency, at which point they were split into 6-well or 12-well plates. To split the cells, media was aspirated and 10ml of PBS was added to wash the remaining media off. This was then aspirated and 3ml of trypsin was applied with an accompanying incubation for 5 minutes at 37ºC with 5% CO₂. Once the cell adherence to the plate was diminished they were removed from the incubator and subject to gentle physical force do ensure the maximal number stayed in suspension. Media was then added (6ml) to inactivate trypsin. The mixture was transferred to a 15ml falcon tube which allowed gentle rotation of the cells to form a homogenous fluid to aliquot into plates or a new flask.
2.7.1 Characterization

The Huh7 cell line has been previously characterized for metabolizing enzymes and transport proteins in the literature (Sivertsson et al., 2010). We reconfirmed that our cells followed the same RNA expression patterns for NCTP, BCRP, and OATP1B1 using real-time PCR. Samples were normalized to 18s rRNA as a housekeeping gene and then compared to human liver expression of the same transcripts.

2.7.2 Uptake Transport and Uremic Toxin Incubation

Cells were cultured for one week post-confluency in order to optimize transporter expression and all plates were replicated in triplicate (Sivertsson et al., 2010). Validation assays for transporter exposure time and rosuvastatin concentration were completed prior to the uremic toxin incubation experiments. Time points of 0.5, 1, 5, 10, 15, and 20 minutes were evaluated, 10 minutes was selected due to linear transport kinetics at this time. To determine the optimal drug concentration an assay was performed to estimate the affinity of OATP1B1 for rosuvastatin. Concentrations of 1, 2, 5, 10 and 20µM were used due to an approximation of OATP1B1 enzyme affinity ($K_m$) for rosuvastatin to be 5µM from the literature (Ho et al., 2006). The last component of this study involved incubating the Huh7 cells with uremic toxins and rosuvastatin concurrently to determine whether there was a change from control. The toxins used and their respective concentrations were determined from the literature to approximate those measured in CKD patients and are as follows: 210µM indoxyl sulfate, 220µM p-cresyl sulfate, 500µM hippuric acid, 40µM CMPF, 2µM methyl guanidate, 2mM creatinine, 80mM urea, 8µM guanidinosuccinic acid, 23µM phenyl sulfate, and 2µM 4-ethyl phenyl sulfate (Vanholder et al., 2003; Duranton et al., 2012). Cells were treated with individual toxins plus rosuvastatin, as well as a cocktail comprised of all the aforementioned toxins in addition to rosuvastatin. All of the toxins and rosuvastatin were dissolved in Hank’s Buffered Salt solution (HBSS).

Cell uptake work was carried out according to the following protocol. The first step was to aspirate media and wash with PBS, the PBS was then removed and uremic toxins listed above added along with 5 µM rosuvastatin for 10 minutes. Subsequent
revisions to the protocol included a 2 hour preincubation with the uremic toxins. Upon completion of the pretreatment, either drug or drug with uremic toxin was added. Rosuvastatin was allowed to incubate for 10 minutes at 37°C, after which the wells were aspirated and ice-cold HBSS was then added to wash and discontinue transport. Cells were then lysed using 0.2N NaOH containing internal standard (D3 Rosuvastatin). Cessation of lysis was achieved by neutralizing the solutions with 1N HCl. Samples were subsequently aliquoted and subjected to sonication to ensure maximal cell lysis. The final step was to perform solid-phase extraction to prepare the samples for quantification by UPLC-MS using the same rosuvastatin quantification conditions as described in section 2.4.3.

2.7.3 Uremic Toxin Incubation and Transcriptional Expression

The expression of CYP2B6 was assessed at the mRNA level after a 24-hour incubation with various uremic toxins including guanidinosuccinic acid, methyl guanidate, CMPF, hippuric acid, urea, creatinine, indole-3-acetic acid, p-cresyl sulfate, indoxyl sulfate, and p-cresol. The incubation of the uremic toxins on the cells was conducted by another student in the lab (Velenosi et al. unpublished). Briefly, the cells were grown until 4 weeks post-confluency using the same medium mentioned previously. The cells were treated with the highest levels of uremic toxins reported in humans: creatinine (2121.6µM), p-cresyl sulfate (186.1µM), CMPF (391.7µM), guanidinosuccinic acid (268.6µM), hippuric acid (2631.3µM), indole-3-acetic acid (51.9µM), indoxyl sulfate (1113.2µM), methylguanidine (24.9µM), urea (76.6µM), and in a cocktail containing all of the toxins at their respective concentrations (Vanholder et al., 2003). The incubation was performed in the previously described maintenance media but excluding fetal bovine serum. An RNA extraction was performed as described above, and then real-time PCR using the CYP2B6 primer listed in table 2.1 with 18s rRNA as the housekeeping gene.

2.8 Statistical Analysis

Statistical analysis was performed using a one-way ANOVA with subsequent use of either Tukey’s or Dunnet’s post-hoc test. Data is displayed mean ± the standard error
of the mean with $p<0.05$ being the threshold for statistical significance. GraphPad Prism version 6.0 software was used for statistical analysis and generation of graphs.
Chapter 3

3 Results

3.1 Drug Transport in a 7-week in vivo CKD Model

3.1.1 Assessment of Kidney Damage

Plasma urea and creatinine were significantly increased in the CKD group compared to control and control+AST-120 \((p<0.0001)\) (Figure 3.1). There was a 5.9 fold increase in urea from CKD animals compared to control and a 6.3 fold increase in creatinine. Urea was significantly higher in CKD compared to CKD+AST-120 \((p<0.05)\), CKD+AST-120 was not significantly greater than control or control+AST-120 however, despite a 3.4 fold increase compared to control. CKD+AST-120 was significantly greater than control \((p<0.01)\) for plasma creatinine and differed from control+AST-120 significantly as well \((p<0.05)\).

3.1.2 Transporter Expression and Function

No significance was seen between groups pertaining to the relative hepatic mRNA expression of Ntcp, P-gp, or Oatp1b2 (Figure 3.2). Bsep (Figure 3.2B) was significantly lower in control+AST-120 compared to control \((p<0.05)\), and significantly higher in CKD+AST-120 compared to control+AST-120 \((p<0.01)\). The relative enteric mRNA expression of P-gp, Bcrp, Mrp2, and CYP3A2 showed no significant differences between groups (Figure 3.3). Hepatic transporter function was determined by calculating the liver to plasma ratio of rosuvastatin in the animals. No differences were observed between groups for the transport of rosuvastatin (Figure 3.4).

There was no difference in the hepatic mRNA expression of Oatp1a4 between groups (Figure 3.5A). CKD animals showed a 25% decrease in expression compared to control that was not observed in CKD+AST-120 however. There was also a significant correlation between increasing serum creatinine and decreasing Oatp1a4 expression \((r^2=0.2095,\) Figure 3.5B).
Figure 3.1 - Plasma urea (A) and creatinine (B) levels in control, CKD, control+AST-120, and CKD+AST-120 wistar rats after a 7 week study. Presented as mean ± SEM, n=10-14 per group, ****p <0.0001, **p<0.01, ††††p<0.0001, †p<0.05. Plasma urea was significantly different between CKD and CKD+AST-120, CKD is also significantly different from control and control+AST-120 following Tukey’s post-hoc test. Creatinine was significantly different when comparing CKD or CKD+AST-120 to control or control+AST-120 using Tukey’s post-hoc test. * is relative to control, † is relative to control+AST-120.
Figure 3.2 - Relative mRNA expression of hepatic transporters in a 7-week CKD model. Panels A through D display control, control+AST-120, CKD and CKD+AST-120 in Wistar rats. Presented as mean ± SEM, n=10-14 per group, *p <0.05, ††p<0.01. β-actin was used as the housekeeping gene for Ntcp (A), Bsep (B), P-gp (C), and Oatp1b2 (D). Control+AST-120 was significantly lower than control for Bsep and CKD+AST-120 was significantly higher than control+AST-120. * is relative to control, † is relative to control+AST-120.
Figure 3.3 - Relative mRNA expression of intestinal P-gp (A), Bcrp (B), Mrp2 (C), and CYP3A2 (D) in control, control+AST-120, CKD and CKD+AST-120 in wistar rats. Presented as mean ± SEM, n=10-14 per group. Villin was used as the housekeeping gene.
**Figure 3.4** - Liver to plasma ratio of rosuvastatin in control, control+AST-120, CKD, and CKD+AST-120 wistar rats. Analyzed by a one-way ANOVA and Tukey’s post-hoc test. Presented as mean ± SEM, n=10-14 per group.
**Figure 3.5** - (A) Relative liver Oatp1a4 mRNA expression in control, control+AST-120, CKD, and CKD+AST-120 in wistar rats. Presented as mean ± SEM, n=10-14 per group. β-actin was used as the housekeeping gene. (B) Linear regression of control and CKD rats displaying the correlation between creatinine and Oatp1a4. Oatp1a4 was significantly correlated with kidney function ($p<0.05$), $r^2 = 0.2095$. 
3.2 Drug Transport in an 8-week *in vivo* Model of CKD

3.2.1 Assessment of Kidney Damage

Serum urea in CKD and CKD+AST-120 groups was significantly higher (*p*<0.01) than both control and control+AST-120 groups (Figure 3.6A). There was a 6.2 fold increase in plasma urea from CKD animals compared to control. Serum creatinine from CKD and CKD+AST-120 was also significantly different (*p*<0.0001) than control and control+AST-120 groups (Figure 3.6B). This includes a 10.8 fold increase in creatinine from CKD animals when compared to control.

3.2.2 Transporter Expression and Function

The mRNA expression of Oatp1a4 and Bcrp were analyzed in hepatic tissue (Figure 3.7A). CKD animals had significantly lower (*p*<0.05) Oatp1a4 expression compared to controls that manifested as a 50% reduction of the transcript. There were no differences between the other groups for Oatp1a4. The relative mRNA expression of hepatic Bcrp was significantly lower (*p*<0.05) in control+AST-120 and CKD+AST-120 compared to both control and CKD groups (Figure 3.7B).

The liver to plasma ratio of rosuvastatin was not significantly different between groups (Figure 3.8). Although there was no significance, a two-fold increase in the liver to plasma ratio of rosuvastatin in CKD compared to control was observed.
Figure 3.6 - Plasma urea (A) and creatinine (B) levels in control, CKD, control+AST-120, and CKD+AST-120 wistar rats after an 8 week study. Presented as mean ± SEM, n=6-8 per group. **p<0.01, ****p<0.0001, ††p<0.01, ††††p<0.0001. Both plasma urea and creatinine were significantly different when comparing CKD or CKD+AST-120 to control or control+AST-120 using Tukey’s post-hoc test. * is relative to control, † is relative to control+AST-120.
Figure 3.7 - Relative mRNA expression of hepatic Oatp1a4 (A) and Bcrp (B) in an 8 week model of adenine induced CKD. Graphs A and B display control, control+AST, CKD and CKD+AST-120 in wistar rats. Presented as mean ± SEM, n=6-8 per group, *p<0.05, †p<0.05. β-actin was used as the housekeeping gene for Oatp1a4 and Bcrp. Oatp1a4 expression in CKD animals was significantly lower than that of control animals. Control+AST-120 and CKD+AST-120 had significantly lower Bcrp expression compared to both control and CKD animals. * is relative to control, † is relative to CKD.
**Figure 3.8** - Liver to plasma ratio of rosvastatin in control, control+AST-120, CKD, and CKD+AST-120 wistar rats. Analyzed by a one-way ANOVA and Tukey’s post-hoc test. Presented as mean ± SEM, n=6-8 per group.
3.3 Drug Metabolism in an 8-week *in vivo* CKD Model

3.3.1 Assessment of Kidney Damage

Serum urea in CKD and CKD+AST-120 groups was significantly different (*p*<0.0001) from both control and control+AST-120 groups (Figure 3.9A). There was an 8.7 fold increase in plasma urea from CKD animals compared to control. Serum creatinine from CKD and CKD+AST-120 was also significantly different (*p*<0.0001) from control and control+AST-120 groups (Figure 3.9B). This includes a 9.1 fold increase in creatinine from CKD animals when compared to control.

3.3.2 Hepatic Oatp Transcriptional Expression

Oatp1a1 mRNA levels were significantly decreased (*p*<0.0001) in CKD relative to control (Figure 3.10A). CKD+AST-120 and control+AST-120 also significantly decreased (*p*<0.001 and *p*<0.05) respectively relative to control expression. The expression of Oatp1a4 was significantly declined (*p*<0.05) by 45% and 43.5% in CKD relative to control and CKD+AST-120 respectively (Figure 3.10B). CKD animals also differed from CKD+AST-120 significantly (*p*<0.01). There was no significance between groups for either of Oatp1a5 or Oatp1b2.

3.3.3 CYP2B Transcriptional Expression

The expression of CYP2B1 showed significant differences (*p*<0.001) between control and both CKD and CKD+AST-120 groups (Figure 3.11A). CKD animals showed an average decrease in expression of 87.4% when compared to control rats. Control+AST-120 mRNA expression was also significantly lower (*p*<0.05) relative to control. CYP2B2 showed a significant decrease (*p*<0.05) in mRNA expression between control+AST-120 and CKD animals (Figure 3.11B). Control+AST-120 and CKD+AST-120 were 70% and 61% lower than control respectively.
Figure 3.9 - Plasma urea (A) and creatinine (B) levels in control, CKD, control+AST-120, and CKD+AST-120 wistar rats after an 8 week study. Presented as mean ± SEM, n=9-12 per group, ****p<0.0001, ††††p<0.0001. Both plasma urea and creatinine were significantly different when comparing CKD or CKD+AST-120 to control or control+AST-120 using Tukey’s post-hoc test. * is relative to control, † is relative to control+AST-120.
Figure 3.10 - Relative mRNA expression of hepatic transporters in an 8-week CKD model. Panels A through D display control, control+AST, CKD and CKD+AST-120 in wistar rats. Presented as mean ± SEM, n=9-12 per group, *p <0.05, ***p<0.001, ****p<0.0001, ††p<0.01. β-actin was used as the housekeeping gene for Oatp1a1 (A), Oatp1a4 (B), Oatp1a5 (C), and Oatp1b2 (D). Oatp1a1 expression was significantly lower in all groups relative to control, determined by Tukey’s post-hoc test. The expression of Oatp1a4 in CKD was significantly lower than all other groups according to Tukey’s post-hoc test. * is relative to control, † is relative to control+AST-120.
**Figure 3.11** - Relative mRNA expression of CYP2B1 (A) and CYP2B2 (B) in an 8-week CKD model. Panels A and B display control, control+AST, CKD and CKD+AST-120 in wistar rats. Presented as mean ± SEM, n=9-12 per group, *p* < 0.05, ***p* < 0.001, †p* < 0.05. β-actin was used as the housekeeping gene for CYP2B1 and CYP2B2. CYP2B1 expression was significantly lower in all other groups relative to control, as determined by Tukey’s post-hoc test. The expression of CYP2B2 in the control+AST-120 group was significantly lower than the CKD rats according to Tukey’s post-hoc test. * is relative to control, † is relative to control+AST-120.
3.3.4 CYP2B Protein Expression and Activity in CKD

No significance differences were observed between groups regarding CYP2B protein expression (Figure 3.12A). The conversion of bupropion to OH-bupropion was significantly lower in CKD and CKD+AST-120 animals compared to control and control+AST-120 (Figure 3.13). Calculation of Michaelis-Menten parameters and subsequent analysis with a one-way ANOVA and Tukey’s Post-hoc test revealed differences with maximal enzyme velocity ($V_{max}$), $K_m$, and intrinsic clearance between groups (Figure 3.14, Table 3.1). CKD and CKD+AST-120 had a significantly lower $V_{max}$ when compared to control and control+AST-120 ($p<0.0001$ and $p<0.01$ respectively). Enzyme affinity was shown to be lower in CKD and CKD+AST-120 when compared to control and control+AST-120 as the $K_m$ was higher ($p<0.0001$). Intrinsic clearance of bupropion was shown to be significantly lower in CKD and CKD+AST-120 when compared to control and control+AST-120 ($p<0.0001$).
Figure 3.12 - A) Relative protein expression of CYP2B from an 8-week model of CKD in wistar rats. Control, control+AST, CKD and CKD+AST-120 groups are presented as mean ± SEM, n=9-12 per group. β-actin was used as the housekeeping gene. (B) Representative blots with LC= Loading Control, C=Control, C+A=Control+AST-120, CKD+A=CKD+AST-120, CKD=CKD.
**Figure 3.13** - Bupropion metabolism to OH-bupropion as a measure of CYP2B activity in rat liver microsomes from control, control+AST-120, CKD, and CKD+AST-120 animals. $V_{\text{max}}$ and $K_m$ were determined using the Michaelis-Menten model. They were further analyzed using column statistics and an ordinary one-way ANOVA with Tukey’s post-hoc test, $n=9-12$ per group and *$P <0.05$. $V_{\text{max}}$ was significantly higher in control and control+AST-120 as compared to CKD and CKD+AST-120 while $K_m$ followed the opposite trend of being lower in control and control+AST-120 as opposed to CKD and CKD+AST-120.
**Figure 3.14** - Michaelis-Menten parameters derived from figure 3.13 and displayed as $V_{\text{max}}$ (A), $K_m$ (B), and intrinsic clearance (C). Control, control+AST, CKD and CKD+AST-120 groups are presented as mean ± SEM, n=9-12 per group, ****$p<0.0001$, ††$p<0.01$, ††††$p<0.0001$. $V_{\text{max}}$ and intrinsic clearance in CKD and CKD+AST-120 animals were significantly lower than control and control+AST-120. The $K_m$ in CKD and CKD+AST-120 rats was significantly greater than control and control+AST-120. * is relative to control, † is relative to control+AST-120.
<table>
<thead>
<tr>
<th>Sample</th>
<th>Model Equation</th>
<th>$K_m$ ($\mu$M)</th>
<th>$V_{max}$ (pmol/min/mg CYP)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>Michaelis-Menten</td>
<td>60.34±2.615</td>
<td>60.68 ±3.713</td>
</tr>
<tr>
<td>Control+AST-120</td>
<td>72.13±8.303</td>
<td>52.23±4.469</td>
<td></td>
</tr>
<tr>
<td>CKD</td>
<td>312.2±30.29</td>
<td>32.66±2.654</td>
<td></td>
</tr>
<tr>
<td>CKD+AST-120</td>
<td>303.2±39.24</td>
<td>35.45±3.546</td>
<td></td>
</tr>
</tbody>
</table>

**Table 3.1** - Michaelis-Menten parameters for $K_m$ and $V_{max}$ pertaining to the data in Figure 13. Values are presented as mean ± SEM.
3.3.5 mRNA Expression of Nuclear Receptors and ChIP for CYP2B

The transcriptional expression of hepatic CAR and RXR were evaluated and showed no differences between groups (Figure 3.15). The binding of RNA polymerase II to the CYP2B1 promoter was not different between control, CKD, or IgG groups (Figure 3.16A). The positive control was RNA polymerase II binding to the CYP3A2 promoter, which showed a significant decrease ($p<0.05$) in CKD compared to control (Figure 3.16B).
Figure 3.15 - Relative mRNA expression of hepatic transcription factors in an 8 week model of CKD. Graphs A and B display control, control+AST, CKD and CKD+AST-120 in wistar rats. Presented as mean ± SEM, n=9-12 per group. β-actin was used as the housekeeping gene for CAR (A) and RXR (B).
**Figure 3.16** - Binding of RNA Polymerase II to hepatic CYP2B1 (A) and CYP3A2 (B) promoters in an 8 week model of CKD. Graphs A and B display control and CKD RNA Polymerase II binding as well as pooled IgG pulldowns from ChIP performed in tissue from male Wistar rats. Presented as mean ± SEM, n=4-6 per group, *p<0.05. All genes were normalized to RNA Pol II binding to GAPDH. RNA Polymerase II binding to the 3A2 promoter in CKD animals was significantly lower than those from control, according to Tukey’s post-hoc test.
3.4 Uremic Toxins as a Mechanism of Down-Regulation \textit{in vitro}

3.4.1 Huh7 Cell Line Validation

Expression of hepatic transporters normalized to 18s rRNA in both human liver samples and Huh7 cells were comparable to previously reported levels by Sivertsson et al in 2010 (Figure 3.17). The expression of OATP1B1 was 63.5% lower in Huh7 cells compared to human livers. The expression of NTCP was 58.5% higher in Huh7 cells compared to human liver controls. The expression of BCRP was 97.2% lower than normal human hepatic levels.

3.4.2 Rosuvastatin Uptake

Co-incubation of Huh7 cells with rosuvastatin and various uremic toxins showed no difference from control in regards to intracellular rosuvastatin concentrations (Figure 3.18). Pre-incubation of the cells with uremic toxins for 2 hours followed by a co-incubation with rosuvastatin and the same toxins resulted in no differences from control except for wells treated with CMPF (Figure 3.19). CMPF was significantly higher than control levels \((p<0.05)\) with a 50% increase in rosuvastatin uptake. The positive control of 100µM rifampin showed a significantly lower \((p<0.01)\) intracellular concentration of rosuvastatin compared to control levels.

3.4.3 CYP2B6 Expression

Uremic toxins did not cause any significant differences in the expression of CYP2B6 mRNA expression using Dunnett’s multiple comparisons test (Figure 3.20).
Figure 3.17 - Huh7 cell line characterization. Expression of mRNA is relative to human liver for each transporter. 18S rRNA was used to control for individual sample RNA expression levels.
**Figure 3.18** - Rosuvastatin uptake in Huh7 cells after co-incubation with various uremic toxins. The cocktail was a combination of all uremic toxins presented. Analyzed using ordinary one-way ANOVA with Dunnett’s Post-hoc test. Presented as mean ± SEM, n=3 per group.
Figure 3.19 - Rosuvastatin uptake in Huh7 cells after pre and co-incubation with various uremic toxins. The cocktail was a combination of all uremic toxins presented except phenyl sulfate and 4-ethyl phenyl sulfate. Analyzed using ordinary one-way ANOVA with Dunnett’s Post-hoc test. Presented as mean ± SEM, n=3 per group, *p<0.05, **p<0.01.
Figure 3.20 - CYP2B6 mRNA expression in Huh7 cells after a 24 hour incubation with various uremic toxins. Presented as mean ± SEM, n=3 per group. DMSO served as the vehicle control. The cocktail included all the uremic toxins presented in the figure at their respective concentrations.
Chapter 4

4 Discussion

There has been a recent emergence of studies evaluating the effect of chronic kidney disease on drug transport and metabolism. This stems from the increasing prevalence of the disease and the numerous comorbidities that follow its onset. The glaring repercussion of multiple severe comorbidities is that there will be a need for prescriptions of a multitude of drugs in order to combat the extensive pathogenic symptoms. This is typical for CKD patients, and this alone can cause drug-drug interactions and adverse drug reactions. To make matters worse it is well understood and obvious that the renal excretory pathway is compromised in CKD, meaning altered drug pharmacokinetics will be abundant and need to be accounted for. This has been included in a draft to be implemented into the Food and Drug Administration’s guideline for drug development (FDA 2010). What has piqued the interest of investigators recently is the impairment of hepatic and enteric drug transport and metabolism. This will clearly have a large effect on drug kinetics as well since the majority of currently marketed drugs are metabolized in the liver and enteric uptake is necessary for most orally administered drugs to exert an effect.

The current putative mechanism for the altered function and expression of hepatic and enteric metabolism and transport revolves around the uremic condition that is secondary to the loss of renal function (Nolin, 2008; Velenosi et al., 2012; Naud et al., 2012). Studies have shown how inflammatory reactions and cytokines are capable of affecting CYP expression and function, however it is believed that the accumulated metabolites and molecules in uremia play a larger role in CKD patients (Abdel-Razzak et al., 1993; Nolin et al., 2008). In this project we have removed gut-derived uremic toxins in order to elucidate whether they play an integral role in hepatic transport and metabolic irregularities. The oral adsorbent AST-120 fulfills this objective and is a drug that is prescribed to slow the progression to ESRD in Japan. AST-120 was employed in a rat model of CKD, induced by 0.7% adenine. To further uncover the role of uremic toxins in the alteration of hepatic transport, a study in the human hepatoma cell line was conducted.
with the implementation of incubation periods with individual uremic toxins and a probe for transport activity.

4.1 Conclusions

4.1.1 Drug Transport in a 7-week Model of CKD

Due to the large increases in serum urea and creatinine there is sufficient evidence to say that the wistar rats indeed had CKD induced by adenine using the 7-week protocol. Contrary to our hypothesis however we did not notice changes in the transcriptional expression of hepatic transporters, including Ntcp, P-gp, and Oatp1b2. The assessment of the transcriptional expression of these genes in CKD is novel in the literature. The translational expression and activity of P-gp has been evaluated previously and has been showed to be up-regulated in CKD (Naud et al., 2008). Our results showed no significant difference in transcriptional expression between CKD and control for P-gp, although there was a 6.4 fold increase in CKD, which aligns with the aforementioned study. The CKD+AST-120 group was 36% lower than CKD, possibly implying a partial role of uremic metabolites in the upregulation of P-gp and could warrant further investigation.

The mRNA expression of enteric transporters P-gp, Bcrp, and Mrp2 were unchanged from control in our study. A previous study has shown enteric P-gp and Mrp2 to have diminished activity and decreased translational expression in CKD (Naud et al., 2007). This discontinuity may suggest that only translation and downstream propagation are affected or it may be due to differences in the model used as the previous study employed a 5/6 nephrectomy to induce CKD.

Prior to starting formal experiments, I performed initial work on hepatic Oatp1a4 in tissue obtained from another student in our lab. This demonstrated a very promising result as we saw a significant decrease in Oatp1a4 mRNA expression in CKD animals with a subsequent recovery in those treated with AST-120 (Figure 3.10). This was found in rats that were subject to the 8-week protocol, however, the level of CKD in this model led to the need for premature euthanasia for multiple animals and therefore loss of power for the study. We devised the 7-week model due to the irreversible kidney damage incurred by week 4 on the 0.7% adenine diet, and with the expectation that it would cause
a similar level of kidney damage while improving mortality rates (Tamagaki et al., 2006; Hewitson et al., 2008). Hepatic Oatp1a4 showed no difference in transcriptional expression and the transport of rosuvastatin was unimpaired in the 7-week model. Due to its correlation with decreased kidney function and reports on decreased translational expression, we deemed it worthwhile to further pursue this in a more severe model of CKD (Naud et al., 2008). Based on the results the previous 8-week protocol provided, we decided that a more severe level of CKD would help to test our hypothesis.

4.2 Drug Transport in an 8-week model

Again the plasma markers of CKD indicated that the model was sufficient to induce severe kidney damage. The transcriptional expression of Oatp1a4 was decreased by 50% and showed a significant difference from control animals. Interestingly the animals from the CKD+AST-120 groups showed no significant decrease compared to control. This suggests the Oatp1a4 down-regulation may be mediated by uremic toxins that are removed by AST-120. Although both 8-week animal studies showed no difference between control and CKD-AST, one did display a 46.5% decrease in expression. Further studies in this area may be necessary for a definitive result as to the role of uremic toxins on the impairment of Oatp1a4 transcription.

The transport of rosuvastatin was not significantly different between groups for the 7-week or 8-week model of CKD. This evidence is not conclusive to state that the activity of Oatp1a4 is not decreased in CKD however. Although Oatp1a4 has a high affinity for rosuvastatin, Oatp1a1, Oatp1a5, and Oatp1b2 all have affinity for it as well (Ho et al., 2006). Due to the empirical evidence that the Oatp1a4 transcript and protein expression are decreased there is reason to believe that the activity is affected too, however, it is not seen with rosuvastatin as a probe because there may be compensation by the unaffected Oatps. Oatp1a1 was also significantly decreased in CKD, but we speculate that Oatp1a5 and Oatp1b2 are compensating for the transport due to their lack of significance between groups. Oatp1a5 was expressed at 43% relative to control despite a lack of significance, but Oatp1b2 was expressed at 130% of control and may be the main compensatory transporter. Oatp1b2 is also considered a rat orthologue of human OATP1B1 and OATP1B3 (Cattori et al., 2000; Smith et al., 2005; Knauer et al., 2010). The liver to
plasma concentration of rosuvastatin showed a twofold increase between CKD and control suggesting that the uptake is not being impeded, but it may in fact be the efflux of rosuvastatin that is altered. We subsequently analyzed the main rat efflux transporter for rosuvastatin, Bcrp. The mRNA expression patterns for Bcrp did not explain the increases in the liver to plasma ratio as it was only decreased in the groups that received AST-120. This is an interesting finding and may suggest that there is a low molecular weight (100-10000Da) mediator for Bcrp expression in the gut that is being removed by AST-120. It is most likely present in both control and the pathological CKD condition as the decrease in expression is seen in both control+AST-120 and CKD+AST-120 treatments. This warrants further exploration.

4.3 Altered Expression and Function of CYP2B

We observed a significant decrease in the transcriptional expression of CYP2B1 in both CKD and CKD+AST-120 compared to control. Rats from the control+AST-120 group also significantly differed from control in regards to CYP2B1 expression. This pattern is similar to that of the expression of Bcrp, where AST-120 administration appears to be linked to decreased transcriptional expression. CKD and CKD+AST-120 animals were 30% and 24% lower than control+AST-120 respectively, suggesting that the pathologic state of CKD plays a role in CYP2B1 inhibition. It is unlikely that gut-derived uremic toxins are the perpetrator in this instance as there is only a 5.8% recovery in the CKD+AST-120 group when compared to CKD. Other potential mechanisms could be from the increased levels of middle molecules such as the parathyroid hormone, which circulates at higher levels in CKD and as a previous study has shown, it plays a role in the downregulation of CYP1A1, CYP2C11, and CYP3A2 (Michaud et al., 2006). Cytokines are also middle molecules that have been shown to play a role in the altered expression of CYP enzymes as they have increased abundance in CKD (Amdur et al., 2016). The adenine-induced model of CKD also shows increases in inflammatory cytokines (Ali et al., 2016). Increased cytokine production increases the activity of inducible nitric oxide synthase, which in turn increases nitric oxide output, this has been shown to downregulate CYP expression and function (Abdel-Razzak et al., 1993; Aitken et al., 2006; Morgan et al., 2008). A study from 1996 describes the role of nitric oxide in
the down regulation of CYP2B1 and CYP2B2 in rat primary hepatocyte cultures (Carlson and Billings, 1996). Treatment with TNFα, IL-1β, and IFN-γ (all increased in CKD) caused high levels of nitric oxide self-generation in the culture and subsequent loss of total CYP expression with the most affected isoenzymes being CYP2B1 and CYP2B2 (Carlson and Billings, 1996). Nitric oxide synthase inhibitors attenuated this downregulation, suggesting nitric oxide may be responsible (Carlson and Billings, 1996).

CKD patients typically do not have high systemic nitric oxide levels however, nitric oxide synthase is impaired by asymmetric dimethylarginine and potentially indoxyl sulfate, two abundant uremic toxins (Figure 4.1) (Baylis, 2008). Treatment with AST-120 has been shown to increase nitric oxide levels through the removal of indoxyl sulfate (Niwa, 2013). Inflammatory factors increase the hepatic production of nitric oxide through inducible nitric oxide synthase, therefore causing potentially inhibitory levels of nitric oxide with persistent inflammation (Aitken et al., 2006; Niwa, 2013). TNFα and IL-1β may play independent roles in the decrease in CYP2B1 transcription as well, as they are upregulated in CKD as well as in the adenine induced model of CKD (Morgan et al., 2002; Aitken et al., 2006; Ali et al., 2016).

The translational expression of CYP2B was unaffected between groups. This is likely due to the lack of specificity of the antibody and the inherent difficulty of separating proteins with 97% sequence homology using antibody based techniques (Pekthong et al., 2012). We believe the result shows no difference because the expression of CYP2B2 in CKD is unchanged and may be masking the decrease in CYP2B1. To further investigate this we looked at the activity of CYP2B1 using bupropion metabolism as a selective marker for enzyme function (Pekthong et al., 2012). CYP2B1 is considered more catalytically active and we have shown decreased mRNA expression in CKD so we decided to focus on it (Pekthong et al., 2012). The conversion of BUP to OH-BUP showed differences between control and control+AST-120 when compared to CKD and CKD+AST-120. The decreased V_{max} and intrinsic clearance suggests that CYP2B1 activity is impaired. The pronounced increase observed in K_{m} suggests that there are other CYP enzymes playing a part in OH-BUP production. The residual metabolic function and the respective K_{m} is indicative of the affinity of CYP2E1 for BUP. CYP2B1 is responsible for 75% of OH-
Figure 4.1 - Proposed mechanism for the decreased mRNA expression and function of CYP2B1 in CKD.
BUP formation and CYP2C11 accounts for 8.7% as noted by Pekthong and colleagues in 2012. In the present study we have shown that CYP2B1 expression is decreased and likely its function too. CYP2C11 has been shown to have a significant loss of function in CKD as well (Leblond et al., 2000; Velenosi et al., 2012). CYP2E1 normally accounts for 10.9% of BUP metabolism and is likely responsible for the residual creation of OH-BUP seen in the CKD and CKD+AST-120 groups (Pekthong et al., 2012). The mRNA and protein expression of CYP2E1 are not affected in primary rat hepatocytes incubated with serum from uremic patients for 24 hours which likely means its activity is preserved in CKD (Michaud et al., 2005).

The impairment of CYP2B1 activity in CKD is probably not due to gut-derived uremic toxins as no recovery was observed when animals were treated with AST-120. A putative mechanism may be inhibition due to reactive oxygen species, high levels of pro-inflammatory markers, and subsequent nitric oxide production through inducible nitric oxide synthase. A study has shown peroxynitrite is able to cause nitration of tyrosine residues in CYP2B1, conferring a loss of function of the protein (Roberts et al., 1998). Peroxynitrite is high in CKD conditions due to excessive superoxide levels reacting with nitric oxide and subsequent production of peroxynitrate (Zalba et al., 2006).

The next step in the assessment of the down regulation of CYP2B1 in CKD was to evaluate the nuclear regulation. The mRNA expression of CAR and RXR showed no difference between groups so we moved to ChIP in an attempt to examine whether CAR or RNA Pol II binding were altered in the PBREM promoter region. We again saw no difference between groups however a concern over the high IgG background signal of the experiment was unable to be solved, leaving our data for this experiment inconclusive. The positive control of RNA Pol II binding to the CYP3A2 promoter showed the same results as previously reported in the literature, decreased binding in CKD (Velenosi et al., 2014).
4.4 Role of Uremic Toxins on Rosuvastatin Transport in vitro

Validation of the Huh7 cell line consisted of a comparison to human liver mRNA expression levels. Sufficient OATP1B1 and NTCP mRNA expression was displayed for it to be a valid system for rosuvastatin uptake. The main efflux transporter for rosuvastatin in humans was not highly expressed in this cell line, suggesting that most of the rosuvastatin taken up by the cells will remain internalized, allowing for more accurate assessment of uptake.

No differences were seen between groups for the uptake of rosuvastatin in the Huh7 cell line when incubated with various uremic toxins. This study shows that uremic toxins are unlikely to cause an alteration in rosuvastatin pharmacokinetics. Neither the specific individual toxins or the cocktail of toxins showed a difference with a co-incubation or a preincubation with the toxins. There is the possibility that other uremic toxins that were not tested may disrupt the activity of OATP1B1 and NTCP but we have been unable to show this with the ones we selected. The concentrations for the uremic toxins were the highest reported amount recorded from patients in the literature (Vanholder et al., 2003). Higher concentrations would compromise the physiological relevancy of the experiment and have also resulted in substantial levels of cell death.

The transcriptional expression of CYP2B6 was unchanged when Huh7 cells were treated with various uremic toxins for 24 hours. This is the human orthologue of CYP2B1 and CYP2B2 so based on the AST-120 data from the in vivo portion of the study it would be reasonable to conclude that the gut-derived uremic toxins are not the direct cause for the decreased activity and expression seen in the rats. A study from 2014 strengthens this theory as they noticed no difference in CYP2B6 activity when human liver microsomes were incubated with serum from CKD patients pre- and post-hemodialysis (Volpe et al., 2014). Conclusions from this could also be that the uremic toxins are not involved in direct inhibition but may play a regulatory role on CYP2B6 expression as it has been shown to have a loss of activity in CKD (Turpeinen et al., 2007). This study shows a 66% decrease in the conversion of BUP to OH-BUP in renally impaired patients compared to control (Turpeinen et al., 2007). The conversion to OH-BUP is a selective marker for
CYP2B6 function in humans (Faucette et al., 2000). CYP2B6 is also renally expressed so if its hepatic expression is not affected this study’s findings may also be a result of impaired renal CYP2B6 (Gervot et al., 1999).

4.5 Limitations

We were not able to use absolute quantification of rosuvastatin in the assessment of transport function as there were inconsistencies due to human error involving the injection of the drug. The liver to plasma ratio controls for this but a direct quantification and comparison between matrices would have been more appropriate.

Uremic toxins other than urea and creatinine were not quantified in this study. The increase of gut-derived uremic toxins in CKD and the ability of AST-120 to remove them are well described in the literature (Niwa et al., 1991; Yamamoto et al., 2015; Velenosi et al., 2016). We believed that the reproducibility of these processes would subvert the need to quantify them for the purposes of this project.

An attempt was made to use fexofenadine as a transporter function probe for the CKD in vivo rat model as there have been reports on its clearance decreasing in CKD (Nolin et al., 2009; Joy et al., 2014; Thomson et al., 2015). We were unable to achieve adequate fexofenadine solubility for the purpose of IV injection in our initial trials. This portion of the project was discontinued to focus on rosuvastatin transport and CYP2B.

Attempts to assess the translational expression of Oatp1a4 were unsuccessful despite repeated attempts at various antibody dilutions. The western blots for CYP2B produced an unexpected result based on mRNA and activity data. It is most likely because of the high sequence homology between CYP2B1 and 2B2 combined with a poor specificity antibody (the only one available). There are two bands in some of the lanes when probing for 2B1 and 2B2 and this is shown on the antibody product sheet as well. We were unable to separate these bands using different voltages and run-times for the gels. We were also unable to elucidate which band belongs to which protein as supersomes for CYP2B1 and CYP2B2 are not available.
The difficulties with ChIP are obvious when looking at our data (Figure 3.16). Due to the high sequence homology of CYP2B1 and CYP2B2 (98-100% in the promoter region), it was difficult to develop CYP2B1 specific primers. With the limited options available, the primer specificity was not optimal. The CAR binding site primer was well designed and validated however no signal was observed in any of the replicates of the experiment. This may be because of the lack of phenobarbital induction results in no binding of CAR to the PBREM. We were unable to acquire phenobarbital induced tissue in order to test this theory. A study from 1998 showed that there was constitutive activation of CAR in the absence of PB in transformed primary mouse hepatocytes (Honkakoski et al., 1998). The same group also points out how this is unusual and may result from incomplete signaling pathways or absent inhibitors in the transformed cell line (Honkakoski et al., 1998).

4.6 Future Studies

An interesting finding with the administration of the endogenous flavonoid chrysin, showed reduced TNFα in adenine treated rats (Ali et al., 2016). Increased TNFα is a potential factor in the increased peroxynitrite seen in CKD as inducible nitric oxide synthase can be activated during an inflammatory response. Peroxynitrite has been shown to affect the activity of CYP2B1 (Roberts et al., 1998). Administration of chrysin may be able to alleviate the downregulation of CYP2B1 in CKD conditions through the amelioration of pathologic nitric oxide species.

A lot of focus is put on the regulation of CYP enzymes in CKD, a slightly overlooked area is the effect of the pathologic state on cytochrome P450 oxidoreductase (POR). POR facilitates the transfer of electrons from NADPH to CYP enzymes, providing the oxidizing power necessary for metabolism (Jin et al., 2015). Without proper POR function the CYP enzymes will be unable to function as efficiently, leading to altered metabolism and drug pharmacokinetics. Analysis of POR expression and function in CKD could reveal a mechanism for the loss of CYP function.

Human CYP2B6 has been shown to retain its activity in microsomal metabolism assays when incubated with pre and post-dialysis uremic serum (Volpe et al., 2014). Uremic serum alone may not encapsulate the inhibitory markers that are responsible for the
observed downregulation seen for CYP2B1. Analysis of hepatic CYP2B6 expression and function in livers from CKD patients may reveal a result more similar to what we have observed in vivo for CYP2B1.

If hepatic CYP2B6 shows no difference in expression or function in CKD patients another avenue to explore would be renal CYP2B6. Bupropion clearance has been shown to be decreased in CKD and CYP2B6 is the metabolic enzyme with the highest reported affinity for it (Faucette et al., 2000; Turpeinen et al., 2007). To understand how BUP pharmacokinetics are altered a logical next step would be to analyze renal CYP2B6. Observed changes here could explain the work by Turpeinen et al from 2007. To confirm the conclusion that the residual metabolism of bupropion in the rat liver microsomes is due to the catalytic activity of hepatic CYP2E1, a metabolism assay using supersomes could be completed. Impairment of individual isozymes or the use of Eadie-Hofstee analysis could also elucidate which CYP enzyme is responsible for the residual conversion of BUP to OH-BUP in CKD conditions.

The gut microbiome plays an integral role in the propagation of CKD through the production of uremic toxins. The administration of AST-120 removes toxins from the GI tract to help reduce the effects of the uremic syndrome. It would be interesting to know if there is a direct effect of AST-120 on the inhabitants of the gut, if there is any change in the enteric milieu for patients taking AST-120 compared to CKD patients who are not.

The expression of CYP2B1 and CYP2B2 is inducible via PB, this is due to the promoter region, PBREM (Czekaj, 2000). It is unknown if CAR binding to PBREM is constitutive in the absence of PB. A study to elucidate the binding state of CAR to PBREM in control and CKD as well as control+PB and CKD+PB would allow conclusions to be drawn about the endogenous interaction of CAR and the PBREM and the regulation of CYP2B1 and 2B2.

4.7 Overall Conclusions

There is an abundance of evidence demonstrating the pharmacokinetics of transported drugs are altered in CKD patients. Our animal model did not accurately reproduce
previous results as we did not observe any differences in rosuvastatin disposition in this study. Future studies are needed in this area but they should pursue alternative methods to test this. This is the first report to my knowledge of altered CYP2B1 expression and function in CKD. The mechanism of downregulation requires additional investigation as our initially proposed pathway did not appear to play a role. Work on hepatic CYP2B6 from CKD patients should also be completed as I would anticipate it follows a similar trend to CYP2B1 in regards to function and expression in CKD. Further knowledge of the activity of CYP2B6 in CKD would allow for more informed drug dosing to patients in the disease state.
References


CRESTOR (rosuvastatin calcium, tablets) Product Monograph. AstraZeneca Canada Inc. Date of Revision: November 14, 2012 (Control #158206).


FDA. Food and drug administration (2010) Pharmacokinetics in Patients with Impaired Renal Function — Study Design, Data Analysis, and Impact on Dosing and Labeling

Feere DA, Velenosi TJ, and Urquhart BL (2015) Effect of erythropoietin on hepatic


Hesse LM, Venkatakrishnan K, Court MH, Moltke von LL, Duan SX, Shader RI, and Greenblatt DJ (2000) CYP2B6 mediates the in vitro hydroxylation of bupropion:
Potential drug interactions with other antidepressants. *Drug Metabolism and Disposition* **28:**1176–1183, American Society for Pharmacology and Experimental Therapeutics.


Pekthong D, Desbans C, Martin H, and Richert L (2012) Bupropion hydroxylation as a


Tsujimoto T, Sairenchi T, Iso H, Irie F, Yamagishi K, Watanabe H, Tanaka K, Muto T,


Appendix A – Animal Ethics Approval

September 10, 2009

*This is the Original Approval for this protocol*
*A Full Protocol submission will be required in 2013*

Dear Dr. Urquhart:

Your Animal Use Protocol form entitled:
The Effect of Kidney Failure and Kidney Transplantation on the Expression and Activity of Drug Metabolizing Enzymes and Drug Transport Proteins
Funding Agency: UWO Start-up/NSERC Applied For

has been approved by the University Council on Animal Care. This approval is valid from September 10, 2009 to September 30, 2010. The protocol number for this project is #2009-058.

1. This number must be indicated when ordering animals for this project.
2. Animals for other projects may not be ordered under this number.
3. If no number appears please contact this office when grant approval is received.
   If the application for funding is not successful and you wish to proceed with the project, request that an internal scientific peer review be performed by the Animal Use Subcommittee office.
4. Purchases of animals other than through this system must be cleared through the ACVS office. Health certificates will be required.

<table>
<thead>
<tr>
<th>ANIMALS APPROVED FOR 4 YEARS</th>
</tr>
</thead>
<tbody>
<tr>
<td>SPECIES &amp; SECT.D.5.1 GROUP ID#</td>
</tr>
<tr>
<td>Rat ID# 1-36</td>
</tr>
<tr>
<td>An Above ID# 37</td>
</tr>
</tbody>
</table>

REQUIREMENTS/COMMENTS
Please ensure that individual(s) performing procedures on live animals, as described in this protocol, are familiar with the contents of this document.

c.c. Approved Protocol Approval Letter - B. Urquhart, W. Lagerwerf

The University of Western Ontario
Animal Use Subcommittee / University Council on Animal Care
Health Sciences Centre, London, Ontario N6A 5C1
PH: 519-661-2111 ext. 86770 FL 519-661-2028 www.uwo.ca/animal
Curriculum Vitae

Andrew Stanton Kucey

Post-secondary Education and Degrees:
Western University
London, Ontario, Canada
2010-2014 Bachelor of Science, Honours Double Major in Biology and Medical Sciences

Western University
London, Ontario, Canada
2014-2016 MSc. Physiology and Pharmacology

Honours and Awards:
Western Graduate Research Scholarship
2014-2016

Related Work Experience
Teaching Assistant – Western University, London, ON
Department of Physiology and Pharmacology
2014-2016: Pharmacology 4660A – Human Toxicology
2016: Pharmacology 2060A – Introduction to Pharmacology and Therapeutics

Manuscripts:

Natasha Gill MD, Natalie Sirizzotti BSc, David Johnson MD, Andrew S Kucey BSc, Alvin Tieu BMSc, Brad L Urquhart PhD, Melanie Columbus PhD, Rodrick Lim MD, Michael Rieder MD PhD, Shruti Mehrotra MD, Naveen Poonai MSc MD. Endogenous glucocorticoid response to single dose dexamethasone for croup in children: a pharmacodynamic study. In submission to Pediatric Emergency Care, February 17, 2016.

Presentations:


cause adrenal suppression?: A prospective study. Pediatric Academic Societies in Baltimore, Maryland, USA, April 30-May 3, 2016. (Poster)

