

**UCSF**

**UC San Francisco Electronic Theses and Dissertations**

**Title**

Effects of Chronic Kidney Disease on Drug Disposition: Transport, Metabolism, and Pharmacokinetics of Different Biopharmaceutics Drug Disposition Classification System (BDDCS) Drugs

**Permalink**

<https://escholarship.org/uc/item/4df87254>

**Author**

Reyes, Maribel

**Publication Date**

2011

Peer reviewed|Thesis/dissertation

Effects of Chronic Kidney Disease on Drug Disposition: Transport, Metabolism and  
Pharmacokinetics of Different Biopharmaceutics Drug Disposition Classification System  
(BDDCS) Drugs

by

MARIBEL REYES

DISSERTATION

Submitted in partial satisfaction of the requirements for the degree of

DOCTOR OF PHILOSOPHY

in

Pharmaceutical Sciences and Pharmacogenomics

in the

GRADUATE DIVISION

of the

UNIVERSITY OF CALIFORNIA, SAN FRANCISCO



This dissertation is dedicated to my family

—

Esta tesis la dedico a mi familia

## ACKNOWLEDGMENTS

I would like to express my infinite gratitude to my mentor, advisor, and pharmacokinetics *guru* Leslie Z. Benet. Thanks for always giving me complete freedom to explore different areas of my research and nurturing my development as a scientist. Thanks for always having a great sense of humor when the research did not work out as we had hoped, but always finding a positive side to the situation. Thanks for those times of criticism, which were not comfortable one bit, but I know it is part of the development as a scientist. Best of all is the genuine interest Les has for the well being of his past and present graduate students. On that note, I must give thank to Les for not forgetting that graduate students also have a life outside of the laboratory, and thanks to that I was able to take some trips around the world, that made graduate school a lot more pleasant. Being in Les' laboratory is definitely an experience that has changed my life, and from which I have learned so much, both in the professional level and personal level. I will forever be grateful to Les for all I have learned from him and for his support throughout these years. Thank you.

Next I would like to thank my family and friends. Thanks to my parents Maria and Pedro for always being loving and supportive of their stubborn child, me. Thank you for all the sacrifices you have made for my brothers, sister, and me in order to give us the opportunity for a better life. I think it's paying off! Thanks to my Mom for words of wisdom and being there when I was going through some rough times during graduate school. I would like to thank my parents for always supporting my crazy plans of traveling to distant lands, and encouraging me to take advantage of opportunities that

came my way. Thanks to my Dad for his support throughout graduate school and for showing me how to enjoy the simple things in life. Thanks to my brother Hugo, with whom I had a wonderful time living with during some of my graduate school years. Thanks to Hugo for cooking excellent dinners and making the Armour days and Olive days feel like a real home. Thanks to my brother Raul and Veronica for always helping me in whatever I needed and being supportive all the time. I would also like to thank Miguel, who has become part of the family and from whom I've learned good sports and environmental habits. I know I can always count on my family for anything and everything. I love you all and thank you deeply for everything you share with me.

*Quiero agradecer a mis padres María y Pedro por todo el amor, cariño, y apoyo que siempre me han dado. Desde que nací creo que sabían que sería la más terca de la familia, pero a pesar de todo siempre me han apoyado en todo lo que hago. Les agradezco todos los sacrificios que han hecho a para que mis hermanos y yo tengamos oportunidades de una mejor vida. Creo que los sacrificios han dado resultados! Quiero agradecer a mi mamá por siempre apoyarme cuando las cosas no fueron tan fáciles mientras estave en el doctorado, y por darme palabras de aliento. También quiero agradecer a mis padres por siempre apoyarme cuando tenía ideas locas de viajar a lugares distantes, y gracias por apoyarmarme y aconsejarme a tomar las oportunidades que se me presentaran. Quiero agradecer a mi papá por siempre apoyarme en todo y por enseñarme a disfrutar las cosas sencillas de la vida, como los vegetales cosechados en el jardín de la casa. También quiero agradecer a mi hermano Hugo por cocinar cuando vivimos juntos durante mis años del doctorado. Gracias a el, vivir en Armour y en Olive se sintió como un hogar y no solo una casa. Quiero agradecer a mi hermano*

*Raúl y mi hermana Verónica por siempre apoyarme y estar ahí cuando los necesito. Por último, quiero agradecer a Miguel, quien ha llegado a ser parte de la familia, y de quien he aprendido buenos hábitos deportivos y ambientalistas. Les agradezco a todos y cada uno profundamente por todo lo que comparten conmigo. Los amo a todos.*

I would like to thank all of my friends in graduate school and outside of graduate school, in no particular order. Thanks to Sarah and Kim for your friendship, advice, and the fun times we had throughout these years. Thanks to my friends from all over the world for teaching me about your culture and life. My friends in Chile: Marcelo, who I know I can count on despite everything; Jenny and Ximena, who never forget our friendship despite the distance; Lio and Lalo, my great friends and who always make me laugh and listen to my complains. My Mexican friend Paty whom I met in the U building of UCSF, we had a blast in San Francisco, and became lifelong friends. My distinguished Chilean engineers, David and Diego, who made the weekend outings fun and with whom I would laugh until I cried. My Egyptian friends, Dalia and Mahmoud, opposite worlds in the same country, but both gave me great insight into the wonderful Egyptian culture and life. My friend Diana from Ukraine and Mari from Barcelona, it was a pleasure meeting you last year and thank you for inviting me into your homes when I visited Europe. My friend Marcel with whom I've shared memorable experiences and I hope we share more. Thanks to my friends from the belly dance community, for sharing your enthusiasm for the art. Thanks to my friend and teacher Amina, Marcela, and fellow dancers for your friendship.

Last but not least, I would like to thank my colleagues. Thanks to Hideaki for his help with the IPRL studies, and for the great scientific conversations. Thanks to Anita,

Howard, Wei, Caroline, Alan, Emilio, Frances, and all other Benet Lab members who have made it a great work environment and even fun. I would like to thank my thesis committee members, Kathy Giacomini and Tony McDonagh for their advice and input into my thesis work. I would also like to thank Lynda Frassetto for her work and dedication in the clinical study.

Thanks to everyone who in one way or another has been part of my life during the past 5 years of graduate school. Everyone has enriched my experiences in different ways and has added a special touch to my life. Graduate school would have not been the same without you.



## ABSTRACT

### **Effects of Chronic Kidney Disease on Drug Disposition: Transport, Metabolism, and Pharmacokinetics of Different Biopharmaceutics Drug Disposition Classification System (BDDCS) Drugs**

**Maribel Reyes**

Pharmacokinetic changes of non-renally excreted drugs have been observed in chronic kidney disease (CKD) patients. Typically, dose adjustment is implemented in CKD patients for drugs that are renally excreted, but not for non-renally excreted drugs. I hypothesized that uremic toxins in CKD patients alter the transport of drugs and consequently alter drug disposition. Since BDDCS categorize drugs based on drug transporter involvement for drug disposition, I also hypothesized that BDDCS can be used as a tool to predict which drugs (Class 2, 3, and 4 but not Class 1) would have changes in drug disposition in CKD patients.

Uremic toxins in CKD may alter drug metabolism; however, uremic toxins have not been investigated for their effect on drug transporters. In this thesis I investigated if uremic toxins affect drug transport in transfected cells (transfected with human hepatic transporters), and rat and human hepatocytes. I observed no change in drug transport for the Class 1 drug propranolol, but reduction in transport for Class 2 losartan and Class 4 eprosartan in transfected cells. On the other hand, in rat and human hepatocytes, changes were only observed for losartan. There was no change in metabolism in rat or human microsomes for propranolol or losartan in the presence of hemodialysis (HD) serum.

Studies have shown that hepatic transporters play an important role in drug disposition, but changes in hepatic drug transporters in CKD have not been explored. I used a rat model chronic kidney disease and investigated the hepatic drug disposition of propranolol, losartan, and eprosartan in the isolated perfused rat liver system by perfusing the liver with HD serum or normal serum. The results from these studies were inconclusive, the variability was high and the number of animals used was small.

A human clinical study was carried out in CKD patients and healthy volunteers. I investigated the pharmacokinetic changes of propranolol, losartan, and erythromycin. As predicted there were no changes in propranolol PK, but there were significant changes for losartan metabolite and erythromycin. This showed that BDDCS can be a useful tool for predicting changes in drug disposition in CKD patients.

**TABLE OF CONTENTS**

**CHAPTER 1: CHRONIC KIDNEY DISEASE, TRANSPORTERS, AND METABOLIZING ENZYMES: CONSEQUENCES ON DRUG DISPOSITION .... 1**

1.1 INTRODUCTION ..... 2

1.2 KIDNEYS, LIVER, AND IMPORTANCE IN DRUG DISPOSITION ..... 3

    1.2.1 The Kidneys..... 3

    1.2.2 The Liver ..... 6

1.3 CHRONIC KIDNEY DISEASE..... 8

    1.3.1 Chronic Kidney Disease Statistics ..... 8

    1.3.2 Definition of Chronic Kidney Disease..... 9

1.4 DRUG DISPOSITION IN CHRONIC KIDNEY DISEASE..... 10

1.5 UREMIC TOXINS ..... 13

1.6 CYTOCHROMES P450 METABOLIZING ENZYMES ..... 14

1.7 DRUG TRANSPORTERS ..... 15

    1.7.1 Drug Transporter Localization and Structure ..... 15

    1.7.2 Transporters and Drug-Drug Interactions..... 17

1.8 BIOPHARMACEUTICS DRUG DISPOSITION CLASSIFICATION SYSTEM..... 19

1.9 RESEARCH PROJECT ..... 23

    1.9.1 Rationale..... 23

    1.9.2 Hypothesis..... 23

    1.9.3 Specific Aims ..... 24

1.10 REFERENCES ..... 25

**CHAPTER 2: EFFECTS UREMIC TOXINS AND HEMODIALYSIS SERUM ON DRUG TRANSPORT AND METABOLISM: IN VITRO STUDIES ..... 30**

2.1	INTRODUCTION .....	31
2.1	MATERIALS AND METHODS .....	35
2.2.1	Chemicals and Reagents .....	35
2.2.2	Hemodialysis Serum .....	36
2.2.3	Cell Transfection and Uptake Assay .....	36
2.2.4	Microsome Incubations .....	37
2.2.5	Hepatocyte Isolation.....	38
2.2.6	Hepatocyte Incubations.....	38
2.2.7	LC/MS-MS Measurement of Propranolol, Losartan, and Eprosartan and their Metabolites.....	40
2.2.8	Data/Statistical Analysis .....	41
2.3	RESULTS.....	41
2.3.1	Effect of Uremic Toxins on Uptake of [ <sup>3</sup> H]-Estrone Sulfate into Transfected Cells and Rat Hepatocytes.....	41
2.3.2	Uptake of BDDCS Drugs by Rat Hepatocytes in the Presence of Uremic Toxins.....	45
2.3.3	Effects of Hemodialysis Serum on Drug Uptake.....	48
2.3.4	Time-course Studies in Rat Hepatocytes and Microsome Studies .....	50
2.4	DISCUSSION.....	56
2.5	CONCLUSION.....	61
2.6	REFERENCES .....	63

**CHAPTER 3: BIDIRECTIONAL STUDIES IN MDR1-MDCK, MDCK, CMOAT-MDCKII, AND MDCKII CELLS: EFFECTS OF UREMIC TOXINS AND HUMAN HEMODIALYSIS SERUM ON BDDCS DRUGS AND MODEL SUBSTRATES ..... 68**

3.1 INTRODUCTION ..... 69

3.2 MATERIALS/METHODS ..... 73

    3.2.1 Cell Culture ..... 73

    3.2.2 Bidirectional Assay ..... 74

    3.2.3 LC/MSMS Assays ..... 75

    3.2.4 Data Analysis ..... 75

3.3 RESULTS ..... 76

    3.3.1 Bidirectional Studies: MDR1-MDCK and MDCK Cells ..... 76

    3.3.2 Bidirectional Studies: cMOAT-MDCKII and MDCKII cells ..... 78

    3.3.3 Propranolol Bidirectional Studies: MDR1-MDCK and MDCK Cells ..... 81

    3.3.4 Losartan Bidirectional Studies: MDR1-MDCK and MDCK Cells ..... 83

3.4 DISCUSSION ..... 86

3.5 CONCLUSION ..... 88

3.6 REFERENCES ..... 90

**CHAPTER 4: HEPATIC DISPOSITION OF BDDCS DRUGS IN CHRONIC KIDNEY DISEASE RATS: ISOLATED PERFUSED RAT LIVER (IPRL) WITH HEMODIALYSIS SERUM AND NORMAL SERUM ..... 92**

4.1 INTRODUCTION ..... 94

4.2 MATERIALS/METHODS ..... 98

    4.2.1 Surgery and Perfusion of Isolated Rat Livers ..... 98

    4.2.2 Western Blot ..... 100

4.3	RESULTS.....	101
4.3.1	Nephrectomized Rats vs. Sham Operated Rats.....	101
4.3.2	Propranolol IPRL.....	102
4.3.3	Losartan IPRL.....	107
4.3.4	Eprosartan IPRL.....	109
4.3.5	Western Blot.....	111
4.4	DISCUSSION.....	114
4.5	CONCLUSION.....	117
4.5	REFERENCES.....	119

**CHAPTER 5: PHARMACOKINETIC STUDIES OF PROPRANOLOL, LOSARTAN, AND ERYTHROMYCIN IN HEALTHY SUBJECTS AND CHRONIC KIDNEY DISEASE PATIENTS: COMPARISON OF THREE BDDCS DRUGS..... 121**

5.1	INTRODUCTION.....	122
5.2	METHODS.....	124
5.2.1	Study Subjects.....	124
5.2.2	Study Procedures.....	124
5.2.3	Analytical Procedures.....	125
5.2.4	Pharmacokinetic Analyses.....	126
5.2.5	Statistical Analyses.....	126
5.3	RESULTS.....	127
5.3.1	Pharmacokinetics of Propranolol in Healthy vs. CKD Subjects.....	128
5.3.2	Pharmacokinetics of Losartan in Healthy vs. CKD Subjects.....	132
5.3.2	Pharmacokinetics of Erythromycin in Healthy vs. CKD Subjects.....	135

5.4	DISCUSSION.....	138
5.5	CONCLUSION.....	142
5.6	REFERENCES .....	144
<b>CHAPTER 6: CONCLUSIONS AND PERSPECTIVES .....</b>		<b>146</b>

## LIST OF TABLES

TABLE 1.1: STAGES OF CHRONIC KIDNEY DISEASE (NATIONAL KIDNEY FOUNDATION, 2002).....	9
TABLE 2.1: PROPRANOLOL AND 4-OH-PROPRANOLOL AUC 0→60 <sub>MIN</sub> FOR TIME-COURSE STUDIES AND /FOLD INCREASE FOR SIGNIFICANT CHANGES VS. NORMAL SERUM IN RAT HEPATOCYTES..	51
TABLE 2.2: LOSARTAN AND EXP3174 AUC 0→60 <sub>MIN</sub> FOR TIME-COURSE STUDIES AND /FOLD INCREASE OR DECREASE FOR SIGNIFICANT CHANGES VS. NORMAL SERUM IN RAT HEPATOCYTES.....	53
TABLE 2.3: EPROSARTAN AUC 0→60 <sub>MIN</sub> FOR TIME-COURSE STUDIES AND /FOLD INCREASE FOR SIGNIFICANT CHANGES VS. NORMAL SERUM IN RAT HEPATOCYTES..	55
TABLE 3.1: BIDIRECTIONAL STUDY OF [ <sup>3</sup> H]-VINBLASTINE IN MDR1-MDCK AND MDCK.....	77
TABLE 3.2: BIDIRECTIONAL STUDY OF [ <sup>3</sup> H]-PRAVASTATIN (PRAV.) IN CMOAT-MDCKII AND MDCKII CELLS.....	79
TABLE 3.3: BIDIRECTIONAL STUDY OF 10 <sub>μ</sub> M PROPRANOLOL IN MDR1-MDCK AND MDCK.....	81
TABLE 3.4: BIDIRECTIONAL STUDY OF 10 <sub>μ</sub> M LOSARTAN IN MDR1-MDCK AND MDCK.....	84
TABLE 4.1: GROUPS FOR IPRL EXPERIMENTS: LIVER TYPE AND PERFUSATE TYPE.....	97
TABLE 4.2: 5/6 NEPHRECTOMY RAT AND SHAM OPERATED RAT BIOCHEMICAL PARAMETERS .....	102



TABLE 4.3: ISOLATED PERFUSED RAT LIVER PROPRANOLOL (1 $\mu$ M) CONCENTRATION IN VARIOUS MATRICES .....	104
TABLE 4.4: P VALUES OF COMPARISONS BETWEEN GROUPS USING T-TEST .....	104
TABLE 4.5: ISOLATED PERFUSED RAT LIVER 4-OH PROPRANOLOL AUCs AND AMOUNTS IN THE LIVER .....	106
TABLE 4.6: COMPARISON OF EXTRACTION FRACTION ( $E_H$ ) AND ESTIMATED INTRINSIC CLEARANCE ( $CL_{INT}$ ) OF L-PROPRANOLOL IN IPRL FROM TERAO AND SHEN (1985) .....	107
TABLE 4.7: ISOLATED PERFUSED RAT LIVER LOSARTAN (1 $\mu$ M) CONCENTRATION IN VARIOUS MATRICES .....	108
TABLE 4.8: P VALUES OF COMPARISONS BETWEEN LOSARTAN GROUPS USING T-TEST .....	109
TABLE 4.9: EPROSARTAN CONCENTRATION IN VARIOUS MATRICES FROM IPRL.....	110
TABLE 4.10: P VALUES OF COMPARISONS BETWEEN EPROSARTAN GROUPS USING T-TEST .....	111
TABLE 5.1: PATIENT DEMOGRAPHIC DATA AND CHEMISTRIES .....	128
TABLE 5.2: PHARMACOKINETIC PARAMETERS OF PROPRANOLOL AND 4-OH PROPRANOLOL .....	131
TABLE 5.3: PHARMACOKINETIC PARAMETERS OF LOSARTAN AND EXP3174 .....	134
TABLE 5.4: PHARMACOKINETIC PARAMETERS OF ERYTHROMYCIN AND N-DEMETHYL ERYTHROMYCIN .....	137

TABLE 5.5: PERCENT OF DOSE EXCRETED IN THE URINE ..... 138

## LIST OF FIGURES

FIGURE 1.1: THE KIDNEY (BRENNER AND RECTOR, 8 <sup>TH</sup> EDITION) .....	4
FIGURE 1.2: ILLUSTRATION OF THE NEPHRON, ITS COMPONENTS, AND IONIC MOVEMENT TO FORM URINE ( <a href="http://faculty.washington.edu.htm">HTTP://FACULTY.WASHINGTON.EDU.HTM</a> , 2003).....	5
FIGURE 1.3: DIAGRAM DEPICTING THE LIVER, BILIARY SYSTEM, STOMACH AND PANCREAS ( <a href="http://www.mainlinehealth.org/stw/images/125543.jpg">HTTP://WWW.MAINLINEHEALTH.ORG/STW/IMAGES/125543.JPG</a> , 2010).....	6
FIGURE 1.4: HEPATOCYTE AND OTHER HEPATIC CELLS (SLEISENGER ET AL., 2006) .....	7
FIGURE 1.5: PHARMACOKINETICS OF EPROSARTAN IN PATIENTS WITH DIFFERENT DEGREES OF RENAL IMPAIRMENT, FIGURE FROM MARTIN ET AL. (1998)...	12
FIGURE 1.6: HEPATOCYTE AND TRANSPORTER LOCALIZATION .....	16
FIGURE 1.7: BCS ACCORDING TO AMIDON ET AL. (1995).....	20
FIGURE 1.8: BIOPHARMACEUTICS DRUG DISPOSITION CLASSIFICATION SYSTEM (BDDCS). (A) INCLUDING EXTENT OF METABOLISM, (B) TRANSPORTER EFFECTS FOLLOWED BY ORAL DOSING. ....	20
FIGURE 1.9: CHEMICAL STRUCTURES OF COMPOUNDS USED .....	22
FIGURE 2.1A: UPTAKE OF [ <sup>3</sup> H]- ESTRONE SULFATE IN OATP1B1 AND EMPTY VECTOR CONTROL TRANSFECTED CELLS IN THE PRESENCE OF UREMIC TOXINS...	42
FIGURE 2.1B: UPTAKE OF [ <sup>3</sup> H]- ESTRONE SULFATE IN OATP1B3 AND EMPTY VECTOR CONTROL TRANSFECTED CELLS IN THE PRESENCE OF UREMIC TOXINS .....	43

FIGURE 2.1C: UPTAKE OF [ <sup>3</sup> H]- ESTRONE SULFATE IN OATP2B1 AND EMPTY VECTOR CONTROL TRANSFECTED CELLS IN THE PRESENCE OF UREMIC TOXINS .....	44
FIGURE 2.2: UPTAKE OF [ <sup>3</sup> H]-ESTRONE SULFATE IN RAT HEPATOCYTES IN THE PRESENCE OF UREMIC TOXINS. ....	45
FIGURE 2.3A: UPTAKE OF PROPRANOLOL 10 $\mu$ M IN RAT HEPATOCYTES IN THE PRESENCE OF UREMIC TOXINS OR UPTAKE TRANSPORTER INHIBITORS. ....	46
FIGURE 2.3B: UPTAKE OF LOSARTAN 10 $\mu$ M IN RAT HEPATOCYTES IN THE PRESENCE OF UREMIC TOXINS OR UPTAKE TRANSPORTER INHIBITORS.....	47
FIGURE 2.3C: UPTAKE OF EPROSARTAN 10 $\mu$ M IN RAT HEPATOCYTES IN THE PRESENCE OF UREMIC TOXINS OR UPTAKE TRANSPORTER INHIBITORS.. ....	47
FIGURE 2.4A: UPTAKE OF PROPRANOLOL 10 $\mu$ M, LOSARTAN 10 $\mu$ M, OR EPROSARTAN 10 $\mu$ M IN RAT HEPATOCYTES IN THE PRESENCE OF NORMAL SERUM, HEMODYALYSIS SERUM, RIFAMPIN 10 $\mu$ M, OR CYP INHIBITOR. ....	49
FIGURE 2.4B: UPTAKE OF PROPRANOLOL 10 $\mu$ M, LOSARTAN 10 $\mu$ M, OR EPROSARTAN 10 $\mu$ M IN HUMAN HEPATOCYTES IN THE PRESENCE OF NORMAL SERUM, HEMODYALYSIS SERUM, RIFAMPIN 10 $\mu$ M, OR CYP INHIBITOR.....	49
FIGURE 2.5A: METABOLISM OF PROPRANOLOL 10 $\mu$ M IN POOLED RAT MICROSOMES IN THE PRESENCE OF NOMAL SERUM, HEMODIALYSIS SERUM, UPTAKE TRANSPORTER INHIBITOR, OR CYP INHIBITOR.....	52
FIGURE 2.5B: METABOLISM OF PROPRANOLOL 10 $\mu$ M IN POOLED HUMAN MICROSOMES IN THE PRESENCE OF NOMAL SERUM, HEMODIALYSIS SERUM, UPTAKE TRANSPORTER INHIBITOR, OR CYP INHIBITOR. ....	52
FIGURE 2.6A: METABOLISM OF LOSARTAN 10 $\mu$ M IN POOLED RAT MICROSOMES IN THE PRESENCE OF NOMAL SERUM, HEMODIALYSIS SERUM, UPTAKE TRANSPORTER INHIBITOR, OR CYP INHIBITOR VERSUS NORMAL SERUM	54

FIGURE 2.6B: METABOLISM OF LOSARTAN 10 $\mu$ M IN POOLED HUMAN MICROSOMES IN THE PRESENCE OF NORMAL SERUM, HEMODIALYSIS SERUM, UPTAKE TRANSPORTER INHIBITOR, OR CYP INHIBITOR VERSUS NORMAL SERUM.	54
FIGURE 3.1: DIAGRAM DEPICTING CELL MONOLAYER CULTURED IN TRANSWELL INSERT AND PLATE. APICAL (A) SIDE AND BASOLATERAL (B) SIDE CONTAIN BUFFER WITH OR WITHOUT DRUG AND INHIBITOR.	70
FIGURE 3.2: SCHEMATIC OF TWO HEPATOCYTES JOINED BY BILE CANALICULAR MEMBRANE. EFFLUX TRANSPORTERS DEPICTED IN THE APICAL CANALICULAR MEMBRANE AND BASOLATERAL MEMBRANE. UPTAKE TRANSPORTERS DEPICTED IN THE BASOLATERAL MEMBRANE.	71
FIGURE 3.3: BIDIRECTIONAL STUDY OF [ $^3$ H]-VINBLASTINE IN MDR1-MDCK CELLS. CONTROL IS HANKS BUFFER AND [ $^3$ H]-VINBLASTINE, GG918 IS P-GP INHIBITOR.	78
FIGURE 3.4: BIDIRECTIONAL STUDY OF [ $^3$ H]-PRAVASTATIN IN cMOAT-MDCKII CELLS. CONTROL IS HANK'S BUFFER PLUS [ $^3$ H]-PRAVASTATIN. MK-571 IS cMOAT INHIBITOR.	80
FIGURE 3.5: INTRACELLULAR ACCUMULATION OF [ $^3$ H]-PRAVASTATIN IN cMOAT-MDCKII CELLS. CONTROL IS HANK'S BUFFER PLUS [ $^3$ H]-PRAVASTATIN. MK-571 IS cMOAT INHIBITOR. A-C IS INTRACELLULAR ACCUMULATION FROM APICAL (A) TO INTRACELLULAR (C), AND B-C IS ACCUMULATION FROM BASOLATERAL (B) TO INTRACELLULAR (C).	80
FIGURE 3.6: BIDIRECTIONAL STUDY OF PROPRANOLOL 10 $\mu$ M IN MDR1-MDCK (A) CELLS AND MDCK (B) CELLS.	82
FIGURE 3.7: BIDIRECTIONAL STUDY OF LOSARTAN 10 $\mu$ M IN MDR1-MDCK (A) CELLS AND MDCK (B) CELLS.	85

FIGURE 4.1: SEQUENCE OF EVENTS FROM FIRST SURGERY ON RATS TO ANALYSIS OF SAMPLES FROM IPRL.....	96
FIGURE 4.2: DIAGRAM DEPICTING ISOLATED PERFUSED RAT LIVER (IPRL) SETUP. YONG LIU AND ONUA (2004).....	97
FIGURE 4.3: CONCENTRATION OF PROPRANOLOL IN IPRL PERFUSATE OVER TIME. EFFECT OF NEPHRECTOMY AND SERUM FROM HEMODIALYSIS PATIENTS ON HEPATIC DISPOSITION. ....	103
FIGURE 4.4: CONCENTRATIONS OF 4-OH PROPRANOLOL IN IPRLs. EFFECT OF NEPHRECTOMY AND SERUM FROM HEMODIALYSIS PATIENTS ON HEPATIC DISPOSITION. ....	105
FIGURE 4.5: CONCENTRATIONS OF LOSARTAN IN IPRLs. EFFECT OF NEPHRECTOMY AND SERUM FROM HEMODIALYSIS PATIENTS ON HEPATIC DISPOSITION. ....	108
FIGURE 4.6: CONCENTRATION TIME CURVE OF EPROSARTAN IN IPRL. EFFECT OF NEPHRECTOMY AND SERUM FROM HEMODIALYSIS PATIENTS ON HEPATIC DISPOSITION. ....	110
FIGURE 4.7: WESTERN BLOTS DEPICTING HEPATIC TRANSPORTER EXPRESSION IN SHAM AND NEPHRECTOMIZED RAT LIVERS.....	112
FIGURE 4.8: GRAPH DEPICTING RELATIVE INTENSITY (RELATIVE TO B-ACTIN) OF BANDS OF TRANSPORT PROTEINS IN LIVERS FROM NEPHRECTOMIZED RATS.....	113
FIGURE 5.1: AUC PROFILE FOR PROPRANOLOL IN HEALTHY SUBJECTS AND CKD SUBJECTS.....	129
FIGURE 5.2: AUC PROFILE FOR 4-OH PROPRANOLOL IN HEALTHY SUBJECTS AND CKD PATIENTS .....	130

FIGURE 5.3: AUC PROFILE FOR LOSARTAN IN HEALTHY SUBJECTS AND CKD PATIENTS ..... 132

FIGURE 5.4: AUC PROFILE FOR EXP3174 IN HEALTHY SUBJECTS AND CKD PATIENTS ..... 133

FIGURE 5.5: AUC PROFILE FOR ERYTHROMYCIN IN HEALTHY SUBJECTS AND CKD PATIENTS ..... 135

FIGURE 5.6: AUC PROFILE FOR N-DEMETHYL ERYTHROMYCIN IN HEALTHY SUBJECTS AND CKD PATIENTS ..... 136

## **Chapter 1: Chronic Kidney Disease, Transporters, and Metabolizing Enzymes: *consequences on drug disposition***

### **Summary**

This chapter outlines the importance of investigating the effects of chronic kidney disease in drug disposition. The role of the kidney in important physiological functions as well as drug disposition is described to indicate the importance of the kidney as a major organ that regulates body homeostasis as well as its importance in drug disposition. A brief discussion of chronic kidney disease statistics in the United States and the public health impact is included. The definition of chronic kidney disease and its stages is described to place a perspective on how the disease progresses and leads to accumulation of uremic toxins, which are central to the hypothesis for this project. The importance of metabolizing enzyme and transporters in drug disposition and how these are affected in chronic kidney disease is also discussed. Next, the biopharmaceutics drug disposition classification system (BDDCS) will be described and its utility for predicting the disposition of different classes of drugs is discussed. Finally, the rationale, hypothesis, and aims of this research project are summarized.



## 1.1 Introduction

Pharmacokinetics and pharmacology have made great advances over the last 50 years, leading to the discovery of natural and synthetic compounds to treat a variety of diseases. However, there are still many challenges facing drug development, from finding suitable targets for active compounds, developing formulations for poorly permeable drugs, to deciphering the extent of involvement of drug transporters in pharmacokinetics, and effectively utilizing pharmacogenomics to help address issues in drug development. Drug absorption, distribution, metabolism, and elimination (ADME) drive drug disposition in the body, and consequently drug efficacy. These parameters are central to pharmacokinetics (PK) and pharmacodynamics (PD) making it essential to study changes in ADME in order to determine how the pharmacokinetics and pharmacodynamics of a drug will be affected. The organs that are most relevant to these parameters are the intestine, the liver, and the kidney. Moreover, the changes in these parameters in disease states compared to healthy volunteers need to be considered for effective drug treatment. Such is the case of chronic kidney disease (CKD), in which patients show alterations in pharmacokinetics of some medications that are not eliminated by the kidney, yet it is not known why these alterations occur. Understanding these changes in the absorption, distribution, metabolism, and elimination in CKD, and understanding what mediates these changes, can lead to better dosing of these drugs and prevent toxicities, drug-drug interactions, and improve efficacy. In the next sections the relevant function of the kidney and liver will be discussed, as well as CKD, metabolizing enzymes, transporters, the biopharmaceutics drug disposition classification system (BDDCS), and scientific findings on these topics will be discussed. This will set the

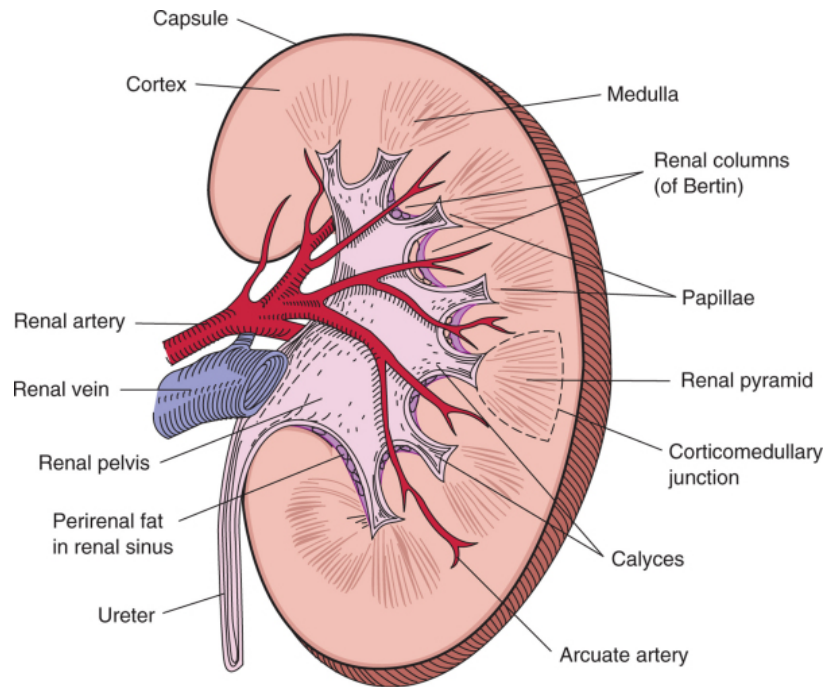
stage for the rationale of the hypothesis tested in this thesis and the approach used to study the hypothesis.

## **1.2 Kidneys, Liver, and Importance in Drug Disposition**

### ***1.2.1 The Kidneys***

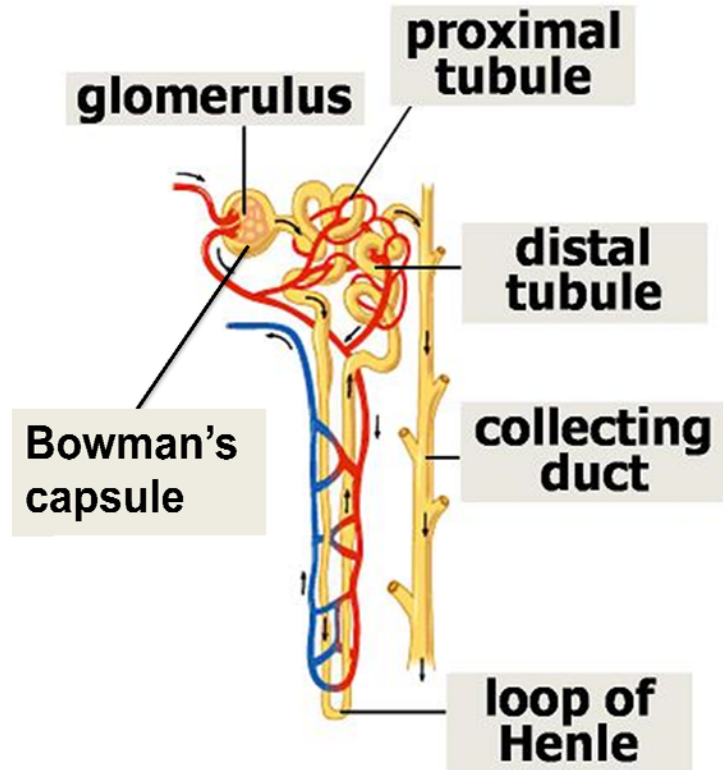
The kidneys play a central role in drug elimination. In addition, the kidney expresses metabolizing enzymes and transporters responsible for drug metabolism and transport (see section 1.6 and 1.7). As an excretory organ, the kidney is a major route of drug elimination, and can also be a site of reabsorption for drugs. Hence, in the presence of renal impairment, drug elimination or reabsorption will be altered.

The kidneys (Figure 1.1) are paired organs located in the posterior part of the abdomen on each side of the vertebral column. The weight of each human kidney ranges from 125 g to 170 g in the adult male and from 115 g to 155 g in the adult female (Brenner and Rector, 2008).



**Figure 1.1:** The kidney (Brenner and Rector, 8<sup>th</sup> Edition)

The functional unit of the kidney is the nephron. Each human kidney contains about  $0.6 \times 10^6$  to  $1.4 \times 10^6$  nephrons. The essential components of the nephron are depicted in Figure 1.2 and include the glomerulus, Bowman's capsule, the proximal tubule, the distal tubule, the collecting duct, and the loop of Henle. The filtration of a nearly protein-free fluid from the glomerular capillaries into the Bowman space is the first step in urine formation. Electrolytes, amino acids, glucose, and other endogenous or exogenous compounds smaller than  $20\text{\AA}$  ( $< 60,000$  daltons) are freely filtered while molecules larger than  $\approx 50\text{\AA}$  are virtually excluded from filtration (Brenner and Rector, 2008).



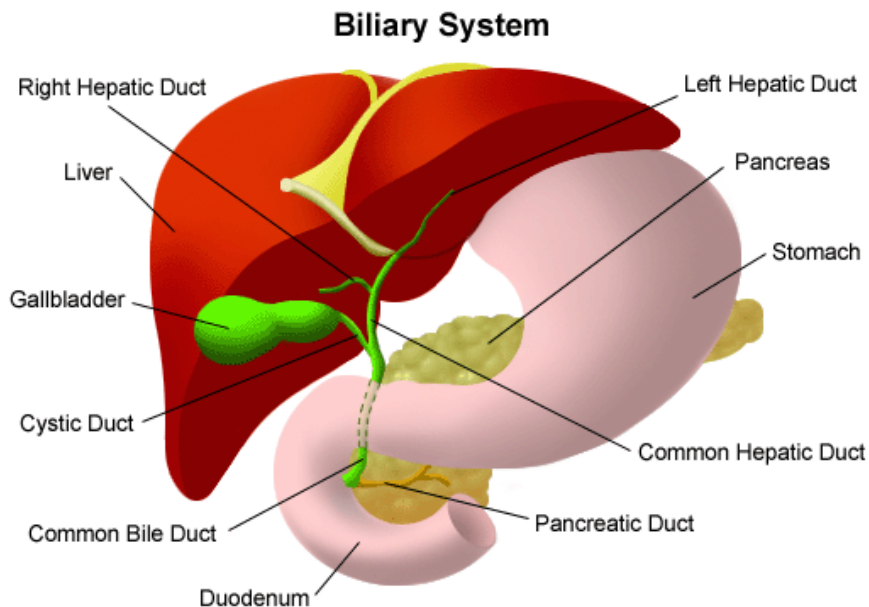
**Figure 1.2:** Illustration of the nephron, its components, and ionic movement to form urine (<http://faculty.washington.edu.htm> , 2003)

The kidneys are involved in important physiological processes that regulate body homeostasis. The kidneys, in addition to removing waste and xenobiotics from the bloodstream, are also responsible for secreting three important hormones: erythropoietin, which stimulates the bone marrow to make red blood cells; renin, which regulates blood pressure; and calcitriol, which is the active form of vitamin D and helps maintain calcium homeostasis. The kidney is responsible for maintaining a balance of electrolytes, glucose, amino acids, and organic cations and anions through reabsorption into the bloodstream. Due to all the critical physiological roles of the kidney, it is important to understand how alterations in the kidney may affect processes in other organs, as well as understanding

how the kidney function may affect drug pharmacokinetics (PK) and/or metabolism for non-renally excreted drugs (those metabolized and excreted by the liver).

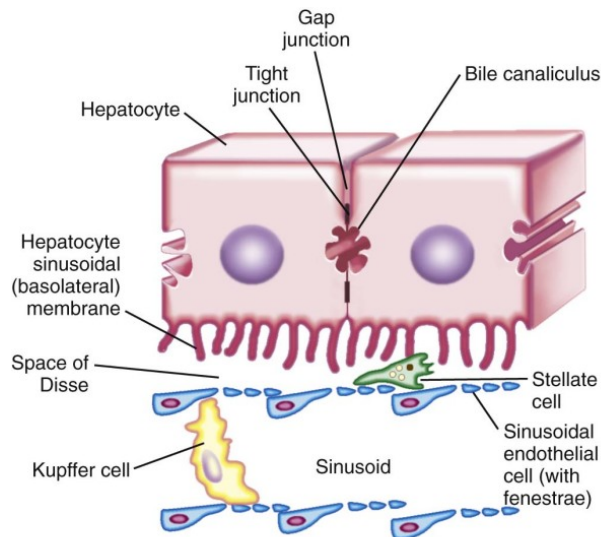
### 1.2.2 *The Liver*

Along with the kidneys, the liver is another vital organ for drug elimination, via metabolism and biliary excretion (Figure 1.3). The liver also expresses drug transporters and a variety of metabolizing enzymes, which play an important role in ADME and affect drug PK/PD. The liver is a vital organ responsible for a wide range of functions, including metabolism, glycogen storage, decomposition of blood cells, hormone production, detoxification, protein synthesis, and production of bile acids. The liver lies below the diaphragm in the thoracic region of the abdomen. The production of bile, an alkaline fluid, aids in digestion via the emulsification of lipids.



**Figure 1.3:** Diagram depicting the liver, biliary system, stomach and pancreas (<http://www.mainlinehealth.org/stw/images/125543.jpg>, 2010).

The liver is comprised of approximately 70-80% hepatocytes, the cell type responsible for metabolism of xenobiotics and endogenous compounds. Hepatocytes are polarized epithelial cells (Figure 1.4). Their plasma membranes have three distinct domains—(1) the sinusoidal surface (~37% of the cell surface) that comes in direct contact with plasma through the fenestrae of the specialized hepatic sinusoidal endothelial cells; (2) the canalicular surface (~13% of the cell surface) that encloses the bile canaliculus; and (3) contiguous surfaces (Sleisenger et al., 2006).



**Figure 1.4:** Hepatocyte and other hepatic cells (Sleisenger et al., 2006)

The hepatocyte contains important Phase I and Phase II metabolizing enzymes that modify xenobiotics into more polar compounds, making them more readily available for elimination. The enzymes include the cytochromes P450 (CYP450) metabolizing enzymes, the epoxide hydrolases, methyltransferases, glutathione-S-transferases, N-acetyltransferases, UDP-glucuronosyltransferases, etc. The most abundant isoform of

CYP450 enzymes in humans is CYP3A4. This enzyme along with other CYP isoforms is important in drug metabolism and drug-drug interactions (see section 1.6). In addition to the metabolizing enzymes, the hepatocyte also expresses transporters in the basolateral and canalicular surface of the cell (see section 1.7). Due to the major role the liver plays in xenobiotic metabolism, it is important to know the consequences of CKD on liver CYPs and transporters in order to understand the consequences of CKD on drug disposition.

## **1.3 Chronic Kidney Disease**

### ***1.3.1 Chronic Kidney Disease Statistics***

The physiological processes that the kidney carries out are essential for homeostasis and drug disposition; thus, it is essential to investigate the consequences of chronic kidney disease (CKD) on these processes. CKD occurs when the kidneys suffer from gradual and usually permanent loss of function over time. The disease afflicts millions of Americans every year, and according to data collected through the National Health and Nutrition Examination Survey from 1994-2004, 11.5% of adults ages 20 or older (23 million adults) have physiological evidence of chronic kidney disease (Levey et al., 2009). Chronic kidney disease is typically a secondary disease to hypertension, diabetes, glomerulonephritis, cystic kidney, or urologic diseases. One of the major consequences of CKD is impaired body homeostasis, leading to electrolyte imbalance, decreased ability to excrete phosphate and potassium, uremia, abnormalities in calcium, parathyroid hormone, and vitamin D metabolism. The increase in worldwide obesity

projects that the incidence of diabetes and hypertension will increase over the next decade, and with that the increase in CDK as a consequence of these primary diseases. Other chronic kidney disease includes polycystic kidney disease, glomerulonephritis, and genetic diseases that affect the renal function and lead to CKD.

### ***1.3.2 Definition of Chronic Kidney Disease***

The glomerular filtration rate (GFR) is one of the indications of renal function and is used to categorize the stage of CKD (Table 1.1). Chronic kidney disease is categorized into 5 stages, with stage 1 having evidence of kidney damage and with normal or increased GFR and stage 5 being the most severe with a GFR below 15ml/min/1.73m<sup>2</sup> and requiring renal replacement therapy such as hemodialysis and eventually transplantation.

**Table 1.1:** Stages of chronic kidney disease (National Kidney Foundation, 2002).\

<b>Stages</b>	<b>Description</b>	<b>GFR (mL/min/1.73m<sup>2</sup>)</b>
1	Kidney damage with normal GFR	≥ 90
2	Kidney damage with mild ↓ GFR	60-89
3	Moderate ↓ GFR	30-59
4	Severe ↓ GFR	15-29
5	Kidney failure (ESRD)	< 15 (or dialysis)
ESRD: end stage renal disease GFR: glomerular filtration rate		



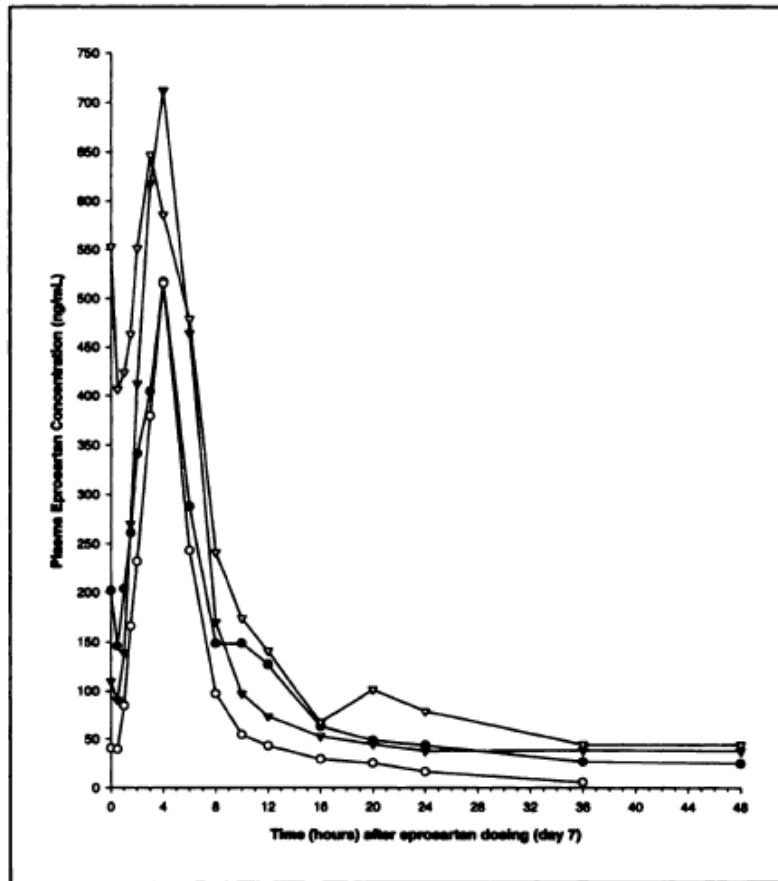
Knowing occurrence of kidney damage and CKD in the general population is important because only a minority of patients progress to kidney failure (often referred as end stage renal disease), and only the patients that advance to end stage renal disease (ESRD) are reported in national registries. The majority of patients with CKD die from a co-morbidity or else maintain relatively stable but reduced renal function, and suffer the consequences of CKD without ever progressing to the need for dialysis (Brenner and Rector, 2008). Thus, ESRD registries provide only limited insight into the true burden of morbidity and mortality associated with CKD. The earlier stages of kidney dysfunction are often clinically silent; when symptoms do arise, they are often nonspecific and commonly attributed to co-morbidities or age-related frailty.

#### **1.4 Drug Disposition in Chronic Kidney Disease**

Chronic kidney disease is a condition that deserves special consideration when prescribing and developing drugs. Two major routes of elimination of xenobiotics are urinary excretion (kidneys) or metabolism (liver/intestine). Metabolism and transport are major factors that contribute to drug pharmacokinetics (PK) and pharmacodynamics (PD). It is well established that for drugs eliminated via the kidneys, the dose needs to be adjusted for patients with CKD in order to prevent toxicities and significant changes in PK/PD due to the impaired renal function. These dose adjustments are typically now determined when a new drug is developed. On the other hand, for drugs that undergo hepatic metabolism, there is no standard adjustment for dose for CKD patients. Furthermore, there is no standard clinical study in drug development that determines the

changes in pharmacokinetics of non-renally excreted drugs in patients with CKD or end stage renal disease (ESRD) in which hemodialysis (HD) is initiated. A study of metoprolol in chronic kidney disease patients has shown that in patients with chronic kidney disease there is an accumulation of the 4  $\alpha$ -hydroxymetoprolol isomers and of both metoprolol acidic metabolite enantiomers, when compared with the patients without kidney disease. There was also a 50% reduction in renal clearance, but the accumulation of the metoprolol acidic metabolite was 4-fold, indicating that renal elimination was not the only pathway being affected in the elimination process (Cerqueira et al., 2005).

Another study in chronic kidney disease patients has investigated the PK of eprosartan, a non-metabolized drug that primarily undergoes biliary excretion. In this study, patients with different stages of chronic kidney disease and on hemodialysis received multiple doses of eprosartan and PK parameters were determined (Figure 1.5). The total maximum concentration ( $C_{max}$ ) and the area under the concentration-time curve (AUC) were not different in healthy volunteers and mild kidney disease patients. However, for patients with stage 4 CKD, the  $C_{max}$  increased 25-35% and AUC increased 51-55% , the renal clearance ( $CL_R$ ) decreased 41% in patients with moderate renal impairment and 91% in those with severe renal impairment (Martin et al., 1998).



*Figure 1. Mean plasma concentrations of eprosartan on day 7 after repeated oral administration of eprosartan 200 mg twice daily in subjects with normal renal function (●), patients with mild renal impairment (○), patients with moderate renal impairment (▼), and patients with severe renal impairment (▽).*

**Figure 1.5:** Pharmacokinetics of eprosartan in patients with different degrees of renal impairment, figure from Martin et al. (1998).

Although the reports of this study indicate that the medication was well tolerated, this is still an example of how non-renal excreted drugs show altered PK in patients with CKD. Furthermore, the more profound the CKD (in terms of GFR reduction), which also correlates with greater accumulation of uremic toxins, the more profound the change in PK parameters.

Although clinical evidence has shown that patients with chronic kidney disease show altered pharmacokinetics of non-renally excreted drugs (Churchwell and Mueller, 2007), there are few studies that have investigated how CKD affects drug metabolism, disposition, and elimination for drugs that undergo hepatic metabolism and elimination. Specifically, there is little research investigating how uremic toxins affect drug disposition, i.e. through inhibition of metabolizing enzymes and/or transporters.

### **1.5 Uremic Toxins**

Uremic toxins are suspected to play a role in the pharmacokinetic changes of drugs in CKD. Uremic toxins are byproducts of metabolism that accumulate in CKD patients due to the decreased renal function and the inability of the kidneys to effectively remove these waste products from the blood. The most common uremic toxin is urea, which if accumulated to high concentrations becomes fatal. At stage 5 of CKD, more commonly known as ESRD, the patient must undergo hemodialysis in order to remove these life-threatening uremic toxins by artificial filtration of the blood through a dialysis unit. However, these uremic toxins accumulate in the earlier stages of CKD to a lesser degree. The uremic toxins have been classified into three groups, the water soluble-small molecules, the middle molecules, and the protein-bound molecules (Vanholder et al., 2003). These toxins vary in their characteristics, and even include cytokines and hormones like parathyroid hormone (PTH). It is not known how the accumulation of these toxins affects other physiological functions in CKD patients. Studies have shown

that the uremic toxins affect the metabolism and uptake of erythromycin and digoxin (Sun et al., 2004; Tsujimoto et al., 2008).

## **1.6 Cytochromes P450 Metabolizing Enzymes**

Cytochromes P450 (CYP450) metabolizing enzymes play a major role in metabolism of endogenous compounds as well as xenobiotics. In humans, these enzymes are responsible for 70%-80% of phase I metabolism (Wijnen et al., 2007). The importance of CYP450 metabolizing enzymes in drug metabolism has been very well established, and considerations of inhibition or activation of these enzymes are important in drug-drug interactions, drug pharmacokinetics, pharmacodynamics, and drug development. Furthermore, in the last 15 years, with the sequencing of the human genome, and further advances in genetic technologies, scientist have been able to study the genetic mutations in the genes that encode the CYP450s (van der Weide and Steijns, 1999). These genetic mutations known as polymorphisms have been shown to alter the function of CYP450 and in turn alter the metabolism of drugs, which leads to pharmacokinetic and pharmacodynamic changes and ultimately changes in drug efficacy (Wang et al., 2005). There are identified populations in certain regions of the world that show a higher prevalence of specific mutations in certain CYP450 alleles, which have been shown to alter the activity of CYP450s important for drug metabolism (Maekawa et al. 2010). There is a great effort to discover how genetic changes in genes that encode CYP450 enzymes affect drug metabolism and disposition, and the consequences on clinical outcomes.

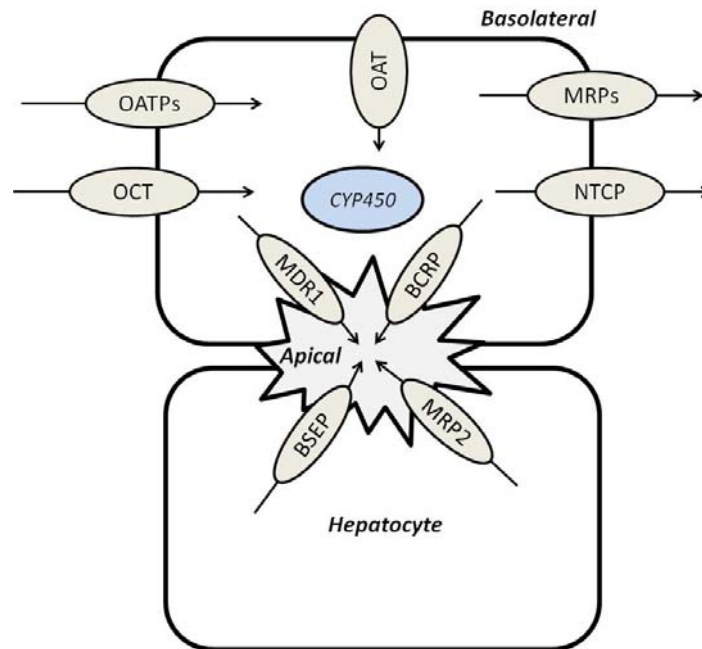
Studies that have explored the role of uremic toxins on the effects of drug disposition have investigated the effect of uremic toxins on CYP450 metabolizing enzymes (Leblond et al., 2000; Guevin et al., 2002; Michaud et al., 2008). These studies have shown that uremic toxins affect the activity of CYP450 metabolizing enzymes by reducing the metabolism of substrates for CYP450 (Dreisbach et al., 2003). The expression of these enzymes has also been studied in CKD rat models, and it has shown been that some isoforms of CYP450 show altered expression in CKD (Leblond et al., 2001). More recently, with the advance in the knowledge of drug transporters, a focus has been shifted to investigating how drug transporters are affected in CKD.

## **1.7 Drug Transporters**

### **1.8 1.7.1 *Drug Transporter Localization and Structure***

Hepatic uptake transporters have a significant impact on drug disposition and drug-drug interactions, particularly for metabolized drugs. Hepatic uptake transporters are localized at the basolateral membrane of the hepatocyte and facilitate the transport of endogenous substances. These transporters also facilitate the uptake of drug into the cell, subsequently exposing drug to metabolizing enzymes and to canalicular membrane transporters for elimination. There are three major uptake transporter families that have been well characterized for their role in drug disposition and impact on pharmacokinetics (Figure 1.6), the SLCO family of transporters also known as OATPs (organic anion transporter polypeptide), and the SLC22A family transporters, which include the OATs (organic anion transporters) and OCTs (organic cation transporters). Other transporters

like MRPs (multidrug resistance protein), MDR1 (multidrug resistance transporter, also known as P-glycoprotein, P-gp), BCRP (breast cancer resistance protein), BSEP (bile salt export pump), and NTCP (sodium-taurocholate cotransporting polypeptide) are also expressed in the hepatocyte and are currently being studied for their impact on clinically significant outcomes.



**Figure 1.6:** Hepatocyte and transporter localization

Hepatic efflux transporters are located in the bile canalicular (apical) membrane of the hepatocyte as well as at the basolateral membrane and facilitate the efflux of endogenous substances and xenobiotics. The first transporter to be discovered and known to transport drugs was P-glycoprotein (P-gp or MDR1). Other efflux transporters include MRP (multi drug resistance-associated protein), BCRP (breast cancer resistant protein), and BSEP (bile salt transporter). Each transporter family contains different members with a wide range and sometimes overlapping substrate specificity, different

inhibitory substances, and different degrees of expression in different tissues (Chandra and Brouwer, 2004). The structure of mouse P-gp transporter was recently solved by Aller et al., (2009) at the Scripps Institute and it revealed the molecular basis for specific drug binding. However, the structure of other transporters is still unresolved. Knowing the three dimensional structures of the transporters of interest will be helpful in designing specific inhibitors and studying their role in drug transport and drug-drug interactions. Currently the lack of specific inhibitors poses an inconvenience for studying specific transporters in cell systems such as hepatocytes or cell lines that endogenously express these transporters.

### ***1.7.2 Transporters and Drug-Drug Interactions***

Studies investigating drug transporters have shed light on their importance on drug interactions that many times have clinical significance. Not all drugs require an uptake transporter and not all drugs are metabolized. Thus, it is particularly important to determine the role of uptake transporters for metabolized drugs and how these affect drug interactions. If a drug is metabolized and requires an uptake transporter for exposure to the metabolizing enzyme and a second drug inhibits uptake of the first drug, the consequence will be decreased metabolism of the first drug. This can in turn increase the concentration of the parent drug in the systemic circulation. Multiple pharmacodynamic consequences can result: 1) If the parent drug is the active species then an interaction resulting in increased systemic concentrations can lead to increased efficacy and potential toxicity if the receptor for activity/toxicity is not in the liver (i.e. glyburide) or decreased effects if the receptor for activity is in the liver (i.e. statins) and 2) if a metabolite of the drug is the active species and less metabolite is formed due to inhibition of hepatic uptake



then decreased activity may be observed. It has become increasingly important to investigate the role of hepatic transporters on drug interactions and how these transporters play a role in drug disposition and therapeutic effects. Our laboratory and other research groups have investigated the impact of drug transporters in drug interactions and the results have made it more evident that drug transporters play a role in drug pharmacokinetics and drug-drug interactions and drug development (Giacomini et al., 2010 ; Lau et al., 2004; Lau et al., 2007; Zamek-Gliszczyński et al., 2009; Zheng et al., 2009). There has also been a great focus on pharmacogenetics, and investigating how polymorphisms in transporters have an effect on drug disposition, pharmacokinetics, and drug response (Kroetz et al., 2010 ; Giacomini et al., 2007; Kivisto and Niemi, 2007). Several studies have shown that single nucleotide polymorphisms of transporters alter the function of the transporter resulting in altered drug response. A clinical study in patients with OATP polymorphism showed that plasma concentrations of the HIV protease inhibitor lopinavir increased compared to patients without the polymorphism (Hartkoorn et al., 2010).

The important role of drug transporters in drug disposition has also brought into question how these drug transporters are affected in disease states. The role of drug transporters in chronic kidney disease and how they can affect drug disposition has been a question that has not been extensively explored. Few studies to date have attempted to study the role of transporters in CKD, although some studies in 5/6 nephrectomized rats (model for CKD) have reported changes in transporter expression in the liver and intestine (Naud et al., 2007; Naud et al., 2008). However, no studies have been done comparing different drugs that are classified by the biopharmaceutics drug disposition

classification system (BDDCS) in CKD rat models or *in vitro* studies to assess drug transport in the presence of uremic serum from hemodialysis patients or uremic toxins.

### **1.9 Biopharmaceutics Drug Disposition Classification System**

The biopharmaceutics drug disposition classification system (BDDCS) was developed by Wu and Benet (2005) in order to classify drugs by the extent of metabolism and solubility to be able to predict drug disposition. The BDDCS system (Figure 1.8a) was an adaptation from the biopharmaceutics classification system (BCS) (Figure 1.7), developed by Amidon et al. (1995). BCS is an experimental model, in which solubility and permeability are the main parameters taken into consideration to predict *in vivo* pharmacokinetic performance of drug products from measurements of permeability and solubility. The BCS was adopted by the Food and Drug Administration (FDA) to be used as a science based approach to allow waiver of *in vivo* bioavailability and bioequivalence testing of immediate-release solid dosage forms for Class 1 drugs (Figure 1.7).

**Figure 1.7:** BCS according to Amidon et al. (1995).

	High Solubility	Low Solubility
High Permeability	<b>Class 1</b> High Solubility High Permeability (Rapid Dissolution for Biowaiver)	<b>Class 2</b> Low Solubility High Permeability
Low Permeability	<b>Class 3</b> High Solubility Low Permeability	<b>Class 4</b> Low Solubility Low Permeability

BDDCS incorporated the extent of metabolism as a parameter to predict drug disposition (Figure 1.8a) and to predict the effects of transporters on drug disposition (Figure 1.8b).

**Figure 1.8:** Biopharmaceutics Drug Disposition Classification System (BDDCS). (a) including extent of metabolism, (b) transporter effects followed by oral dosing.

(a) Classification by extent of metabolism according to BDDCS

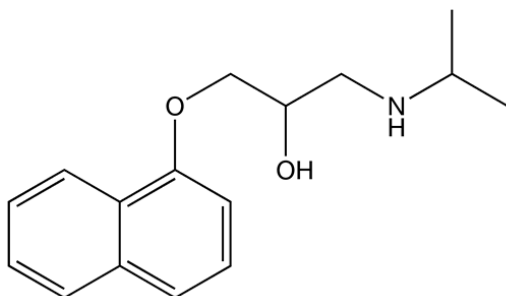
	High Solubility	Low Solubility
Extensive Metabolism	<b>Class 1</b> High Solubility Extensive Metabolism (Rapid dissolution and $\geq 70\%$ extent of metabolism)	<b>Class 2</b> Low Solubility Extensive Metabolism
Poor Metabolism	<b>Class 3</b> High Solubility Poor Metabolism	<b>Class 4</b> Low Solubility Poor Metabolism

(b) Transporter effects predicted by BDDCS following oral dosing (Shugarts and Benet, 2010).

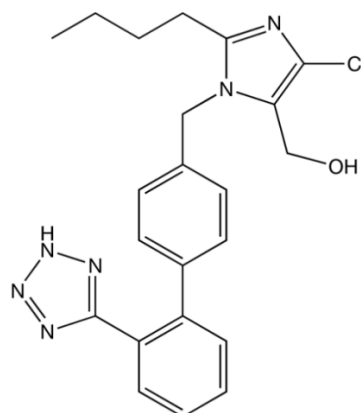
	High Solubility	Low Solubility
Extensive Metabolism	<b>Class 1</b> Transporter effects minimal	<b>Class 2</b> Efflux transporter effects predominate in the gut, while absorptive and efflux transporter effects occur the in liver
Poor Metabolism	<b>Class 3</b> Absorptive transporter effects predominate (but may be modulated by efflux transporters)	<b>Class 4</b> Absorptive and efflux transporter effects could be important

The differential effect of transporters on drug disposition in the BDDCS system and the changes in pharmacokinetics observed in CKD, led us to investigate how uremic toxins and CKD may affect drug disposition for different BDDCS drugs. For our studies, the three drugs under investigation were propranolol (class 1), losartan (class 2), eprosartan (class 4) for in vitro studies, and erythromycin (class 3) for human clinical studies, whose structures are depicted below.

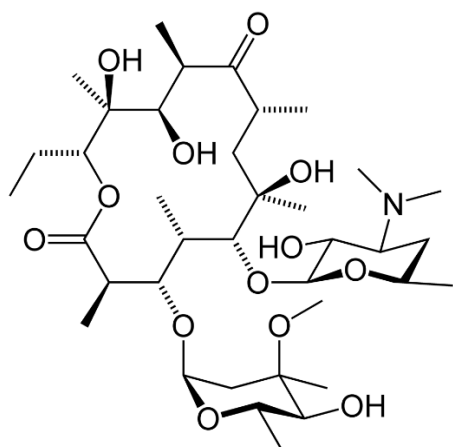
**Figure 1.9:** Chemical structures of compounds used



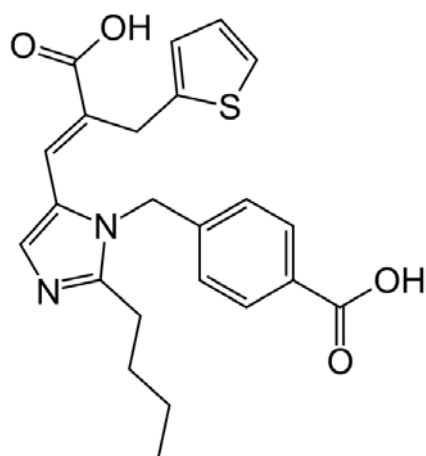
Propranolol (Class 1) MW: 259.35



Losartan (Class 2) MW: 422.91



Erythromycin (Class 3) MW: 733.94



Eprosartan (Class 4) MW: 424.51

## **1.10 Research Project**

### ***1.10.1 Rationale***

The overall goal of this project was to understand the effects of uremic toxins present in patients with chronic kidney disease on hepatic transporters and metabolizing enzymes. We investigated the non-renal effects of uremic toxins on the pharmacokinetics of four non-renally excreted drugs in cellular systems, in isolated perfused liver, and finally in vivo in healthy volunteers and patients with chronic kidney disease (CKD). We suspected that inhibition of hepatic transporters and/or enzymes by uremic toxins may account for the observed changes in the non-renal pharmacokinetics of some drugs taken by patients with renal failure.

### ***1.10.2 Hypothesis***

The recent and previous studies on transporters, metabolizing enzymes and CKD led to the hypothesis that uremic toxins have inhibitory effects on hepatic transporters as well as metabolizing enzymes, and this accounts for the decrease in clearance of drugs that are metabolized/eliminated through the hepatobiliary system. Furthermore, we hypothesized that the pharmacokinetics of drugs from different BDDCS classes will be affected differently by uremic toxins depending on the transporter and enzyme influence on drug disposition.

### ***1.10.3 Specific Aims***

1. Determine the *in vitro* effects of uremic toxins on hepatic transporters in transfected cell systems, using known substrates and propranolol, losartan and eprosartan as model compounds. Investigate the uptake of these drugs in rat and human hepatocytes and the inhibition of uptake by uremic toxins. Investigate the metabolism of the selected drugs in rat and human microsomes and the inhibition of CYPs by uremic toxins.
2. Investigate the changes in pharmacokinetics in the chronic kidney disease rat model for the selected drugs using the isolated perfused liver model. Also, analyze changes in protein expression of transporters and metabolizing enzymes.
3. Conduct a clinical study to investigate the pharmacokinetics of the three selected Class 1, 2 and 3 drugs in CKD patients not undergoing hemodialysis versus healthy volunteers.

## 1.11 References

- Aller SG, Yu J, Ward A, Weng Y, Chittaboina S, Zhuo R, Harrell PM, Trinh YT, Zhang Q, Urbatsch IL and Chang G (2009) Structure of P-glycoprotein reveals a molecular basis for poly-specific drug binding. *Science* **323**:1718-1722.
- Amidon GL, Lennernas H, Shah VP and Crison JR (1995) A theoretical basis for a biopharmaceutic drug classification: the correlation of in vitro drug product dissolution and in vivo bioavailability. *Pharm Res* **12**:413-420.
- Brenner BM and Rector FC (2008) *Brenner & Rector's The Kidney*. Saunders Elsevier, Philadelphia.
- Cerqueira PM, Coelho EB, Geleilete TJ, Goldman GH and Lanchote VL (2005) Influence of chronic renal failure on stereoselective metoprolol metabolism in hypertensive patients. *J Clin Pharmacol* **45**:1422-1433.
- Chandra P and Brouwer KL (2004) The complexities of hepatic drug transport: current knowledge and emerging concepts. *Pharm Res* **21**:719-735.
- Churchwell MD and Mueller BA (2007) Selected pharmacokinetic issues in patients with chronic kidney disease. *Blood Purif* **25**:133-138.
- Dreisbach AW, Japa S, Gebrekal AB, Mowry SE, Lertora JJ, Kamath BL and Rettie AE (2003) Cytochrome P4502C9 activity in end-stage renal disease. *Clin Pharmacol Ther* **73**:475-477.
- Giacomini KM, Brett CM, Altman RB, Benowitz NL, Dolan ME, Flockhart DA, Johnson JA, Hayes DF, Klein T, Krauss RM, Kroetz DL, McLeod HL, Nguyen AT, Ratain MJ, Relling MV, Reus V, Roden DM, Schaefer CA, Shuldiner AR, Skaar T, Tantisira K, Tyndale RF, Wang L, Weinshilboum RM, Weiss ST and Zineh I



(2007) The pharmacogenetics research network: from SNP discovery to clinical drug response. *Clin Pharmacol Ther* **81**:328-345.

Giacomini KM, Huang SM, Tweedie DJ, Benet LZ, Brouwer KL, Chu X, Dahlin A, Evers R, Fischer V, Hillgren KM, Hoffmaster KA, Ishikawa T, Keppler D, Kim RB, Lee CA, Niemi M, Polli JW, Sugiyama Y, Swaan PW, Ware JA, Wright SH, Yee SW, Zamek-Gliszczynski MJ and Zhang L (2010) Membrane transporters in drug development. *Nat Rev Drug Discov* **9**:215-236.

Guevin C, Michaud J, Naud J, Leblond FA and Pichette V (2002) Down-regulation of hepatic cytochrome p450 in chronic renal failure: role of uremic mediators. *Br J Pharmacol* **137**:1039-1046.

Hartkoorn RC, Kwan WS, Shallcross V, Chaikan A, Liptrott N, Egan D, Sora ES, James CE, Gibbons S, Bray PG, Back DJ, Khoo SH and Owen A (2010) HIV protease inhibitors are substrates for OATP1A2, OATP1B1 and OATP1B3 and lopinavir plasma concentrations are influenced by SLCO1B1 polymorphisms. *Pharmacogenet Genomics* **20**:112-120.

Kivisto KT and Niemi M (2007) Influence of drug transporter polymorphisms on pravastatin pharmacokinetics in humans. *Pharm Res* **24**:239-247.

Kroetz DL, Yee SW and Giacomini KM (2010) The pharmacogenomics of membrane transporters project: research at the interface of genomics and transporter pharmacology. *Clin Pharmacol Ther* **87**:109-116.

Lau YY, Huang Y, Frassetto L and Benet LZ (2007) effect of OATP1B transporter inhibition on the pharmacokinetics of atorvastatin in healthy volunteers. *Clin Pharmacol Ther* **81**:194-204.

- Lau YY, Wu CY, Okochi H and Benet LZ (2004) Ex situ inhibition of hepatic uptake and efflux significantly changes metabolism: hepatic enzyme-transporter interplay. *J Pharmacol Exp Ther* **308**:1040-1045.
- Leblond F, Guevin C, Demers C, Pellerin I, Gascon-Barre M and Pichette V (2001) Downregulation of hepatic cytochrome P450 in chronic renal failure. *J Am Soc Nephrol* **12**:326-332.
- Leblond FA, Giroux L, Villeneuve JP and Pichette V (2000) Decreased in vivo metabolism of drugs in chronic renal failure. *Drug Metab Dispos* **28**:1317-1320.
- Levey AS, Stevens LA, Schmid CH, Zhang YL, Castro AF, 3rd, Feldman HI, Kusek JW, Eggers P, Van Lente F, Greene T and Coresh J (2009) A new equation to estimate glomerular filtration rate. *Ann Intern Med* **150**:604-612.
- Maekawa K, Harakawa N, Yoshimura T, Kim SR, Fujimura Y, Aohara F, Sai K, Katori N, Tohkin M, Naito M, Hasegawa R, Okuda H, Sawada JI, Niwa T and Saito Y (2010) CYP3A4\*16 and CYP3A4\*18 Alleles Found in East Asians Exhibit Differential Catalytic Activities for Seven CYP3A4 Substrate Drugs. *Drug Metab Dispos*.
- Martin DE, Chapelsky MC, Ilson B, Tenero D, Boike SC, Zariffa N and Jorkasky DK (1998) Pharmacokinetics and protein binding of eprosartan in healthy volunteers and in patients with varying degrees of renal impairment. *J Clin Pharmacol* **38**:129-137.
- Michaud J, Nolin TD, Naud J, Dani M, Lafrance JP, Leblond FA, Himmelfarb J and Pichette V (2008) Effect of hemodialysis on hepatic cytochrome P450 functional expression. *J Pharmacol Sci* **108**:157-163.

- Naud J, Michaud J, Boisvert C, Desbiens K, Leblond FA, Mitchell A, Jones C, Bonnardeaux A and Pichette V (2007) Down-regulation of intestinal drug transporters in chronic renal failure in rats. *J Pharmacol Exp Ther* **320**:978-985.
- Naud J, Michaud J, Leblond FA, Lefrancois S, Bonnardeaux A and Pichette V (2008) Effects of chronic renal failure on liver drug transporters. *Drug Metab Dispos* **36**:124-128.
- Sleisenger MH, Feldman M, Friedman LS and Brandt LJ (2006) *Sleisenger & Fordtran's Gastrointestinal and Liver Disease : Pathophysiology, Diagnosis, Management*. Saunders, Philadelphia.
- Sun H, Huang Y, Frassetto L and Benet LZ (2004) Effects of uremic toxins on hepatic uptake and metabolism of erythromycin. *Drug Metab Dispos* **32**:1239-1246.
- Tsujimoto M, Kinoshita Y, Hirata S, Otagiri M, Ohtani H and Sawada Y (2008) Effects of uremic serum and uremic toxins on hepatic uptake of digoxin. *Ther Drug Monit*.
- van der Weide J and Steijns LS (1999) Cytochrome P450 enzyme system: genetic polymorphisms and impact on clinical pharmacology. *Ann Clin Biochem* **36 ( Pt 6)**:722-729.
- Vanholder R, De Smet R, Glorieux G, Argiles A, Baurmeister U, Brunet P, Clark W, Cohen G, De Deyn PP, Deppisch R, Descamps-Latscha B, Henle T, Jorres A, Lemke HD, Massy ZA, Passlick-Deetjen J, Rodriguez M, Stegmayr B, Stenvinkel P, Tetta C, Wanner C and Zidek W (2003) Review on uremic toxins: classification, concentration, and interindividual variability. *Kidney Int* **63**:1934-1943.

- Wang R, Chen K, Wen SY, Li J and Wang SQ (2005) Pharmacokinetics of glimepiride and cytochrome P450 2C9 genetic polymorphisms. *Clin Pharmacol Ther* **78**:90-92.
- Wijnen PA, Op den Buijsch RA, Drent M, Kuijpers PM, Neef C, Bast A, Bekers O and Koek GH (2007) Review article: The prevalence and clinical relevance of cytochrome P450 polymorphisms. *Aliment Pharmacol Ther* **26 Suppl 2**:211-219.
- Wu CY and Benet LZ (2005) Predicting drug disposition via application of BCS: transport/absorption/ elimination interplay and development of a biopharmaceutics drug disposition classification system. *Pharm Res* **22**:11-23.
- Zamek-Gliszczyński MJ, Kalvass JC, Pollack GM and Brouwer KL (2009) Relationship between drug/metabolite exposure and impairment of excretory transport function. *Drug Metab Dispos* **37**:386-390.
- Zheng HX, Huang Y, Frassetto LA and Benet LZ (2009) Elucidating rifampin's inducing and inhibiting effects on glyburide pharmacokinetics and blood glucose in healthy volunteers: unmasking the differential effects of enzyme induction and transporter inhibition for a drug and its primary metabolite. *Clin Pharmacol Ther* **85**:78-85.

## **Chapter 2: Effects Uremic Toxins and Hemodialysis Serum on Drug Transport and Metabolism: *in vitro* studies**

### **Summary**

Chronic kidney disease (CKD) is recognized to cause pharmacokinetic changes in renally excreted drugs; however, pharmacokinetic changes are also reported for drugs that are non-renally eliminated. Few studies have investigated how uremic toxins may affect drug transporters and metabolizing enzymes and how these may result in pharmacokinetic/metabolic changes in CKD. Here, we investigated the effects of uremic toxins and human uremic serum on the transport of the prototypical transporter substrate [<sup>3</sup>H]-estrone sulfate and three BDDCS drugs, propranolol, losartan, and eprosartan. We observed a significant decrease in [<sup>3</sup>H]-estrone sulfate uptake with some uremic toxins in both transfected cells and rat hepatocytes. Also, the uptake of losartan was decreased in rat and human hepatocytes (28% and 48% respectively) in the presence of hemodialysis (HD) serum. In the time-course studies of propranolol, HD serum and rifampin did not cause a significant change in AUC. Time-course studies of losartan showed a 27%, 65% and 68% increase in AUC in the presence of HD serum, rifampin, and sulfaphenazole, respectively. The intracellular losartan AUC also increased significantly in the treatment groups and the metabolite AUC decreased by 41% and 26% in rifampin and sulfaphenazole treated groups, respectively. The intracellular AUC of eprosartan increased by 190% in the presence of HD serum but there was no significant change in the presence of rifampin. These studies indicate that the uremic toxins contained in HD

serum play an important role in drug disposition through drug transporters. Furthermore, these studies suggest that different drugs will be affected differently in chronic kidney disease.

## **2.1 Introduction**

One of the consequences of chronic kidney disease is the accumulation of waste products from metabolic processes, called uremic solutes, in the blood. Uremic solutes increase with a decline in renal function. Not all uremic solutes are known to cause toxicity; however, some can be fatal if not removed. Urea is quantitatively the most important solute excreted by the kidney, and levels rise higher than those of any other solute when the kidney fails. But early studies indicated that urea causes only a minor part of uremic illness, and that the full expression of uremia may require accumulation of urea plus other solutes (Brenner and Rector, 2008).

At stage five of chronic kidney disease (CKD) also called end stage renal disease (ESRD), the concentrations of uremic solutes are high enough to require renal replacement therapy through hemodialysis. Hemodialysis removes the most threatening uremic toxins like urea and creatinine. Hemodialysis involves diffusion of solutes across a semipermeable membrane and utilizes counter current flow, where the dialysate is flowing in the opposite direction to blood flow. Counter current flow maintains the concentration gradient across the membrane at a maximum and increases the efficiency of dialysis (Brenner and Rector, 2008). A negative aspect of renal replacement therapy like hemodialysis is that it removes solutes indiscriminately; thus, the improvement in the

patient cannot be attributed to the removal of a specific compound. Hemodialysis and the membranes used in this process can remove solutes at different rates based on molecular size, protein binding, and sequestration within cells. However, many solutes are not efficiently removed. For this reason, it is suspected that the solutes or uremic toxins may have an impact on different biological processes, including drug metabolism (Michaud et al., 2005). Studies from Michaud et al. (2005) investigated the effects of human hemodialysis serum on the expression and activity of CYP450 enzymes in rat hepatocytes. When the rat hepatocytes were incubated for 24hr, the P450 level and protein expression, as well as mRNA levels of P450 isoforms (CYP1A2, 2C6, 2C11, 2D1/2D2, 3A2 and 4A1/4A3), were decreased by more than 45% compared to control serum. CYP3A and CYP1A activities were decreased by 51 and 59% respectively. Michaud et al. (2005) carried out experiments using serum from patients who had been under hemodialysis for 6 months, serum from new hemodialysis patients and from patients that had undergone transplantation. They found that the time a patient had been on hemodialysis did not have an effect on the CYP450 activity in the rat hepatocytes, but when they used the serum from patients who had undergone transplantation, CYP450 activity returned to normal. Thus, it was concluded that human uremic serum contains solutes that decrease rat hepatic P450 activity and expression secondary to reduced gene expression.

Drug metabolism and transport have not been extensively studied in renal failure. Chronic kidney disease (CKD) causes pharmacokinetic changes of drugs that are renally excreted unchanged; thus, the dose for these drugs is usually adjusted for this patient population. However, dose adjustments for drugs that are eliminated primarily via

hepatic metabolism or biliary excretion are rarely considered in CKD. In recent years, drug transporters have been recognized to have an important role in drug disposition. Several studies in our laboratory have shown that hepatic uptake transporters play a pivotal role in drug disposition by facilitating the uptake of drug into the hepatocyte and exposing the drug to the metabolizing enzyme (Lau et al., 2004; Lam et al., 2006; Lau et al., 2006). These studies demonstrated the importance of transporter-enzyme interplay in drug pharmacokinetics of drugs like atorvastatin, erythromycin and digoxin.

Uremic toxins are known to accumulate in patients with CKD, and they are categorized based on their size, protein binding, and water solubility (Vanholder et al., 2003). Indoxyl sulfate and 3-carboxy-4-methyl-5-propyl-2-furanpropanoic acid (CMPF) are two uremic toxins that have been previously studied for their involvement in hepatic drug metabolism and disposition (Sun et al., 2004; Sun et al., 2010; Tsujimoto et al., 2008). Human uremic serum from patients on hemodialysis has also been studied *in vitro* to determine how the toxins contained in uremic serum alter metabolizing enzymes. Studies have shown that uremic serum can inhibit CYPs *in vitro* in cultured rat hepatocytes (Guevin et al., 2002; Michaud et al., 2005). Organic anion transporters (OATs) have been investigated for their involvement in the transport of uremic toxins in the kidney (Deguchi et al., 2002). The effect of uremic toxins on hepatic drug transporters, however, has not been extensively investigated. Moreover, the effects of uremic toxins on different drugs based on the Biopharmaceutics Drug Disposition Classification System (BDDCS) have never been investigated.

BDDCS is a drug classification system based on solubility, permeability (from the FDA's Biopharmaceutics Classification System, BCS), and the extent of metabolism



that predicts the effects of drug transporters and metabolizing enzymes on drug disposition (Wu and Benet, 2005; Shugarts and Benet, 2009). In this classification system, the route of elimination (metabolism or renal and biliary clearance), the involvement of transporters, and the permeability rate will place a drug in a certain class and predict the importance of transporters and enzyme-transporter interplay in the gut and the liver for that class of drug. For a Class 1 highly permeable and extensively metabolized drug, transporter effects in the liver will be minimal, (they would not require a transporter to enter or exit the hepatocyte). For a Class 2 drug, the low solubility of the drug may limit the amount of drug available, thus uptake transporters may be required to make more of the drug available to the cell and efflux transporters can affect efflux from the hepatocyte. For Class 3 and 4 drugs, transporters would be required because of the low permeability of the drug. Therefore, uptake and efflux transporters could become important in Class 2, 3, and 4 drugs in the liver. We hypothesized that if uremic toxins have effects on hepatic transporters, we may expect to see a difference in drug uptake and/or efflux of drug according to its BDDCS class. For a class 1 drug, since transporters are not clinically relevant, the presence of uremic toxins, even if they are affecting transporters, would not have an effect on uptake or efflux of the drug. On the other hand, uremic toxins could have an effect on drug uptake and/or efflux of Class 2, 3, and 4 drugs. In our studies we investigated the effects of various uremic toxins, on the uptake of the model compound [<sup>3</sup>H]-estrone sulfate, which is a known substrate for uptake transporters, and three BDDCS (propranolol Class1, losartan Class 2, eprosartan Class 4) drugs in transfected cells and rat hepatocytes. Losartan has been shown to be a substrate for OATP2B1 uptake transporter (Flynn et al., 2010), and eprosartan has been shown to

be a substrate for OATP1B1 (Sun et al., 2005). We also studied the effects of human hemodialysis (HD) serum on drug uptake in transfected cells, rat and human hepatocytes, and the effect of HD serum on the uptake and metabolism of propranolol, losartan, and eprosartan in rat hepatocytes in time course studies. We analyzed the total concentration of parent compound and their major metabolite (propranolol metabolite was 4-OH propranolol and losartan metabolite was EXP3174, eprosartan is not a metabolized drug), and also the intracellular concentration of the parent compound.

## **2.1 Materials and Methods**

### ***2.2.1 Chemicals and Reagents***

Propranolol, rifampin, quinidine, sulfaphenazole, indoxyl sulfate, endothelin, quinolinic acid, indole-3-acetic acid, *p*-cresol, homocysteine, tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ), interleukin-6 (IL-6), and parathyroid hormone (PTH) as well as HPLC-grade dimethyl sulfoxide, *tert*-butyl-methyl-ether (MTBE), and acetonitrile (ACN) were purchased from Sigma-Aldrich (St. Louis, MO). Losartan potassium salt, EXP3174, 4-OH propranolol, and eprosartan were purchased from Toronto Research Chemicals (Ontario, Canada). GG918 (GF120918) was graciously donated by GlaxoSmithKline (Research Triangle Park, NC). [ $^3\text{H}$ ]-estrone sulfate was purchased from Pekin Elmer (Boston, MA). Plasmids containing transporter inserts or empty vector (pcMV6) were purchased from Origene Technologies Inc. (Rockville, MD). Lipofectamine was purchased from Invitrogen Corporation (Carlsbad, CA). CMPF was purchased from Cayman Chemicals (Ann Arbor, Michigan). Cell culture media was purchased from UCSF Cell

Culture Facility (San Francisco, CA). Pooled male rat liver microsomes were acquired from BD Biosciences (Woburn, MA). Pooled human cryopreserved hepatocytes were obtained from Cellz Direct (Dallas, TX). Male Sprague-Dawley rats (200–350 g) from Charles River Laboratories (Wilmington, MA) were housed in the UCSF animal care facility with a 12-h light/dark cycle and allowed free access to water and food. The animal studies were approved by Committee on Animal Research, UCSF.

### **2.2.2 Hemodialysis Serum**

A protocol to obtain blood from patients on hemodialysis was approved by the UCSF Committee on Human Research. Blood was obtained from eight patients from the outpatient hemodialysis unit at the UCSF Mount Zion clinic. All patients had been on hemodialysis for more than a year. The blood was obtained on dialysis day before initiating dialysis. The serum was separated from the blood and the serum from all patients was pooled into a single batch. The pooled serum was then used to carry out the *in vitro* experiments. All experiments were carried out with 10% human serum (normal or hemodialysis).

### **2.2.3 Cell Transfection and Uptake Assay**

HEK293 cells were cultured in Eagle's minimal essential medium with Eagle's balanced salt solution and L-glutamine (2mM) plus 10% heat-inactivated fetal bovine serum (FBS), penicillin/streptomycin (1µg/ml). A day before transfection, cells were seeded at a density of  $0.5 \times 10^6 / \text{cm}^2$ . The next day they were transiently transfected with OATP1B1-pcMV5, OATP1B3-pcMV6, OATP2B1-pcMV6 or pcMV6 empty vector control using Lipofectamine 2000 per the manufacturer's protocol. After 24 hours the media was replaced, and 48 hours after transfection the cells were used for uptake

studies. Before initiation of the uptake study, cells were washed once with Hanks buffer pre-warmed to 37°C. The uptake study was initiated by adding 1ml of Hanks buffer containing 10% of FBS plus 17.45µM [<sup>3</sup>H]-estrone sulfate (1:4000 dilution) alone as a control or with 10µM rifampin, 100µM MK-571, or a uremic toxin as inhibitors and incubating at 37°C for 2 min. Preliminary experiments had shown that the uptake rate was linear over this time period (data not shown). For the inhibition studies, inhibitors and substrates were added simultaneously. Uremic toxin concentration was chosen based on a preliminary dose-response curve and literature review of concentrations previously used. After two minutes, the buffer was removed to terminate the reaction and the cells were washed three times with ice-cold PBS. Cells were scraped from the wells and homogenized. An aliquot of 200 µL was transferred to a scintillation vial and quenched with liquid scintillation counting solution. The intracellular radioactivity was measured using a scintillation counter (LS6000TA; Beckman Coulter, Fullerton, CA). The results are reported as pM/cell count.

#### **2.2.4 *Microsome Incubations***

The incubation conditions were as described previously (Salphati and Benet, 1999). In brief, each reaction mixture contained 0.5 mg/ml microsomes, 5 mM NADPH, phosphate buffer, 10 µM losartan or 10 µM propranolol, plus 10 µM of rifampin, or 1 µM of sulfaphenazole as a CYP2C9 inhibitor (negative control for losartan metabolism), or 30µM of quinidine as CYP2D6 inhibitor (negative control for propranolol metabolism). The total DMSO concentration used to solubilize rifampin was less than 1% for all in vitro studies; all other compounds were solubilized in methanol. The total reaction volume was 250 µl. The reaction period was 30 minutes at 37°C. For each

sample, the reaction was stopped via protein precipitation by addition of an equal volume of ACN containing the internal standard (IS), warfarin (1  $\mu$ M). The supernatants were stored at -80°C for LC/MS-MS analysis.

### ***2.2.5 Hepatocyte Isolation***

Hepatocyte isolation was carried out in our laboratory after protocol approval from UCSF Institutional Animal Care and Use Committee (IACUC). Anesthesia was induced in rats by intraperitoneal injection with a 1 ml/kg dose of ketamine:xylazine (80 mg/ml:12 mg/ml) before surgery (Lau et al., 2006). The portal vein was cannulated with an i.v. catheter (catalog number 2007-04; Becton Dickinson, Sandy, UT) and perfused with oxygenated liver perfusion buffer (Invitrogen, Carlsbad, CA) for 5 min at 30 ml/min, followed by perfusion with an oxygenated hepatocyte washing buffer (Invitrogen) modified with 2 mM L-glutamine, 10 mM HEPES, and 1.2 U/ml collagenase (Sigma-Aldrich) for 5 min at 20 ml/min. The digested liver was excised and homogenized in a beaker. Hepatocytes were then washed twice with an ice-cold hepatocyte wash buffer containing 2 mM L-glutamine and 10 mM HEPES and were centrifuged at 50g for 2 to 3 min. Cell viability was determined using the trypan blue exclusion method. Cells with viability of greater than 80% were used for further studies.

### ***2.2.6 Hepatocyte Incubations***

Hepatocyte incubations were carried out immediately after cell isolation. Cells were resuspended and diluted to  $2 \times 10^6$  per ml in Krebs-Henseleit buffer (pH 7.4) containing 0.21 g/l sodium bicarbonate and supplemented with 1% BSA and 10 mM glucose. Cell suspensions for all hepatocyte incubations were pre-warmed for 5 min in a 37°C shaking water bath before initiation of incubations. For the uptake studies, 10 $\mu$ M

propranolol, 10  $\mu$ M losartan, or 10  $\mu$ M eprosartan with and without 10  $\mu$ M rifampin or 100  $\mu$ M MK571, was added to the cell suspensions in the 37°C water bath. At 2 min, the reactions were terminated by transferring  $1 \times 10^6$  hepatocytes into a centrifuge tube containing 700  $\mu$ l of a mixture of silicone oil and mineral oil (Shitara et al., 2003) and centrifuged at 13,000g for 10 seconds. After removing the buffer layer and the oil layer, each cell pellet was resuspended in 100  $\mu$ l of water and sonicated for 15 min to ensure a thorough cell lysis. This was followed by adding 200  $\mu$ l of ACN containing IS and spinning at 13,000g for 15 min to precipitate protein. The supernatant was then transferred into a HPLC vial (Hewlett Packard, Palo Alto, CA) for LC-MS/MS analysis. The same experimental conditions were used with radiolabeled compound, and uremic toxins. Intracellular radioactivity was measured with a liquid scintillation counter (LS6000TA; Beckman Coulter, Fullerton, CA).

For the time course studies, 15 ml of hepatocytes ( $2 \times 10^6$  cells/ml) were warmed in a 50 ml flask and shaken in a 37°C water bath for 5 min. Each study was initiated by concomitantly adding 1  $\mu$ M propranolol, 1  $\mu$ M losartan, or 10  $\mu$ M eprosartan with DMSO (control), 10  $\mu$ M rifampin, 1  $\mu$ M sulfaphenazole, or 30  $\mu$ M of quinidine. Sulfaphenazole was used as the CYP2C9 inhibitor for losartan, and quinidine was used as the CYP2D6 inhibitor for propranolol. The drug concentrations were chosen based on the maximum plasma concentration ( $C_{\max}$ ) reported in human clinical studies. Rifampin was used as the OATP inhibitor. At 5, 10, 15, 20, 30, 45, and 60 min, 0.5 ml of cells were transferred into a centrifuge tube containing 700  $\mu$ l of a mixture of silicone oil and mineral oil and centrifuged at 13,000g for 10 s to stop the reaction. Ten seconds later, 1 ml of cells was transferred into a glass tube containing MTBE and IS followed by

vortexing to stop the reaction. Sample preparation for the intracellular measurement of propranolol and losartan was the same as for the uptake studies. Sample preparation for the measurement of propranolol and losartan metabolism was the same as that for the inhibition studies.

For evaluating the inhibition of metabolism, propranolol or losartan was co-incubated with 30  $\mu\text{M}$  quinidine or 1  $\mu\text{M}$  sulfaphenazole and a 0.5ml sample was taken at 5, 10, 15, 20, 30, 45, and 60 minutes. The reaction was stopped by transferring 1 ml of cells to a fresh glass tube containing MTBE and IS followed by vortexing. All samples were spun down at 2000g for 10 min. After quick-freezing the aqueous layer in a methanol/dry ice bath, the organic layer was poured into a new tube and evaporated under nitrogen gas. Each sample was reconstituted with 300  $\mu\text{l}$  of ACN/water (50/50, v/v) for LC/MS-MS analysis of the parent compound and its primary metabolite.

### ***2.2.7 LC/MS-MS Measurement of Propranolol, Losartan, and Eprosartan and their Metabolites***

A PESCIEX triple quadrupole instrument (PESCIEX API4000) was used with electro spray-positive ionization mode. The multiple reaction monitor was set at transitions 260.1-116.0  $m/z$  for propranolol, 276.0-116.0  $m/z$  for 4-OH-propranolol, 423.0-207.0  $m/z$  for losartan, 473.0-235.0  $m/z$  for losartan metabolite (EXP3174), 425.1-207.3  $m/z$  for eprosartan, and 309.1-251.1  $m/z$  for warfarin.

The ionspray voltage was set at 5500 and the temperature at 450°C. The collision energy was set at 27eV for propranolol, 25eV for 4-OH-propranolol and EXP3174, 50eV for losartan, and 35eV for eprosartan. An analytical Waters Symmetry Column C18 (2.1x50mm, 5 $\mu\text{m}$  particle size; Symmetry Columns, Milford, Massachusetts) was used in

a Shimadzu liquid chromatography system. The mobile phase for propranolol and 4-OH-propranolol consisted of 70% methanol:30% water containing 0.1% formic acid. For losartan and EXP3174, the mobile phase consisted of 55% acetonitrile:45% water containing 0.2% of formic acid. Finally, for eprosartan, the mobile phase consisted of 28% acetonitrile:72% water containing 0.5% of formic acid. Twenty microliter aliquots were injected, and the flow rate was set at 0.3 ml/min into the mass system.

### **2.2.8 Data/Statistical Analysis**

ANOVA statistical analysis was used to compare the differences between groups, a difference was considered statistically significant different if  $p < 0.05$ . Area under the concentration-time curve (AUC) for parent and metabolite was determined from time 0 to  $t_{last}$  by the trapezoidal rule.

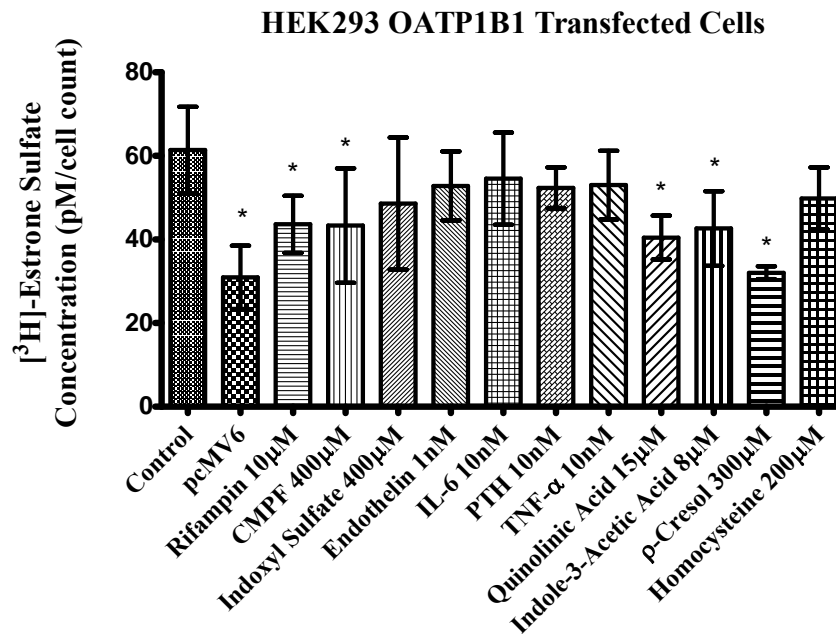
## **2.3 Results**

### **2.3.1 Effect of Uremic Toxins on Uptake of [<sup>3</sup>H]-Estrone Sulfate into Transfected Cells and Rat Hepatocytes.**

To determine the effect of uremic toxins on drug transport we carried out uptake studies on transfected cells systems and rat hepatocytes, using [<sup>3</sup>H]-estrone sulfate (4.3nM) as a prototypical substrate for uptake transporters. Rifampin (10 $\mu$ M), a potent inhibitor of OATPs was used as a positive control. MK-571 (100 $\mu$ M), an inhibitor of OATP and MRPs, was also used as a positive control. Uptake assays of [<sup>3</sup>H]-estrone sulfate in transient transfection of HEK293 cells with OATP1B1 and OATP2B1 showed that the uptake of the radiolabeled compound was decreased with the OATP inhibitor

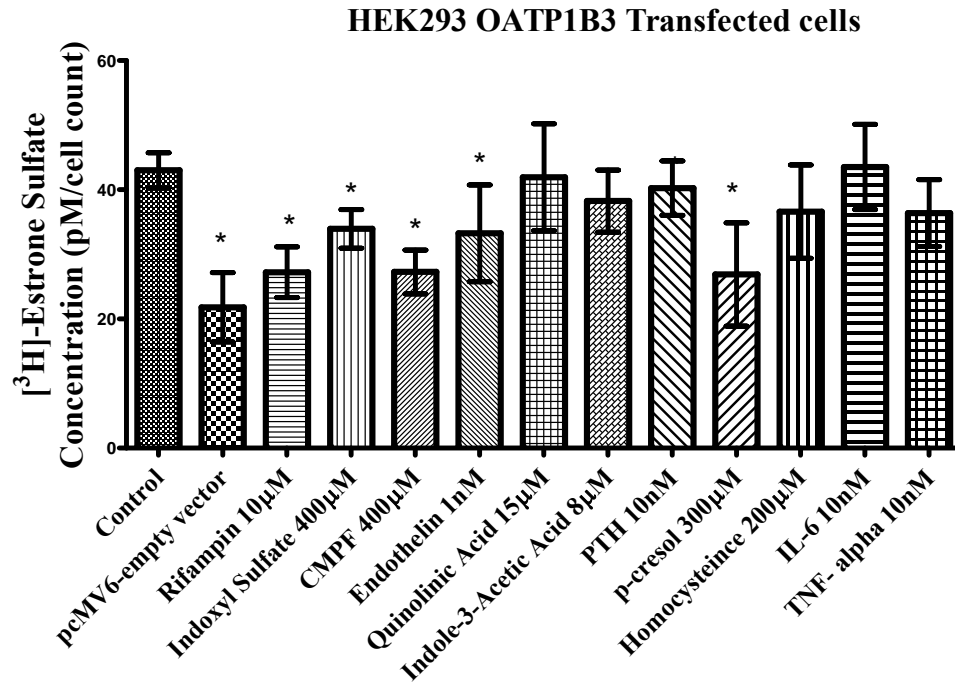


rifampin, and with some of the uremic toxins. The uptake of [<sup>3</sup>H]-estrone sulfate in OATP1B1 transfected cells (Figure 2.1a) was decreased (p<0.05) on average by 50% in the pcMV6 empty vector transfected cells, and by 30%, 30%, 34%, 30%, and 48% in the presence of rifampin (10 μM), CMPF (400 μM), quinolinic acid (15 μM), indole-3-acetic acid (8 μM), and *p*-cresol (300μM), respectively.



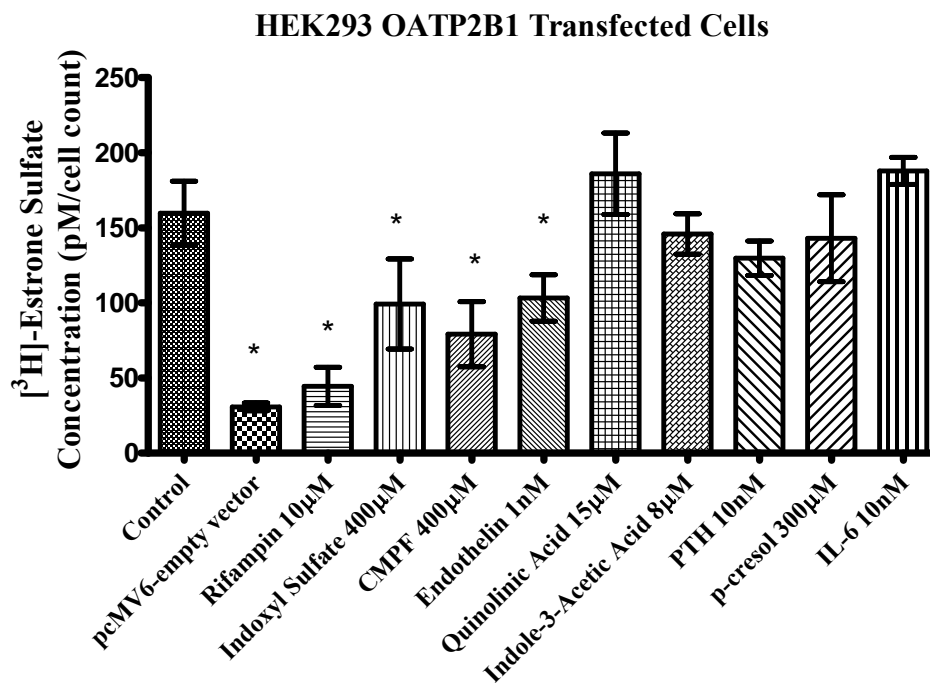
**Figure 0.1a:** Uptake of [<sup>3</sup>H]- estrone sulfate in OATP1B1 and empty vector control transfected cells in the presence of uremic toxins. \* p<0.05, n=6

The uptake of [<sup>3</sup>H]-estrone sulfate in OATP1B3 (Figure 2.1b) transfected cells was decreased (p<0.05) on average by 50% in pcMV6 empty vector control, by 37%, 21%, 37%, 23%, and 37% in the presence of rifampin (10μM), indoxyl sulfate(400μM), CMPF (400μM), endothelin (1nM), and *p*-cresol (300μM), respectively.



**Figure 2.1b:** Uptake of [<sup>3</sup>H]- estrone sulfate in OATP1B3 and empty vector control transfected cells in the presence of uremic toxins. \* p<0.05 n=6

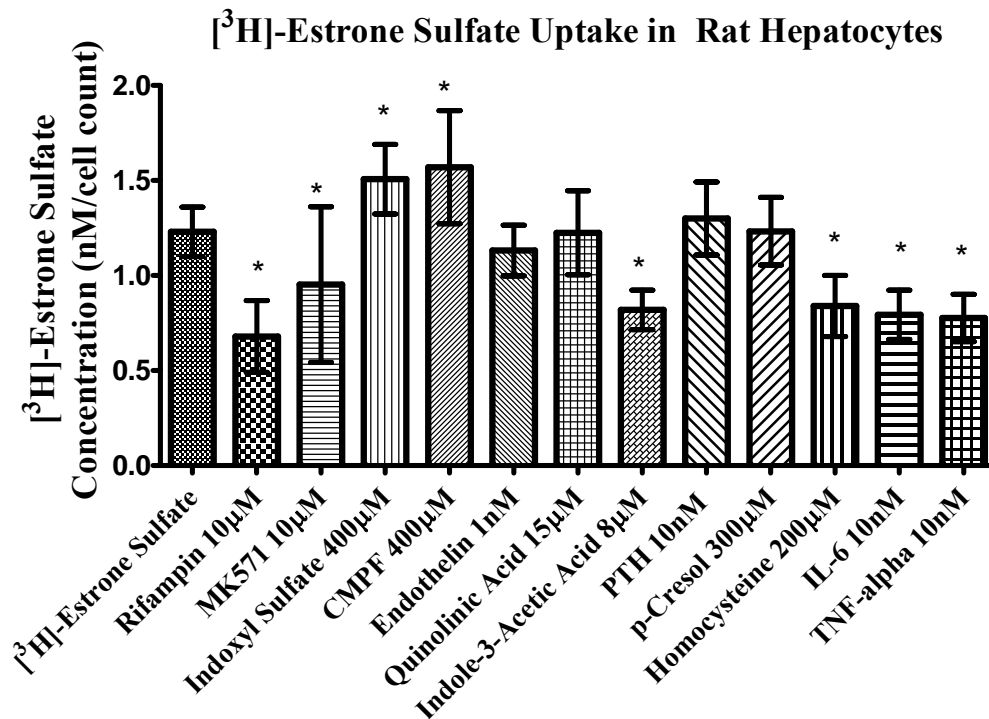
The uptake of [<sup>3</sup>H]-estrone sulfate in OATP2B1 (Figure 2.1c) transfected cells was decreased (p<0.05) on average by 80% in pcMV6 empty vector control, by 72%, 38%, 50%, and 36%, in the presence of rifampin (10µM), indoxyl sulfate(400µM), CMPF (400µM), and endothelin (1nM), respectively.



**Figure 2.1c:** Uptake of [<sup>3</sup>H]- estrone sulfate in OATP2B1 and empty vector control transfected cells in the presence of uremic toxins. \* p<0.05 n=2

In contrast, the uptake of [<sup>3</sup>H]-estrone sulfate (Figure 2.2) in rat hepatocytes exhibited both an increase and a decrease in uptake in the presence of uremic toxins. Rifampin and MK-571 were used as positive controls for inhibition of uptake transporters. There was on average a 45%, 23%, 33%, 32%, 36%, and 37% decrease (p<0.05) in uptake in the presence rifampin, MK-571, indole-3-acetic acid, homocysteine, interleukin 6 (IL-6), TNF-alpha, respectively, and there was 21% and 30% increase (p<0.05) in uptake in the presence of indoxyl sulfate and CMPF. The decrease in uptake in the presence of uremic toxins indicates that uptake transporters are being inhibited by some uremic toxins as was seen in the transfected cells. The increase in uptake of [<sup>3</sup>H]-estrone sulfate in the presence of some uremic toxins suggests that efflux

transporters may also be significantly inhibited by uremic toxins since in the transfected cells indoxyl sulfate and CMPF caused increased uptake.

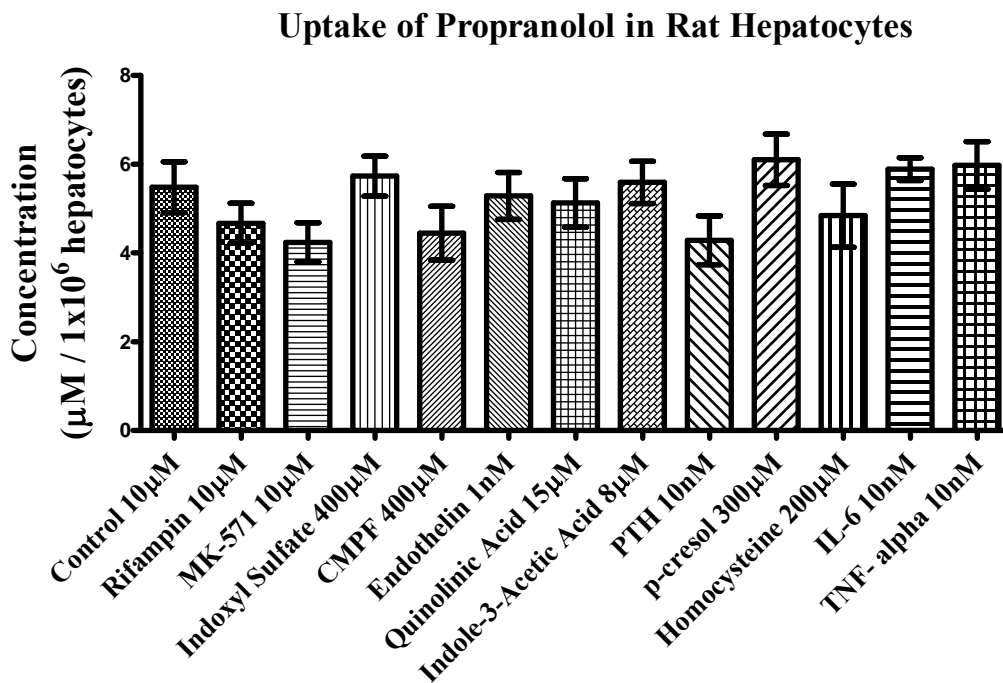


**Figure 0.2:** Uptake of [<sup>3</sup>H]-estrone sulfate in rat hepatocytes in the presence of uremic toxins. \*p<0.05, n=6

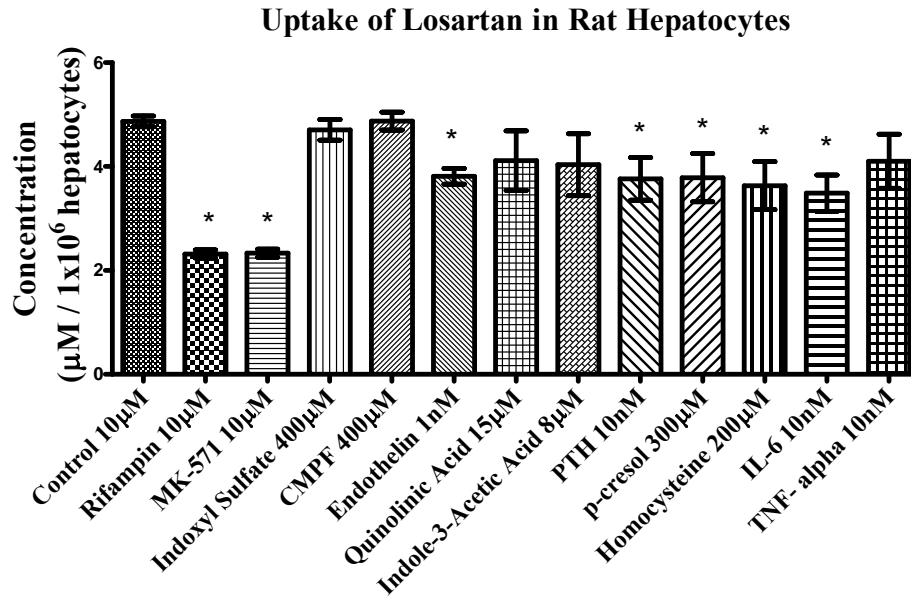
### 2.3.2 Uptake of BDDCS Drugs by Rat Hepatocytes in the Presence of Uremic Toxins

Using a prototypical substrate such as [<sup>3</sup>H]-estrone sulfate allowed us to investigate the possible involvement of drug transporters and the effects of uremic toxins. Next, we investigated the effects of uremic toxins on different BDDCS drugs. We used propranolol (10 µM) as our Class 1 drug (highly soluble, highly permeable, and extensively metabolized), losartan (10 µM) as our Class 2 drug (low solubility, high permeability, and extensively metabolized), and eprosartan (10 µM) as our Class 4 drug (high solubility, low permeability, and poorly metabolized). The uptake of propranolol in

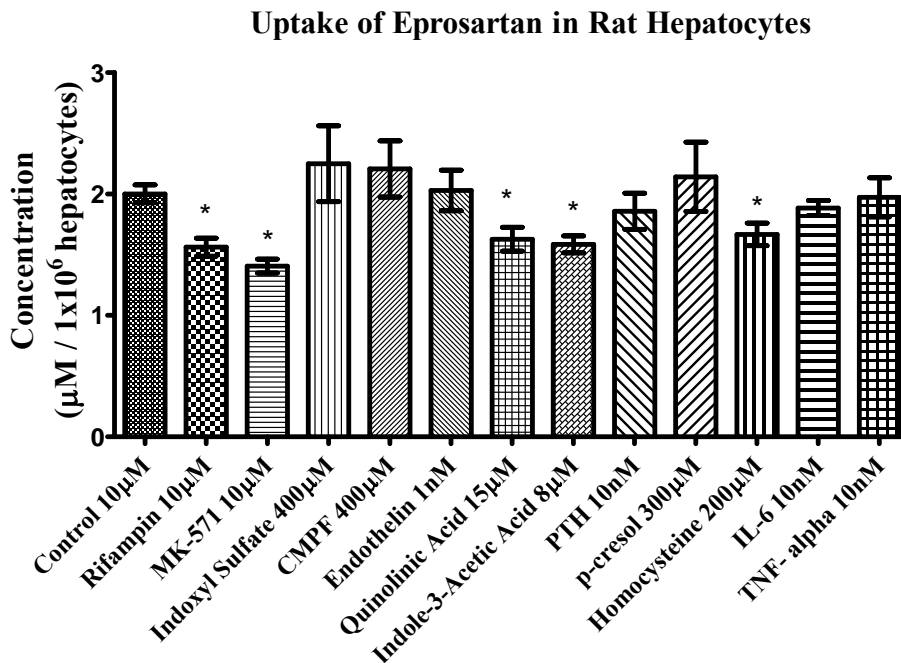
rat hepatocytes (Figure 2.3a) was not significantly changed with any of the uremic toxins or with the known uptake inhibitors rifampin and MK-571. The uptake of losartan 10 $\mu$ M (Figure 2.3b) was inhibited ( $p < 0.05$ ) by 52%, 48%, 22%, 23%, 22%, 26%, and 28% in the presence of rifampin, MK-571, endothelin, para thyroid hormone (PTH), *p*-cresol, homocysteine, and IL-6, respectively. This indicates that losartan is a substrate for uptake transporters since rifampin and MK-571 inhibited the uptake of losartan and the uremic toxins also had inhibitory effects. Lastly, the uptake of eprosartan (Figure 2.3c) was also shown to be inhibited by ( $p < 0.05$ ) 22%, 30%, 19%, 21%, and 17% in the presence of rifampin, MK-571, quinolinic acid, indole-3-acetic acid, and homocysteine, respectively. This suggests that the uptake of eprosartan is also mediated by uptake drug transporters expressed in hepatocytes.



**Figure 2.3a:** Uptake of propranolol 10 $\mu$ M in rat hepatocytes in the presence of uremic toxins or uptake transporter inhibitors. \* $p < 0.05$ ,  $n = 4$ .



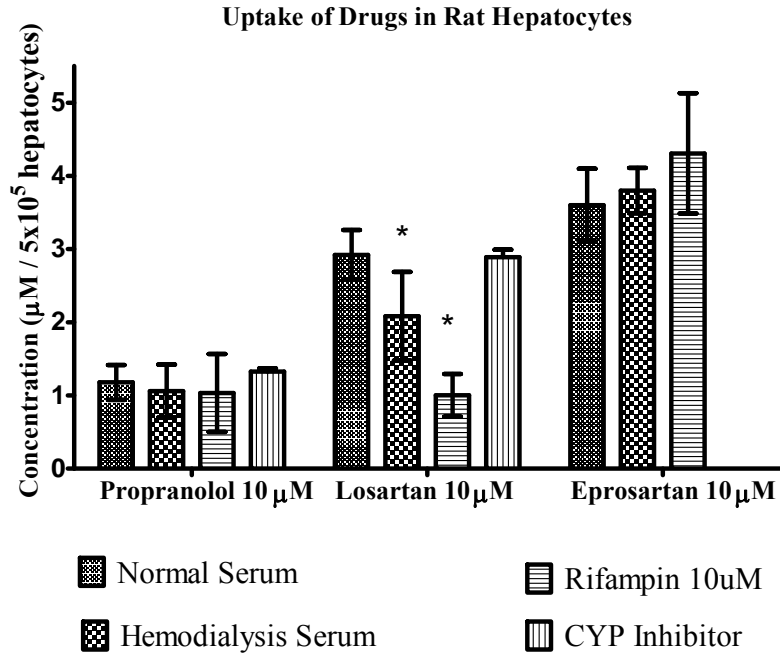
**Figure 2.3b:** Uptake of losartan 10 μM in rat hepatocytes in the presence of uremic toxins or uptake transporter inhibitors. \*p<0.05, n=4.



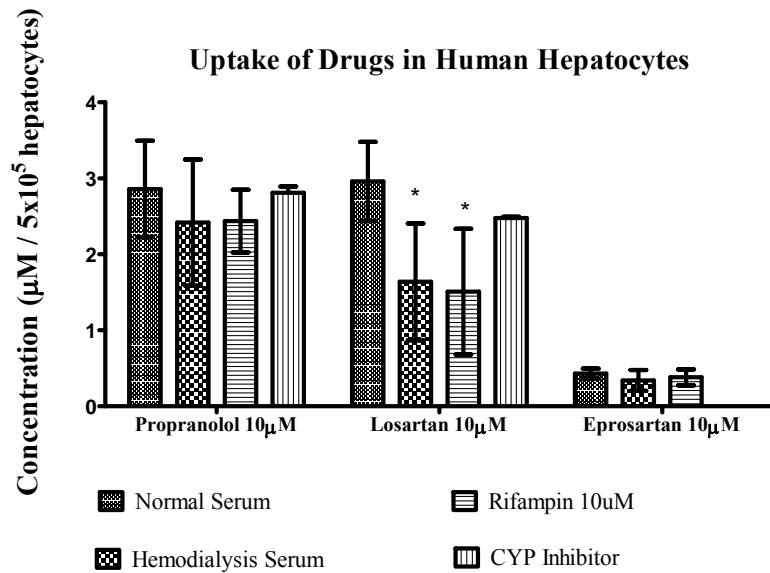
**Figure 2.3c:** Uptake of eprosartan 10 μM in rat hepatocytes in the presence of uremic toxins or uptake transporter inhibitors. \*p<0.05, n=4.

### 2.3.3 *Effects of Hemodialysis Serum on Drug Uptake*

Serum from patients on hemodialysis was obtained from the outpatient hemodialysis unit at UCSF to carry out *in vitro* uptake studies, to determine the collective effects of uremic toxins on the uptake of the three BDDCS drugs. Propranolol (10 $\mu$ M), losartan (10 $\mu$ M), and eprosartan (10 $\mu$ M) were assayed for their uptake into rat hepatocytes (Figure 2.4a) in the presence of normal serum, hemodialysis serum or normal serum plus rifampin (10 $\mu$ M) and the CYP inhibitors quinidine (30 $\mu$ M) for propranolol and sulfaphenazole (1 $\mu$ M) for losartan. There were no significant differences in uptake of propranolol in the presence of hemodialysis serum or rifampin, which agrees with our *in vitro* studies of the uptake of propranolol in the presence of uremic toxins. The uptake of losartan was decreased by 28% and 66% in the presence of HD serum and rifampin respectively, compared to normal serum. There were also no significant differences in the uptake of eprosartan in the presence of HD serum or rifampin. The same experiment was repeated using cryopreserved human hepatocytes (Figure 2.4b). We observed the same results, with no significant changes in uptake of propranolol or eprosartan in the presence of HD serum or rifampin, but the uptake of losartan was decreased ( $p < 0.05$ ) by 48% and 55% in the presence of HD serum and rifampin, respectively. However, the uptake of eprosartan in human hepatocytes was much lower than that observed in rat hepatocytes, while the opposite result was seen with propranolol. These studies indicated that the uremic toxins contained in the HD serum do have an effect on uptake of a Class 2 drug, therefore we investigated the effects of HD serum in longer time-course studies to determine the effects it may have in metabolism and uptake over an hour.



**Figure 2.4a:** Uptake of propranolol 10µM, losartan 10µM, or eprosartan 10µM in rat hepatocytes in the presence of normal serum, hemodialysis serum, rifampin 10µM, or CYP inhibitor. \*p<0.05, n=4.



**Figure 2.4b:** Uptake of propranolol 10µM, losartan 10µM, or eprosartan 10µM in human hepatocytes in the presence of normal serum, hemodialysis serum, rifampin 10µM, or CYP inhibitor. \*p<0.05, n=4.



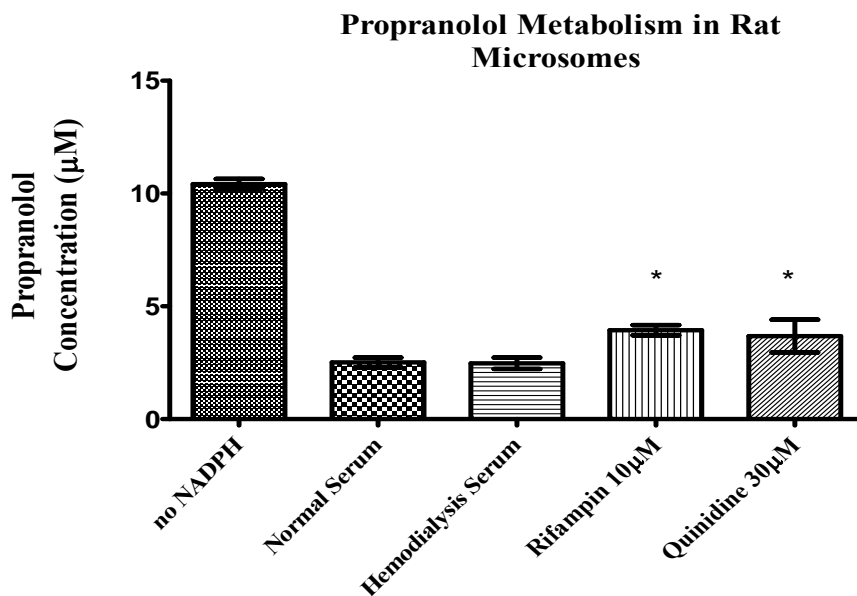
#### **2.3.4 Time-course Studies in Rat Hepatocytes and Microsome Studies**

Time-course studies were carried out using freshly isolated rat hepatocytes over an hour for propranolol (10 $\mu$ M), losartan (1 $\mu$ M), or eprosartan (10 $\mu$ M). These concentrations were chosen since they are representative of the plasma concentrations in humans. We analyzed the total drug concentration over time, the intracellular drug concentration, and the total metabolite concentration over time. The intracellular metabolite concentrations were below the limit of quantitation. Quinidine (30 $\mu$ M) was used as a CYP2D6 inhibitor for propranolol and sulfaphenazole (1 $\mu$ M) was used as a CYP2C9 inhibitor for losartan. For the total concentration of propranolol over time, we saw no significant change in parent drug in the presence of rifampin or HD serum. We observed a 37% increase in AUC (area under the curve) in the quinidine treated time course samples vs. those treated with normal serum (Table 2.1). The intracellular AUC of propranolol in hepatocytes (Table 2.1) increased over time by 243% in the quinidine treated group. The metabolite AUC (Table 2.1) did not change significantly in any of the groups. The microsomes studies showed that rifampin and quinidine both inhibit metabolism in rat microsomes (Figure 2.5a), whereas only quinidine inhibited propranolol metabolism in human microsomes (Figure 2.5b).

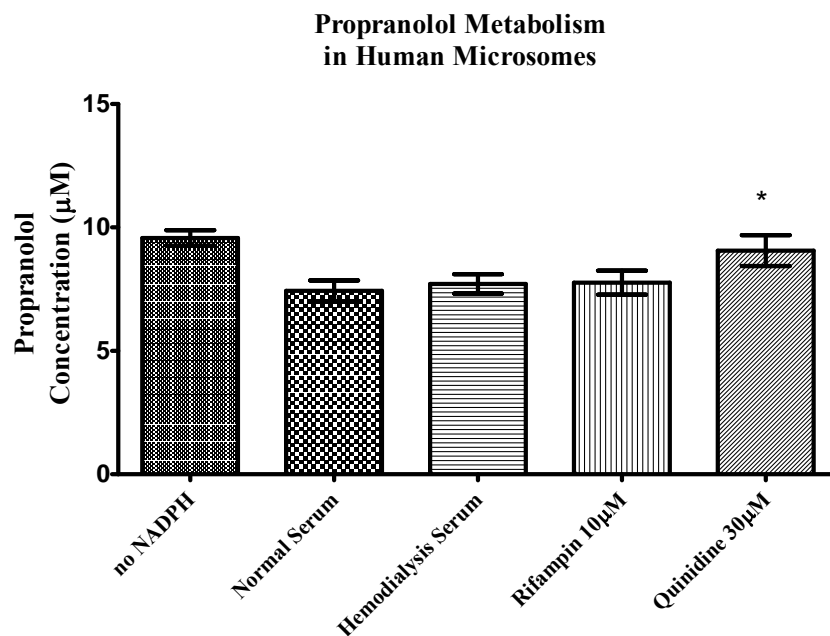
**Table 2.1:** Propranolol and 4-OH-propranolol AUC 0→60<sub>min</sub> for time-course studies and /fold increase for significant changes vs. normal serum in rat hepatocytes. \*p<0.05, n=4.

<b>Propranolol 10μM</b>	<b>Normal Serum</b>	<b>Hemodialysis Serum</b>	<b>Rifampin 10μM</b>	<b>Quinidine 30μM</b>
<b>AUC of</b>				
<b>Cumulative Drug (μM*min)</b>	<b>410 ± 38</b>	<b>403 ± 87</b>	<b>421 ± 39</b>	<b>*561 ± 18 /1.37</b>
<b>AUC of</b>				
<b>Intracellular Drug (μM*min)</b>	<b>2.67 ± 1.14</b>	<b>3.48 ± 2.49</b>	<b>3.20 ± 0.46</b>	<b>*6.51±0.44 /2.44</b>
<b>AUC of</b>				
<b>Cumulative Metabolite (μM*min)</b>	<b>31.9 ± 17</b>	<b>40.3 ± 26.0</b>	<b>36.1 ± 24.0</b>	<b>31.0 ± 10.0</b>

Results shown as average ± SD, /ratio compared to normal serum, \* p<0.05 by ANOVA



**Figure 2.5a:** Metabolism of propranolol 10µM in pooled rat microsomes in the presence of normal serum, hemodialysis serum, uptake transporter inhibitor, or CYP inhibitor. \*p<0.05, n=4.



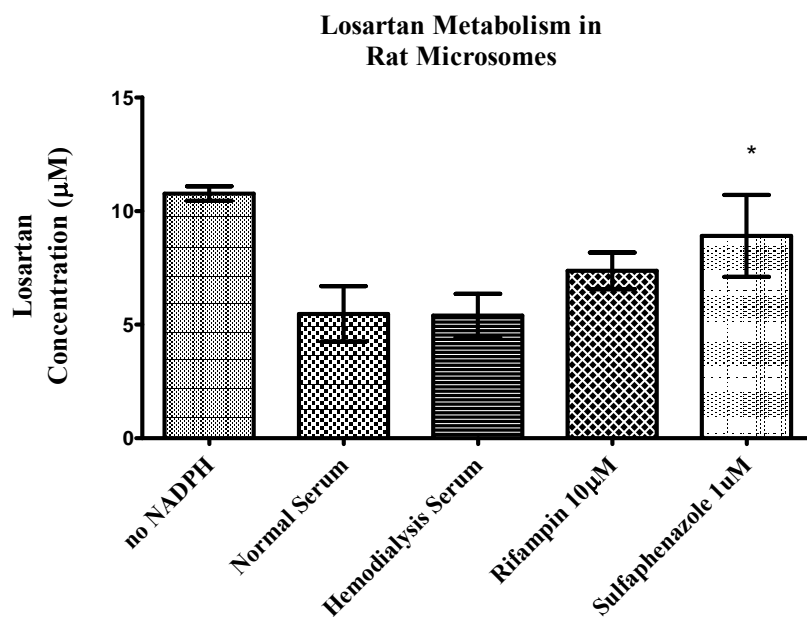
**Figure 2.5b:** Metabolism of propranolol 10µM in pooled human microsomes in the presence of normal serum, hemodialysis serum, uptake transporter inhibitor, or CYP inhibitor. \*p<0.05, n=4.

The losartan time-course study showed a 27%, 65%, and 68% increase ( $p<0.05$ ) in AUC in the presence of hemodialysis serum, rifampin, and sulfaphenazole, respectively (Table 2.2). The intracellular AUC of losartan (Table 2.2) decreased ( $p<0.05$ ) by 26%, 47%, and 33% in the presence of HD serum, rifampin and sulfaphenazole, respectively. The metabolite AUC (Table 2.2) decreased ( $p<0.05$ ) 41% and 26% in the presence of rifampin, and sulfaphenazole. The rat microsome studies showed a decrease in metabolism in the presence of sulfaphenazole (Figure 2.6a), i.e. significant increase in parent drug. Sulfaphenazole is reported to be a strong inhibitor of CYP2C9. In contrast, human microsome studies showed no inhibition of metabolism of losartan in the presence of sulfaphenazole at 1 $\mu$ M and 100 $\mu$ M (Figure 2.6b).

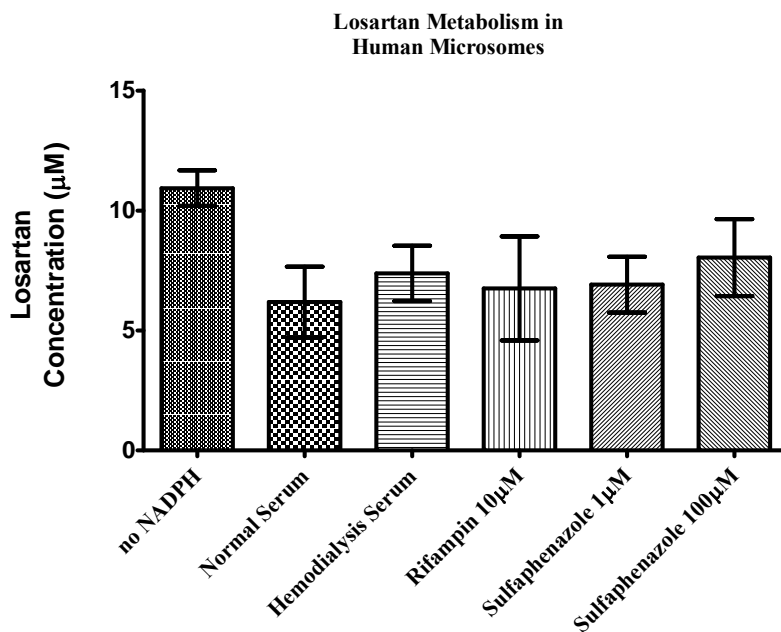
**Table 2.2:** Losartan and EXP3174 AUC 0 $\rightarrow$ 60<sub>min</sub> for time-course studies and /fold increase or decrease for significant changes vs. normal serum in rat hepatocytes, n=4.

<b>Losartan 1<math>\mu</math>M</b>	<b>Normal Serum</b>	<b>Hemodialysis Serum</b>	<b>Rifampin 10<math>\mu</math>M</b>	<b>Sulfaphenazole 1<math>\mu</math>M</b>
AUC of Cumulative Drug ( $\mu$ M*min)	<b>32.1 <math>\pm</math> 9.4</b>	<b>*40.8 <math>\pm</math> 6.2</b> /1.27	<b>*53.0 <math>\pm</math> 18.7</b> /1.65	<b>*54.0 <math>\pm</math> 19.8</b> /1.68
AUC of Intracellular Drug ( $\mu$ M*min)	<b>44.6 <math>\pm</math> 17.1</b>	<b>*33.1 <math>\pm</math> 20.4</b> /0.74	<b>*23.7 <math>\pm</math> 14.1</b> /0.53	<b>*30.1 <math>\pm</math> 20.9</b> /0.67
AUC of Cumulative Metabolite ( $\mu$ M*min)	<b>1.47 <math>\pm</math> 0.97</b>	<b>1.66<math>\pm</math>1.05</b>	<b>*0.87 <math>\pm</math> 0.47</b> /0.59	<b>*1.09 <math>\pm</math> 0.66</b> /0.74

Results shown as average  $\pm$  SD, /ratio compared to normal serum, \*  $p<0.05$  by ANOVA, n=3



**Figure 2.6a:** Metabolism of losartan 10 µM in pooled rat microsomes in the presence of normal serum, hemodialysis serum, uptake transporter inhibitor, or CYP inhibitor. \*p<0.05 versus normal serum, n=4.



**Figure 2.6b:** Metabolism of losartan 10 µM in pooled human microsomes in the presence of normal serum, hemodialysis serum, uptake transporter inhibitor, or CYP inhibitor. \*p<0.05 versus normal serum, n=4.

The eprosartan time-course study showed no change in cumulative AUC of drug in the presence of hemodialysis serum and rifampin (Table 2.3), which corresponds to the drug not undergoing metabolism. The intracellular AUC increased by 91% in the presence of hemodialysis serum (Table 2.3). This indicates that eprosartan was taken up into the cell either actively and/or passively, but once inside the cell, it did not readily exit the cell in the presence of HD serum.

**Table 2.3:** Eprosartan AUC 0→60<sub>min</sub> for time-course studies and /fold increase for significant changes vs. normal serum in rat hepatocytes. \*p<0.05, n=4.

<b>Eprosartan 10μM</b>	<b>Normal Serum</b>	<b>Hemodialysis Serum</b>	<b>Rifampin 10μM</b>
<b>AUC of Cumulative Drug (μM*min)</b>	<b>575 ± 32</b>	<b>615 ± 60</b>	<b>572 ± 37</b>
<b>AUC of Intracellular Drug (μM*min)</b>	<b>59.7 ± 18.2</b>	<b>*114 ± 23 /1.91</b>	<b>74.7 ± 27.2</b>

Results shown as average ± SD, /ratio compared to normal serum, \* p<0.05 by ANOVA

## 2.4 Discussion

Hepatic drug transporters have been widely recognized to play a significant role in the disposition of drugs that undergo hepatic metabolism and elimination (Yamazaki et al., 1996; Shugarts and Benet, 2009). These transporters are known to transport endogenous substances such as hormones and other compounds that are classified as cations and anions. In the last 15 years transporters have been studied for their role in transporting xenobiotics. In the liver, uptake transporters facilitate the drug's transport from the basolateral side of the cell membrane (the blood side) into the hepatocyte, and efflux transporters facilitate the exit of the drug from inside the cell back into the blood through a basolateral efflux transporter or into the bile through an apical (canalicular) efflux transporter. Studies have shown that these transporters play a role in drug-drug interactions and also affect the metabolism of drugs by altering the access of the drug to the enzyme (Smith et al., 2005; Lu et al., 2006; Shugarts and Benet, 2009; Zamek- Gliszczynski et al., 2009; Zheng et al., 2009). Polymorphisms in these transporters have been shown to be of clinical relevance, by which alterations in the transporter expression or function due to mutations can lead to a clinically significant change in drug pharmacokinetics or drug response (Kivisto and Niemi, 2007; Oswald et al., 2008; Degorter and Kim, 2009). These transporters are of clinical relevance due to their effects on drug pharmacokinetics, drug pharmacodynamics, and drug-drug interactions.

The role of drug transporters in disease, such as chronic kidney disease or renal failure, has not been investigated extensively. Studies show that in patients with chronic kidney disease there are pharmacokinetic changes of non-renally eliminated drugs (Gonzalez-Martin et al., 1992; Martin et al., 1998; Nolin, 2008; Hilger et al., 2009; Nolin

et al., 2009; Small et al., 2009). Studies have investigated the effect of uremic toxins on drug metabolism, and it has been reported that some uremic toxins inhibit some CYP450 metabolizing enzymes but not others (Leblond et al., 2001; Guevin et al., 2002; Dreisbach and Lertora, 2003; Michaud et al., 2008; Nolin, 2008). Since drug transporters have a significant effect on drug disposition, we investigated whether the uremic toxins and HD serum from patients with chronic kidney disease have an effect on drug transporters. Furthermore, we investigated whether different drugs would be differentially affected by uremic toxins depending on the drug's properties that would likely require them to be transported by a hepatic drug transporter. In the BDDCS classification system, the drug's solubility, permeability and extent of metabolism, predicts whether a transporter could have a significant effect on drug disposition. In these studies, we showed that some uremic toxins have an inhibitory effect on the prototypical OATP substrate [<sup>3</sup>H]-estrone sulfate, in cells transfected with OATP1B1 and OATP1B3. Although pc-MV6 transfected cells still show transport of these substrates, this can be explained by endogenous expression of drug transporters in these cell lines. We also showed the inhibition of uptake of radiolabeled substrates as well as the BDDCS drugs by uremic toxins on uptakes studies in rat hepatocytes. Interestingly, in rat hepatocytes, some uremic toxins caused increased uptake into hepatocytes. This may indicate that uremic toxins not only have an effect on uptake transporters but also on efflux transporters and this can affect drug disposition for drugs that are substrates for efflux transporters (basolateral or apical). Uptake studies were carried out using the drugs propranolol, losartan, and eprosartan, in the presence of uremic toxins in rat hepatocytes. In these studies we found no significant inhibition or increase of uptake for



propranolol in the presence of uremic toxins or rifampin. Since propranolol is a Class 1 drug, highly soluble and permeable, BDDCS predicts that transporters do not play a significant role in the drug's intestinal and hepatic disposition. Hence, if uremic toxins are having an effect on hepatic transporters, this would not affect the uptake of propranolol, which is consistent with our results. On the other hand, the uptakes of losartan and eprosartan were inhibited by some uremic toxins as well as rifampin (OATP inhibitor). This suggests that both are substrates for uptake transporters and that uremic toxins have an inhibitory effect on these transporters.

In these studies, uremic toxins were tested separately, however; since some uremic toxins had an effect on one transporter but not other, we obtained human HD serum from patients on hemodialysis to account for *in vivo* protein binding of the uremic toxins and for any synergistic effects due to uremic toxins. When we tested propranolol, losartan, and eprosartan in the presence of HD serum in rat hepatocytes, we observed a significant decrease in losartan uptake in the presence of HD serum and rifampin. However we did not see this inhibitory effect on the uptake of propranolol or eprosartan. We repeated the experiment using human cryopreserved hepatocytes and observed the same results. We predicted that there would be no inhibitory effect in the uptake of the Class 1 drug propranolol in the presence of HD serum; however, we had expected to see an inhibitory effect in the uptake of eprosartan. These results may be due to the fact that eprosartan might be a substrate for both basolateral uptake and efflux transporters, or a substrate for efflux transporters both basolateral and/or apical, as indicated by the time-course studies, and further studies are necessary to understand this result.

In the hepatocyte time-course studies of the Class 1 drug, propranolol, the cumulative propranolol AUC increased only in the quinidine treated group compared to the normal serum group. This was due to the inhibition in metabolism by quinidine, which would increase the parent compound/metabolite ratio and increase the parent AUC. Also, the AUC of intracellular propranolol increased only in the quinidine treated group, but there were no changes in the cumulative AUC of 4-OH-propranolol. In the quinidine treated groups, the increase in intracellular propranolol is due to the decrease in metabolism as indicated by the rat and human microsome studies. The rat and human hepatocyte 2 min uptake studies indicate that HD serum, rifampin, or quinidine, have no effect in the uptake of propranolol (Figure 2.4a,b). In the quinidine treated group, we suspect that we did not see a change in metabolite formation due to the increase in intracellular parent compound (secondary to the inhibition of metabolism by quinidine). The increase in the amount of intracellular propranolol apparently was sufficient to produce enough metabolite and not cause a significant difference in metabolite AUC compared to the normal serum treated group. Also, 4-OH propranolol undergoes phase II metabolism by glucuronidation, which is another explanation on why we did not observe a significant difference in the metabolite AUC. As the metabolite was formed, it was subject to further metabolism by phase II enzymes, which could explain no significant difference in metabolite AUC. These findings are evidence that in the presence of hemodialysis serum or rifampin there is no change in uptake or metabolism of propranolol.

In the time-course studies of the class 2 drug, losartan, the AUC of cumulative losartan was higher in the presence of hemodialysis serum, rifampin, and sulfaphenazole

compared to the normal serum group. This suggests that the metabolism in those groups was decreased either due to a decrease in CYP450 activity or decrease in uptake of the drug, which would lead to less drug being exposed to the enzyme for metabolism. The rat microsome studies showed that there was no change in metabolism in the presence of HD serum or rifampin, but there was inhibition of metabolism in the presence of sulfaphenazole. Also, the intracellular AUC of losartan decreased in the presence of HD serum, rifampin, and sulfaphenazole. These observations suggest that HD serum decreases the uptake of losartan into the hepatocytes but does not affect the metabolism of losartan. Also, sulfaphenazole affects only the metabolism and not the uptake of losartan (as indicated by the rat and human hepatocyte 2 min uptake results in the presence of sulfaphenazole, Figure 2.4a,b). The AUC of the cumulative metabolite showed that there was a decrease in metabolite in the presence of rifampin and sulfaphenazole. However there was no change in metabolite formation in the presence of hemodialysis serum. The rat microsome studies indicate a slight inhibition (but not statistically significant) of metabolism of losartan in the presence of rifampin. The decrease in metabolite in the rifampin treated group is due to the inhibition of uptake, which in turn will expose less of the drug to the metabolizing enzymes and decrease the metabolite formation. In the sulfaphenazole treated group, the decrease in metabolite formation is a direct effect of metabolism inhibition but not uptake. The HD treated group showed no significant change in the metabolite formation, although there was a significant decrease in uptake.

For the time course studies of the Class 4 drug, eprosartan, the cumulative AUC of the drug in all groups remained the same since this drug is not metabolized. The

intracellular AUC of eprosartan increased in the presence of hemodialysis serum. This indicates that eprosartan was transported into the cell or passively diffused into the cell, but once inside the cell it was unable to be effluxed, leading us to believe that efflux transporters are being inhibited by hemodialysis serum and not allowing the drug to readily exit the cell.

## 2.5 Conclusion

We have demonstrated that certain uremic toxins decreased the uptake of the prototypical radiolabeled substrate [<sup>3</sup>H]-estrone sulfate into transfected cells, which indicated that uremic toxins can inhibit uptake transporters. The studies with hemodialysis serum and BDDCS drugs showed that, in fact, for a class 1 drug there was no difference in uptake or metabolism in cell or hepatocyte studies. For the class 2 drug we observed a decrease in uptake in the cell and hepatocyte studies as predicted, and no difference in metabolism. In contrast, for the class 4 drug, we did not observe a difference in the uptake in cell or hepatocytes, which is contrary to our hypothesis. However, for the class 4 drug, we observed an increase in the intracellular concentration in the hepatocyte time-course studies, suggesting that efflux rather than uptake transporters were affected by the hemodialysis serum and this could be important for drugs that are substrate for efflux transporters. These studies suggest that BDDCS is a tool that can be used to predict the effects of CKD on drug disposition for different classes of drugs *in vitro*. However, for class 3 or 4 drugs, since the absorptive transporters effects predominate, but may be modulated by efflux transporters, assays should be carried out

for uptake and efflux transporters to better predict the effects of CKD in this class of drugs. The significant changes found in the *in vitro* studies are evidence that this change may have clinical relevance, and *in vivo* studies should be carried out to further investigate the consequences.

New recommendations from the Food and Drug Administration suggest a full pharmacokinetic study for new drugs in chronic kidney disease patients, even when renal excretion is minimal (Zhang et al., 2009). Being able to predict based on BDDCS class if a drug will be affected by uremic toxins can be useful in deciding what studies may be necessary for a new drug with respect to various patient populations.

## 2.6 References

- Brenner BM and Rector FC (2008) *Brenner & Rector's The Kidney*. Saunders Elsevier, Philadelphia.
- Degorter MK and Kim RB (2009) Hepatic drug transporters, old and new: pharmacogenomics, drug response, and clinical relevance. *Hepatology* **50**:1014-1016.
- Deguchi T, Ohtsuki S, Otagiri M, Takanaga H, Asaba H, Mori S and Terasaki T (2002) Major role of organic anion transporter 3 in the transport of indoxyl sulfate in the kidney. *Kidney Int* **61**:1760-1768.
- Dreisbach AW and Lertora JJ (2003) The effect of chronic renal failure on hepatic drug metabolism and drug disposition. *Semin Dial* **16**:45-50.
- Flynn C, BA H and Reed G (2010) Losartan is a substrate of organic anion transporting polypeptide 2B1. *FASEB Meeting abstract, #758.2*. Anaheim , California. April24-28.
- Gonzalez-Martin G, Thambo S, Paulos C, Vasquez I and Paredes J (1992) The pharmacokinetics of nifurtimox in chronic renal failure. *Eur J Clin Pharmacol* **42**:671-673.
- Guevin C, Michaud J, Naud J, Leblond FA and Pichette V (2002) Down-regulation of hepatic cytochrome P450 in chronic renal failure: role of uremic mediators. *Br J Pharmacol* **137**:1039-1046.
- Hilger RA, Richly H, Grubert M, Kredtke S, Thyssen D, Eberhardt W, Hense J, Schuler M and Scheulen ME (2009) Pharmacokinetics of sorafenib in patients with renal impairment undergoing hemodialysis. *Int J Clin Pharmacol Ther* **47**:61-64.

- Kivisto KT and Niemi M (2007) Influence of drug transporter polymorphisms on pravastatin pharmacokinetics in humans. *Pharm Res* **24**:239-247.
- Lam JL, Okochi H, Huang Y and Benet LZ (2006) In vitro and in vivo correlation of hepatic transporter effects on erythromycin metabolism: characterizing the importance of transporter-enzyme interplay. *Drug Metab Dispos* **34**:1336-1344.
- Lau YY, Okochi H, Huang Y and Benet LZ (2006) Multiple transporters affect the disposition of atorvastatin and its two active hydroxy metabolites: application of in vitro and ex situ systems. *J Pharmacol Exp Ther* **316**:762-771.
- Lau YY, Wu CY, Okochi H and Benet LZ (2004) Ex situ inhibition of hepatic uptake and efflux significantly changes metabolism: hepatic enzyme-transporter interplay. *J Pharmacol Exp Ther* **308**:1040-1045.
- Leblond F, Guevin C, Demers C, Pellerin I, Gascon-Barre M and Pichette V (2001) Downregulation of hepatic cytochrome P450 in chronic renal failure. *J Am Soc Nephrol* **12**:326-332.
- Lu C, Gallegos R, Li P, Xia CQ, Pusalkar S, Uttamsingh V, Nix D, Miwa GT and Gan LS (2006) Investigation of drug-drug interaction potential of bortezomib in vivo in female Sprague-Dawley rats and in vitro in human liver microsomes. *Drug Metab Dispos* **34**:702-708.
- Martin DE, Chapelsky MC, Ilson B, Tenero D, Boike SC, Zariffa N and Jorkasky DK (1998) Pharmacokinetics and protein binding of eprosartan in healthy volunteers and in patients with varying degrees of renal impairment. *J Clin Pharmacol* **38**:129-137.

- Michaud J, Dube P, Naud J, Leblond FA, Desbiens K, Bonnardeaux A and Pichette V (2005) Effects of serum from patients with chronic renal failure on rat hepatic cytochrome P450. *Br J Pharmacol* **144**:1067-1077.
- Michaud J, Nolin TD, Naud J, Dani M, Lafrance JP, Leblond FA, Himmelfarb J and Pichette V (2008) Effect of hemodialysis on hepatic cytochrome P450 functional expression. *J Pharmacol Sci* **108**:157-163.
- Nolin TD (2008) Altered nonrenal drug clearance in ESRD. *Curr Opin Nephrol Hypertens* **17**:555-559.
- Nolin TD, Frye RF, Le P, Sadr H, Naud J, Leblond FA, Pichette V and Himmelfarb J (2009) ESRD impairs nonrenal clearance of fexofenadine but not midazolam. *J Am Soc Nephrol* **20**:2269-2276.
- Oswald S, Konig J, Lutjohann D, Giessmann T, Kroemer HK, Rimmbach C, Roskopf D, Fromm MF and Siegmund W (2008) Disposition of ezetimibe is influenced by polymorphisms of the hepatic uptake carrier OATP1B1. *Pharmacogenet Genomics* **18**:559-568.
- Salphati L and Benet LZ (1999) Metabolism of digoxin and digoxigenin digitoxosides in rat liver microsomes: involvement of cytochrome P4503A. *Xenobiotica* **29**:171-185.
- Shitara Y, Li AP, Kato Y, Lu C, Ito K, Itoh T and Sugiyama Y (2003) Function of uptake transporters for taurocholate and estradiol 17beta-D-glucuronide in cryopreserved human hepatocytes. *Drug Metab Pharmacokinet* **18**:33-41.
- Shugarts S and Benet LZ (2009) The role of transporters in the pharmacokinetics of orally administered drugs. *Pharm Res* **26**:2039-2054.



- Small DS, Wrishko RE, Ernest CS, 2nd, Ni L, Winters KJ, Farid NA, Li YG, Brandt JT, Salazar DE, Borel AG, Kles KA and Payne CD (2009) Prasugrel pharmacokinetics and pharmacodynamics in subjects with moderate renal impairment and end-stage renal disease. *J Clin Pharm Ther* **34**:585-594.
- Smith NF, Figg WD and Sparreboom A (2005) Role of the liver-specific transporters OATP1B1 and OATP1B3 in governing drug elimination. *Expert Opin Drug Metab Toxicol* **1**:429-445.
- Sun H, Frassetto LA, Huang Y and Benet LZ (2010) Hepatic clearance, but not gut availability, of erythromycin is altered in patients with end-stage renal disease. *Clin Pharmacol Ther* **87**:465-472 .
- Sun H, Huang Y, Frassetto L and Benet LZ (2004) Effects of uremic toxins on hepatic uptake and metabolism of erythromycin. *Drug Metab Dispos* **32**:1239-1246.
- Sun H, Huang Y, Okochi H, Frassetto L and Benet LZ (2005) Uremic toxins inhibit hepatic uptake of eprosartan. (Abstract PD-6) *Clin Pharmacol Ther* **77**:P2.
- Tsujimoto M, Kinoshita Y, Hirata S, Otagiri M, Ohtani H and Sawada Y (2008) Effects of uremic serum and uremic toxins on hepatic uptake of digoxin. *Ther Drug Monit* **30**:576-582.
- Vanholder R, De Smet R, Glorieux G, Argiles A, Baurmeister U, Brunet P, Clark W, Cohen G, De Deyn PP, Deppisch R, Descamps-Latscha B, Henle T, Jorres A, Lemke HD, Massy ZA, Passlick-Deetjen J, Rodriguez M, Stegmayr B, Stenvinkel P, Tetta C, Wanner C and Zidek W (2003) Review on uremic toxins: classification, concentration, and interindividual variability. *Kidney Int* **63**:1934-1943.

- Wu CY and Benet LZ (2005) Predicting drug disposition via application of BCS: transport/absorption/ elimination interplay and development of a biopharmaceutics drug disposition classification system. *Pharm Res* **22**:11-23.
- Yamazaki M, Suzuki H and Sugiyama Y (1996) Recent advances in carrier-mediated hepatic uptake and biliary excretion of xenobiotics. *Pharm Res* **13**:497-513.
- Zamek-Glisczynski MJ, Kalvass JC, Pollack GM and Brouwer KL (2009) Relationship between drug/metabolite exposure and impairment of excretory transport function. *Drug Metab Dispos* **37**:386-390.
- Zhang Y, Zhang L, Abraham S, Apparaju S, Wu TC, Strong JM, Xiao S, Atkinson AJ, Jr., Thummel KE, Leeder JS, Lee C, Burckart GJ, Lesko LJ and Huang SM (2009) Assessment of the impact of renal impairment on systemic exposure of new molecular entities: evaluation of recent new drug applications. *Clin Pharmacol Ther* **85**:305-311.
- Zheng HX, Huang Y, Frassetto LA and Benet LZ (2009) Elucidating rifampin's inducing and inhibiting effects on glyburide pharmacokinetics and blood glucose in healthy volunteers: unmasking the differential effects of enzyme induction and transporter inhibition for a drug and its primary metabolite. *Clin Pharmacol Ther* **85**:78-85.

### **Chapter 3: Bidirectional studies in MDR1-MDCK, MDCK, cMOAT-MDCKII, and MDCKII Cells: *effects of uremic toxins and human hemodialysis serum on BDDCS drugs and model substrates***

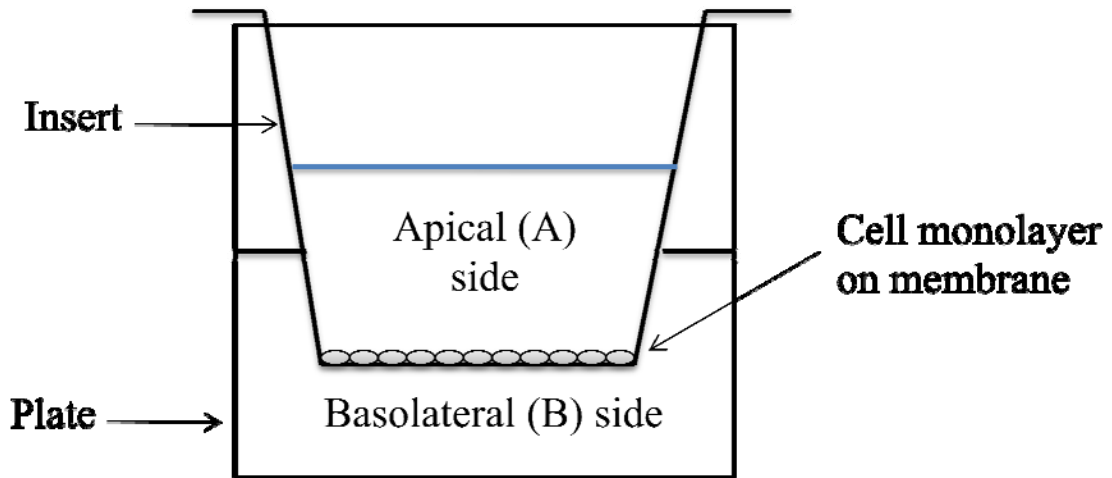
#### **Summary**

Efflux transporters play an important role in drug disposition and can play an important role in drug-drug interactions. In these studies we investigated the effect of uremic toxins and human hemodialysis (HD) serum on the transport of model substrates as well as BDDCS drugs in transfected cell systems. Bidirectional studies were carried out in MDR1-MDCK, MDCK, cMOAT-MDCKII, and MDCKII cells to determine the permeability of [<sup>3</sup>H]-vinblastine, [<sup>3</sup>H]-pravastatin in the presence of uremic toxins and of propranolol and losartan in the presence of human HD serum. The results showed that in the presence of CMPF, indoxyl sulfate, and endothelin, the net flux of [<sup>3</sup>H]-vinblastine and [<sup>3</sup>H]-pravastatin was reduced by 50% or more compared to cells exposed to Hank's buffer with no toxins. The bidirectional studies also showed that HD serum had no effect on the net flux of propranolol, and the net flux of losartan was increased in the presence of HD serum. The losartan results suggest that uptake and efflux transporters are involved in its transport, and that HD serum can inhibit both uptake and efflux transporters as shown by the inhibition of net flux of [<sup>3</sup>H]-vinblastine in the presence of uremic toxins. In conclusion, uremic toxins have inhibitory effects on efflux transporters, and decrease the net flux of prototypical radiolabeled compounds. Also, since the Class 1

drug propranolol does not require a transporter, the presence of uremic toxins does not affect the net flux of this drug.

### **3.1 Introduction**

Madin-Darby Canine Kidney (MDCK) epithelial cells and colon carcinoma (Caco-2) cells are widely used for permeability studies and efflux transporter studies. Caco-2 cells are the main model system to study gut permeability in vitro by measuring the permeability of a compound across a monolayer of cells. MDCK and Caco-2 cells have also been extensively used to measure the permeability of drugs mediated by efflux transporters by over expressing efflux transporters in these cell lines. These cells are ideal systems since they form a monolayer with tight junctions when plated, which allow for measurement of movement of compounds across the cells instead of paracellular transport. The permeability is measured by conducting bidirectional studies using a transwell system (Figure 3.1), in which the drug or drug plus transporter inhibitor is dosed in either the apical or basolateral compartment, and the samples are taken from the opposite compartment at different time points.



**Figure 3.1:** Diagram depicting cell monolayer cultured in transwell insert and plate. Apical (A) side and basolateral (B) side contain buffer with or without drug and inhibitor.

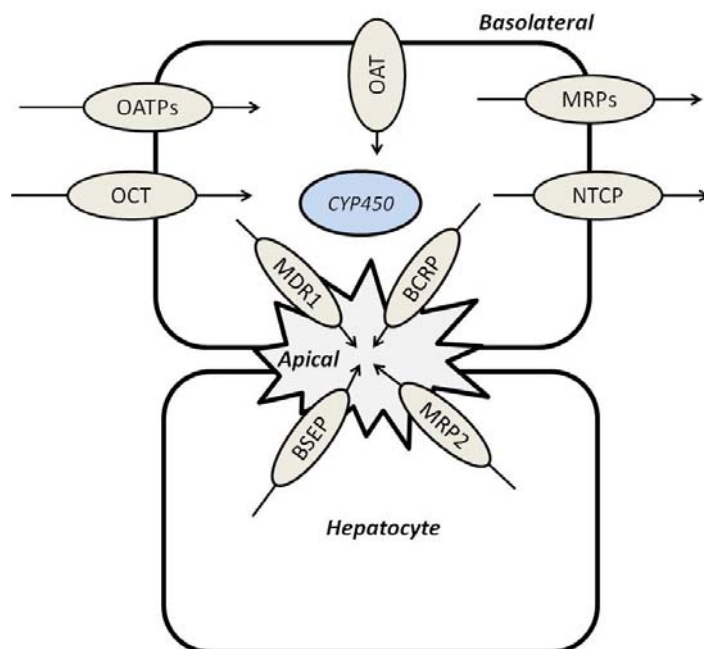
The apparent permeability ( $P_{app}$ ) of a compound is calculated by the equation\

$$P_{app} = \frac{dQ/dt}{S \cdot (C_0 - C_t)}$$

where  $P_{app}$  = apparent permeability;  $S$  = membrane surface area,  $C_0$  = donor concentration at time 0, and  $dQ/dt$  = amount of drug transported per time. The net flux of a compounds is calculated by the ratio of the  $P_{app}(B-A)/P_{app}(A-B)$ , and compound is considered to have a transporter mediated efflux if the ratio is greater than 2.

Uptake and efflux transporters are expressed in the liver. The efflux transporters are located in both the basolateral and apical membranes (Figure 3.2). The efflux transporters expressed in the canalicular membrane of the hepatocyte include P-gp

(MDR1), MRP2 (cMOAT), BCRP, and BSEP. In the basolateral membrane, the efflux transporters expressed are MRP3, MRP4, MRP5 and NTCP.



**Figure 3.2:** Schematic of two hepatocytes joined by bile canalicular membrane. Efflux transporters depicted in the apical canalicular membrane and basolateral membrane. Uptake transporters depicted in the basolateral membrane.

Bidirectional studies using transfected cells over expressing efflux transporters have been well established in our laboratory (Flanagan and Benet, 1999; Cummins et al., 2002; Flanagan et al., 2002). These studies have shown the enzyme-transporter interplay and the involvement of efflux transporters in drug disposition *in vitro*. It has also been very well established that inhibition of efflux transporters can lead to clinically important effects in drug disposition (Tanigawara, 2000; Matheny et al., 2001; Chen et al., 2003;

Drescher et al., 2003). These studies have shown that since P-gp is expressed in liver, intestine, and brain; alterations of this transporter play a major role in drug pharmacokinetics and disposition.

Few studies have investigated the effects of chronic kidney disease on efflux transporters. Also, there have been only a couple of studies that investigated the expression of P-gp in chronic kidney disease rat models (Naud et al., 2007). A study by Huang et al. (2000) has shown that acute renal failure affects P-gp expression and function. In these studies, the serum from renal failure rats was used in Caco-2 assays to measure the activity of P-gp and its expression. Here they found that in the presence of the renal failure serum, the activity of P-gp was reduced regardless of the protein expression.

After investigating the effects of hemodialysis serum and uremic toxins in cells transfected with uptake transporters, we wanted to investigate if uremic toxins and/or hemodialysis serum also affected the transport of drugs in cells transfected with efflux transporters. We investigated the effects of uremic toxins on transport of [<sup>3</sup>H]-vinblastine, a substrate for P-gp in MDR1-MDCK cells and MDCK cells, and the transport of [<sup>3</sup>H]-pravastatin on cMOAT-MDCKII and MDCKII cells in the presence of selected uremic toxins that had shown some effect in the uptake studies. We also investigated the transport of propranolol and losartan in MDR1-MDCK and MDCK cells in the presence of normal serum, HD serum, or inhibitor. This allowed us to investigate if transporters in the canalicular membrane of the hepatocyte could be affected by the uremic toxins or uremic serum. The study of Sun et al. (2004) had reported that erythromycin was a

substrate for P-gp but there was no inhibition in transport of erythromycin with selected uremic toxins.

### **3.2 Materials/Methods**

Propranolol and HPLC-grade dimethyl sulfoxide, *tert*-butyl-methyl-ether (MTBE), and acetonitrile (ACN) were purchased from Sigma-Aldrich (St. Louis, MO). Losartan potassium salt was purchased from Toronto Research Chemicals (Ontario, Canada). GG918 (GF120918) was graciously donated by GlaxoSmithKline (Research Triangle Park, NC). Radiolabeled [<sup>3</sup>H]-vinblastine and [<sup>3</sup>H]-pravastatin were obtained from Perkin Elmer (Waltham, MA). Transwell inserts and poly-D-lysine coated 6-well plates were obtained from BD Biosciences (Bedford, MA). Cells were obtained from the UCSF cell culture facility. Approval for obtaining serum from hemodialysis patients and normal serum from healthy volunteers was obtained from the Committee on Human Research at UCSF. Serum from seven subjects in each group was pooled, to obtain pooled HD serum and pooled normal serum.

#### **3.2.1 Cell Culture**

MII-cMOAT and MII cells were cultured at 37°C and humidified 5% CO<sub>2</sub> atmosphere in Dulbecco's modified Eagle's medium supplemented with 10% heat-inactivated FBS, 100 U/ml penicillin, and 100 U/ml streptomycin. M-MDR1 cells were cultured in the same medium containing 80 mg/ml colchicine for selected growth of transfected cells (Pastan et al., 1988). Cells were grown to confluence in culture flasks, harvested and seeded into transwell inserts in six-well plates at a density of 1x10<sup>6</sup>



cells/insert. Studies were conducted 5 to 6 days post seeding for the two cell lines. Medium was changed once every 2 days and 24 h before the experiment.

### **3.2.2 Bidirectional Assay**

The transport experiments were adapted with modifications from Lau et al. (2006). In brief, plates with cell monolayer were pre-incubated in transport buffer (Hanks' balanced salt solution containing 25 mM HEPES and 1% FBS or 10% normal serum or 10% hemodialysis serum, pH 7.4) for 10 min at 37°C. Transepithelial electrical resistance was measured in each well using a Millicell ERS voltohmmeter (Millipore Corporation, Bedford, MA) to assess the integrity of monolayer. The average transepithelial electrical resistance values obtained from MII, MIIcMOAT, and M-MDR1 cells were  $170 \pm 15 \Omega \cdot \text{cm}^2$  ( $n = 12$ ),  $160 \pm 10 \Omega \cdot \text{cm}^2$  ( $n = 12$ ), and  $1780 \pm 20 \Omega \cdot \text{cm}^2$  ( $n = 12$ ), respectively. For measuring drug secretion (B)asolateral  $\rightarrow$  (A)apical, 2.5 ml of transport buffer containing 0.1  $\mu\text{M}$  [ $^3\text{H}$ ]-vinblastine, 10 nM [ $^3\text{H}$ ]-pravastatin, 10  $\mu\text{M}$  propranolol, or 10  $\mu\text{M}$  losartan was put into the B side and 1.5 ml of buffer was put into the A side. At the selected times (30min, 60min, and 90min or 120min), 150- $\mu\text{l}$  samples were taken from the A side and replaced with fresh buffer. For measuring drug absorption (A  $\rightarrow$  B), the drug solution was put into the A side and samples were taken from the B side. For inhibition studies, the inhibitors MK571 (10  $\mu\text{M}$ ) or GG918 (0.5  $\mu\text{M}$ ) were put into both the A and B sides. During the studies, the cells were incubated in a shaking incubator (Boekel Scientific, Feasterville, PA). After the last time point (1.5 h), the apical solutions were removed by suction and each filter was dipped twice in ice-cold PBS. Intracellular measurements of the drug were obtained by solubilizing the cells on each culture insert with 0.4 ml of ice-cold MeOH/H<sub>2</sub>O [7/3 (v/v)] and sonicating for 10

min. The samples were mixed with 150µl of ACN for protein precipitation and the homogenate was centrifuged for 5 min at 13,000g, and the resulting supernatant was analyzed by LC/MS-MS. For radiolabeled compounds, the samples were placed in scintillation vials and radioactivity determined by scintillation counting ((LS6000TA; Beckman Coulter, Fullerton, CA).

### **3.2.3 LC/MSMS Assays**

The concentration measurement of propranolol and losartan were performed by LC/MSMS as described in Chapter 2

### **3.2.4 Data Analysis**

The apparent permeability ( $P_{app}$ ) values were calculated as follows, where the rate of transport was measured from the flux of drug across the cells.

$$P_{app} = \frac{1}{S \times C_0} \times \left( \frac{dQ}{dt} \right)$$

$P_{app}$  = apparent permeability,  $S$  = membrane surface area,  $C_0$  = donor concentration at time 0, and  $dQ/dt$  = amount of drug transported per time. The net flux of a compounds is calculated by the ratio of the  $P_{app}(B-A)/P_{app}(A-B)$ . Value are expressed as mean  $\pm$  SD, n=3.

### 3.3 Results

#### 3.3.1 Bidirectional Studies: MDR1-MDCK and MDCK Cells

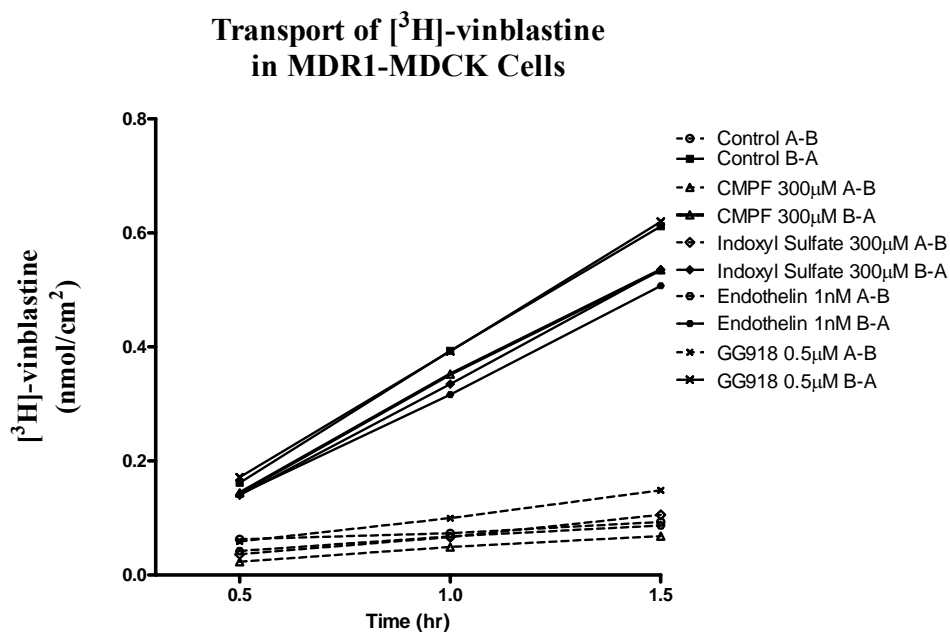
A series of bidirectional studies were carried out in MDR1(P-gp) over expressing cell lines, and the MDCK controls, as well as cMOAT (MRP2) overexpressing cell lines and MDCKII controls. The radiolabeled substrates [ $^3\text{H}$ ]-vinblastine for MDR1 and [ $^3\text{H}$ ]-pravastatin for cMOAT were used in the presence of uremic toxins to determine the effect of selected uremic toxins on the transport of known substrates for MDR1 and cMOAT. The bidirectional studies of [ $^3\text{H}$ ]-vinblastine showed that the net flux decreased from  $17.5 \pm 7.4$  in MDR1-MDCK cells with [ $^3\text{H}$ ]-vinblastine alone to  $8.76 \pm 1.14$ ,  $6.74 \pm 3.29$ ,  $8.26 \pm 0.97$  and  $5.03 \pm 0.72$  in the presence of CMPF, indoxyl sulfate, endothelin, and GG918, respectively (Table 3.2, Figure 3.3). However, the B $\rightarrow$ A apparent permeability or the A $\rightarrow$ B apparent permeability in the groups containing uremic toxins did not change significantly compared to the control group (Table 3.2); nonetheless, the difference in each direction was enough to cause a significant different in net flux.

The bidirectional study in the control cell line MDCK did not show this inhibitory effect in net flux by uremic toxins. The B $\rightarrow$ A apparent permeability did not show any significant changes, except in the presence of indoxyl sulfate and GG918. There were no significant changes in the A $\rightarrow$ B apparent permeability for any group. Other uremic toxins were tested but did not show a significant change in transport (data not shown). Furthermore, there was no significant change in intracellular accumulation from B $\rightarrow$ C (C inside the cell), or from A $\rightarrow$ C in the presence of uremic toxins (data not shown).

**Table 3.1:** Bidirectional study of [<sup>3</sup>H]-vinblastine in MDR1-MDCK and MDCK cells.

<b>Cell Type</b>	<b>Condition</b>	<b>P<sub>app</sub>(A→B)x10<sup>-6</sup> cm/sec</b>	<b>P<sub>app</sub>(B→A)x10<sup>-6</sup> cm/sec</b>	<b>Net Flux Ratio (B→A/A→B)</b>
<b>MDR1-MDCK</b>	[ <sup>3</sup> H]-Vin	0.20 ± 0.11	3.00 ± 0.14	17.5 ± 7.5
	[ <sup>3</sup> H]-Vin +CMPF	0.30 ± 0.037	2.61 ± 0.11	8.76 ± 1.14*
	[ <sup>3</sup> H]-Vin +Indoxyl Sulfate	0.46 ± 0.21	2.61 ± 0.42	6.74 ± 3.29*
	[ <sup>3</sup> H]-Vin + endothelin	0.30 ± 0.05	2.44 ± 0.11	8.26 ± 0.97*
	[ <sup>3</sup> H]-Vin +GG918	0.60 ± 0.04*	2.99 ± 0.20	5.03 ± 0.72*
<b>MDCK</b>	[ <sup>3</sup> H]-Vin	1.47 ± 0.35	2.80 ± 0.23	2.55 ± 0.52
	[ <sup>3</sup> H]-Vin +CMPF	1.24 ± 0.49	2.83 ± 0.23	2.46 ± 0.70
	[ <sup>3</sup> H]-Vin +Indoxyl Sulfate	1.21 ± 0.13	3.47 ± 0.06*	2.87 ± 0.29
	[ <sup>3</sup> H]-Vin+ endothelin	0.93 ± 0.25	2.70 ± 0.41	3.05 ± 0.83
	[ <sup>3</sup> H]-Vin +GG918	1.16 ± 0.37	1.32 ± 0.13*	1.24 ± 0.50

Values expressed as mean ± SD. \* p<0.05, n=3



**Figure 3.3:** Bidirectional study of [<sup>3</sup>H]-vinblastine in MDR1-MDCK cells. Control is Hanks buffer and [<sup>3</sup>H]-vinblastine, GG918 is P-gp inhibitor.

### 3.3.2 Bidirectional Studies: cMOAT-MDCKII and MDCKII cells

The bidirectional studies in cMOAT-MDCKII cells also showed a significant inhibition in the net flux of [<sup>3</sup>H]-pravastatin in the presence of the uremic toxin CMPF and indoxyl sulfate (Figure 3.4). The net flux decreased from  $7.78 \pm 0.74$  with [<sup>3</sup>H]-pravastatin alone to  $3.82 \pm 0.34$ ,  $4.85 \pm 0.86$ ,  $5.85 \pm 0.43$ , and  $1.19 \pm 0.037$  in the presence of CMPF, indoxyl sulfate, endothelin, and MK-571, respectively (Table 3.3). In the MDCKII cells, which do not overexpress cMOAT, there were no significant changes in net flux, except in the group exposed to CMPF (Table 3.3). There was no significant change in the B→A apparent permeability of [<sup>3</sup>H]-pravastatin in either cell line, except for the endothelin treated group in the cMOAT-MDCKII cells. The A→B did not change significantly except for the CMPF group in the MDCKII cells. Also, intracellular

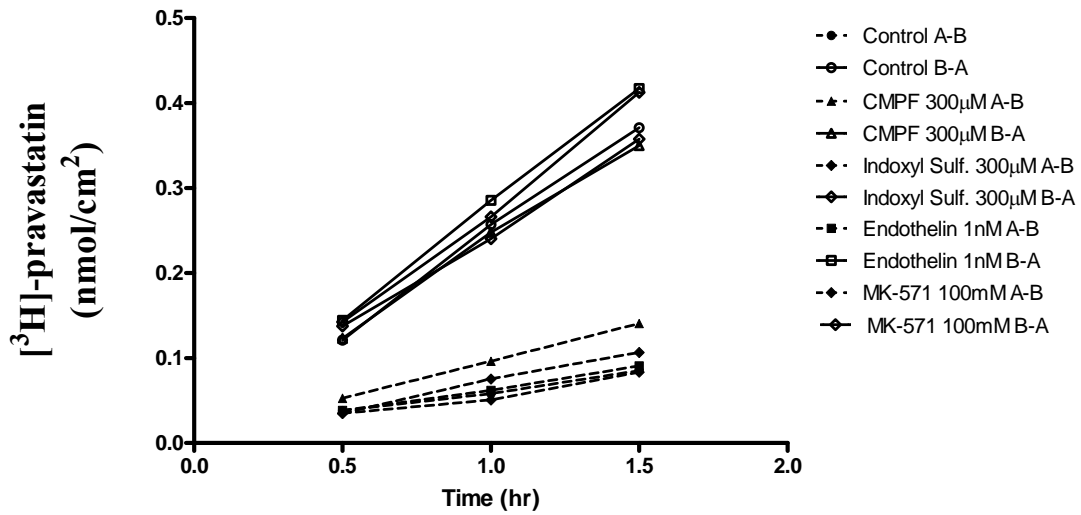
accumulation of [<sup>3</sup>H]-pravastatin from B→C and from A→C showed to be significantly decreased in the presence of uremic toxins, but increased back to control group levels in the MK-571 group (Figure 3.5).

**Table 3.2:** Bidirectional study of [<sup>3</sup>H]-pravastatin (Prav.) in cMOAT-MDCKII and MDCKII cells

<b>Cell Type</b>	<b>Condition</b>	<b>P<sub>app</sub>(A→B)x10<sup>-6</sup> cm/sec</b>	<b>P<sub>app</sub>(B→A)x10<sup>-6</sup> cm/sec</b>	<b>Net Flux Ratio (B→A/A→B)</b>
<b>cMOAT- MDCKII</b>	[ <sup>3</sup> H]- Prav.	1.17 ± 0.20	9.01 ± 0.81	7.78 ± 0.74
	[ <sup>3</sup> H]- Prav. +CMPF	2.10 ± 0.34	7.94 ± 0.67	3.82 ± 0.34*
	[ <sup>3</sup> H]- Prav. +Indoxyl Sulf.	1.58 ± 0.24	7.53 ± 0.67	4.85 ± 0.86*
	[ <sup>3</sup> H]- Prav. + endothelin	1.17 ± 0.19	6.82 ± 0.84*	5.85 ± 0.43*
	[ <sup>3</sup> H]- Prav.+MK- 571	6.07 ± 0.84*	7.72± 0.81	1.19 ± 0.037*
<b>MDCKII</b>	[ <sup>3</sup> H]-Prav.	1.31 ± 0.26	6.95 ± 1.00	5.46 ± 1.16
	[ <sup>3</sup> H]- Prav. +CMPF	2.44 ± 0.24*	6.30 ± 0.49	2.59 ± 0.36*
	[ <sup>3</sup> H]- Prav. +Indoxyl Sulf.	1.94 ± 0.47	6.26 ± 0.22	3.35 ± 0.84
	[ <sup>3</sup> H]- Prav. + endothelin	1.24 ± 0.32	5.81 ± 0.89	4.86 ± 1.28
	[ <sup>3</sup> H]- Prav. +MK- 571	1.99 ± 0.32	7.5± 0.72	3.87 ± 0.97

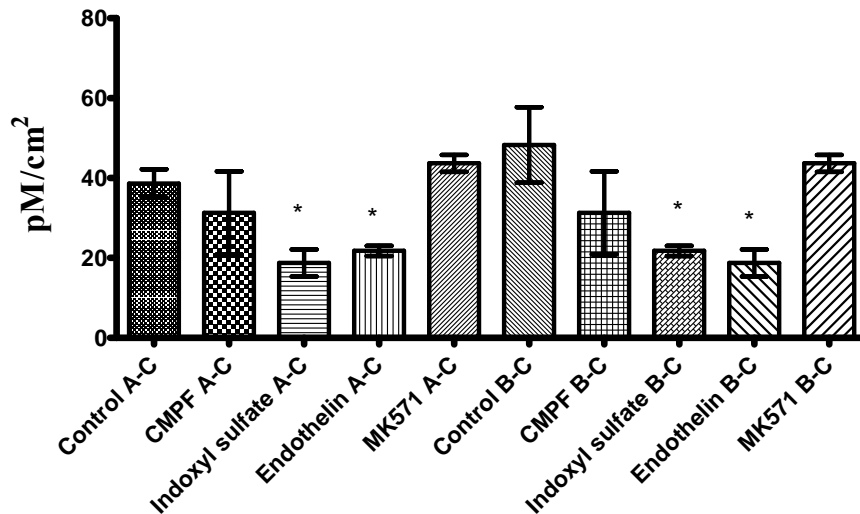
Values expressed as mean ± SD. \* p<0.05, n=3

### Transport of [<sup>3</sup>H]-pravastatin in cMOAT-MDCKII Cells



**Figure 3.4:** Bidirectional study of [<sup>3</sup>H]-pravastatin in cMOAT-MDCKII cells. Control is Hank's buffer plus [<sup>3</sup>H]-pravastatin. MK-571 is cMOAT inhibitor.

### Intracellular Concentration of [<sup>3</sup>H]-pravastatin in cMOAT-MDCKII Cells



**Figure 3.5:** Intracellular accumulation of [<sup>3</sup>H]-pravastatin in cMOAT-MDCKII cells. Control is Hank's buffer plus [<sup>3</sup>H]-pravastatin. MK-571 is cMOAT inhibitor. A-C is intracellular accumulation from apical (A) to intracellular (C), and B-C is accumulation from basolateral (B) to intracellular (C). \* p<0.05, n=3

### 3.3.3 Propranolol Bidirectional Studies: MDR1-MDCK and MDCK Cells

In the bidirectional studies of propranolol and losartan, we investigated the effect of hemodialysis serum (HD) on the transport of these drugs. The bidirectional studies of propranolol 10 $\mu$ M in MDR1-MDCK and MDCK cells showed that there was no inhibition of propranolol net flux or B $\rightarrow$ A transport in either cell line in the presence of HD serum (Figure 3.6). The apparent permeability of propranolol (Table 3.3) was also high compared to the permeability of vinblastine, pravastatin, or losartan (Table 3.4). Propranolol was not a substrate for MDR1 (P-gp) since the efflux ratios (net efflux) were not 2 or greater. For all samples, experimental and control in MDR1-MDCK cells as well as MDCK cells, the net flux ratio was below 2, and essentially 1.

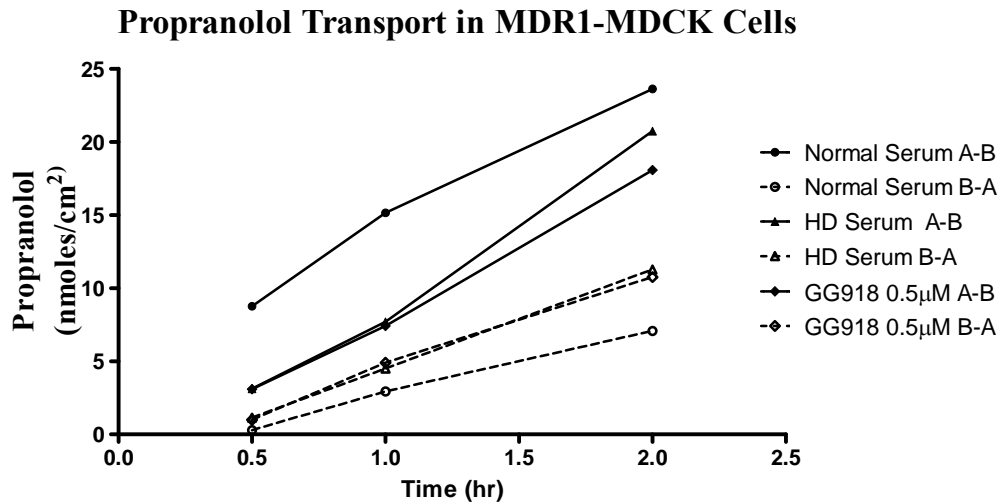
**Table 3.3:** Bidirectional study of 10 $\mu$ M propranolol in MDR1-MDCK and MDCK cells

Cell Type	Condition	$P_{app}(A \rightarrow B) \times 10^{-6}$ cm/sec	$P_{app}(B \rightarrow A) \times 10^{-6}$ cm/sec	Net Flux Ratio (B $\rightarrow$ A/A $\rightarrow$ B)
MDR1- MDCK	Propranolol + NS	26.3 $\pm$ 10.5	20.4 $\pm$ 0.7	0.89 $\pm$ 0.43
	Propranolol + HDS	36.8 $\pm$ 5.5	23.8 $\pm$ 2.6	0.66 $\pm$ 0.17
	Propranolol +GG918	32.5 $\pm$ 3.3	25.2 $\pm$ 4.8	0.77 $\pm$ 0.07
MDCK	Propranolol + NS	23.9 $\pm$ 4.2	23.1 $\pm$ 1.4	0.99 $\pm$ 0.21
	Propranolol + HDS	21.0 $\pm$ 1.1	22.6 $\pm$ 1.8	1.08 $\pm$ 0.03
	Propranolol +GG918	15.8 $\pm$ 6.6	17.9 $\pm$ 2.8*	1.36 $\pm$ 0.84

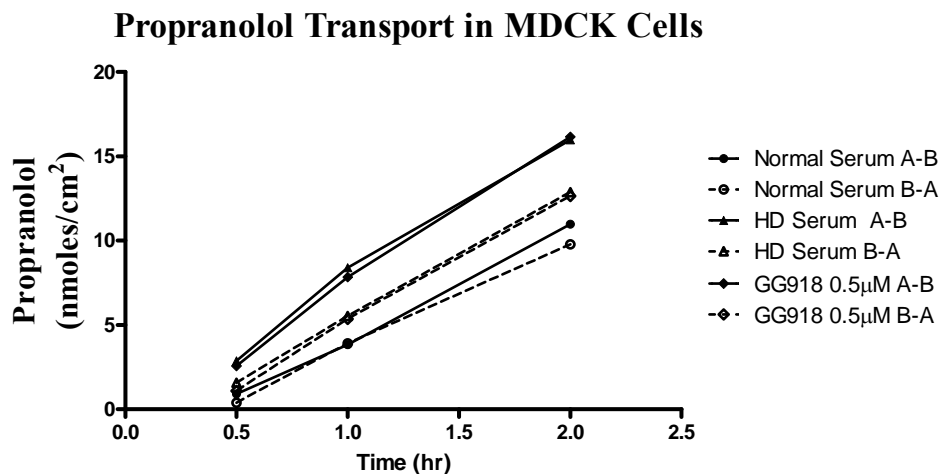
Values express as mean  $\pm$  SD. \* p<0.05, n=3  
NS: Normal serum    HDS: Hemodialysis serum



(a)



(b)



**Figure 3.6:** Bidirectional study of propranolol 10µM in MDR1-MDCK (a) cells and MDCK (b) cells.

### **3.3.4 Losartan Bidirectional Studies: MDR1-MDCK and MDCK Cells**

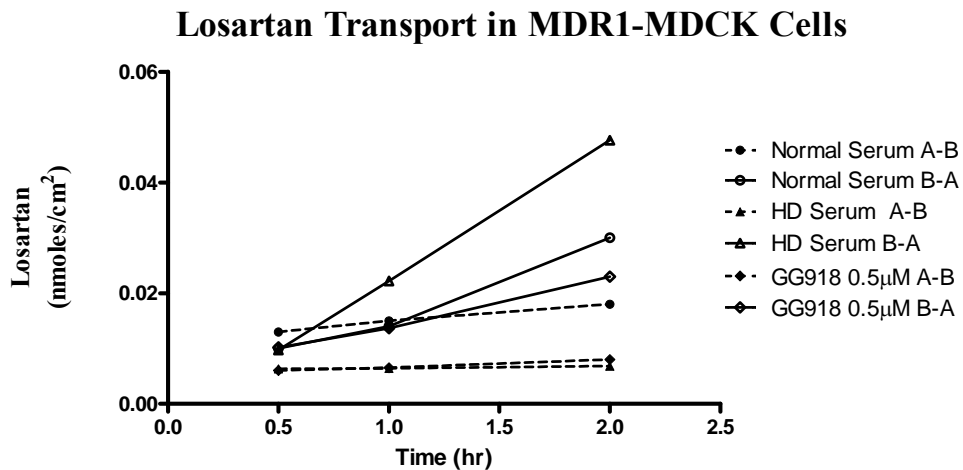
The losartan bidirectional studies in MDR1-MDCK and MDCK cell lines showed that losartan is a substrate for MDR1, as shown by the net flux ratio greater than two. Furthermore, in the presence of HD serum compared to normal serum, the net flux increased from  $6.69 \pm 1.82$  to  $83.9 \pm 35.2$ , and decreased to  $2.42 \pm 0.95$  in the presence of GG918 (Table 3.4). In the MDCK cell line (Figure 3.7b) similar results were observed with an increase in net flux in the presence of HD serum compared to control, from  $0.38 \pm 0.60$  to  $0.88 \pm 0.17$  and a decrease to  $0.23 \pm 0.08$  in the presence of GG918 (Figure 3.7, Table 3.4). On the other hand, when analyzing the apparent permeability rate of  $B \rightarrow A$  and  $A \rightarrow B$  in the MDR1-MDCK cells, the  $B \rightarrow A$  increased in the HD serum group and in the GG918 group; however, the  $A \rightarrow B$  permeability rate decreased in the HD serum group and increased in the GG918 group (Table 3.4). In the MDCK cells, the  $B \rightarrow A$  rate was only significantly different from the control in the GG918 treated group; the variation in the  $A \rightarrow B$  control group was large, and did not show significance when compared to other groups.

**Table 3.4:** Bidirectional study of 10 $\mu$ M losartan in MDR1-MDCK and MDCK cells

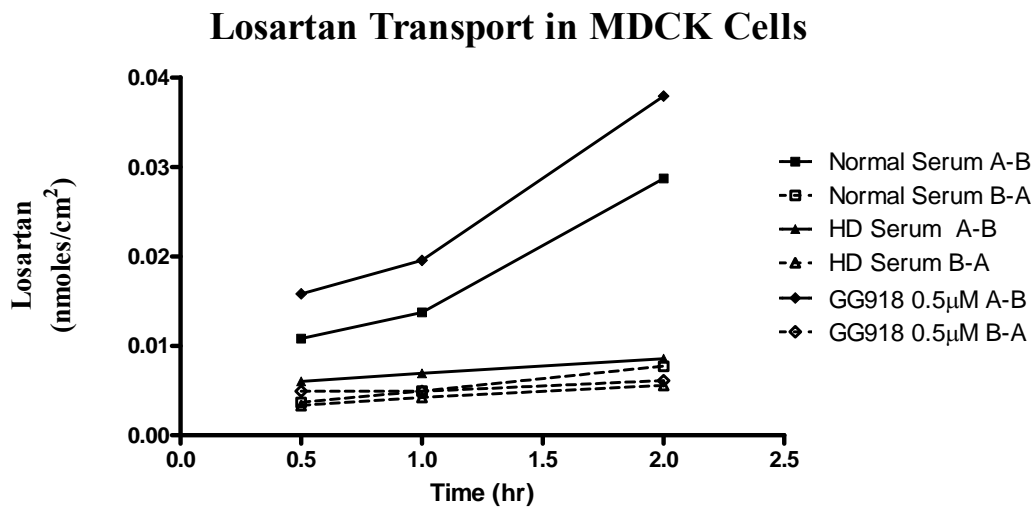
<b>Cell Type</b>	<b>Condition</b>	<b>P<sub>app</sub>(A→B)x10<sup>-6</sup> cm/sec</b>	<b>P<sub>app</sub>(B→A)x10<sup>-6</sup> cm/sec</b>	<b>Net Flux Ratio (B→A/A→B)</b>
<b>MDR1- MDCK</b>	<b>Losartan + NS</b>	<b>0.04 ± 0.01</b>	<b>0.24 ± 0.02</b>	<b>6.69 ± 1.82</b>
	<b>Losartan + HDS</b>	<b>0.009 ± 0.003*</b>	<b>0.70 ± 0.07*</b>	<b>83.8 ± 35.15*</b>
	<b>Losartan + GG918</b>	<b>0.17 ± 0.06*</b>	<b>0.37 ± 0.05 *</b>	<b>2.42 ± 0.95*</b>
<b>MDCK</b>	<b>Losartan + NS</b>	<b>0.56 ± 0.74</b>	<b>0.024 ± 0.01</b>	<b>0.38 ± 0.60</b>
	<b>Losartan + HDS</b>	<b>0.05 ± 0.01</b>	<b>0.041 ± 0.003</b>	<b>0.88 ± 0.17*</b>
	<b>Losartan +GG918</b>	<b>0.34 ± 0.07</b>	<b>0.075 ± 0.009 *</b>	<b>0.23 ± 0.08*</b>

Values expressed as mean ± SD. \* p<0.05, n=3  
NS: Normal serum HDS: Hemodialysis serum

(a)



(b)



**Figure 3.7:** Bidirectional study of losartan 10µM in MDR1-MDCK (a) cells and MDCK (b) cells.

### 3.4 Discussion

Expression of P-gp has been reported to be altered in chronic renal failure rats. The *in vitro* function of P-gp in the presence of serum from renal failure rats has been shown to be reduced. In our studies, we investigated a series of uremic toxins on the transport of radiolabeled substrates on MDR1-MDCK and cMOAT expressing cell lines. We also studied the effects of hemodialysis serum on the permeability of the BDDCS drugs propranolol and losartan in MDR1-MDCK cells. Due to eprosartan's adsorption to the transwell system we were unable to test this compound.

The [<sup>3</sup>H]-vinblastine bidirectional studies demonstrated that the uremic toxins CMPF, indoxyl sulfate, and endothelin significantly reduced the net flux ratio by at least 50%. In contrast, in the MDCK cells, which do not over-express the P-gp transporter, there was no significant inhibition of [<sup>3</sup>H]-vinblastine transport. This suggests that the P-gp transporter was inhibited by uremic toxins in the MDR1-MDCK cell line. However, the [<sup>3</sup>H]-vinblastine  $P_{app}$  (B→A) or  $P_{app}$  (A→B) was not statistically significant in either cell line, but there was an increase in the  $P_{app}$  (A→B) and a decrease in the  $P_{app}$  (B→A) in the MDR1-MDCK cells, which overall showed a statistically significant difference in the net flux ratio of [<sup>3</sup>H]-vinblastine. Also, the  $P_{app}$  (B→A) was significantly higher compared to the  $P_{app}$  (A→B), indicating the presence of active transport in the B→A direction.

The transport of [<sup>3</sup>H]-pravastatin in cMOAT-MDCKII also showed significant inhibition by uremic toxins CMPF, indoxyl sulfate, and endothelin, along with the inhibitor MK-571. The reduction in transport was at least 50% with the uremic toxins

and 85% with MK-571. However, the transport of [<sup>3</sup>H]-pravastatin was also significantly reduced by 40% with CMPF and indoxyl sulfate, but not endothelin, and by 30% with the inhibitor MK-571 in MDCKII cells. The transport of [<sup>3</sup>H]-pravastatin in the non-over expressing cell line may be due to the uptake by OATP transporters. Pravastatin has also been shown to be a substrate for the basolateral transporters OATP1B1 (Nakai et al., 2001) and this transporter has been shown to be endogenously expressed in MDCK cells (Goh et al., 2002). This suggest that pravastatin being a substrate for basolateral and apical transporter may account for the significant difference in [<sup>3</sup>H]-pravastatin transport on both cMOAT-MDCKII and MDCKII cell lines. Experiments using MRP2 substrate with less overlap with uptake transporters need to be carried out to determine the role of uremic toxins on MRP2 alone.

Next, the bidirectional studies showed the effect of hemodialysis (HD) serum on the transport of propranolol and losartan in MDR1-MDCK and MDCK cells. The propranolol transport in MDR1-MDCK and MDCK cells was in agreement with our uptake studies. As hypothesized, there was no significant change in propranolol net flux in either cell line. Furthermore, the  $P_{app}$  (B→A) was high for all groups: treated with normal serum, HD serum, and GG918, indicating that propranolol is highly permeable and not a substrate for P-gp. On the other hand, the transport of losartan was significantly decreased in the presence of GG918, but significantly increased in the presence of HD serum. The  $P_{app}$  (B→A) for losartan in the presence of HD serum increased 5-fold, and the  $P_{app}$  (A→B), decreased 18-fold. These results suggest that since losartan is a substrate for uptake and efflux transporters, hemodialysis serum is affecting the endogenous uptake transporters to a greater extent compared to the efflux

transporters, resulting in a marked increase in net flux. In the MDCK cells, an increase of 3-fold in net flux was observed in the presence of HD serum, which supports that endogenous transporters play a role in the transport of losartan.

### **3.5 Conclusion**

Bidirectional studies using MDR1-MDCK, MDCK, cMOAT-MDCKII, and MDCKII cells suggest that uremic toxins apart from affecting uptake transporters, also affect efflux transporters that are expressed in the canalicular membrane of hepatocytes. Human hemodialysis serum also exhibited an effect in the transport of losartan; however, this change was likely due by the dual action on uptake and efflux transporters. As shown, losartan is a substrate for uptake transporters (Chapter 2) and for efflux transporters as shown here. The transport of propranolol is not affected in the presence of hemodialysis serum since it's a highly permeable drug and not a substrate for transporters. These studies indicate that chronic kidney disease may play a role in drug transport in tissues expressing drug transporters such as the liver. This can have a complex effect for drugs that are substrates for uptake and efflux transporters. If a drug is a substrate for both uptake and efflux transporters, and CKD affects both, then, increased efflux will occur if the drug is a strong substrate for an uptake transporter but weaker for the efflux transporter. Similarly, decreased efflux will occur if a drug is a strong substrate for an efflux transporter but weaker for an uptake transporter. More experiments using cMOAT-MDCKII need to be carried out to investigate the effects of HD serum on the transport of propranolol and losartan. In conclusion, these studies suggest that the different classes of drugs would also be affected differently by uremic

toxins. If a compound is a substrate for an efflux transporter, it is possible that the uremic toxins can affect the efflux of the compound, which in turn can affect the drug's disposition. Both uptake and efflux transporters may be affected in CKD and need to be considered when studying changes in drug disposition.



### 3.6 References

- Chen C, Hanson E, Watson JW and Lee JS (2003) P-glycoprotein limits the brain penetration of nonsedating but not sedating H1-antagonists. *Drug Metab Dispos* 31:312-318.
- Cummins CL, Jacobsen W and Benet LZ (2002) Unmasking the dynamic interplay between intestinal P-glycoprotein and CYP3A4. *J Pharmacol Exp Ther* 300:1036-1045.
- Drescher S, Glaeser H, Murdter T, Hitzl M, Eichelbaum M and Fromm MF (2003) P-glycoprotein-mediated intestinal and biliary digoxin transport in humans. *Clin Pharmacol Ther* 73:223-231.
- Flanagan SD and Benet LZ (1999) Net secretion of furosemide is subject to indomethacin inhibition, as observed in Caco-2 monolayers and excised rat jejunum. *Pharm Res* 16:221-224.
- Flanagan SD, Cummins CL, Susanto M, Liu X, Takahashi LH and Benet LZ (2002) Comparison of furosemide and vinblastine secretion from cell lines overexpressing multidrug resistance protein (P-glycoprotein) and multidrug resistance-associated proteins (MRP1 and MRP2). *Pharmacology* 64:126-134.
- Goh LB, Spears KJ, Yao D, Ayrton A, Morgan P, Roland Wolf C and Friedberg T (2002) Endogenous drug transporters in in vitro and in vivo models for the prediction of drug disposition in man. *Biochem Pharmacol* 64:1569-1578.
- Huang ZH, Murakami T, Okochi A, Yumoto R, Nagai J and Takano M (2000) Expression and function of P-glycoprotein in rats with glycerol-induced acute renal failure. *Eur J Pharmacol* 406:453-460.

- Lau YY, Okochi H, Huang Y and Benet LZ (2006) Multiple transporters affect the disposition of atorvastatin and its two active hydroxy metabolites: application of in vitro and ex situ systems. *J Pharmacol Exp Ther* 316:762-771.
- Matheny CJ, Lamb MW, Brouwer KR and Pollack GM (2001) Pharmacokinetic and pharmacodynamic implications of P-glycoprotein modulation. *Pharmacotherapy* 21:778-796.
- Nakai D, Nakagomi R, Furuta Y, Tokui T, Abe T, Ikeda T and Nishimura K (2001) Human liver-specific organic anion transporter, LST-1, mediates uptake of pravastatin by human hepatocytes. *J Pharmacol Exp Ther* 297:861-867.
- Naud J, Michaud J, Boisvert C, Desbiens K, Leblond FA, Mitchell A, Jones C, Bonnardeaux A and Pichette V (2007) Down-regulation of intestinal drug transporters in chronic renal failure in rats. *J Pharmacol Exp Ther* 320:978-985.
- Pastan I, Gottesman MM, Ueda K, Lovelace E, Rutherford AV and Willingham MC (1988) A retrovirus carrying an MDR1 cDNA confers multidrug resistance and polarized expression of P-glycoprotein in MDCK cells. *Proc Natl Acad Sci U S A* 85:4486-4490.
- Sun H, Huang Y, Frassetto L and Benet LZ (2004) Effects of uremic toxins on hepatic uptake and metabolism of erythromycin. *Drug Metab Dispos* 32:1239-1246.
- Tanigawara Y (2000) Role of P-glycoprotein in drug disposition. *Ther Drug Monit* 22:137-140.

## **Chapter 4: Hepatic Disposition of BDDCS Drugs in Chronic Kidney Disease Rats: *isolated perfused rat liver (IPRL) with hemodialysis serum and normal serum***

### **Summary**

Drug disposition is altered in renal failure; however, it is not known if the alteration in drug disposition is the same for all drugs. Using the isolated perfused rat liver system, we investigated the effects of uremic toxins in hemodialysis serum, and the effects of renal failure (using livers from rats that underwent a 5/6 nephrectomy) on the hepatic disposition of propranolol, losartan, and eprosartan (3 BDDCS drugs). We hypothesized that the disposition of losartan and eprosartan would be affected by livers perfused with hemodialysis serum. We also hypothesized that the hepatic disposition of propranolol would not be affected in livers perfused with hemodialysis serum. The results obtained showed that hepatic disposition of propranolol was affected. However; the disposition of propranolol appears to be affected not by inhibition of transporters, but rather by changes in metabolism. The IPRL studies with propranolol showed a decrease in AUC and an increase in clearance, and the metabolite also showed the same changes, in the presence of hemodialysis serum and in livers from renal failure rats.

The IPRL studies showed significant changes in AUC and clearance, as well as biliary clearance for losartan and accumulation in the liver for eprosartan. These changes indicate that losartan may be a substrate for basolateral efflux transporters and that this transport is altered by hemodialysis serum. There was also an increase in biliary

clearance of losartan with no change in liver accumulation, suggesting that losartan is substrate for both uptake and efflux transporters. If basolateral efflux transporters were inhibited, then losartan would be eliminated via the bile. In comparison, eprosartan's AUC increased and clearance decreased as with losartan, but the amount in the liver increased in the presence of hemodialysis serum. This suggested that eprosartan was not exiting the hepatocyte via increased biliary clearance (since this was unchanged), but that the basolateral efflux was being inhibited. It is still not known if basolateral efflux transporters like Mrps are inhibited by hemodialysis serum, but previous studies in MRP2 transfected cells (Chapter 3) showed inhibition of MRP2 by hemodialysis serum and uremic toxins. Clinical studies as described in Chapter 5, will reveal if renal failure affects drug pharmacokinetics and if the alteration in drug disposition is clinically significant.

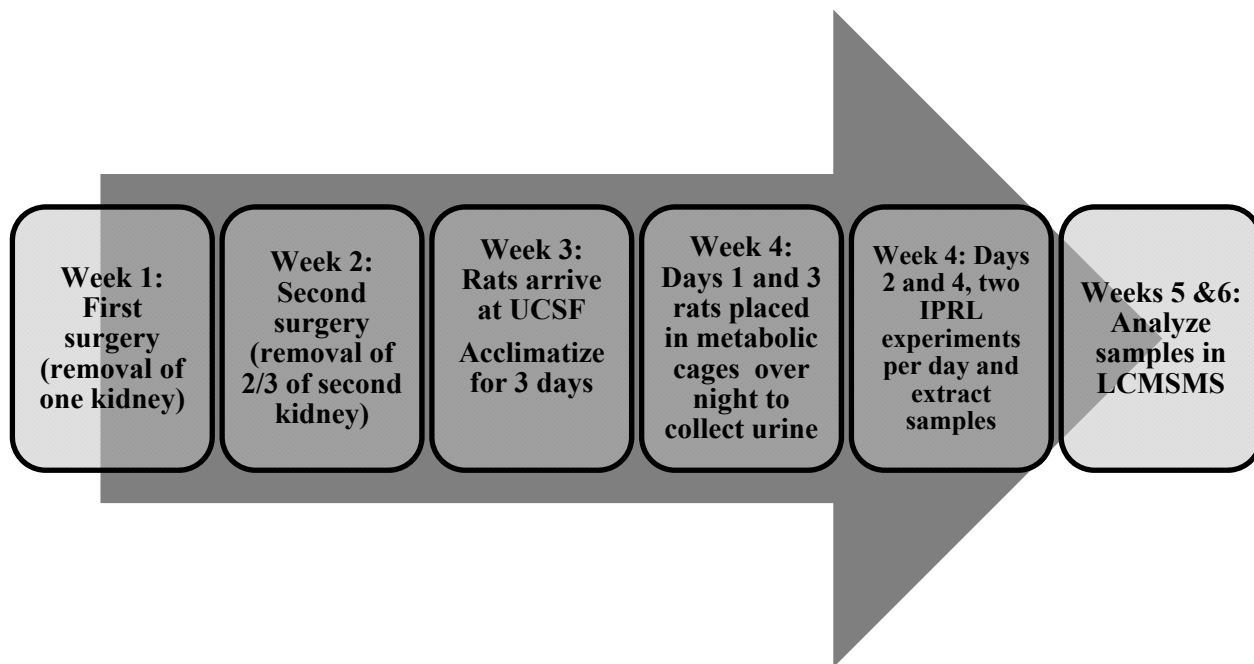
## 4.1 Introduction

Several reports in the literature indicate that normal drug disposition is altered in chronic kidney disease patients. Previous research has investigated the effects of uremic toxins and hemodialysis (HD) serum, which contains uremic toxins (also referred to it as uremic serum), on drug metabolism (Michaud et al., 2005) and, to some extent, on the effect on drug transporters (Naud et al., 2007; Naud et al., 2008). The chronic kidney disease rat model has also been used to study drug disposition through the isolated perfused rat liver (IPRL) system. In 1985, Terao and Shen investigated the effects of uremic blood in the extraction of *l*-propranolol in the perfused rat liver (Terao and Shen, 1985) after results from a pharmacokinetic study of *l*-propranolol in uremic rats showed that there was a 2.5-fold increase in the systemic availability of *l*-propranolol (Terao and Shen, 1983). In the pharmacokinetic study, the extent of absorption was found to be the same in both groups of rats, and the *in vitro* and *in vivo* protein binding was the same in renal failure and normal rats. In the IPRL studies from 1985, it was demonstrated that uremic serum inhibited the hepatic extraction of propranolol. The authors employed the uranyl nitrate injection method to induce renal failure, and then used the livers and/or blood from these rats to perform single pass IPRL. The IPRL studies included uremic livers perfused with uremic blood, uremic livers perfused with normal blood, normal livers perfused with uremic blood and normal livers perfused with normal blood. This allowed the authors to investigate whether the changes in extraction were due to uremic mediators in the blood, or intrinsic changes in the liver due to the renal failure. Terao and Shen (1985) reported that the hepatic extraction of propranolol was inhibited only in the presence of uremic blood, which was a result of a significant decrease in intrinsic

clearance. In a uremic liver perfused with normal blood, there was no inhibition of hepatic extraction. In that study the authors speculated uremic mediators might act as inhibitors of transport through the hepatocyte membrane. Importantly, in the Terao and Shen studies (1983 and 1985) the model of renal failure is chemically induced and produces an acute renal failure model rather than a chronic renal failure model. Also, in those studies, livers were perfused with rat blood not human serum.

Our *in vitro* studies, reported in Chapter 2, using uremic toxins and human uremic serum, suggested that uremic toxins/serum change drug transport in rat and human. The studies also indicated that the transport of losartan, eprosartan, and the model compound, [<sup>3</sup>H]-estrone sulfate is altered in transfected cell systems in the presence of uremic toxins and uremic serum from hemodialysis (HD) patients. Neither propranolol's metabolism nor transport was affected by the presence of uremic toxins/serum. Since our *in vitro* studies were done in isolated systems using only cells, we extended the studies to the liver using the IPRL system. IPRL (Figure 4.2) has been extensively used as an intact organ model for determining hepatic clearance and metabolism of drugs. It is also used to model physiologically based pharmacokinetics of hepatic uptake associated with transport. IPRL avoids neural and hormonal interference and excludes influence from absorption and non-hepatic elimination routes such as renal excretion; thus, it provides a relatively clean hepatic system to study metabolism and pharmacokinetics (Yong Liu, 2004). To study the effects of chronic kidney disease (CKD) on hepatic drug disposition, we used a 5/6 nephrectomy (Nx) rat model. This is the most widely used model for chronic renal dysfunction, in which 5/6 of the kidneys are surgically removed in a two stage surgery. The first surgery removes one kidney and after a one week

recovery, 2/3 of the second kidney is removed (Figure 4.1). Two weeks after the second surgery, the rats reached a moderate stage of renal failure (measured by creatinine clearance) and the IPRL was performed.



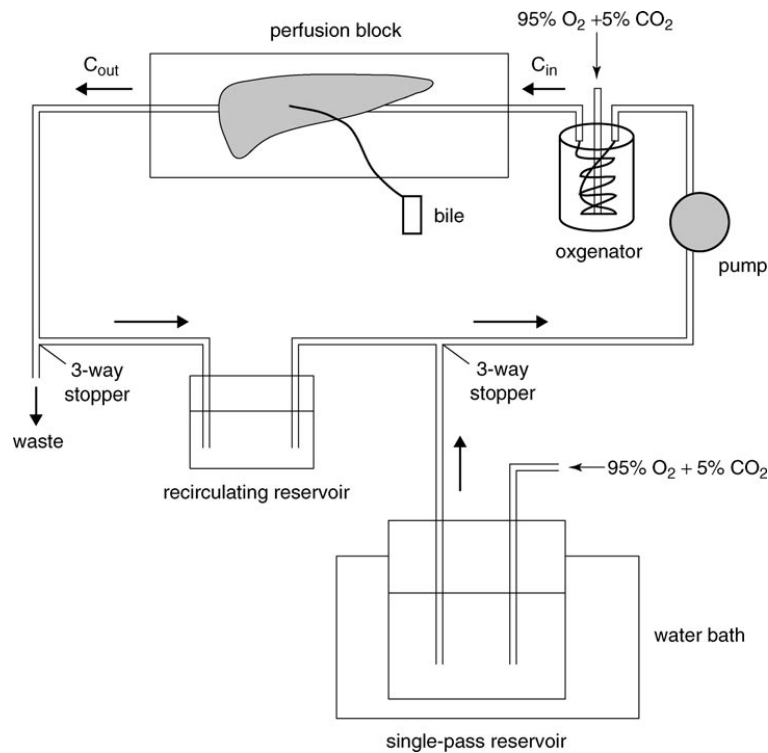
**Figure 4.1:** Sequence of events from first surgery on rats to analysis of samples from IPRL

For each drug we used at least four rats in each group, and four groups were used in all possible combinations (Table 4.1) to determine if the changes in drug disposition and clearance were due to changes in the liver in CKD and/or uremic toxins from hemodialysis uremic serum. In previous nephrectomized rat studies, Naud et al. (2008) reported that there were protein expression changes in P-gp and Oatp2; hence, we investigated the protein expression of several transporters via Western blotting.

**Table 4.1:** Groups for IPRL experiments: liver type and perfusate type.

	<b>Group 1</b>	<b>Group 2</b>	<b>Group 3</b>	<b>Group 4</b>
<b>Liver</b>	Liver from Sham Rat	Liver from Sham Rat	Liver from Nx Rat	Liver from Nx Rat
<b>Serum in perfusate</b>	Normal Serum	HD Serum	Normal Serum	HD Serum

Nx: 5/6 nephrectomy      HD: hemodialysis serum (uremic serum)      Sham: sham surgery



**Figure 4.2:** Diagram depicting Isolated Perfused Rat Liver (IPRL) setup. Yong Liu and Onua (2004)



## 4.2 Materials/Methods

Propranolol, rifampin, as well as HPLC-grade dimethyl sulfoxide, *tert*-butyl-methyl-ether (MTBE), and acetonitrile (ACN) were purchased from Sigma-Aldrich (St. Louis, MO). Losartan potassium salt and eprosartan were purchased from Toronto Research Chemicals (Ontario, Canada). Male spontaneously hypertensive (SH) Sprague-Dawley rats (200–350 g) with a 5/6 nephrectomy or sham nephrectomy were purchased from Charles River Laboratories (Wilmington, MA) and housed in the UCSF animal care facility with a 12-h light/dark cycle and allowed free access to water and food. Urine was collected for 24 hours to determine the urine creatinine; a blood sample was collected to determine serum creatinine. Approval of the studies was obtained from the Committee on Animal Research, UCSF. Hemodialysis (HD) serum was obtained from patients on hemodialysis from the UCSF Mt Zion Outpatient Dialysis Unit. Approval for obtaining serum from patients and normal serum from healthy volunteers was obtained from the Committee on Human Research at UCSF. Serum from seven patients in each group was pooled to obtain pooled HD serum and pooled normal serum.

### ***4.2.1 Surgery and Perfusion of Isolated Rat Livers***

Rats were anesthetized with ketamine:xylazine (80 mg/ml:12 mg/ml) before surgery. The hepatic portal vein and superior vena cava were cannulated after the livers were isolated for perfusion *ex situ* using standard techniques as described previously by our laboratory (Wu and Benet, 2003; Lau et al., 2004). In brief, a catheter was inserted via the hepatic portal vein and perfused, in a recirculating manner, with 80 ml of perfusate composed of Krebs-Henseleit buffer at 37°C, pH 7.4, supplemented with sodium taurocholate (220 nmol/min), 10% human serum from healthy volunteers or

patients on hemodialysis (HD), and glucose (10 mM). The perfusate in the reservoir was oxygenated directly using carbogen (95% O<sub>2</sub>/5% CO<sub>2</sub>) and stirred continuously.

Measures of liver viability included oxygen consumption, portal vein pressure (20–30 mmHg), pH 7.35–7.45, and metabolic capability. Livers were allowed to stabilize for 20 min prior to addition of either propranolol, losartan, or eprosartan. Perfusate samples (0.5 ml) were collected immediately (0min) and at 3, 5, 10, 15, 20, 30, 45, and 60 min after the addition of the compound of interest, and accumulative bile samples were collected for up to 60 min.

At the end of experiment, the liver was removed, blotted dry, and weighed. An aliquot of liver was homogenized with ice-cold PBS in a 1:2 ratio and maintained frozen at -80°C before analysis. To examine the effects of HD serum and CKD on hepatic disposition, 48 rats were divided equally into the following groups: a) liver from sham operated rat perfused with normal serum b) liver from sham rats perfused with HD serum c) liver from Nx rats perfused with normal serum, and d) liver from Nx rats perfused with HD serum. Each of the four groups received 1µM propranolol, 1µM losartan, or 10µM eprosartan. Group (a) for each drug served as a control and groups b-d served as treatment groups. Propranolol and losartan samples for analysis were prepared by liquid-liquid extraction as described previously (Lau et al., 2004). In brief, 3 ml of methyl tertiary butyl ether with internal standard warfarin (1µM) was added to each liver, bile, and perfusate sample. After centrifugation, separation of the organic layer in a methanol dry ice bath, and evaporation of the organic layer under nitrogen gas, the dried solutes were reconstituted with methanol for analysis by LC/MS-MS. Eprosartan samples were extracted with acetonitrile and propranolol was added as internal standard (1µM);

samples were vortexed and spun down at 13,000rpm for 10min. The supernatant was analyzed for drug concentrations by LC/MS-MS. Areas under the curve (AUCs) were calculated using the linear trapezoidal rule from time 3 min to  $t_{last}$  (60min).

#### **4.2.2 Western Blot**

Liver samples from 10 rats were used (5 from sham operated rats and 5 from nephrectomized rats) for Western blotting. The livers samples (5mg) were homogenized in a RIPA buffer from Sigma (St. Louis, MO) containing 1X solution of protease inhibitor cocktail from Fermentas (Glen Burnie, MD); all samples were kept on ice during homogenization. The homogenate was centrifuged at 13000rpm for 20min at 4°C. The supernatant was placed in a fresh ice-cold tube and the concentration was determined by BCA assay. Western blot analysis was performed with some modifications as previously described (Cummins et al., 2001). Briefly, 10 $\mu$ l of 5mg/ml solution of supernatant from homogenized livers was diluted into 10 $\mu$ l of Laemmli 2x loading buffer; the mixture was heated at 70°C for 10 minutes, and 5 $\mu$ l of bromophenol blue loading dye was added. Samples were loaded into a 7.5% SDS-polyacrylamide gel. Electrophoresis was run overnight at 30V and 90mA. After electrophoresis, proteins were transferred to a polyvinylidene difluoride membrane (Bio-Rad, Hercules, CA). The blots were blocked with 5% BSA in 1X TBST (Tris-buffered saline containing 0.05% Tween-20) from Teknova (Hollister, CA) for one hour at 4°C. Then the primary antibody was added and incubated at 4°C overnight. The primary antibodies used were: 1:3000 dilution of P-gp from Abcam (Cambridge, MA), 1:2000 dilution of Bcrp from Kamiya (Seattle, WA), 1:3000 dilution of Oatp1 and Oatp2 from Alpha Diagnostics (San Antonio, TX), 1:3000 dilution of Oat2 and Oct1 from Abcam (Cambridge, MA), and

1:3000 dilution of Mrp2 from Kamiya (Seattle, WA). Blots were then washed and incubated with 1:5000 dilution of anti-rabbit or anti-mouse IgG conjugated with horseradish peroxidase from Abcam (Cambridge, MA) at room temperature for 2 hours. A chemiluminescence kit from Amersham Biosciences (Buckinghamshire, UK) was used for detection. Beta-actin from Abcam, at 1:5000 dilution, was used as a positive control and loading control. Protein levels were quantitated by densitometry using the software ImageJ from NIH's free image processing and analysis software. Results were expressed as relative density compared to the  $\beta$ -actin positive control.

## **4.3 Results**

### ***4.3.1 Nephrectomized Rats vs. Sham Operated Rats***

The isolated perfused rat liver, allowed us to investigate the effects of hemodialysis serum (HD), which contains uremic toxins, on the hepatic disposition, hepatic and biliary clearance, as well as metabolism of propranolol, losartan, and eprosartan in the intact organ. Table 4.2 shows the serum creatinine, and creatinine clearance as well as weights of the rats used in these experiments. Sham operated rats maintained a normal weight of  $301 \pm 14$  g, and the nephrectomized rats (Nx) had a lower body weight of  $262 \pm 12$  g; 24 rats were used for each procedure. The serum creatinine increased from  $0.55 \pm 0.04$  in the sham rats to  $1.40 \pm 0.07$  in the Nx rats, consistent with decreased renal function. The urine production also increased in the Nx rats, and the creatinine clearance decreased from  $491 \pm 68$  in the sham rats to  $200 \pm 13$  in the Nx rats. In humans, the normal glomerular filtration (GFR) rate is 120-160ml/min based on creatinine clearance; stage 3 chronic kidney disease (CKD) corresponds to a GFR of 30-

60 ml/min/1.73cm<sup>3</sup> or 25-50% of normal renal function. Correlating this to the rat creatinine clearance in the sham rats vs. Nx rats, we observed a 60% decrease in creatinine clearance in the Nx rats compared to the sham rats, placing the Nx rats at a mid-stage of moderate chronic renal failure.

**Table 4.2:** 5/6 Nephrectomy rat and sham operated rat biochemical parameters and body weight.

	<b>Ave. Wt (g)</b>	<b>Average SrCr (mg/dL)</b>	<b>Average 24hr Urine vol (ml)</b>	<b>Average Cr Clearance (<math>\mu</math>l/100g/min)</b>
<b>Sham Rats</b> N=24	<b>301 <math>\pm</math> 14</b>	<b>0.55 <math>\pm</math> 0.04</b>	<b>16.9 <math>\pm</math> 2.0</b>	<b>491 <math>\pm</math> 68</b>
<b>Nephrectomized (Nx) Rats</b> N=24	<b>262 <math>\pm</math> 12</b>	<b>1.40 <math>\pm</math> 0.07</b>	<b>34.6 <math>\pm</math> 1.6</b>	<b>200 <math>\pm</math> 13</b>

rCr:

serum creatinine

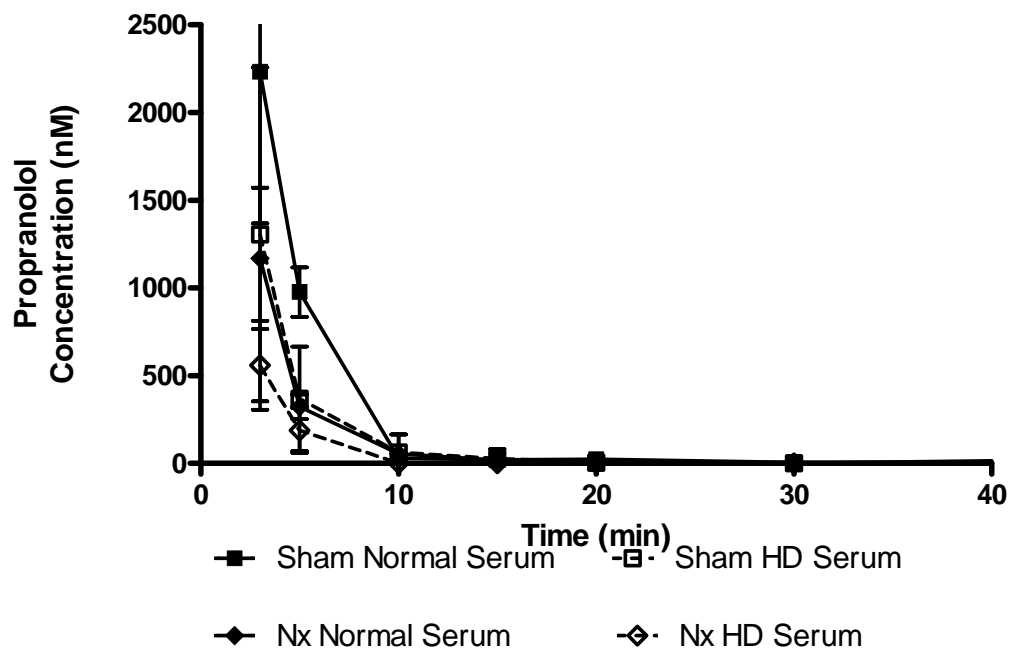
Cr Clearance: creatinine clearance

*Nephrectomized rats at time of IPRL were moderate (mid-stage 3) renal failure*

#### **4.3.2 Propranolol IPRL**

With propranolol, we observed a marked decrease in the perfusate  $AUC_3^{tlast}$  in livers perfused with hemodialysis (HD) serum, and also in both livers from Nx rats perfused with HD serum or normal serum (NS), when compared to the Sham-NS group (Figure 4.3, Table 4.3). The AUC decreased by 50% in the Sham-HD and Nx-NS

groups compared to the Sham-NS group, but because of the large variability and small number of animals in each group, these changes were not statistically significant. The AUC decreased by 80% in the Nx-HD group and this change was significant. There was no significant difference between the Nx liver perfused with normal serum or HD serum. Propranolol was not detected in the bile; thus, no biliary clearance was calculated, and propranolol in the liver was below the limit of quantitation (Table 4.3).



**Figure 4.3:** Concentration of propranolol in IPRL perfusate over time. Effect of nephrectomy and serum from hemodialysis patients on hepatic disposition.

**Table 4.3:** Isolated perfused rat liver propranolol (1  $\mu$ M) concentration in various matrices

<b>Drug</b>	<b>Rat Group</b>	<b>AUC ((ng/ml)*min)</b>	<b>CL biliary (ml/min)</b>	<b>Amount in the liver (ng)</b>
<b>Propranolol 1 <math>\mu</math>M</b>	Sham NS	1646 $\pm$ 560	0	0
	Sham HD	862 $\pm$ 312	0	0
	Nx NS	818 $\pm$ 258	0	0
	Nx HD	383 $\pm$ 100	0	0

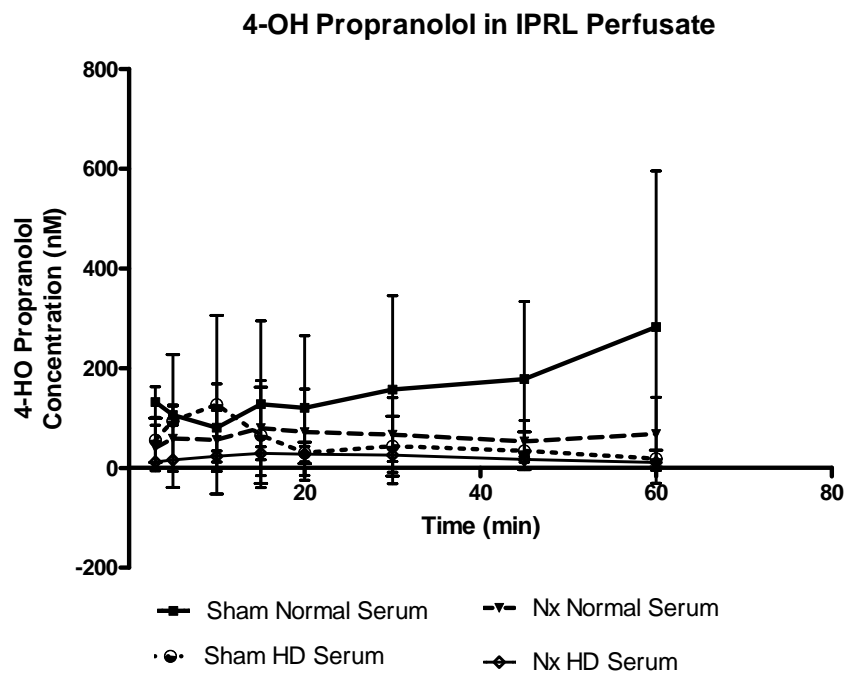
Values expressed as average  $\pm$  SD

**Table 4.4:** P values of comparisons between groups using t-test

<b>Statistical analysis (t-test)</b>	<b>P value for AUC</b>
Sham NS vs Sham HD	0.081
Sham NS vs Nx NS	0.056
Sham NS vs Nx HD	0.026*
Sham HD vs Nx NS	0.837
Sham HD vs Nx HD	0.054*
Nx NS vs Nx HD	0.042*

\*statistically significant

The propranolol metabolite, 4-OH propranolol, was detectable in liver but not in bile (Figure 4.4). The AUCs of 4-OH propranolol followed the same pattern as the parent drug, where HD serum and Nx cause a decrease in metabolite AUC compared to the Sham NS group (Table 4.5). The amount of metabolite in the liver also decreased in these same groups compared to the Sham NS group. However, these effects did not reach statistical significance because of high variability in the data.



**Figure 4.4:** Concentrations of 4-OH propranolol in IPRLs. Effect of nephrectomy and serum from hemodialysis patients on hepatic disposition.



**Table 4.5:** Isolated perfused rat liver 4-OH propranolol AUCs and amounts in the liver  
**Propranolol Metabolite**

<b>Drug</b>	<b>Rat Group</b>	<b>AUC ((ng/ml)*min)</b>	<b>Amount in the liver (µg)</b>
<b>4-OH Propranolol</b>	Sham	2557 ± 2641	0.86 ± 0.87
	NS		
	Sham	805 ± 952	0.31 ± 0.16
	HD		
	Nx NS	1000 ± 1037	0.12 ± 0.05
	Nx HD	358 ± 102	0.16 ± 0.62

Using uremic livers and uremic blood (Table 4.6) Terao and Shen (1985) observed a significant change in hepatic intrinsic clearance only in groups perfused with uremic serum, regardless of the type of liver being perfused (normal or uremic). They concluded that only uremic serum has a significant effect in the intrinsic clearance of *l*-propranolol which is inconsistent with our findings.

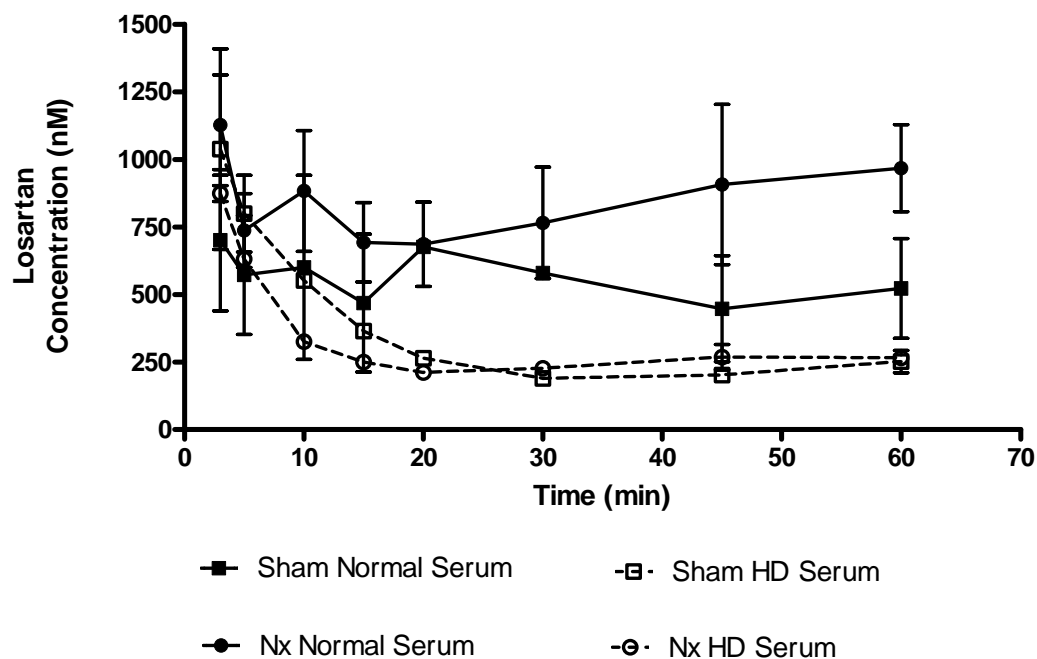
**Table 4.6:** Comparison of extraction fraction ( $E_h$ ) and estimated intrinsic clearance ( $CL_{int}$ ) of *l*-propranolol in IPRL from Terao and Shen (1985).

<b>Experiment</b>	<b><math>E_h</math></b>	<b><math>CL_{int}</math></b>
<b>Normal/NB</b>	0.974 ± 0.005	37.0 ± 6.8
<b>Uremic/UB</b>	0.906 ± 0.017*	10.2 ± 1.15*
<b>Normal/UB</b>	0.927 ± 0.009*	13.6 ± 1.7*
<b>Uremic/NB</b>	0.970 ± 0.010	37.8 ± 12.3

Liver type/perfusion substance,  
 NB= normal blood, UB= uremic blood  
 Mean ± SD (n=4), \*P<0.05

### 4.3.3 Losartan IPRL

The losartan isolated perfused rat liver experiments showed statistically significant changes in groups treated with HD serum (Figure 4.5). There was an approximate 53% and 69% decrease in  $AUC_3^{tlast}$  in the Sham-HD and Nx-HD groups, respectively compared to Sham-NS group, while no difference was observed for the Nx-NS group. The biliary clearance did not show statistically significant differences due to the variability; however, there was a trend towards an increase in biliary clearance in the groups with HD serum, which corresponds to the significant decrease in AUC seen for the HD groups. There were no significant changes in liver content in any group. The metabolite for losartan was produced in small quantities and was below the limit of quantitation.



**Figure 4.5:** Concentrations of losartan in IPRLs. Effect of nephrectomy and serum from hemodialysis patients on hepatic disposition.

**Table 4.7:** Isolated perfused rat liver losartan (1  $\mu$ M) concentration in various matrices

Rat Group	AUC (( $\mu$ g/ml)*min)	CL biliary (ml/min)	Amount in the liver ( $\mu$ g)
Sham NS	13.0 $\pm$ 3.9	0.0031 $\pm$ 0.0031	7.04 $\pm$ 3.85
Sham HD	6.1 $\pm$ 1.9	0.0166 $\pm$ 0.018	6.32 $\pm$ 1.75
Nx NS	19.9 $\pm$ 6.6	0.0021 $\pm$ 0.0012	3.63 $\pm$ 2.14
Nx HD	6.13 $\pm$ 1.44	0.0136 $\pm$ 0.0116	5.48 $\pm$ 0.83

Values expressed as average  $\pm$  SD

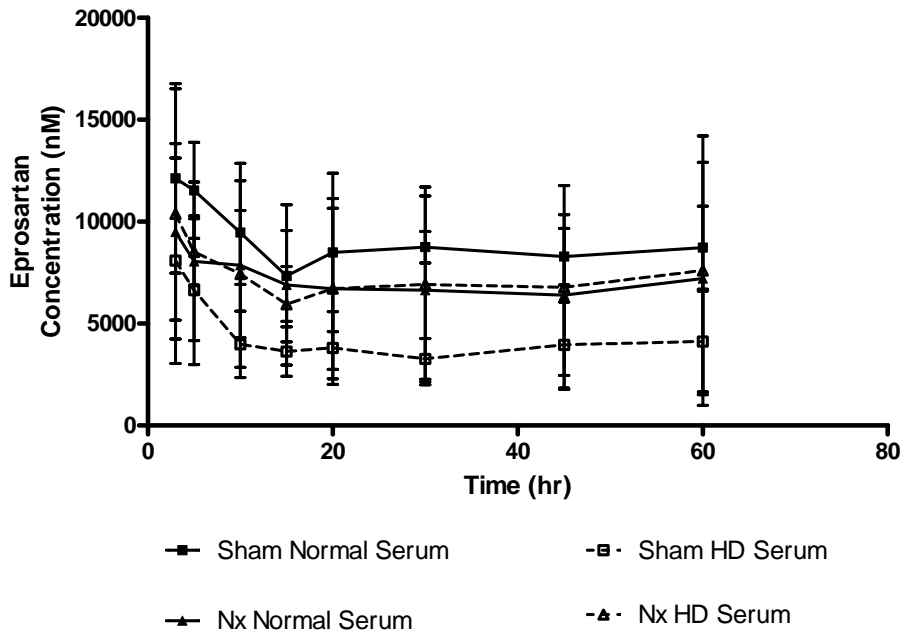
**Table 4.8:** P values of comparisons between losartan groups using t-test

<b>Statistical analysis (t-test)</b>	<b>P value for AUC</b>
Sham NS vs Sham HD	0.024*
Sham NS vs Nx NS	0.221
Sham NS vs Nx HD	0.020*
Nx NS vs Sham HD	0.012*
Sham HD vs Nx HD	0.981
Nx NS vs Nx HD	0.011*

\*statistically significant

#### ***4.3.4 Eprosartan IPRL***

In the eprosartan IPRL experiments, perfusion with HD serum showed a highly significant 64% decrease in AUC in the Sham-HD group vs. the Sham-NS group (Figure 4.6, Table 4.9) and a highly significant 55% decrease in AUC in the Nx-HD group. The biliary clearances and amounts in the liver showed some changes but were not statistically significant.



**Figure 4.6:** Concentration time curve of eprosartan in IPRL. Effect of nephrectomy and serum from hemodialysis patients on hepatic disposition.

**Table 4.9:** Eprosartan concentration in various matrices from IPRL

Drug	Rat Group	AUC ( $\mu\text{g}/\text{ml}\cdot\text{min}$ )	CL biliary (ml/min)	Amount in the liver ( $\mu\text{g}$ )
<b>Eprosartan 10<math>\mu\text{M}</math></b>	Sham NS	212 $\pm$ 27	0.0021 $\pm$ 0.0018	10.0 $\pm$ 1.7
	Sham HD	76.2 $\pm$ 16.7	0.0163 $\pm$ 0.0178	15.1 $\pm$ 7.7
	Nx NS	119 $\pm$ 46	0	7.46 $\pm$ 2.08
	Nx HD	95.8 $\pm$ 15.0	0.00017 $\pm$ 0.00010	17.2 $\pm$ 9.5

Values expressed as average  $\pm$  SD

**Table 4.10:** P values of comparisons between eprosartan groups using t-test

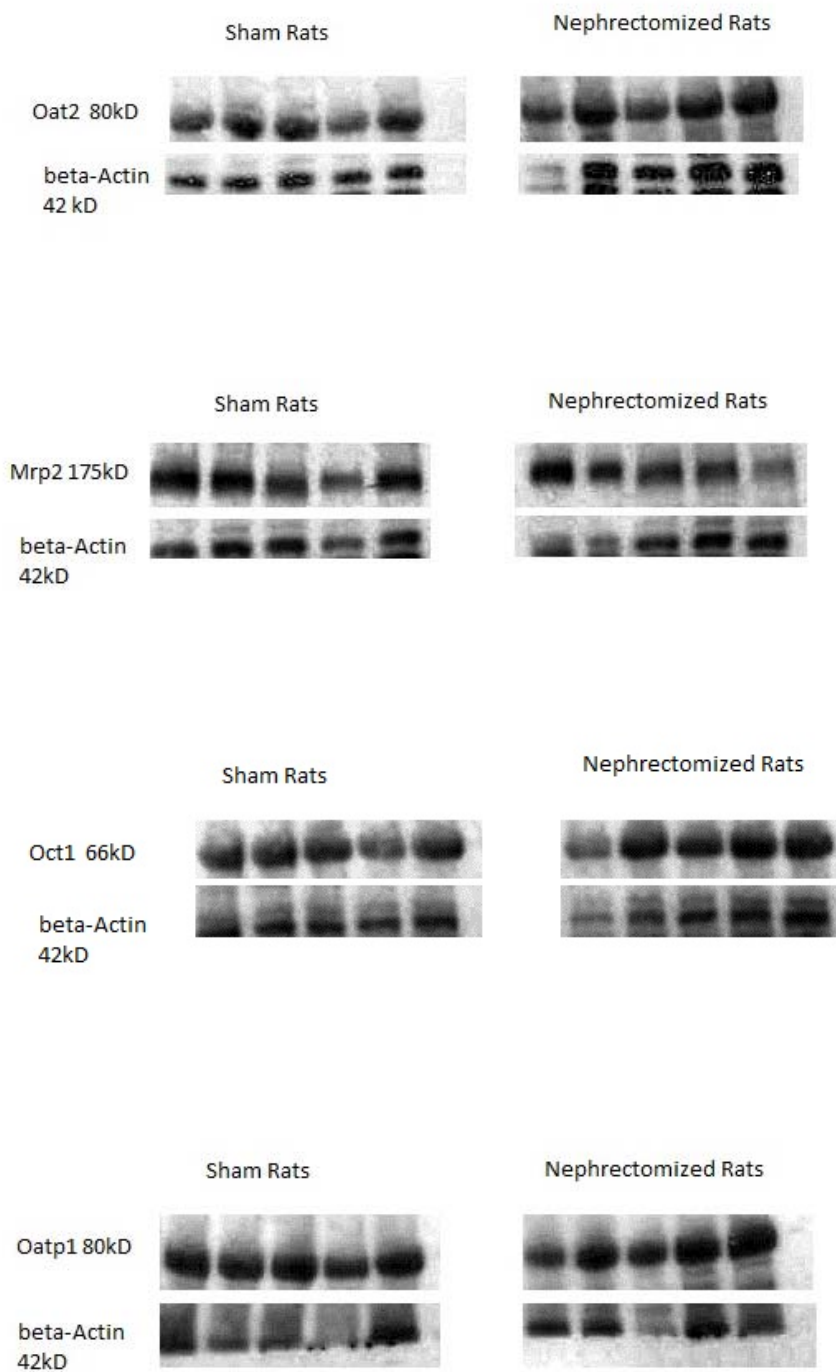
<b>Statistical analysis (t-test)</b>	<b>P value for AUC</b>
Sham NS vs Sham HD	0.0006*
Sham NS vs Nx NS	0.0191*
Sham NS vs Nx HD	0.0012*
Sham HD vs Nx NS	0.2087
Sham HD vs Nx HD	0.2042
Nx NS vs Nx HD	0.4619

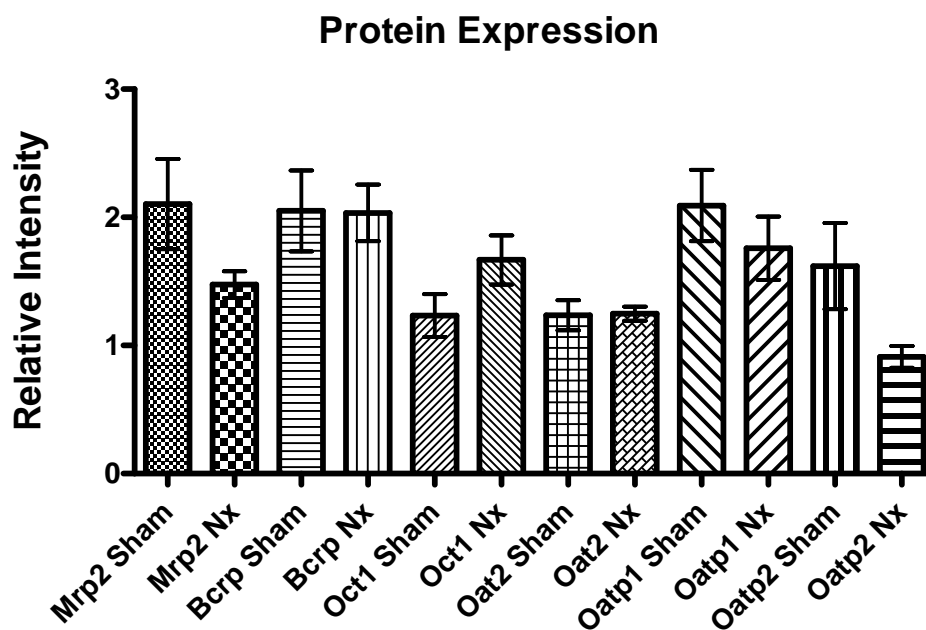
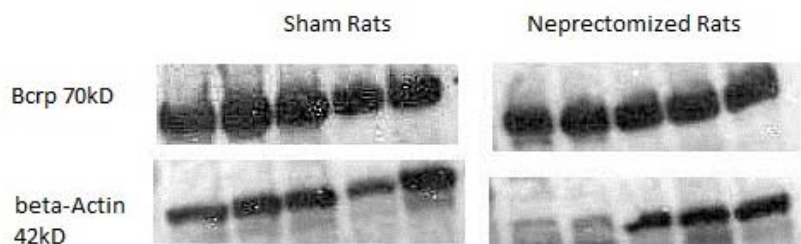
\*statistically significant

#### **4.3.5 Western Blot**

The changes in AUC, biliary clearance, and amount in the liver of the drugs used in the isolated perfused rat liver could be due in part to changes in drug transporters in the liver. After the perfusion, liver samples were frozen and analyzed for expression of uptake and efflux transporters: Oatp1, Oatp2, Oat2, Oct1, Mrp2, and Bcrp. Figure 4.7 shows the blots from the sham rats and nephrectomized rats (n=5), with  $\beta$ -actin as the control. The intensity of bands showing transporters relative to the  $\beta$ -actin loading control intensity were calculated and calculations are depicted as a graph in Figure 4.8. No statistically significant changes were observed. Bcrp and Oat2 expression was the same in livers from nephrectomized rats and sham operated rats. Oct1 expression in the liver from nephrectomized rats increase slightly in intensity compared to sham rats. Mrp2, Oatp1, and Oatp2 expression in nephrectomized rats livers decreased compared to livers of sham operated rats.

**Figure 4.7:** Western blots depicting hepatic transporter expression in sham and nephrectomized rat livers.





**Figure 4.8:** Graph depicting relative intensity (relative to  $\beta$ -actin) of bands of transport proteins in livers from nephrectomized rats.



#### 4.4 Discussion

The effect of chronic kidney disease on drug disposition and pharmacokinetics is not well understood; however, there have been studies that point to uremic toxins accumulated in these patients causing some changes. Uremic toxins have been studied for their effect on drug metabolism in renal failure (Elston et al., 1993; Leblond et al., 2000; Leblond et al., 2001; Guevin et al., 2002; Dreisbach and Lertora, 2003). However, there have been few studies of the effects of uremic toxins/renal failure on drug disposition via hepatic transporters ( Sun et al., 2004; Sun et al., 2010; Dreisbach and Lertora, 2008; Naud et al., 2008). No studies have compared the effects of uremic toxins/renal failure on the disposition of different BDDCS drugs. The ability to predict if the disposition of particular BDDCS drug class is affected by renal failure would be clinically useful. The metabolism of drugs that do not require transporters to enter and/or exit the cell would not be expected to be affected by renal failure whereas the opposite would be true for drugs that require a transporter if transporters are affected by renal failure.

This rationale was the premise for our isolated perfused rat liver studies, in which we studied the class 1 drug propranolol, class 2 drug losartan, and class 4 drug eprosartan. With propranolol (Class 1 drug) showed statistically significant difference in AUC in the Nx-HD groups compared to Sham-HD, Sham-NS, or Nx-NS groups. This indicates that the AUC of propranolol may be altered due to physiological changes in the liver due to renal failure as well as the effect of uremic toxins. The HD serum alone did not show an effect on AUC. In the Terao and Shen (1985) studies with *l*-propranolol, intrinsic clearance decrease in livers perfused with uremic blood but not in uremic livers

with normal blood. They concluded that the uremic toxins are responsible for the clearance decreases and that uremic liver has no effect on this parameter. In contrast, our results show AUC decreasing, possibly because increased clearance. Direct comparison of the earlier studies and the present one may not be valid because the previous study used an acute renal failure model induced by urinary nitrate and rat blood to perfuse the livers, whereas we used nephrectomy chronic renal failure model and human serum for perfusion. Species differences and different physiological changes in our chronic renal failure model may also account for some differences in the results. However, it is difficult to provide a concise rational explanation for our results.

In previous *in vitro* studies we determined that CYP450 metabolizing enzymes are not inhibited by HD serum, and that the metabolism of propranolol is not affected by HD serum (Chapter 2). In the IPRL studies, propranolol metabolite areas appeared to change in parallel with parent drug AUCs, but metabolite concentrations between and within groups were not significantly different. Propranolol is metabolized mainly by CYP2D6, which, unlike other CYPs such as hepatic CYP1A1 and CYP1A2 is not known to be affected by renal failure. Various other CYPs may metabolize propranolol, and CYP1A2 may account for as much as 10% of its metabolism (Li and Zeng, 2000). However, we also observed (Chapter 2) that its metabolism in rat and human hepatocytes is not inhibited by HD serum and we would have expected to see no difference in metabolite or AUC for propranolol in the IPRL.

The losartan (class 2) isolated perfused rat liver experiments showed that renal failure was insufficient to alter hepatic disposition of losartan, but uremic toxins (in the

HD serum) did have an effect. The decrease in AUC in the groups perfused with HD serum was 50%. However, this decrease was not seen in the Nx-NS group, indicating that for losartan disposition, renal failure was not the only factor that would alter hepatic disposition. Since no uremic toxins were present in the Nx-NS group, this indicated that uremic toxins present in the HD serum were responsible for altering the drug disposition. As the *in vitro* studies (Chapter 2) indicated, HD serum did not affect losartan metabolism, but did inhibit losartan uptake in transfected cells and hepatocytes. Yet the results seen here with the IPRL studies show a decrease in AUC in the perfusate, which is opposite to what would be expected for a decrease in hepatic uptake with HD serum. Similarly, increases in biliary clearance were with HD serum, consistent with the decrease in AUC with HD serum. Although HD may be reducing the uptake of losartan into the hepatocyte, the results obtained suggest that HD serum may have an even greater inhibitory effect on unidentified basolateral efflux transporters, than on apical biliary efflux transporters. Studies investigating the effects of HD serum on basolateral Mrps in transfected cell systems and Oct transfected cells would need to be carried out to determine if HD serum inhibits these transporters. If uptake transporters are inhibited, but losartan is a substrate for multiple uptake and efflux transporters, this could account for the unexpected results for AUC and biliary clearance noted here. As shown in the bidirectional studies in MDR1-MDCK cells described in Chapter 3, losartan transport was affected by HD serum, and the net flux increased significantly. The  $P_{app}(B \rightarrow A)$  increased and  $P_{app}(A \rightarrow B)$  decreased, suggesting that both uptake and efflux transporters in MDCK cells are affected by HD serum.

The eprosartan (class 4) IPRL studies, showed a significant decrease in AUC in the Sham-HD, Nx-NS, and Nx-HD groups compared to Sham-NS, following a similar pattern to that seen with losartan. Also, there was no change in the Nx-HD group compared to the Nx-NS group, suggesting that Nx as well as HD both had an effect in AUC since the Nx-NS group also showed a significant difference when from the Sham-NS group. In addition, the eprosartan concentration in the liver seemed to increase in these groups though the increase was not statistically significant. This may indicate that eprosartan is substrate for canalicular efflux transporters and that the HD serum is inhibiting these transporters, causing the hepatic accumulation of eprosartan.

#### **4.5 Conclusion**

The isolated perfused rat liver system showed uremic serum can affect the hepatic disposition of drugs. Different effects were observed for the drugs depending on their BDDCS classification. Although we expected to see no difference in propranolol (class 1) disposition, we observed changes in AUC in parent and metabolite in the presence of HD serum. Since *in vitro* studies have shown that the uptake, efflux, and metabolism of propranolol are not affected by HD serum, the results were unexpected and not consistent with the earlier work of Terao and Shen (1983; 1995). However, for the class 2 and 4 drugs, we observed changes that were not due to metabolism since the metabolism in losartan was not affected *in vitro*, and eprosartan is not metabolized. The changes observed in losartan and eprosartan disposition are potentially due to inhibition (by hemodialysis serum) of basolateral transporters (uptake and/or efflux) in the case of losartan, and canalicular efflux transporters in the case of eprosartan.

A great deal of effort and expense were involved in the studies described in this chapter. However, the results were not consistent with our expectations based on the in vitro results of the earlier chapters. It may be that IPRL studies are not a consistent model for examining the effects of uremic toxins on hepatic uptake processes. Although the IPRL system is useful in determining hepatic drug disposition for different BDDCS classes, the IPRL model here does not support the hypothesis of BDDCS drug disposition changes in CKD. Fortunately, our human studies, described in the next chapter do provide strong support for the hypothesis upon which this thesis work was premised

## 4.5 References

- Cummins CL, Mangravite LM and Benet LZ (2001) Characterizing the expression of CYP3A4 and efflux transporters (P-gp, MRP1, and MRP2) in CYP3A4-transfected Caco-2 cells after induction with sodium butyrate and the phorbol ester 12-O-tetradecanoylphorbol-13-acetate. *Pharm Res* **18**:1102-1109.
- Dreisbach AW and Lertora JJ (2003) The effect of chronic renal failure on hepatic drug metabolism and drug disposition. *Semin Dial* **16**:45-50.
- Dreisbach AW and Lertora JJ (2008) The effect of chronic renal failure on drug metabolism and transport. *Expert Opin Drug Metab Toxicol* **4**:1065-1074.
- Elston AC, Bayliss MK and Park GR (1993) Effect of renal failure on drug metabolism by the liver. *Br J Anaesth* **71**:282-290.
- Guevin C, Michaud J, Naud J, Leblond FA and Pichette V (2002) Down-regulation of hepatic cytochrome p450 in chronic renal failure: role of uremic mediators. *Br J Pharmacol* **137**:1039-1046.
- Leblond F, Guevin C, Demers C, Pellerin I, Gascon-Barre M and Pichette V (2001) Downregulation of hepatic cytochrome P450 in chronic renal failure. *J Am Soc Nephrol* **12**:326-332.
- Leblond FA, Giroux L, Villeneuve JP and Pichette V (2000) Decreased in vivo metabolism of drugs in chronic renal failure. *Drug Metab Dispos* **28**:1317-1320.
- Li X and Zeng S (2000) Stereoselective propranolol metabolism in two drug induced rat hepatic microsomes. *World J Gastroenterol* **6**:74-78.

- Michaud J, Dube P, Naud J, Leblond FA, Desbiens K, Bonnardeaux A and Pichette V (2005) Effects of serum from patients with chronic renal failure on rat hepatic cytochrome P450. *Br J Pharmacol* **144**:1067-1077.
- Naud J, Michaud J, Boisvert C, Desbiens K, Leblond FA, Mitchell A, Jones C, Bonnardeaux A and Pichette V (2007) Down-regulation of intestinal drug transporters in chronic renal failure in rats. *J Pharmacol Exp Ther* **320**:978-985.
- Naud J, Michaud J, Leblond FA, Lefrancois S, Bonnardeaux A and Pichette V (2008) Effects of chronic renal failure on liver drug transporters. *Drug Metab Dispos* **36**:124-128.
- Sun H, Frassetto LA, Huang Y and Benet LZ (2010) Hepatic clearance, but not gut availability, of erythromycin is altered in patients with end-stage renal disease. *Clin Pharmacol Ther* **87**:465-472.
- Sun H, Huang Y, Frassetto L and Benet LZ (2004) Effects of uremic toxins on hepatic uptake and metabolism of erythromycin. *Drug Metab Dispos* **32**:1239-1246.
- Terao N and Shen DD (1983) Effect of experimental renal failure on the disposition kinetics of l-propranolol in rats. *J Pharmacol Exp Ther* **227**:295-301.
- Terao N and Shen DD (1985) Reduced extraction of I-propranolol by perfused rat liver in the presence of uremic blood. *J Pharmacol Exp Ther* **233**:277-284.
- Yong Liu SJW and Onua ET (2004) Hepatic clearance and drug metabolism using isolated perfused rat liver. *Current Protocols in Pharmacology*. Suppl. 26: 7.9-7.9.10.

## **Chapter 5: Pharmacokinetic studies of propranolol, losartan, and erythromycin in healthy subjects and chronic kidney disease patients: comparison of three BDDCS drugs**

### **Summary**

The pharmacokinetic study of propranolol, losartan, and erythromycin in chronic kidney disease (CKD) patients and healthy controls reported here shows different changes in pharmacokinetics for the three drugs in CKD patients vs. healthy controls. We had hypothesized that due to the involvement of transporters in drug disposition, and the possible inhibition/alteration of transporters in CKD, there may be different PK changes in different drugs based on their Biopharmaceutics Drug Disposition Classification System class. This study is in progress, and for statistical comparison here we report the results from 5 CKD patients and 5 healthy volunteers. Our studies showed that for the class 1 drug, propranolol, there were no significant changes in pharmacokinetics in CKD patients vs. healthy subjects. In contrast, in the losartan study, although there were no statistically significant changes for the parent drug in CKD subjects, there were significant 58%, 48% and 55% decreases in the  $C_{max}$ ,  $t_{1/2}$ , and  $AUC_{0-\infty}$ , respectively for the primary metabolite, EXP3174. In the pharmacokinetics of erythromycin we observed significant differences in both parent and metabolite. There was a 48%, 3.2-fold increase in  $t_{1/2}$  and  $AUC_{0-\infty}$ , respectively, and a 66% and 49% decrease in  $CL/F$  and  $V_{ss}/F$ , respectively for erythromycin in CKD compared to controls. The metabolite showed a 1.6-fold and 3.1-fold increase in  $t_{1/2}$  and  $AUC_{0-\infty}$ , respectively. These results suggest that there may be alterations in transporters in CKD



that can cause differences in PK parameters compared to healthy controls. Furthermore, our studies indicate that BDDCS may be used as a classification system to make predictions on drug disposition changes in CKD for different classes of drugs.

## 5.1 Introduction

Drug disposition of different non-renally excreted drugs has been reported to be altered in chronic kidney disease (CKD) patients. The non-renal clearance for drugs such as phenytoin and bumetanide has been reported to increase in CKD, whereas the non-renal clearance for some drugs such as acyclovir, captopril, verapamil and warfarin, among others, has been reported to decrease in CKD (Dreisbach and Lertora, 2003). Some of these drugs are metabolized by CYP450 metabolizing enzymes, while others are not-metabolized or undergo other non-CYP450 mediated metabolism. Other studies have investigated the pharmacokinetic (PK) changes of a drug in healthy volunteers vs. CKD or hemodialysis patients (Vinik et al., 1983; Martin et al., 1998; Nolin et al., 2009). Previously in our laboratory, the pharmacokinetics of erythromycin was investigated in healthy volunteers vs. end-stage renal disease (ESRD) volunteers on hemodialysis (Sun et al.). In that study, the hepatic clearance but not gut bioavailability was found to be altered in ESRD patients. When an oral dose of erythromycin was administered, the AUC,  $t_{1/2}$ , and  $C_{max}$  increased significantly in ESRD subjects compared to the healthy controls. From the IV administration, the bioavailability (F) was determined, and it was not significantly different in the ESRD subjects.

These prior studies have demonstrated that CKD affects the non-renal clearance of these drugs; however, none of the prior studies have investigated different drugs in the same patients. As discussed in Chapters 2 and 3, we have hypothesized that drug transporters may play a role in drug disposition in CKD patients. Furthermore, we have hypothesized that BDDCS may be a useful tool to predict for which orally administered drugs we would expect to see changes in disposition in CKD patients. Thus, it was important to investigate three different Biopharmaceutics Drug Disposition Classification System (BDDCS) drugs in the same patient, in order to see if the changes in PK would occur in Class 2 and 3 drugs and not Class 1. Since transporters are important in Class 2, 3, and 4 drug dispositions, but not Class 1, we predicted that we would see a change in PK for Class 2 and 3 in CKD patients compared to healthy controls. In this study we investigated the pharmacokinetics following a single dose oral administration of propranolol (Class 1), losartan (Class 2), and erythromycin (Class 3) in patients with stage 4 CKD and in healthy controls. Although in this Chapter we report the PK parameters for 5 patients in each group, this study is on-going and will finally include 12 subjects in each group, which will present more accurate results for each drug. At this time the study has been completed in 12 CKD patients and is ongoing in 3 further healthy volunteers. The analysis here was carried out using an equal number of CKD patients to the 5 completed healthy controls using the plasma and urine concentrations.

This study allowed us to investigate if the Biopharmaceutics Drug Disposition Classification System is a useful tool for predicting the pharmacokinetic changes of non-renally excreted drugs in patients with CKD. The study also allowed us to compare *in vitro* data obtained with the drugs that obtained with the same drugs to that obtained in

the *in vivo* human study. This translational aspect of the project is important in assessing the feasibility of using *in vitro* studies to predict (or pre-assess) drug disposition of existing drugs and/or new molecular entities, in CKD patients.

## **5.2 Methods**

### **5.2.1 Study Subjects**

Twelve patients with chronic kidney disease (CKD) with a creatinine clearance  $\leq 40$  ml/min and 12 healthy controls were studied. Normal renal function was defined as creatinine clearance  $\geq 80$  ml/min. Creatinine clearance was estimated using the MDRD (modification of diet in renal disease) formula. Patients who were taking drugs that would interfere with the metabolizing enzymes of the drugs being studied or who were taking drugs that are known inhibitors of hepatic uptake transporters were excluded, if possible. Subjects with evidence of liver disease or of abuse of alcohol or illicit drugs within the 6 months prior to the study and those having a serum albumin level  $< 3.0$  g/dl, hematocrit level  $< 30$  mg/dl, or allergy to propranolol, losartan, or erythromycin were also excluded. Subjects were matched by sex and age  $\pm 5$  years. Due to the high prevalence of chronic kidney disease in African Americans, most of the CKD patients were African American; however, we were unable to match the ethnicity to the healthy subjects.

### **5.2.2 Study Procedures**

The study protocol was approved by the University of California, San Francisco, Human Research institutional review board. Written informed consent was obtained from all study participants. The study was conducted at the university's General Clinical Research Center from May 2010-present. The subjects were admitted to the research

center in the morning on each of the study days. Each subject underwent three pharmacokinetic studies, one for propranolol, one for losartan, and one for erythromycin. The study was randomized and separated by at least 7 days. Each subject received either oral administration of propranolol (40mg), losartan (50mg) or erythromycin (150mg). The subjects were asked to fast from the midnight prior to each study day until 3 h after administration of the dose. For patients with CKD, all other medications were taken  $\geq 3$  h after study drug dosing. Blood samples were obtained for pharmacokinetic analysis before the dose (0) and at 0.5, 1, 1.5, 2, 4, 6, 8, 10, 12, 14, and 24 h after the dose. Plasma samples were kept at  $-80$  °C until analysis. Urine was collected for 24 h at 4 h intervals from 0-4 h, 4-8 h, 8-12 h, and then 12-24 h. Samples were aliquoted into 20-ml bottles and stored at  $-80$  °C until analysis.

### ***5.2.3 Analytical Procedures***

Plasma samples were assayed for propranolol, 4-OH propranolol, losartan, and EXP3174, using LC-MS/MS as previously described in Chapter 2. For erythromycin and *N*-demethyl-erythromycin, the multiple reaction monitor for plasma and urine sample analyses was set at transitions 576.4-158.1 *m/z* and 720.2-562.2 *m/z*, respectively. The ionspray voltage was set at 5500 and the temperature at 450°C. The collision energy was set at 39eV for erythromycin and *N*-demethyl-erythromycin. The mobile phase for erythromycin and *N*-demethyl-erythromycin consisted of 50% acetonitrile: 50% water containing 0.1% formic acid and 2 mM sodium acetate.

Parameters and mobile phase for other drugs (propranolol and losartan) are described in Chapter 2. Twenty microliter aliquots were injected, and the flow rate was set at 0.15 ml/min into the mass system. The methods for propranolol, 4-OH propranolol,

losartan, EXP3174, erythromycin and *N*-demethyl-erythromycin were validated from 0.5ng/ml to 250ng/ml, 0.5ng/ml to 100ng/ml, 10ng/ml to 2500ng/ml, 25ng/ml to 1000ng/ml, 10ng/ml to 2500ng/ml, 0.5ng/ml to 100ng/ml, respectively. The curves were linear over the validated ranges, and  $1/x^2$  weighting was used.

#### ***5.2.4 Pharmacokinetic Analyses***

Pharmacokinetic parameters were estimated from plasma concentration data by noncompartmental analysis using WinNonlin Phoenix Professional software, (Pharsight, Mountain View, CA). The AUC was estimated using the linear trapezoidal method up to the last measured concentration and extrapolated to infinite time by dividing this last measured concentration by the terminal rate constant. Renal clearance was calculated by dividing the amount excreted unchanged in urine by the plasma AUC over 24 h. Weight-adjusted AUCs (AUC/weight) were also calculated. The elimination half-life was obtained by linear regression analysis of the last data points after semi log transformation of the data.

#### ***5.2.5 Statistical Analyses***

The differences in pharmacokinetic parameters between patients with chronic kidney disease and healthy controls were analyzed using unpaired Student's *t*-tests. A sample size of 12 subjects per group was calculated as providing  $\geq 80\%$  power to detect AUC differences of  $\geq 50\%$  between the groups. Results are expressed as mean  $\pm$  SD. Logarithmic transformation of  $T_{\max}$ ,  $C_{\max}$ ,  $t_{1/2}$ ,  $V_{ss}/F$ ,  $CL/F$ , and AUC values was performed before statistical analysis, and 95% confidence intervals were calculated for the geometric mean ratios.

### 5.3 Results

The data shown represent 5 subjects in each group. The study is on-going and when completed will contain 12 subjects in each group. To date, all the CKD subjects have completed the study; however, only 5 healthy have completed the study. For this reason here, we have only included the first 5 subjects in each group to provide a balanced statistical analysis of the results.

Patient demographic data and clinical chemistry values are given in Table 1. The healthy and CKD subjects were matched by age  $\pm$  5 years to avoid confounding effects of age. The body weight differences were not statistically significant. The serum creatinine, aspartate aminotransferase (AST), blood urea nitrogen (BUN), and creatinine clearance were significantly higher in CKD patients as expected (Table 1). Although we did our best to match by ethnicity, due to the high prevalence of CKD in African Americans, we enrolled 5 African Americans in the CKD group and one African American and 4 Caucasians in the healthy group. However, the  $CL_{cr}$  estimate adjusts for ethnic differences in its calculation.

**Table 5.1:** Patient demographic data and chemistries

	Healthy (mean ± SD)	CKD (mean ± SD)
<b><i>Basic characteristics</i></b>		
<b>N</b>	<b>5</b>	<b>5</b>
<b>Age (yrs)</b>	<b>60.2 ± 11.7</b>	<b>57.8 ± 14.7</b>
<b>Weight (kg)</b>	<b>74.5 ± 10.6</b>	<b>90.5 ± 18.3</b>
<b>Sex (M/F)</b>	<b>3/2</b>	<b>4/1</b>
<b>Ethnicity (AA/C/AP/H)</b>	<b>1/4/0/0</b>	<b>5/0/0/0</b>
<b><i>Clinical chemistry</i></b>		
<b>AST (U/L)</b>	<b>21.6 ± 6.30</b>	<b>*39.6 ± 11.1</b>
<b>ALT (U/L)</b>	<b>17.8 ± 7.80</b>	<b>37.8 ± 18.6</b>
<b>Serum creatinine (mg/dL)</b>	<b>0.75 ± 0.20</b>	<b>*2.88 ± 0.59</b>
<b>BUN (mg/dL)</b>	<b>12.2 ± 3.30</b>	<b>*37.0 ± 9.30</b>
<b>Serum albumin (g/L)</b>	<b>4.24 ± 0.30</b>	<b>3.78 ± 0.47</b>
<b>CL<sub>cr</sub> (mL/min/1.73m<sup>2</sup>)<sup>a</sup></b>	<b>110 ± 27.8<sup>a</sup></b>	<b>*27.2 ± 4.76<sup>a</sup></b>

AA, African American; ALT, alanine transaminase; AP, Asian/Pacific Islander; AST, aspartate aminotransferase; BUN, blood urea nitrogen; C, Caucasian; CL<sub>cr</sub>, creatinine clearance; H, Hispanic

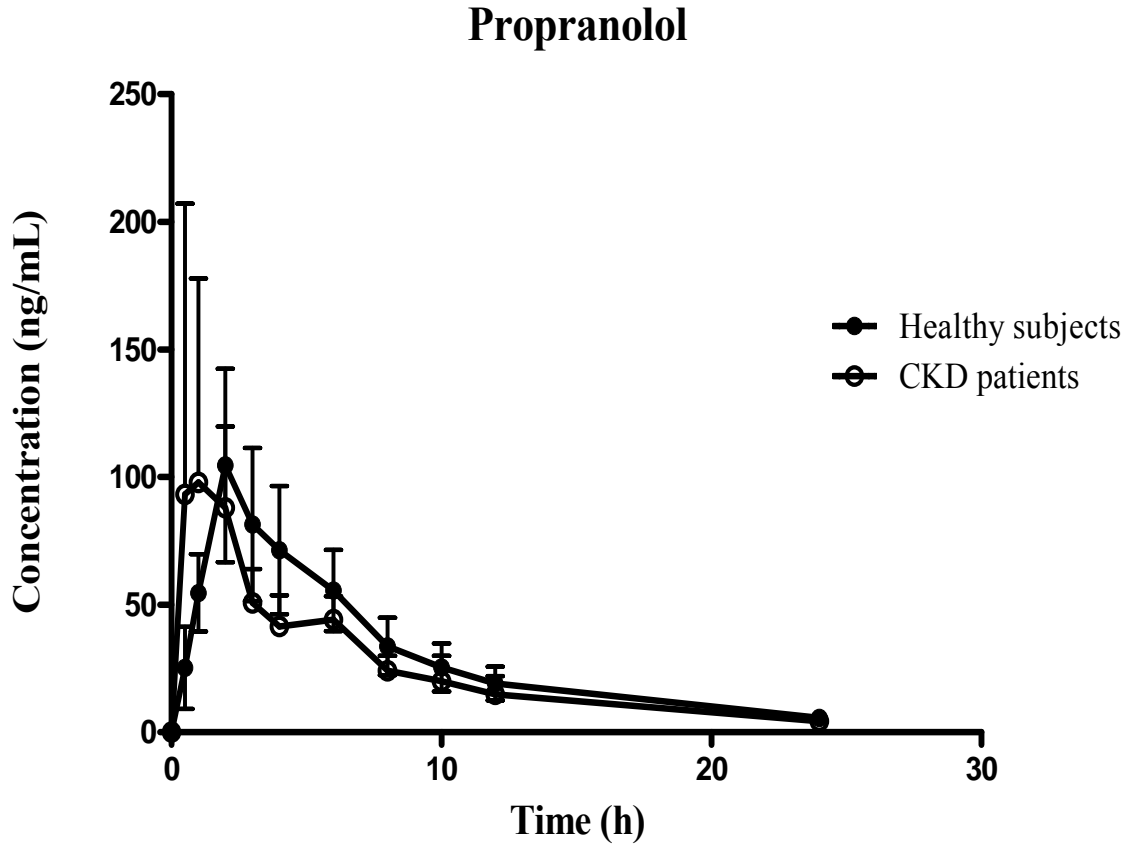
<sup>a</sup>Estimated from the MDRD (modification of diet in renal disease) equation.

\*p<0.05

### 5.3.1 Pharmacokinetics of Propranolol in Healthy vs. CKD Subjects

The pharmacokinetics of propranolol in chronic kidney disease patients were not different from those in healthy subjects. The AUC profile of propranolol showed a slight, but statistically insignificant decrease in CKD subjects (Figure 5.1). The C<sub>max</sub> was slightly higher in CKD subjects, while the T<sub>max</sub>, CL/F, and V<sub>ss</sub>/F were lower. However, these differences were not statistically significant (Table 2).

**Figure 5.1:** AUC profile for propranolol in healthy subjects and CKD subjects

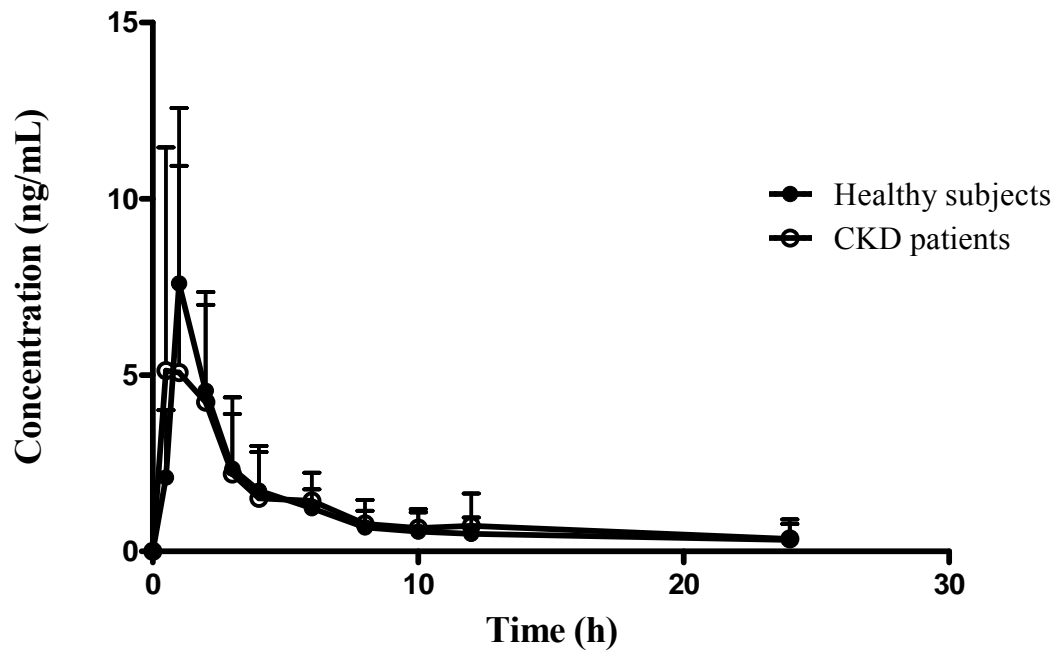


The propranolol metabolite (4-OH propranolol) also exhibited small differences in CKD patients vs. healthy subjects. The AUC profile (Figure 5.2) showed a slight decrease in  $C_{max}$  in CKD subjects, however, these differences were not significant. There were no significant changes in other pharmacokinetic parameters in the propranolol metabolite, except for renal clearance,  $CL_R$  (Table 2).



Figure 5.2: AUC profile for 4-OH propranolol in healthy subjects and CKD patients

### 4-OH propranolol



**Table 5.2:** Pharmacokinetic parameters of propranolol and 4-OH propranolol

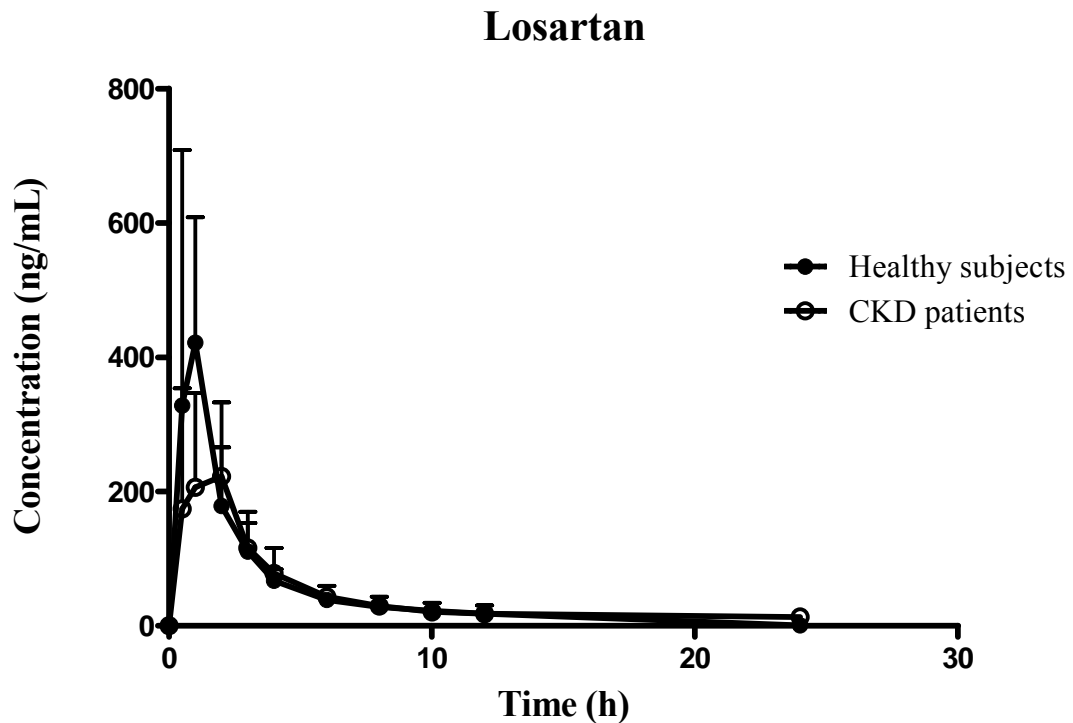
Variable	Propranolol		4-OH propranolol		Mean % of control	95% CI (% of control)	Mean % of control	95% CI (% of control)
	HV	CKD	HV	CKD				
$C_{max}$ (ng/ml)	109 ± 79	126 ± 93.3	7.74 ± 4.82	6.37 ± 5.40	115	96-117	82	0-95
$T_{max}$ (h)	2.60 ± 1.95	1.30 ± 0.67	1.40 ± 0.89	1.40 ± 0.82	50	42-260	100	96-131
$t_{1/2}$ (h)	6.59 ± 1.70	6.36 ± 1.91	11.1 ± 12.7	11.1 ± 11.0	97	89-100	100	55-93
$AUC_{0-24h}$ (ng/ml•h)	743 ± 535	628 ± 175	25.5 ± 9.93	28.0 ± 27.8	85	60-522	110	0-166
$AUC_{0-\infty}$ (ng/ml•h)	800 ± 582	673 ± 201	37.8 ± 20.7	40.5 ± 52.3	84	61-544	107	0-166
$CL_R$ (L/h)	0.002 ± 0.002	0.02 ± 0.03	11.9 ± 14.7	*0.33 ± 0.44	943	0-1086	2.77	0-3.58
$CL/F$ (L/h/kg)	1.23 ± 1.22	0.74 ± 0.29	---	---	60	0-40	---	---
$V_{ss}/F$ (L/kg)	12.0 ± 12.8	6.34 ± 1.46	---	---	53	0-30	---	---

Values expressed as mean ± SD, HV: healthy subjects, CKD: chronic kidney disease patients, \*p<0.05

### 5.3.2 Pharmacokinetics of Losartan in Healthy vs. CKD Subjects

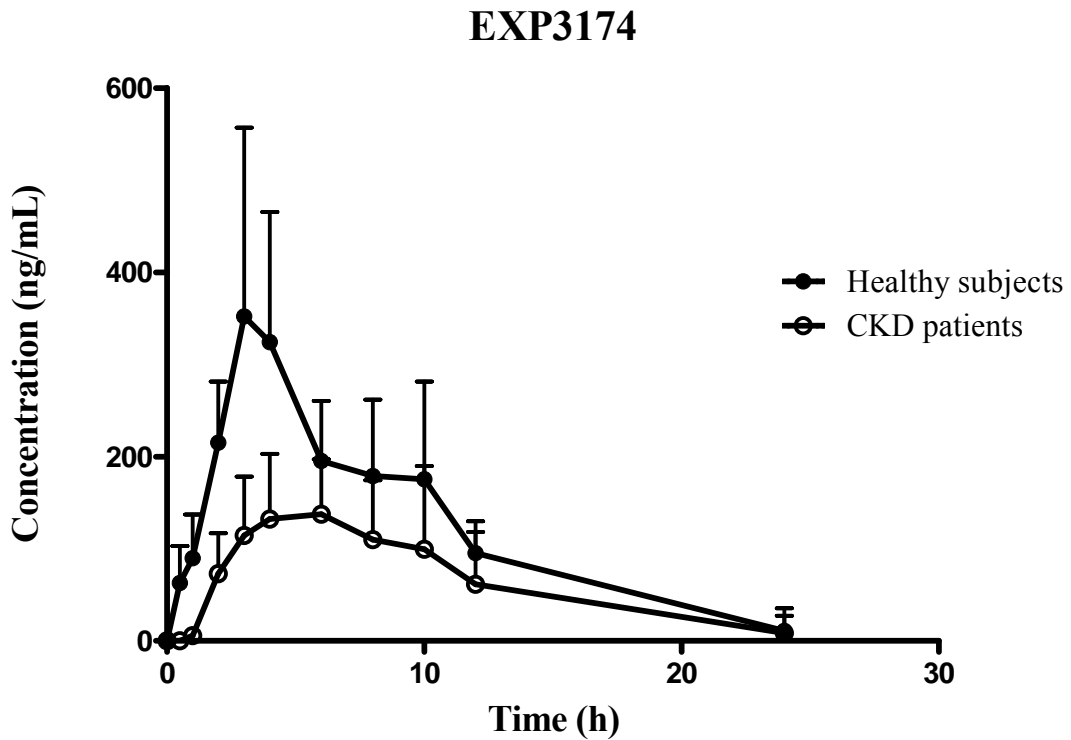
The pharmacokinetic profile of losartan was not statistically significantly different in the CKD subjects compared to the healthy controls. The  $C_{max}$  in the CKD subjects was lower than in the healthy controls (Figure 5.3), however, the variability and low number of subjects did not give a difference with statistical significance. The renal clearance of losartan was significantly lower in CKD subjects.

**Figure 5.3:** AUC profile for losartan in healthy subjects and CKD patients



In contrast, the pharmacokinetic profile of losartan's metabolite, EXP3174, did show a significant difference in the CKD subjects compared to the healthy volunteers (Figure 5.4). The  $C_{max}$  was significantly lower in CKD subjects, with a 58% decrease compared to control, while the  $t_{1/2}$ ,  $CL_R$ , and  $AUC_{0-\infty}$  were significantly lower in CKD subjects at 48%, 72%, and 55%, respectively (Table 3).

**Figure 5.4:** AUC profile for EXP3174 in healthy subjects and CKD patients



**Table 5.3:** Pharmacokinetic parameters of losartan and EXP3174

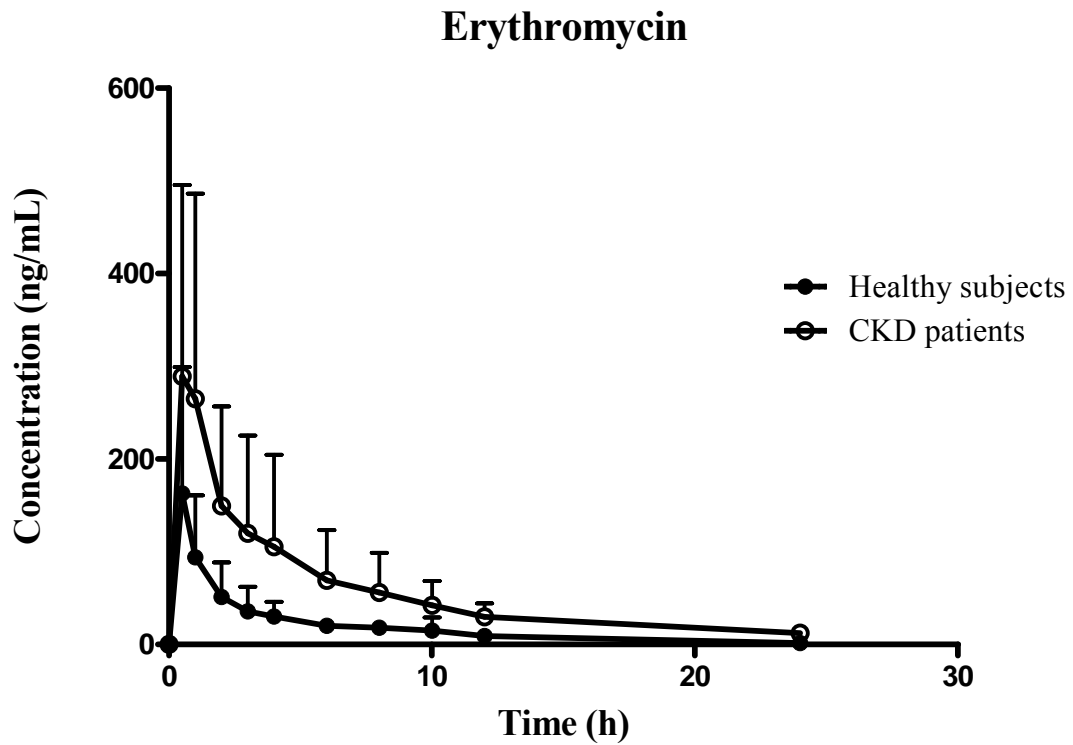
Variable	Losartan		EXP3174					
	HV	Losartan CKD	HV	CKD				
		Mean % of control	95% CI (% of control)	Mean % of control	95% CI (% of control)			
<b>C<sub>max</sub></b> (ng/ml)	554 ± 256	307 ± 257	55	49-77	403 ± 211	*171 ± 56.7	42	36-71
<b>T<sub>max</sub></b> (h)	0.70 ± 0.27	1.30 ± 0.27	186	129-205	3.40 ± 0.55	5.00 ± 2.83	147	55-208
<b>t<sub>1/2</sub></b> (h)	5.33 ± 2.07	8.45 ± 2.07	159	0-213	8.83 ± 4.01	*4.60 ± 1.40	52	48-74
<b>AUC<sub>0-24h</sub></b> (ng/ml•h)	1077 ± 363	1090 ± 363	101	95-117	2553 ± 1264	*1390 ± 794	54	21-55
<b>AUC<sub>0-∞</sub></b> (ng/ml•h)	1216 ± 369	1251 ± 369	103	121-144	3728 ± 1203	*1682 ± 983	44	41-58
<b>CL<sub>R</sub></b> (L/h)	4.22 ± 3.80	*0.61 ± 0.26	14	0-108	5.18 ± 2.71	*1.43 ± 0.36	28	21-54
<b>CL/F</b> (L/h/kg)	0.60 ± 0.20	0.49 ± 0.20	82	78-83	---	---	---	---
<b>V<sub>ss/F</sub></b> (L/kg)	4.64 ± 2.67	5.13 ± 2.67	111	105-147	---	---	---	---

Values expressed as mean ± SD, HV: healthy subjects, CKD: chronic kidney disease patients \* p<0.05

### 5.3.2 Pharmacokinetics of Erythromycin in Healthy vs. CKD Subjects

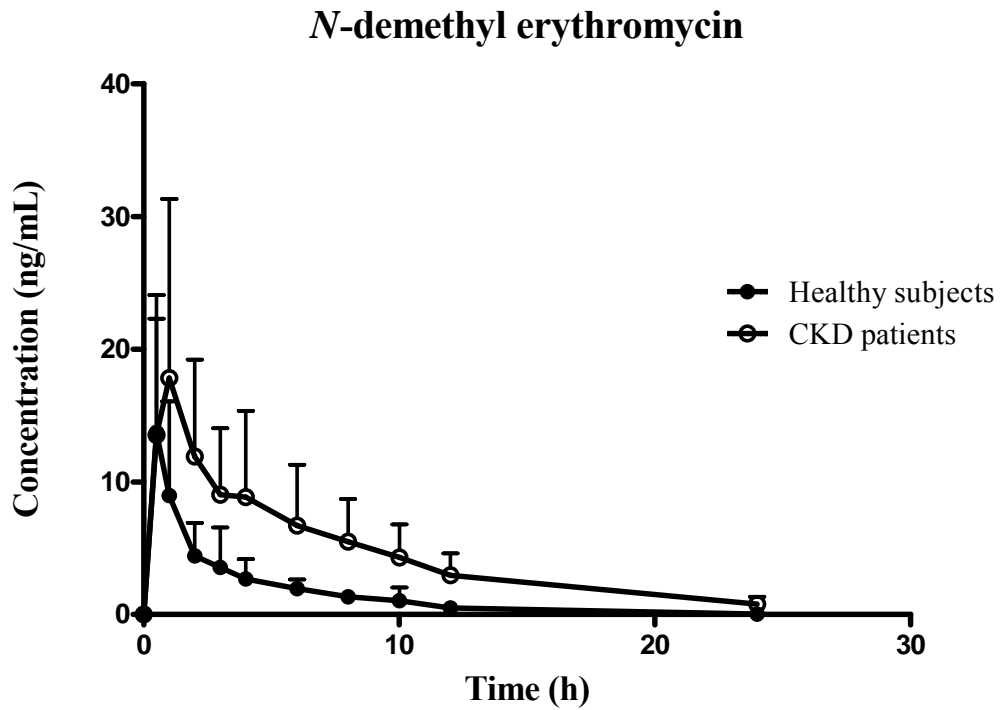
The erythromycin pharmacokinetic profile was significantly different in the CKD patients compared to the healthy controls (Figure 5.5). The  $t_{1/2}$  and  $AUC_{0-\infty}$  increased by 148% and 321%, respectively, while  $CL/F$  and  $V_{ss}/F$  decreased by 66% and 49%, respectively (Table 4).

**Figure 5.5:** AUC profile for erythromycin in healthy subjects and CKD patients



The *N*-demethyl erythromycin AUC was also significantly higher in CKD patients compared to healthy volunteers (Figure 5.6). The pharmacokinetic parameters that showed a significant increase were  $t_{1/2}$  and  $AUC_{0-\infty}$ , at 160% and 310%, respectively (Table 4).

**Figure 5.6:** AUC profile for *N*-demethyl erythromycin in healthy subjects and CKD patients



**Table 5.4:** Pharmacokinetic parameters of erythromycin and N-demethyl erythromycin

Variable	Erythromycin		N-demethyl erythromycin		Mean % of control	95% CI (% of control)	Mean % of control	95% CI (% of control)
	HV	CKD	HV	CKD				
$C_{max}$ (ng/ml)	183 ± 113	299 ± 211	15.2 ± 8.39	20.8 ± 12.2	163	0-117	137	119-141
$T_{max}$ (h)	0.80 ± 0.67	1.00 ± 0.61	0.80 ± 0.67	1.70 ± 1.4	125	0-425	212	103-210
$t_{1/2}$ (h)	4.65 ± 0.49	*6.90 ± 1.55	3.64 ± 1.19	*5.83 ± 0.83	148	107-183	160	134-222
$AUC_{0-24h}$ (ng/ml•h)	433 ± 137	1369 ± 955	35.6 ± 16.7	*112 ± 65.8	316	0-411	315	180-354
$AUC_{0-\infty}$ (ng/ml•h)	474 ± 146	*1522 ± 971	38.9 ± 14.9	*120 ± 67.0	321	0-412	310	202-343
$CL_R$ (L/h)	0.22 ± 0.17	*3.01 ± 2.48	3.71 ± 4.92	0.18 ± 0.12	1368	0-104	5	1-22
$CL/F$ (L/h/kg)	3.69 ± 0.95	*1.26 ± 0.55	---	---	34	23-48	---	---
$V_{ss}/F$ (L/kg)	24.3 ± 4.06	*12.5 ± 6.15	---	---	51	26-78	---	---

Values expressed as mean ± SD, HV: healthy subjects, CKD: chronic kidney disease patients \* =p<0.05



The table below shows the percent of dose administered excreted in the urine over 24 h. There were no statistically significant changes in the percent excreted in the urine for propranolol and its metabolite, or erythromycin and its metabolite because of the large variability observed. There was a statistically significant reduction in percent of losartan and EXP3174 excreted in the urine in CKD patients.

**Table 5.5:** Percent of dose excreted in the urine

	<b>Healthy Volunteers</b>	<b>CKD Patients</b>
<b>Propranolol</b>	0.0031 ± 0.0034	0.033 ± 0.049
<b>4-OH Propranolol</b>	0.434 ± 0.402	0.043 ± 0.068
<b>Losartan</b>	7.53 ± 5.03	*1.24 ± 0.49
<b>EXP3174</b>	24.7 ± 12.5	*4.23 ± 2.98
<b>Erythromycin</b>	1.12 ± 1.07	0.21 ± 0.13
<b>N-desmethyl erythromycin</b>	0.070 ± 0.078	0.013 ± 0.007

Values expressed as average ± SD, \*p<0.05

## 5.4 Discussion

The pharmacokinetic study of three BDDCS drugs in CKD subjects vs. healthy subjects showed that for propranolol (Class 1 drug) there was no significant change in the CKD subjects. It is worth noting that a previous study by Bianchetti et al. (1976), in which propranolol pharmacokinetics were studied in healthy volunteers vs. CKD patients and CKD patients on hemodialysis, the researchers found that the peak concentration ( $C_{peak}$ ) in blood and plasma was 2-3 fold higher in CKD patients compared to healthy and

CKD patients on hemodialysis. The apparent plasma clearance was also reduced in CKD patients and the authors suggested that hepatic extraction in CKD patients was reduced. In contrast, our studies found no significant differences in the PK parameters of propranolol in CKD patients compared to healthy volunteers. We cannot conclude why the results are different. Propranolol is a Class 1 drug, and thus transporters are not important in its disposition, therefore we would expect to see no difference in PK parameters in CKD subjects unless the metabolism of the drug was being affected. We have shown in Chapter 2, that the metabolism of propranolol is not affected by hemodialysis (HD) serum in rat and human hepatocytes or microsomes. Therefore, based on these results we believe that propranolol and most likely other Class 1 drugs would not be affected in CKD, unless changes in metabolism resulting from uremic toxins can be demonstrated.

There are some unusual aspects to the losartan and its primary metabolite EXP3174 reported here. The pharmacokinetic profile of losartan showed no significant difference for the parent but showed a 58% decrease in  $C_{max}$ , 48% decrease in  $t_{1/2}$ , 55% decrease in  $AUC_{0-\infty}$ , and 72% decrease in  $CL_R$  for its metabolite, EXP3174, in CKD patients compared to healthy controls. A previous study by (Sica et al., 1995) reported that there were no significant changes in the PK parameters of losartan or EXP3174 in CKD subjects vs. healthy controls. In that study, the investigators gave multiple doses of losartan for seven days, achieving steady state and used an HPLC analytical method with UV-detection, although reporting a limit of quantitation of 5ng/ml for both losartan and EXP3174. Another study by Dickson et al. (2003) found an increase in AUC of losartan in patients with renal impairment compared to healthy controls, and an increase in the

AUC of EXP3174 but no change in the half life. However, comparing our results in healthy volunteers to those reported by Lo et al. (1995), who characterized the pharmacokinetics of losartan and its active metabolite, the AUC,  $T_{max}$ ,  $t_{1/2}$ ,  $C_{max}$ , and  $CL_R$  yield similar values, although our results have higher variability but for a smaller number of subjects. That study also reported data for EXP3174 following intravenous dosing in which it was concluded that the elimination rate of the metabolite is not limited by its formation rate since the terminal slope of the metabolite was similar when losartan was administered compared to when the metabolite was administered IV, and the half life was longer than that for parent losartan.

From our findings, the reduced AUC for EXP3174 (but no changes in the losartan profile) suggest that there is no change in metabolism or uptake of losartan. If metabolism or uptake of losartan into the liver was inhibited in CKD, we would expect to see a change in AUC of losartan. Our data suggest that losartan is being taken up into the liver and being metabolized, but the metabolite is not being effluxed back into the circulation; hence, there is a reduction in the metabolite AUC in the plasma. We also know from our *in vitro* studies in Chapter 2, that the metabolism of losartan in rat and human hepatocytes and microsomes was not affected by the HD serum. The uptake of losartan was affected by uremic toxins and HD serum; however, the efflux by basolateral transporters was not investigated. It would be relevant to investigate if losartan is a substrate for basolateral efflux transporters in the liver, and if these may be inhibited in CKD.

The  $t_{1/2}$  for EXP3174 in CKD patients ( $4.6 \pm 1.4$  h) is markedly less than that for its parent losartan in CKD patients ( $8.45 \pm 2.07$ ). Theoretically this should not be

possible as the slowest rate limiting step (elimination of the parent drug losartan) should govern the terminal half life of the metabolite. However, the finding here may just reflect the fact that there is an even longer terminal half-life for losartan that is not obvious with 24 h sampling with the limited sensitivity of the assay methods employed. It is obvious in comparing Figure 3 and Figure 2 that during the 12-24 h time period, there is a marked drop in EXP3174 concentrations that is not seen for losartan, although essentially similar concentrations for the parent and the metabolite are observed at 24 h. We attribute this anomaly to the increased assay sensitivity with our LC/MS/MS method vs. the previously employed HPLC method where much shorter half-lives for losartan were reported.

Finally, the pharmacokinetics of both, erythromycin and its metabolite were shown to be altered in CKD compared to healthy controls. As shown previously by Sun et al. (2010), the PK of erythromycin is altered in ESRD subjects. In this study, we found that the AUC of erythromycin significantly increased in CKD patients, and that CL/F is significantly reduced. Also, AUC of the metabolite *N*-demethyl erythromycin is significantly increased in CKD subjects. This suggests that the uptake of erythromycin into the liver is inhibited in CKD, resulting in an increase in AUC, and the metabolite is a substrate for the same transporters, which causes an increase in the AUC of the parent drug in the plasma. In that study the AUC of erythromycin in ESRD subjects was approximately 2400ng/ml\*hr compared to 1040ng/ml\*hr in healthy subjects when given a 250mg oral dose of erythromycin, a 2.3-fold increase in AUC. In our study we observed a 3.2-fold increase in AUC in CKD subjects compared to healthy controls. These results indicate that patients on hemodialysis, as has been previously hypothesized in the literature, would have a reduction in changes in PK parameters compared to CKD

subjects because the hemodialysis procedure removes some of the uremic toxins that may be playing a role in the alteration of PK of non-renally excreted drugs. Our results suggest that this is true for erythromycin, where there was a greater fold increase in AUC in the CKD compared to the ESRD patients from the previous study. Thus, CKD patients, not on dialysis may be more sensitive to the PK changes of drugs than during dialysis, and may require dose adjustment of non-renally excreted drugs.

## **5.5 Conclusion**

The pharmacokinetic clinical study of propranolol, losartan, and erythromycin in CKD subjects vs. healthy controls has shown different changes in PK parameters for each drug in the CKD subjects. The propranolol study showed that no changes in pharmacokinetic parameters are present in CKD subjects compared to healthy controls; thus, agreeing with our hypothesis that for a BDDCS class 1 drug there would be no changes in PK due to uremic toxin accumulation in CKD subjects. Although uremic toxins may affect transporters and metabolizing enzymes, the high solubility and permeability of propranolol allow it to easily cross the cell membrane; hence, not requiring a transporter. The losartan pharmacokinetic study showed no difference in PK parameters for the parent drug. We would have expected to see a difference for a Class 2 drug, since transporters are likely to play a role in drug disposition for this class of drug. However, we did observe significant differences in PK parameters for the metabolite, EXP3174, with a lower AUC,  $C_{\max}$  and  $t_{1/2}$ . Since there was no change in the AUC of the parent, we suspect that the metabolism is not changed, but rather that the metabolite is

not excreted from the liver back into the systemic circulation, and this is causing a reduction in the PK parameters in the metabolite.

The erythromycin PK study exhibited a significant increase in PK parameters for both the parent and metabolite. Since erythromycin is a class 3 drug, we did expect to see a difference in the parameters, which may be caused by the basolateral uptake transporter inhibition in CKD subjects due to the uremic toxins. This inhibition of transporters would cause less of the drug to enter the hepatocyte; thus, increasing the AUC. These results indicate that BDDCS could be used as a tool to predict what type of drug disposition changes may occur for different drugs in CKD patients. Not all drugs are likely to be affected the same in CKD; hence, having a classification system to aid in the prediction of what differences may occur in a drug based on class may be helpful in drug development and drug dosing.

## 5.6 References

- Bianchetti G, Graziani G, Brancaccio D, Morganti A, Leonetti G, Manfrin M, Segà R, Gomeni R, Ponticelli C and Morselli PL (1976) Pharmacokinetics and effects of propranolol in terminal uraemic patients and in patients undergoing regular dialysis treatment. *Clin Pharmacokinet* **1**:373-384.
- Dickson TZ, Zagrobelny J, Lin CC, Ritter MA, Snavely D, Ramjit D, Shahinfar S and Lo MW (2003) Pharmacokinetics, safety, and antihypertensive efficacy of losartan in combination with hydrochlorothiazide in hypertensive patients with renal impairment. *J Clin Pharmacol* **43**:591-603.
- Dreisbach AW and Lertora JJ (2003) The effect of chronic renal failure on hepatic drug metabolism and drug disposition. *Semin Dial* **16**:45-50.
- Lo MW, Goldberg MR, McCrea JB, Lu H, Furtek CI and Bjornsson TD (1995) Pharmacokinetics of losartan, an angiotensin II receptor antagonist, and its active metabolite EXP3174 in humans. *Clin Pharmacol Ther* **58**:641-649.
- Martin DE, Chapelsky MC, Ilson B, Tenero D, Boike SC, Zariffa N and Jorkasky DK (1998) Pharmacokinetics and protein binding of eprosartan in healthy volunteers and in patients with varying degrees of renal impairment. *J Clin Pharmacol* **38**:129-137.
- Nolin TD, Frye RF, Le P, Sadr H, Naud J, Leblond FA, Pichette V and Himmelfarb J (2009) ESRD impairs nonrenal clearance of fexofenadine but not midazolam. *J Am Soc Nephrol* **20**:2269-2276.
- Sica DA, Lo MW, Shaw WC, Keane WF, Gehr TW, Halstenson CE, Lipschutz K, Furtek CI, Ritter MA and Shahinfar S (1995) The pharmacokinetics of losartan in renal insufficiency. *J Hypertens Suppl* **13**:S49-52.

Sun H, Frassetto LA, Huang Y and Benet LZ Hepatic Clearance, but Not Gut Availability, of Erythromycin Is Altered in Patients With End-Stage Renal Disease. *Clin Pharmacol Ther.*

Vinik HR, Reves JG, Greenblatt DJ, Abernethy DR and Smith LR (1983) The pharmacokinetics of midazolam in chronic renal failure patients. *Anesthesiology* **59**:390-394.



## Chapter 6: Conclusions and Perspectives

The work presented in this thesis has for the first time investigated the use of the Biopharmaceutics Drug Disposition Classification System (BDDCS) in predicting drug disposition changes in chronic kidney disease (CKD). Furthermore, I investigated the possible alteration and/or involvement of hepatic drug transporters in CKD, which may lead to changes in drug disposition. I investigated whether uremic toxins, which accumulate in CKD patients, inhibit hepatic drug transporters and thereby inhibit the uptake/efflux of drugs. Due to the involvement of drug transporters in drug disposition we hypothesized that if hepatic drug transporters are affected by uremic toxins, then for those classes (Class 2-4) of drugs that require a transporter there could be a change in drug uptake/disposition in the presence of uremic toxins or in CKD. The *in vitro*, *ex vivo*, and human clinical studies showed results that should help to expand the use of BDDCS for predicting how drug disposition is altered in CKD patients.

The cellular *in vitro* studies demonstrated that uremic toxins and hemodialysis (HD) serum do not have an effect on the uptake or metabolism of the Class 1 drug propranolol. Cells transfected with hepatic uptake transporters did not show a difference in propranolol uptake in the presence of uremic toxins or uremic serum compared to control. However there were significant changes in the uptake of the Class 2 (losartan) and Class 4 (eprosartan) drugs in transfected cells. The rat and human hepatocyte uptake studies showed difference in uptake for propranolol and eprosartan, but a significant difference for losartan. Efflux studies using P-gp transfected cells also showed that there

was a significant reduction in the efflux of model compounds in the presence of uremic toxins, indicating that uremic toxins may not only affect uptake transporters, but also efflux transporters. Finally, the metabolism studies in rat hepatocytes, and rat and liver human microsomes showed no significant changes in metabolism of propranolol or losartan in the presence of HD serum. The expected difference in eprosartan uptake in rat and human hepatocytes was not seen. The presence of uptake and efflux transporters in the hepatocyte may complicate the identification of specific transporters that are affected by uremic toxins since drugs may be substrates for both. Transfected cells were a more useful assay for studying the involvement of drug transporters in the presence of uremic toxins/HD serum. However, hepatocytes and microsomes are useful in determining the effect of uremic toxins/HD serum on drug the metabolism of drugs of different BDDCS classes.

The *ex vivo* studies using CKD rats and sham operated rats for isolated perfused rat liver (IPRL) experiments showed conflicting results. The number of animals per group was low and variability was high, diminishing statistical significance of the results. No significant change in AUC or accumulated propranolol was observed in the presence of HD serum, as expected. However, the predicted increase in perfusate AUC for Class 2 and 4 drugs if drug transporters were being inhibited was not seen and opposite effects for losartan (Class 2) and eprosartan (Class 4) were observed. These confusing observations may be the result of high variability and low number of rats per group and also the use of human serum to perfuse rat livers. The IPRL system is useful for investigating drug disposition, but may require perfusion with serum from the same species to obtain more accurate results.

The human clinical study confirmed that there is no significant change in propranolol pharmacokinetics in CKD patients compared to healthy controls but significant changes were observed for EXP3174, erythromycin, and *N*-demethyl erythromycin. This shows that uremic toxins in CKD patients have no effect on a highly soluble Class 1 drug that does not depend on transporters for membrane transport, but do affect disposition of Class 2 and 3 drugs that depend on transport for uptake and/or elimination.

This project has provided useful information for investigations on drug disposition in CKD patients. Knowledge of which transporters (apical and basolateral) are altered in CKD might have led to a better correlation between *in vitro* and *in vivo* and the availability of liver samples from CKD patients would have helped in defining how hepatic drug transporters vary in this patient population. *In vitro* studies have shown that there is inhibition of transporters by uremic toxins, but it is not known how transporter expression is altered in hepatic tissue. Although *in vitro* data is valuable, human clinical studies remain the best method for investigating the pharmacokinetic changes in CKD. The results of the human clinical studies provide some support for the notion that BDDCS is useful for predicting drug disposition changes in CKD, but further studies are needed. Since non-renal excreted drugs typically have no recommendation for dose adjustment in renal impairment populations, BDDCS could be useful in assessing the need for pharmacokinetic studies in CKD patients during drug development. The Food and Drug Administration (FDA) drafted a guidance for industry for pharmacokinetics in patients with renal impairment; however, the involvement of transporters was not mentioned. Furthermore, the guidance did not mention the differences in PK in different

stages of CKD and dialysis, which may affect the drug exposure. Proper PK studies would facilitate drug dosing recommendations in special populations; thus, preventing or reducing undesirable drug effects, and improving therapeutic effects.

**Publishing Agreement**

*It is the policy of the University to encourage the distribution of all theses, dissertations, and manuscripts. Copies of all UCSF theses, dissertations, and manuscripts will be routed to the library via the Graduate Division. The library will make all theses, dissertations, and manuscripts accessible to the public and will preserve these to the best of their abilities, in perpetuity.*

***Please sign the following statement:***

*I hereby grant permission to the Graduate Division of the University of California, San Francisco to release copies of my thesis, dissertation, or manuscript to the Campus Library to provide access and preservation, in whole or in part, in perpetuity.*

*Mabel Reyes* \_\_\_\_\_ *01-11-2011*  
Author Signature Date