NUCLEOSIDE AND HIV DRUG TRANSPORT AT THE BLOOD-TESTIS BARRIER

by

David Klein

A Dissertation Submitted to the Faculty of the

DEPARTMENT OF PHARMACOLOGY & TOXICOLOGY

In Partial Fulfillment of the Requirements

For the Degree of

DOCTOR OF PHILOSOPHY

In the Graduate College

THE UNIVERSITY OF ARIZONA

2015
THE UNIVERSITY OF ARIZONA
GRADUATE COLLEGE

As members of the Dissertation Committee, we certify that we have read the dissertation prepared by David Klein, titled Nucleoside and HIV Drug Transport at the Blood-Testis Barrier and recommend that it be accepted as fulfilling the dissertation requirement for the Degree of Doctor of Philosophy.

___________________________________________ Date: September 8th 2015
Nathan Cherrington

___________________________________________ Date: September 8th 2015
Stephen Wright

___________________________________________ Date: September 8th 2015
William Dantzler

___________________________________________ Date: September 8th 2015
Patrick Ronaldson

___________________________________________ Date: September 8th 2015
Terrance Monks

Final approval and acceptance of this dissertation is contingent upon the candidate’s submission of the final copies of the dissertation to the Graduate College.

I hereby certify that I have read this dissertation prepared under my direction and recommend that it be accepted as fulfilling the dissertation requirement.

___________________________________________ Date: September 8th 2015
Dissertation Director: Nathan Cherrington
STATEMENT BY AUTHOR

This dissertation has been submitted in partial fulfillment of the requirements for an advanced degree at the University of Arizona and is deposited in the University Library to be made available to borrowers under rules of the Library.

Brief quotations from this dissertation are allowable without special permission, provided that an accurate acknowledgement of the source is made. Requests for permission for extended quotation from or reproduction of this manuscript in whole or in part may be granted by the head of the major department or the Dean of the Graduate College when in his or her judgment the proposed use of the material is in the interests of scholarship. In all other instances, however, permission must be obtained from the author.

SIGNED: David Klein
ACKNOWLEDGEMENTS

First, I would like to thank my mentor, Dr. Nathan Cherrington for his unwavering commitment to the welfare of his students. You’ve helped me learn how to work diligently while also maintaining a charming sense of humor. Thank you for the opportunity to work on an exciting and unique project that allowed me to grow as a scientist. You are an exemplar leader, scientist, and friend.

Secondly, I’d like to thank Dr. Stephen Wright. Despite not being an official member of your lab, or even your department, you always had time to talk with me about data and science in general. You helped me learn what it means to think critically as a scientist. I am grateful for the opportunity to work in your lab so that you could share your enthusiasm of transport with me. I’d also like to extend my thanks to the other members of my committee who constantly guided and shared their experiences so I could become the best scientist I can be.

I would also like to thank lab mates, both past and present, for making the graduate experience enjoyable and valuable. John, Rhiannon, April, Mark, Anika, Hui, and Erica, you have continually challenged how I think about science and provided praise when things went well and shared in the grief when struggles came. I treasure our time together and am proud to call you my colleagues.

Most of all, I would like to express gratitude to my wife, Kaitlyn, your constant support made this possible. You made sure I could keep a healthy balance between school and sanity and motivated me to do my very best. I love you. I’d like to thank my parents for teaching me the importance of hard work and inspiring me to learn more about the world around me.
DEDICATION

I’d like to dedicate this dissertation to my wife, without whom this would never be possible and to my children, Joseph and Zachery (as well as any future children) for providing an endless source of happiness and a reminder to the important things in life. No matter what work you do, I hope this inspires you to put forth your best effort and enjoy it as much as I have enjoyed mine.
Contents

LIST OF FIGURES .......................................................................................................................................................... 10

ABSTRACT ........................................................................................................................................................................ 12

CHAPTER 1: BACKGROUND OF THE MALE GENITAL TRACT AND TRANSPORTERS .............................................. 13

Male Genital Tract Anatomy ........................................................................................................................................ 13

Interstitial Cells of the Testis ........................................................................................................................................ 14

Seminiferous Tubule Cells ........................................................................................................................................... 17

Epididymis ....................................................................................................................................................................... 23

Importance of Toxicology in the Testis ........................................................................................................................ 27

Fertility ........................................................................................................................................................................... 27

Viral Infection and Cancer ............................................................................................................................................ 29

Nucleosides ..................................................................................................................................................................... 31

Blood-Testis Barrier as a Sanctuary Site ....................................................................................................................... 34

Various Definitions of Blood-Testis Barrier ................................................................................................................ 34

Blood-testis Barrier Controversy ................................................................................................................................ 37

Transporters of the Testis ............................................................................................................................................. 39

Organic Solute Transporters ...................................................................................................................................... 44

Inorganic Solute Transporters .................................................................................................................................... 49

Current Study ............................................................................................................................................................... 53

CHAPTER 2: BASOLATERAL UPTAKE OF NUCLEOSIDES BY SERTOLI CELLS IS MEDIATED PRIMARILY
BY EQUILIBRATIVE NUCLEOSIDE TRANSPORTER 1 (ENT1). .................................................................................. 57

Introduction .................................................................................................................................................................... 57

Materials and Methods .................................................................................................................................................. 60

Materials ......................................................................................................................................................................... 60

Branched DNA Assay ................................................................................................................................................... 61

Ex Vivo Transport Experiments with Intact Seminiferous Tubules ........................................................................ 61

Sertoli Isolation ............................................................................................................................................................ 62

Primary Sertoli Cell Transport Experiments ................................................................................................................ 63

Sample Collection ......................................................................................................................................................... 64

Immunohistochemistry .................................................................................................................................................. 64

Statistics: ........................................................................................................................................................................ 65

Results ............................................................................................................................................................................ 65
Sertoli Isolation .................................................................................................................. 121
Primary Sertoli Cell Transport Experiments ..................................................................... 123
Statistics: .......................................................................................................................... 123
Transepithelial Transport of Nucleosides through Primary Rat Sertoli Cells ................. 123
MRP4 Inhibition of Nucleoside and NRTI Transepithelial Transport Through Rat Sertoli Cells .................................................................................................................. 126
Validation of Human Sertoli Cells with TEER and Apical Uptake .............................. 130
Transepithelial Transport of Uridine and AZT in Primary Human Sertoli Cells .......... 132

CHAPTER 6: SUMMARY AND FUTURE DIRECTIONS ......................................................... 139
Summary ............................................................................................................................. 139
Future Studies .................................................................................................................... 145
Specific Aim 1: Establish the molecular mechanisms by which MGT relevant drugs can cross the BTB for clinical use .............................................................. 149
Rationale ............................................................................................................................. 149
Experimental Approach ................................................................................................... 150
Aim 1.1-Determine the Transport of Nucleoside-Based Drugs Across a Monolayer of Human Sertoli Cells ................................................................................................. 151
Aim 1.2- Develop a Model (pharmacophores and Bayesian models) for Penetration of Nucleoside Drugs into the Blood-Testis Barrier ......................................................... 152
Approach: ............................................................................................................................. 152
Experimental strategy ........................................................................................................ 152
Specific aim 2: Determine the impact of epididymal water reabsorption on drug concentration within the MGT in vivo .............................................................................. 153
Rationale ............................................................................................................................. 153
Experimental Approach ................................................................................................... 154
Aim 2.1 - Determine the steady state accumulation of NRTIs within the tissues of the MGT .......................................................................................................................... 156
Aim 2.2 – Test the influence of epididymal water reabsorption on the steady-state distribution of NRTIs within the MGT .............................................................................. 156
Specific Aim 3: Determine the processes that support transepithelial NRTI transport in mouse epididymal cells in vitro .............................................................. 157
Rationale: ............................................................................................................................. 157
Aim 3.1. Determine the location of nucleoside and ABC transporters in mouse and human epididymis .................................................................................................................. 158
Aim 3.2. Determine the transport of NRTI drugs in epididymal cells isolated from mice using knock-down technology to reduce levels of various transporters.......................... 159

Gene knock-down experiments....................................................................................... 160

Aim 3.3. Determine the transport of NRTI drugs in epididymal cells isolated from mice from which selected transporters have genetically eliminated ........................................ 160

Appendix A: LOCALIZATION OF NUCLEOSIDE TRANSPORTERS IN THE EPIDIDYMIS ........ 162

Introduction .................................................................................................................. 162

Materials and Methods ............................................................................................. 164

Materials ...................................................................................................................... 164

Sample Collection ...................................................................................................... 164

Immunohistochemistry ................................................................................................. 165

Results and Discussion ............................................................................................... 165

APPENDIX B: FUNDING ACKNOWLEDGEMENTS ........................................................... 169

REFERENCES .............................................................................................................. 170
LIST OF FIGURES

Figure 1.1: Internal Component of the Rat MGT ................................................................. 15
Figure 1.2: Various Cell Populations within the Testis.......................................................... 19
Figure 1.3: The 6 stages of spermatogenesis in human seminiferous tubules ....................... 22
Figure 1.4: Various Cell Populations within the Epididymis. ................................................ 25
Figure 1.5: Examples of Endogenous and Exogenous Nucleo-Compounds ......................... 33
Figure 1.6: Transporter Expression at the Blood-Testis Barrier ........................................... 52
Figure 2.1: Time course of basolateral transport of [3H]uridine by rodent seminiferous tubules. .......................................................................................................................... 66
Figure 2.2: Ex vivo kinetic analysis of basolateral transport of [3H]uridine by rodent seminiferous tubules ..................................................................................................................... 69
Figure 2.3: Ex vivo analysis of the effects of NBMPR on basolateral transport of [3H]uridine by rodent seminiferous tubules ......................................................................................... 71
Figure 2.4: In vitro analysis of role of rENT1 and rENT2 in [3H]uridine transepithelial transport by primary cultured Sertoli cells ................................................................................................................................. 73
Figure 2.5: Effects of NBMPR on the basolateral uptake of [3H]uridine in primary cultured rat Sertoli cells.......................................................................................................................... 75
Figure 2.6: Branched DNA analysis of nucleoside transporter expression in seminiferous tubules relative to kidney ........................................................................................................... 77
Figure 2.7: Localization of rENT1 and rENT2 in the testis ...................................................... 79
Figure 2.8: Localization of hENT1 and hENT2 in the testis .................................................... 80
Figure 2.9: Inhibition of [3H]uridine transport by NRTI in primary cultured rat Sertoli cells ............................................................................................................................................. 82
Figure 2.10: Model of ENT-Mediated Transport of Nucleosides Across the BTB. .................. 83
Figure 3.1: MRP1 localization in the testis ............................................................................. 90
Figure 3.2: MRP4 localization in the testis ........................................................................... 92
Figure 3.3: MRP5 localization in the testis ........................................................................... 94
Figure 3.4: MRP8 localization in the testis ........................................................................... 96
Figure 4.1: Mdr and BCRP Expression ................................................................................. 104
Figure 4.2: Nucleoside Expression ....................................................................................... 106
Figure 4.3: Mrp Expression ................................................................................................. 107
Figure 4.4: Oat Expression ................................................................................................. 109
Figure 4.5: Oct Expression ................................................................................................. 110
Figure 4.6 Oatp Expression: .................................................................................................................. 112
Figure 5.1: Transepithelial Transport of Nucleosides through Primary Rat Sertoli Cells............ 124
Figure 5.2 MRP4 Inhibition on Nucleoside and NRTI Transepithelial Transport Through Rat Sertoli Cells................................................................................................................................. 127
Figure 5.3: Validation of Human Sertoli Cells with TEER and Apical Uptake. ................................. 129
Figure 5.4 Transepithelial Transport of Uridine and AZT in Primary Human Sertoli Cells.......... 131
Figure 5.5 Models for Nucleoside and NRTI Transport at the BTB in Rats and Humans .......... 134
Figure 6.1: A model demonstrating how NRTIs may bypass the BTB via ENT1 and ENT2 (left) and then become concentrated in the epididymis due to reabsorption of water (right) ................. 142
Figure A.1. Localization of Nucleoside Transporters in the Epididymis........................................... 166
ABSTRACT

The immune-reactive sperm are kept separate from the body by epithelial barriers such as the blood-testis barrier (BTB). While these barriers are beneficial for the protection of sperm from toxicants, they can make treating these areas difficult due to preventing the entry of pharmacological agents. This is especially an issue in the treatment of HIV and Ebola infection based on the ample evidence that these viruses are able to survive and spread from within the male genital tract (MGT), but only a few antiviral drugs are known to access the MGT. Transporters that line the epithelial barriers of the MGT, especially the BTB, are important for determining whether or not a drug is able to penetrate into the MGT through transepithelial transport. Several nucleoside analogs (NSA), which are used to treat HIV infection and leukemias, are known to be able to accumulate in seminal plasma, which makes them a useful tool for understanding transepithelial transport for the BTB. The purpose of these studies is to characterize the transport profile for the MGT, in particular the BTB, to gain a better understanding of how xenobiotics, especially ones based on nucleosides, can access the MGT. The chief finding of this work is the discovery of a transepithelial transport pathway expressed by Sertoli cells that allows for the entry of nucleosides (necessary for germ cell development) and NSA into the MGT. This pathway depends on equilibrative nucleoside transporter (ENT) 1 uptake and ENT2 efflux and occurs in both rats and humans. These studies provide the foundation for being able to predict the penetration of novel drugs into the MGT.
Male Genital Tract Anatomy

The male genital tract (MGT) is a fascinating system capable of the incredible process of propagating a species. Fertility is only used a few times during the course of an organism’s lifetime for humans, yet it has a profound effect on several aspects of quality of life, sociology, and political issues. The MGT is critical for the propagation of mammalian species and involves several coordinated processes for proper fertility. Figure 1.1 is a photograph of the internal components of the MGT from a rat with all the major organs identified. These tissues are important for the generation of sperm (spermatogenesis), sperm storage, and secretions that ultimately comprise semen. Many studies are also performed with seminal plasma, which is semen without the cellular component (primarily sperm cells). The MGT starts within what is likely the most well known organ of the male reproductive system: the testis.

The dynamic nature of the testis can make the tissue appear “messy” when viewed at high magnification. Nonetheless, it is important to appreciate the careful orchestration that occurs that allows for successful spermatogenesis to take place. The testis is the male gonad; typically egg shaped and encapsulated by a tunica layer. In most species, the testis resides outside of the abdominal cavity within the scrotum. The primary functions of this organ are the production of
spermatozoa and testosterone, the hormone responsible for most secondary male characteristics. Both of these functions are key to maintaining male fertility. In very broad terms, the inside of the tunica can be categorized into two regions: the seminiferous tubules and the interstitial area between the tubules (Hess, 1999; Su et al., 2011). The seminiferous tubules are generally responsible for spermatogenesis while the interstitium is responsible for steroid production. The seminiferous tubules can be subdivided into several (~250 in human) distinct lobes. In order to carry out the primary functions, the testis possesses several types of cells that work in concert for proper sperm maturation and hormone production.

**Interstitial Cells of the Testis**

The cell types of the testis are labeled in figure 1.2 to demonstrate their morphology and location within the testis. The testis receives nutrients from the capillaries within the interstitial area. These capillaries are made up of endothelial cells that form tight junctions to limit the diffusion of hydrophobic compounds (Mruk and Cheng, 2010; Mital et al., 2011). It has been suggested that these cells are a component of the BTB due to their ability to limit diffusion via tight junctions and transporter expression (Mital et al., 2011). However, it should be noted that the transepithelial electrical resistance (TEER) for testicular endothelial cells has not been shown to be nearly as high as it is for the endothelial cells in the brain, which are the primary component of the blood-brain barrier (BBB) (Ronaldson et al., 2008; Burkhart et al., 2015).
**Figure 1.1: Internal Component of the Rat MGT.** Rat MGT labeled with the testis, epididymis (caput and cauda), vas deferens (ductus deferens), seminal vesicles and prostate. Note that the bladder is labeled for reference and is not generally considered part of the MGT. The cauda has been pulled away from the testis which is its normal location in order to make visualization easier.
The other predominant cell type within interstitium is the Leydig cell. Leydig cells, also referred to interstitial cells of Leydig, grow in clusters near seminiferous tubules and are easily stained with eosin. There are two populations of Leydig cells that are important for proper steroid biosynthesis; fetal and adult Leydig cells. The fetal Leydig cells are essential for the development of the male genital tract (MGT) including the descending of the testis (Tremblay, 2015). These cells die off shortly after birth and are not thought to contribute to the adult Leydig cell population (Haider, 2004). Adult Leydig cells are stimulated by luteinizing hormone (LH) to produce steroids such as testosterone. Leydig cells are regarded as being the primary source of testosterone in males (Haider, 2004; Morgan et al., 2012; Dankers et al., 2013; Tremblay, 2015). Interestingly, multidrug resistance-associated protein (Mrp) Mrp4−/− mice are reported to have low testicular testosterone production, low testis weight, and impaired gametogenesis but stable circulating levels of testosterone (Morgan et al., 2012). The authors speculate that Mrp4 regulates cAMP homeostasis, which is necessary for the proper signaling of LH to steroidogenesis. The plasma levels of testosterone are suspected of being maintained through upregulation of hepatic cytochrome P450 (Cyp) 2b10 (Morgan et al., 2012).

It is relatively rare for Leydig cells to form tumors, although hyperplasia is common in patients with testicular disorders such as Sertoli-only syndrome (Tremblay, 2015). Hyperplasia involves overactivation of the LH receptor. This is especially interesting since LH receptor can be induced by estrogens compounds in rodents, although these findings have yet to be extended to humans (Dankers
et al., 2013). Leydig cell tumors can occur in young children and induce the characteristic symptom of precocious puberty due to excessive production of testosterone.

**Seminiferous Tubule Cells**

Within the seminiferous tubules, there are three primary cell types; the peritubular myoid cells, the Sertoli cells and the germ cells. The peritubular myoid cells line the outer ring of the BTB and are primarily responsible for muscle contractions of the seminiferous tubules (Virtanen et al., 1986; Carlo, 1988). These cells are in direct contact with the basal lamia (basement membrane) of seminiferous tubules. Recent studies have suggested that these cells are also important for the maintenance of spermatogonial stem cells, the precursor for all germ cells (Chen et al., 2014). This speculation is based on the observation that targeted disruption of the androgen receptor gene in peritubular myoid cells causes a gradual depletion of spermatogonia. Dependence of androgen receptor for spermatogonia maintenance suggests that testosterone is critical for stimulating the peritubular myoid cells to create a microenvironment that is critical for maintaining spermatogonia. There is also evidence that these cells express P-glycoprotein (P-gp) which indicates they may play a role keeping potential toxicants out of the seminiferous tubules (Bart et al., 2002). Peritubular myoid cells are especially thin and in rodents, can be difficult to locate histologically.

Germ cell is a general term that refers to developing spermatozoa and is the most numerous cell type in the testis. However, their morphology and
characteristics can vary tremendously depending on their stage of development (Hess, 1999; Cheng and Mruk, 2011; Qian, Y Cheng, et al., 2013). It is not surprising that these frequently dividing cells represent the vast majority of testicular neoplasms (Dankers et al., 2013; Meyts et al., 2013). Germ cell development begins with the spermatogonia (Also referred to as spermatogonial stem cells), which are located on the basolateral membrane of Sertoli cells outside of the tight junctions. These cells are very important for toxicology because they do not replenish. Once a significant number of them are killed, the male becomes permanently sterile (Liu et al., 2011; Zhou et al., 2012). Spermatogonia are known to be sensitive to several toxicants such as chemotherapeutic agents, immunosuppressive drugs, radiation, alkylating drugs, and even ethanol (Suzuki et al., 2003; Caires et al., 2012; Kanatsu-Shinohara and Shinohara, 2013). There is clinical interest in transplanting these cells to restore fertility and some surgical attempts have been met with moderate success (Gharwan et al., 2014). Once these cells divide via mitosis, one of the daughter cells bypasses the tight junctions through what is called “an airlock mechanism” (Pelletier, 2011). The airlock mechanism describes a germ cell that moves towards the lumen and forms another tight junction behind it towards the basolateral membrane. Once the new tight junction is formed, the old one breaks apart thereby allowing the germ cell to get past the BTB without disrupting the tight junctions in a manner that would allow potential toxicants to enter.

As the germ cell matures, it will generally move towards the lumen and will divide again through meiosis and become a round spermatid. Round
Figure 1.2: Various Cell Populations within the Testis. (Deltagen.com)
A section of testis stained with hemotoxylin demonstrating the localization for Sertoli cells, Leydig cells, germ cells (spermatogonia, spermatid, and spermatocyte).
spermatids are haploid but still possess a large circular morphology, as opposed to the compact flagella-possessing morphology commonly associated with sperm (Gerton and Millette, 1986; Pang et al., 2006; Su, Mruk, and Cheng, 2011; Fietz et al., 2013).

It is at this point in development the germ cell ceases cell division and begins dramatic restructuring to compact the DNA and develop flagella essential for proper sperm function (Hess, 1999). During these changes, the germ cells will become elongated spermatids and eventually be released into the lumen of the seminiferous tubule as spermatozoa. The details for these processes are well described in other review articles (Hess, 1999; Juul et al., 2014; Gunes et al., 2015). It is important to note that the spermatozoa released into the seminiferous tubules are incapable of conception and are immotile (Gerton and Millette, 1986; Aliabadi et al., 2013). Maturation of the spermatozoa occurs downstream in the male genital tract (MGT) within the epididymis (Besançon et al., 1985; Cornwall, 2009). This process of spermatogonia to released spermatozoa is referred to as one cycle of spermatogenesis.

Sertoli cells are the epithelial cells of the testis and represent the bulk of the static cellular mass for the seminiferous tubules. These cells are long and often described as stringy or tree-like (Santiemma et al., 1992; Wang et al., 2015). The twisted shape is due to maintaining adhesion to developing germ cells throughout the dynamic process of spermatogenesis. Sertoli cells are often cited as an example of sustentacular cells, which means they are important for structural support, specifically for the seminiferous tubules. Sertoli cells were first described as “mother” or “nurse” cells for the developing germ cells due to
their critical function of supplying nutrients to the spermatids (Jiang et al., n.d.; Anway, 2002; Fietz et al., 2013). As germ cells develop, they shed large amounts of cytoplasm which the Sertoli cells will engulf (Xiao, Mruk, EWP Wong, et al., 2014). The primary function of Sertoli cells is to support spermatogenesis through secretion of essential nutrients, protection from potential toxicants and creation of a unique microenvironment critical for proper germ cell development. To accomplish this function, Sertoli cells express several proteins that are used to create tight junctions that connect each cell to neighboring Sertoli cells on the basolateral membrane. These tight junctions form a tight barrier sealing the lumen of the seminiferous tubule from the rest of the body. The combination of Sertoli cells and their tight junctions form what is commonly referred to as the anatomical BTB (Bart et al., 2002; Cheng and Mruk, 2011; Su, Mruk, and Cheng, 2011).

An essential feature of Sertoli cells is sensitivity to follicle-stimulating hormone (FSH) and expression of FSH receptor is considered an unique marker for Sertoli cells within the testis (Santiemma et al., 1992; Pineau et al., 1999). FSH stimulation causes the Sertoli cells to secrete inhibin, which is a negative feedback regulator of FSH secretion in the pituitary gland, and androgen-binding proteins. As the name implies, the function of androgen binding proteins is to bind to testosterone decreasing the hydrophobicity of the compound allowing the distribution of testosterone to be limited (Santiemma et al., 1992; Chinta et al., 2015). Regarding Sertoli cells, androgen binding proteins keep the concentration of testosterone within the seminiferous tubules high enough to aid in spermatogenesis (Dohle et al., 2003; Hammond, 2011).
Figure 1.3: The 6 stages of spermatogenesis in human seminiferous tubules. (Sibler, 1991) A rendition of the different type of germ cells that are associated with each other in a particular stage for the human testis.
The orchestration of spermatogenesis has some interesting morphological consequences on the testis. Each Sertoli cells is capable of supporting multiple cycles of spermatogenesis at once. The duration of the cycle is species dependent, but it is on the order of weeks for most mammals (approximately 64 days for humans and 47 days for rats). Since new cycles always begin at the same point of germ cell development, Sertoli cells will always possess a histological pattern of germ cells in a particular developmental stage (Hess, 1999; Amann, 2008).

These stages (sometimes called waves or cellular associations) can be observed histologically and are given a roman numeral designation (i.e. V for stage five). The number of stages varies between species, with humans possessing 6 (see figure 1.3) and rats having 14 (Xiao, Mruk, CKC Wong, et al., 2014). Local Sertoli cells will always be in the same stage and the various stages can be observed along the length of a seminiferous tubule (Hess, 1999). Being able to identify the stages of a section of seminiferous tubule can be very valuable in reproductive toxicology since some toxicants only affect certain stages and the expression of some xenobiotic transporters is known to be stage specific (Mann and Lutwak-Mann, 1982; D M Creasy, 2001; Enokizono et al., 2007; Su et al., 2010; Qian, Y-H Cheng, Jenardhanan, et al., 2013).

**Epididymis**

The seminiferous tubules from the various lobes of the testis pool together into an interconnecting network of tubules in an area named the rete testis. The rete testis connects the large number of seminiferous tubules to the efferent ducts connecting to the epididymis and also is responsible for some water absorption.
The epididymis is a crescent shaped organ attached to the outside of the testis through the efferent ducts. It is a single convoluted tubule responsible for the storage and maturation of sperm and is the section of the MGT between the testis and the ductus deferens (or vas deferens). The epididymis is classically divided into three regions starting from the testis and ending at the ductus deferens; the caput (head), corpus (body), and the cauda (tail), although sometimes the head is further divided into forth section called the initial segment. It is in the initial segment (or head depending on classification) that the efferent ducts connect to the epididymal duct.

The various cell type of the epididymis and their locations can be observed in figure 1.4. The duct is lined with epithelial cells called principal cells (sometimes main cells) that form tight junctions near the apical membrane. These cells are responsible for maintaining the structure of the ducts and secretions that are important for sperm maturation, such as carnitine. To aid in these functions, they form nonmotile stereocillia that reach into the lumen of the epididymal duct (Cornwall, 2009; Alkafafy et al., 2011; Zuo et al., 2011). Also lining the ducts are smaller cells called basal cells. These cells are also connected to the basement membrane, but do not reach the lumen of the duct. They are traditionally thought to be precursor cells for the principal cells, although recent evidence suggests that they are also important for nutrient salvaging (Arrighi, 2014; Mandon and Cyr, 2015). Unlike the testis, the interstitial cells (between the ducts) are not thought to be very active and are primarily for structural support.

There has been a growing interest within the literature in the mechanisms of sperm maturation within the epididymis. It is well established that the luminal
Figure 1.4: Various Cell Populations within the Epididymis. (Dartmouth.edu/anatomy) A section of epididymis stained with hemotoxylin demonstrating the localization for principal cells with the stereocilia, basal cells, and the interstitial smooth muscle cells.
secretions within the epididymal duct are essential for successful sperm development (Bagnis et al., 2001; Hermo and Smith, 2011; Mital et al., 2011). One of the primary processes of the epididymis and rete testis is the removal of water from the MGT. It has been reported that as much as 99% of the seminiferous fluid is reabsorbed in these areas (Cornwall, 2009; Cai et al., 2013). The water reabsorption has potentially interesting and surprisingly unexplored impacts on drug concentration within the MGT. A xenobiotic that gets into the MGT in the testis and flows downstream into the cauda of the epididymis would be expected to see a ~100 fold increase in concentration, assuming it is not reabsorbed by the epididymis. This implies that toxicants below the toxic threshold in the testis could increase to toxic concentrations within the epididymis providing a mechanism for epididymis-specific toxicity.

One of the central players to epididymal water reabsorption is sodium/hydrogen exchanger 3 (NHE3), which is expressed on the apical membranes of principal cells and is inhibited by amiloride (Wong and Yeung, 1976; Bagnis et al., 2001; Zhou et al., 2001). This transporter allows for Na$^+$ to be reabsorbed from the MGT that causes water to follow out of the duct through various aquaporins (Lu et al., 2007; Alkafafy et al., 2011; Hermo and Smith, 2011; Moretti et al., 2012). This transporter is also important for the secretion of H$^+$ which creates an acidic environment within the duct important for sperm storage (Enomoto et al., 2002; Yeung et al., 2004; Pastor-Soler et al., 2005; Cornwall, 2009; Zuo et al., 2010; Arrighi, 2014). Mature sperm is stored within the cauda of the epididymis until ejaculation, during which time ductal contractions push stored sperm into the ductus deferens that ultimately connects
to the urethra. During ejaculation, the ductus deferens contracts, pushing sperm along the MGT collecting secretions from the accessory sex glands (primarily seminal vesicles and prostate) that mix to ultimately form semen.

While the testis and epididymis are responsible for the sperm component of semen, these organs contribute to 2-5% of the total volume of seminal plasma (Cao et al., 2008; Batruch et al., 2012). The majority of seminal plasma is derived from the seminal vesicles (60-70%) and the prostate (20-30%). The remaining seminal plasma originates from the bulbourethral glands (Cao et al., 2008). It is the function of these organs, sometimes referred to as male accessory glands, to provide nutrients that allow for the sperm to be motile and survive in the vaginal cavity.

Importance of Toxicology in the Testis

The reproductive system is one of the only body systems where pharmacological agents for and against function are desired. The field has come to appreciate the complexities of this system including the excessive preparation and the finely tuned orchestration of several tissues that is required for proper fertility to occur. Understanding the testis, in particular the access that xenobiotics have to the BTB, will impact several clinically relevant fields, including fertility, cancer treatment, and virus infection.

Fertility

There is growing interest in understanding the mechanisms of male infertility. Subfertility, which refers to men who have normal sexual function but
low or dysfunctional sperm counts (indicative of a problem within the testis or epididymis), affects 1 out of every 20 men (Hirsh, 2003; Brugh and Lipshultz, 2004). The majority of these cases are idiopathic (Brugh and Lipshultz, 2004). Infertility represents an important quality of life issue for many American couples. A recent survey determined that many couples seeking fertility treatment are willing to pay thousands of dollars (average $5,000) out-of-pocket (Wu et al., 2014). The survey went on to point out that couples seeking in vitro fertilization (IVF), one of the most common methods to treat male infertility, would spend over $15,000 simply for the attempt. Most of the couples surveyed did not have large disposable income and many were willing to borrow money or take loans to pay for the costly treatment (Wu et al., 2013). These data demonstrate the importance of understanding the physiological processes of the male reproductive tract, particularly in the testis and epididymis, to develop more effective and affordable treatments.

There has also been great interest in developing a male contraception, which is essentially a reversible reproductive toxicant. Studies in this field have yet to yield an approved drug. One of the issues that is frequently encountered is delivery of drugs past the BTB, which further highlights the need to understand BTB transport dynamics (Su, Mruk, Lee, et al., 2011). A promising male contraceptive in phase II human trials is adjudin (Qian, Y-H Cheng, Jenardhanan, et al., 2013). The mechanism of action for this drug is to disrupt adhesion molecules between the germ cell and the Sertoli cells, thereby ceasing germ cell development without disrupting precursor cells or affecting testosterone production (Su et al., 2010; Qian, Y Cheng, et al., 2013).
**Viral Infection and Cancer**

Another clinically relevant aspect of the BTB is the shielding of viruses and cancer cells from therapies. The concept is that viruses and tumor cells are able to access the BTB at low quantities and the BTB prevents drugs from reaching a therapeutic threshold within the MGT. Over time, the destructive agents can replicate within the MGT so when therapy is ceased, they can get back to the bloodstream and cause a relapse. This is suspected to occur in human immunodeficiency virus (HIV) infection, ebola infection, and acute lymphoblastic leukemia (ALL).

As devastating as HIV is to developing nations, it also remains a tremendous problem in the United States. The Centers for Disease Control (CDC) estimates more than 1.1 million Americans are infected with HIV, and the number continues to rise every year (Ruela Corrêa et al., 2012). One of the primary reasons for this rise is the failure of highly active antiretroviral therapy (HAART) to eradicate the virus from the body (Le Tortorec and Dejucq-Rainsford, 2010). This creates an epidemiological concern as the longer an HIV positive patient takes HAART, the more likely the virus will acquire resistance to the therapy, which can make resistant strains more prevalent and cause current therapy to become ineffective over time. Another weakness of HAART is that asymptomatic patients may still remain infectious and have the capacity to transmit the virus to others (Shehu-Xhilaga et al., 2005; Le Tortorec and Dejucq-Rainsford, 2010; Ambrosioni et al., 2014). Consequently, there is an urgent need to design new drugs for the treatment of HIV infection that are able to eradicate HIV from patients.
One of the reasons current therapy is unable to clear the virus from the body is the presence of sanctuary sites, which are areas of the body in which the virus can survive, but where drugs cannot reach therapeutic concentrations (Eilers et al., 2008; Ronaldson et al., 2008; Dahl et al., 2010; Palmer et al., 2011). The testis is a classic example of a sanctuary site that is particularly important for HIV transmission considering that nearly 75% of HIV infected patients in the US are men and transmission from men occurs with high efficacy due to the highly infectious nature of semen (Royce et al., 1997). Strategies that improve disposition of HAART to the MGT would not only help treatment of the patient by eliminating a sanctuary site, but would also substantially decrease transmission of the virus from semen which represents a common means of infection.

Recent reports have noted that the Ebola virus is also transmissible through infected seminal plasma in convalescent men (Mackay and Arden, 2015). This is especially alarming since seminal plasma can be infectious for months after the virus undetectable in blood (Rogstad and Tunbridge, 2015). Currently, there are limited treatment options for treating Ebola virus disease (EVD) in patients. The WHO has prioritized the development and retasking of drugs to treat EVD, however in order to optimally reduce transmission, drugs that treat the virus must penetrate the MGT.

In terms of cancer treatment, the impact of the BTB focuses on testicular relapse (Dave et al., 2007). Testicular relapse is the detection of cancerous cells, particularly leukemia, in the testis following chemotherapy treatment, a condition that can lead to relapse. This process is thought to be the result of poor
penetration of chemotherapeutics into the testis because of the BTB. This occurs frequently in patients diagnosed with childhood acute lymphoblastic leukemia (ALL) where testicular relapse has been reported in nearly 40% of boys (20% of all ALL relapse patients) (de Góes et al.; Grundy et al., 1997; Arya et al., 2010; Kulkarni et al., 2010). Current therapy for testicular relapse is either removal of the testicles (orchiectomy) or testicular irradiation, both of which cause infertility and significant hormone disruption (Locatelli et al., 2012). These side effects cause profound quality of life issues for the young patients that underscore the need for medications that can treat the leukemia within the BTB. Improving disposition of these chemotherapy treatments to the testis would be expected to reduce testicular relapse for cancer patients and be a great increase in the quality of life of patients. Therefore, it would be important that ALL treatment regimens include drugs that are able to reach therapeutic concentrations within the seminiferous tubules for male patients. Information regarding which ALL drugs can penetrate the BTB is currently lacking, although there is some evidence that several nucleoside-based drugs are able to accumulate in the MGT (Jeha et al., 2004; Locatelli et al., 2012; Macanas-Pirard et al., 2012).

**Nucleosides**

The current work focuses on nucleoside transport and as such, it is important to describe what a nucleoside is and why there is an interest in these compounds as substrates. A nucleoside is a nucleobase or nitrogenous base (such as adenine) bonded to a 5-carbon sugar, typically ribose or deoxyribose. These compounds can be produced *de novo* by the liver or they can be acquired from
the diet. Nucleosides can be metabolized via phosphorylation into nucleotides. Up to three phosphate groups may be added to the 5’ carbon on the sugar of nucleosides. Nucleotides serve several vital functions within a cell, most notably as building blocks for DNA and RNA although they can also function as energy storage (ATP) or as secondary signaling molecules (cAMP). These functions are especially critical in rapidly dividing entities such as cancer cells or replicating viruses. This is the reason that many drugs have been developed in recent decades to inhibit these functions for the treatment of cancer and viral infections. These drugs are collectively referred to as nucleoside analogs (NSA). As the name suggests, NSA medications resemble nucleosides by possessing a nucleobase like structure bonded to a 5-carbon sugar (or moiety similar to a 5-carbon sugar; see figure 1.5). The structural similarity of NSA drugs allows them to competitively inhibit access to endogenous nucleosides, thereby hindering critical cell functions such as DNA synthesis. The mechanism of action for many of these drugs is incorporation into a growing strand of DNA via dehydration synthesis involving the phosphate group on the 5’ carbon, because the lack of a properly aligned hydroxyl group on the 3’ carbon, they do not allow for the addition of new nucleotides. Many of these compounds have been noted to have a high affinity for reverse transcriptase, an enzyme vital to RNA viruses such as HIV to synthesize DNA. Since there is not a human equivalent for reverse transcriptase, these NSA drugs are used for the treatment of viral infections and are named nucleoside reverse transcriptase inhibitors (NRTI). For these reasons, nucleoside drugs are very important for the treatment of viral MGT diseases and
Figure 1.5: Examples of Endogenous and Exogenous Nucleo-Compounds. Representative structures of endogenous and xenobiotic nucleobase, nucleosides, and nucleotides. Note the addition of the sugar (nucleoside) or phosphate group (nucleotide).
understanding how they are transported into the MGT would represent a significant milestone in the eradication of these diseases from the patient.

**Blood-Testis Barrier as a Sanctuary Site**

**Various Definitions of Blood-Testis Barrier**

Doctors have recognized the inability of many compounds to reach appreciable concentrations within the testis. The term blood-testis barrier (BTB) was originally coined to refer to the observation that most compounds, especially dyes, did not access the testis. Since that time, the field has come a long way in identifying what comprises the BTB and how it functions. The purpose of the BTB is understood to be for the benefit of developing germ cells. It is apparent that the human body is exposed to limitless compounds that are potentially spermatotoxic. Even a genotoxic compound could have devastating reproductive toxicity as it may have deleterious effects for the offspring if the damaged sperm were to conceive (transgenerational toxicology). However, depending on the context, the BTB can refer to different components of the BTB namely the immunological, physiological, and anatomical.

Immunologists often use the term to describe the immune-privileged nature of the BTB. Immune-privilege is required for proper sperm maturation because sperm are immunogenic, which means the immune system will make antibodies against sperm. Males that have antibodies that are reactive to sperm are generally infertile (Diekman and Herr, 1997; Bandivdekar, 2014). This is because immune tolerance is developed prior to puberty, which is before the
testis is capable of spermatogenesis. The Sertoli cell tight junctions are thought to partially account for the immune-privilege, however this cannot be the complete explanation since spermatogonia, which are immunoreactive, reside basal of the tight junctions. Additionally, the testis is well connected with lymph nodes (Li et al., 2012). The testis has several immune suppression mechanisms in place including structure of the testis, anti-inflammatory Sertoli cells secretions, and endocrine cytokines. (Hedger, 2011; Meinhardt and Hedger, 2011; Mital et al., 2011; Li et al., 2012). Interestingly, these immune suppression effects are sufficiently strong enough that xenografts and allografts within the interstitial region of rat testis can thrive without rejection for weeks (Head et al., 1983; Hedger, 2011).

There is also the physiological or transport portion of the BTB (Mital et al., 2011). This aspect refers to efflux transporters that efflux substrates out of the tubules. These transporters line the basolateral membrane of the Sertoli cells and the peritubular myoid cells. Several transporters are known to participate in this function, including P-gp, MRP1, and breast cancer resistance protein (BCRP) (Bart et al., 2002, 2004a). While this process refers to efflux from the seminiferous tubules, it could also include luminal efflux from endothelial cells into the blood as well, although this has not been studied as extensively. The physiological portion of the BTB is effective at pumping out hydrophobic compounds that can diffuse into cells as well as hydrophilic compounds that can access the Sertoli cells via uptake transporters. Since many of these transporters are known to interact with a wide variety of clinical drugs, inhibition of these transporters should improve disposition of several compounds to the testis that,
depending on the drug in question, may be beneficial or harmful for male reproduction.

In the field of pharmacology, the BTB refers to the tight junctions between the Sertoli cells. This is named the anatomical portion of the BTB, although this can also include the tight junctions between endothelial cells as well (Mital et al., 2011). The proteins making up the tight junctions anchor cell membranes close together to greatly limit the extracellular space (which is typically aqueous) between them. This form of barrier is especially effective for hydrophilic compounds as it greatly hinders paracellular diffusion, however these barriers alone are not thought to be as effective for hydrophobic compounds that can presumably cross cell membranes easily (Mruk and Cheng, 2010; Cai et al., 2013). The work presented here will primarily refer to the anatomical portion of the BTB as the BTB, although it should be noted that some of the studies also have implications for the physiological BTB as well.

Since the BTB and the blood-brain barrier (BBB) are two of the most well known tight barriers of the body, comparison between them is inescapable. They share many similarities in that they are both effective at keeping xenobiotics and the immune system from accessing the components they protect through the use of tight junctions, efflux transporters, and various methods of immune suppression. One of the key differences is the BBB is primarily endothelial while the BTB is primarily epithelial. Another difference is the tight junctions at the BBB are suspected to be much more resistant to electrical current compared to the tight junctions expressed by Sertoli cells, indicative of a less leaky barrier. Strategies for bypassing the BBB, at this time, have been more thoroughly
explored compared to the BTB, primarily due to the difficulty in treating CNS cancers and infections. Advanced testicular cancers and infections can be treated by orchiectomy while the CNS equivalent would not be very practical. However, there is an increased interest in understanding the dynamics of the BTB, particularly in relation to transporter expression. The BTB has classically been suspected of creating a sanctuary site for viral infections and cancers (testicular relapse) by limiting the distribution of chemotherapeutics. Developing strategies for accessing these sites through endogenous transporter systems is identified as the top priority by the NIH Therapeutic Targeting, Blood-Brain Barrier, Gene Therapy and Vascular Biology review group. However, the idea that the BTB is a sanctuary site has recently been controversial and so it is worth discussing the literature regarding the controversy.

**Blood-testis Barrier Controversy.**

While the existence of the BTB is well documented, there are some who call to question the idea that it is clinically significant with respect to HIV infection and testicular relapse. Some studies have cited that relapse rate does not decrease significantly in patients that have had orchiectomies or testicular irradiation (Chong et al., 1986; Dave et al., 2007). These studies go on to point out that when a tumor metastasizes to the testis, eradication of the primary tumor is sufficient to prevent relapse. It is important to note that the arguments against the idea that the testis is a chemo-privileged site do not apply to “wet” tumors such as acute lymphoblastic leukemia (ALL) since those types of cancers were not included in the studies. Additionally, several other studies have reported
that viable metastatic tumors can be found in the testis even after successful treatment of the primary cancer furthering the impact of the BTB on cancer treatment (Fowler and Whitmore, 1981; Calvo et al., 1983; Snow et al., 1983; Dave et al., 2007).

There is also a group that argues the BTB is not a sanctuary site of HIV infection (Lowe et al., 2004). The primary thought process behind this argument is that the reservoir for HIV infection is in CD4 T-cells which are not present within the seminiferous tubules. Therefore, HIV should not be able to survive within the testis. This group argues that instead of the testis, HIV accesses the MGT through infection of seminal vesicles. This idea is based on evidence that the virus can be detected in autopsy specimens (Deleage et al., 2011). While these are important points, there are data that address these concerns. First, genetic drift studies have determined that viral genome from seminal plasma is distinct from blood plasma in patients infected with HIV for several years, indicative of a population of HIV within the MGT separate from the blood (Byrn and Kiessling, 1998; Anderson et al., 2010). The seminal vesicles are not capable of limiting disposition of HIV drugs into seminal plasma and therefore cannot account for the distinct viral population (Cao et al., 2008; Else et al., 2011). The concern for the lack of CD4 T-cells fails to address the fact that sperm is capable of transfecting HIV which may represent a potential viral reservoir and the testis is capable of HIV infection ex vivo (Royce et al., 1997; Roulet et al., 2006; Ceballos et al., 2009; Dahl et al., 2010). Within the entirety of the MGT, the seminiferous tubules represent the most exclusive compartment and therefore the most capable of allowing for viral replication without interference from antiviral drugs.
The study that revealed HIV infection in the seminal vesicles was based on tissue extracted from autopsy patients, many of whom died from AIDS related complications, and so it is unclear if seminal vesicles remain infected when blood plasma viral load is undetectable. Although it is not yet completely conclusive that the distinct MGT viral population arises from the seminiferous tubules, current literature suggests that this is the most reasonable location.

**Transporters of the Testis**

In order for a drug or a toxicant to have an effect, it must reach the target site at sufficient concentrations. Whether a xenobiotic will reach the target site depends primarily on how well the compound is absorbed, distributed, metabolized and eliminated from the body. These processes, typically referred to as ADME, largely determine the plasma concentrations of a compound and as such, are critical for understanding the toxicity of a compound (Augustine *et al.*, 2005; Klaassen and Aleksunes, 2010). One group of specialized proteins called transporters are known to play vital roles in the ADME processes for many clinically relevant xenobiotics (Lu *et al.*, 2004; Klaassen and Aleksunes, 2010; Jung *et al.*, 2013). Transporters are proteins that span across lipid bilayers and allow for the passage of chemicals through biological membranes. The physiological function of transporters is to allow endogenous substrates access to cells and biological compartments or to restrict access of potential toxicants within the body (Merrell *et al.*, 2008; Lake *et al.*, 2011). For example, transporters that are expressed in the intestine can either allow for the absorption of ingested compounds into the body, or keep those compounds from
entering the bloodstream (Grandvuinet et al., 2012; Singh et al., 2014). Additionally, a compound’s interaction with transporters in the liver or kidney can drastically affect the metabolism and excretion of the compound (Pelis et al., 2007; Merrell et al., 2008; Rödiger et al., 2010; Pelis and Wright, 2011; Canet et al., 2012; Hardwick et al., 2012).

The histology alone will not determine if xenobiotic transporters are involved in the selective concentration of a toxicant in the seminiferous tubules or interstitium of the testis. Such information can only come about from a disposition study (or high-resolution whole body autoradiography) which isolates the testis into its component parts and analyzes them separately. Such conclusions could be inferred from disposition studies in other tissues that express the same transporter and are shown to impact the concentration of a select xenobiotic. In my review of the literature, I found that there are currently few examples of transporters causing toxicity in the male reproductive tract. However, this should not be misinterpreted to mean that transporters do not play a role in testis pathology and could be responsible the severity of toxicity observed. Indeed, it is known that induction or reduction of xenobiotic transporter expression is associated with severe side effects (Clarke et al., 2014).

Nearly all transporters are associated with one of two superfamilies based on the driving force: ATP-binding cassette (ABC) transporters that use ATP hydrolysis to provide energy for transport against an electrochemical gradient, and solute carrier (SLC) transporters that transport compounds using electrochemical gradients, in some cases coupled to a cosubstrate, typically an ion such as Na⁺ (Baldwin et al., 2004; Augustine et al., 2005; Su et al., 2009;
Klaassen and Aleksunes, 2010; Robillard et al., 2012). This transport coupling can translocate in the same (symport) or in opposing directions (exchange or antiport). As an example, sodium/hydrogen exchanger family (gene SLC9A) couples the efflux of H\(^+\) ions with uptake of Na\(^+\) ions (Zuo et al., 2011; Madonna and De Caterina, 2013; Becker et al., 2014). Since extracellular Na\(^+\) concentrations far exceed intracellular concentrations, the uptake of Na\(^+\) provides a driving force that allows for the efflux of H\(^+\) even into an acidic environment. Within these two superfamilies, transporters are typically named based on their substrates. For example, equilibrative nucleoside transporters (ENT) facilitate the movement of nucleosides, organic cationic transporters (OCT) interact with organic cations, etc (Pastor-Anglada et al., 2005; Molina-Arcas et al., 2008; Erythrocyte et al., 2010; Jung et al., 2013; Müller et al., 2013). The normal nomenclature is to capitalize the name when referring to human isoforms or the species is ambiguous (OCT) and lower case for rodent (Oct). Interestingly, ENT is capitalized no matter the species and so my work follows that naming style. Some families of transporters, such as ENTs, have a narrow selectivity of substrates while others, such as multidrug resistance-associated proteins (MRP), have less selective of substrates (Bart et al., 2004a; Augustine et al., 2005). Transporters can also be characterized based on the directionality, i.e. whether they allow substrates into cells (uptake), out of cells (efflux), or both (bidirectional) at physiologic conditions.

When investigating transport activities in the context of biological systems, it is critical to know the location of the protein as it can greatly impact transporter function for a tissue. Location refers to both which types of cells
express functional protein and, in polarized epithelial cells, whether the transporter resides in the basolateral membrane or the apical membrane into a distinct compartment. If an uptake transporter for a toxicant is located on a basolateral membrane of an epithelial cell and an efflux transporter is on the apical side, then this would represent a transepithelial pathway, shuttling substrate from the blood into the cell and then out through the apical membrane. Such a transepithelial pathway can explain how a particular toxicant can accumulate in an area of the body. A solid understanding of transporter function and location can lead to a better prediction of toxicant exposure.

There is a significant body of data that demonstrates the clinical significance of transporters. A classic example is Dubin-Johnson syndrome which is an increase in conjugated bilirubin due to a defect in multidrug resistance-associated protein 2 (MRP2, gene name ABCC2), a canalicular transporter that is responsible for the efflux of bilirubin glucuronides into bile (Li et al., 2013; Sticova and Jirsa, 2013; Keppler, 2014a; b). There is also increasing evidence that diseases of the liver, such as nonalcoholic steatohepatitis (NASH), can influence transporter profiles in the liver, which is responsible for the breakdown of many drugs (Merrell et al., 2008; Hardwick et al., 2012; Clarke et al., 2014). This can cause altered plasma levels for many commonly prescribed drugs that may, in turn, lead to toxic side effects even at standard therapeutic doses. Transporters can also be important in mediating drug-drug interactions, for example cimetidine and procainamide. Cimetidine inhibits uptake transporters (OCT) and efflux transporters (multidrug and toxin extrusion, MATE) responsible for renal secretion of procainamide (McKinney and Speeg, 1982; Christian et al., 1984;
Coadministration of these drugs results in an increase in procainamide retention which can cause more pronounced side effects (McKinney and Speeg, 1982; Christian et al., 1984).

While most of the transporter research has centered on hepatic and renal transport, there is growing interest in studying other tissues as well, including the testis (Augustine et al., 2005; Klein et al., 2013, 2014a). Testicular transport mechanisms are especially important regarding the blood-testis barrier (BTB). The anatomical portion of the BTB, the basal tight junctions between the Sertoli cells, can impede xenobiotic diffusion between cells (Hedger, 2011; Mital et al., 2011; Su, Mruk, and Cheng, 2011; Su, Mruk, Lee, et al., 2011; França et al., 2012). Due to the tight junctions, transepithelial transport through Sertoli cells is the primary way for a hydrophilic compound to access the seminiferous tubules at significant concentrations. Additionally, efflux transporters along the basal membrane of Sertoli cells may serve a protective function to developing sperm by preventing the germ cells from being exposed to potential toxicants (Bart et al., 2002, 2004a; Robillard et al., 2012). Uptake transporters are important for nutrients, such as lactate, nucleosides and carnitine, that cannot diffuse past the tight junctions but are needed by the germ cells (Enomoto et al., 2002; Kato et al., 2005a; Aliabadi et al., 2013). From a clinical perspective, the basolateral efflux transporters can act as obstacles for drugs requiring entry into the seminiferous tubules in order to achieve full therapeutic effect (see chapter 2). The transepithelial transport pathways can also be potentially problematic by providing a mechanism of access for toxicants to the male genital tract (MGT), especially if the transporters are able to concentrate their substrates inside the
MGT. It is also possible for transport in the seminiferous tubules to have an effect downstream in the epididymis. Fluid in the lumen of the seminiferous tubules flows into the epididymis where up to 99% of the water is reabsorbed, resulting in a dramatic increase in solute concentration inside the epididymal duct, potentially causing toxicity (Leung et al., 2001; Cornwall, 2009). This mechanism of testicular transport followed by epididymal concentration may be occurring with drugs used to treat HIV (see chapter 2). In short, a solid understanding of transport within the testis, and especially in seminiferous tubules, has great potential to positively impact male reproductive health. It can improve the disposition of newer drugs to the testis, predict potential toxicants, present a better understanding of physiological (and potentially, pathological) process, and provide a mechanism for toxicity.

**ABC Efflux Transporters**

Organic transporters are clinically relevant due to their potential impact on drug disposition. The mRNA expression for several xenobiotic transporters of the testis, Sertoli cells and epididymal cells has been analyzed (Augustine et al., 2005; Klaassen and Aleksunes, 2010). Many of the xenobiotic transporters found in testis are ABC efflux transporters (transporting substrates out of the tubules) that have a wide variety of substrates. These include P-glycoprotein (P-gp), breast cancer resistance protein (BCRP), MRP1, MRP4, MRP5, MRP7, and MRP8 (Bart et al., 2002, 2004a; Augustine et al., 2005; Su et al., 2009; Robillard et al., 2012). All of these, except for MRP7, have been localized within the testis (Bart et al., 2002; Enokizono et al., 2007; Qian, Y-H Cheng, Mruk, et al., 2013). BCRP is
highly expressed on the luminal side of interstitial capillaries, and can be expressed by Sertoli cells in a stage-specific (stages VII-VIII) manner at the apical ectoplasmic specialization (ES) (Bart et al., 2002, 2004a; Dankers et al., 2013; Qian, Y-H Cheng, Mruk, et al., 2013). The localization of P-gp is known to be in the luminal membrane of capillaries, Leydig cells, and along the BTB, although whether it is expressed by Sertoli cells or myoid cells remains controversial (Bart et al., 2002; Su et al., 2009). MRP1 and MRP4 have also been shown to be expressed by Leydig cells and on the basolateral membrane of Sertoli cells in rodents (Bart et al., 2002, 2004a; Evans, 2011; Morgan et al., 2012; Dankers et al., 2013).

Interestingly, MRP4 is localized to the basolateral membrane in humans and macaques testis. MRP5 has only been detected in Leydig cells and MRP8 has been localized to round spermatids (see chapter 3). The physiological function of these multispecific efflux transporters is primarily thought to be cytoprotective, i.e. keep potential toxicants from reaching developing sperm (Bart et al., 2004a; Bortfeld et al., 2006; Kruh et al., 2007; Su et al., 2009; Klein et al., 2014a). However, the locations of MRP5 and MRP8 indicate that these transporters have some other function. While there is speculation that these transporters participate in steroid (MRP5) or growth hormone (MRP8) efflux, the physiological role of these proteins within the testis remains to be determined (see chapter 3).

**Nucleoside Transporters**
In addition to ABC efflux transporters, several organic solute transporters from the SLC super family have been investigated in the testis. These include ENT1, ENT2, several organic anion transporting polypeptides (OATP), organic anion transporter 2 (OAT2), organic cation transporter 3 (OCT3), and OCTN2 (Augustine et al., 2005; Kato et al., 2006; R Kato et al., 2009; Klaassen and Aleksunes, 2010; Zhou et al., 2012; Klein et al., 2013). ENT proteins are thought to be important for allowing vital nucleosides to reach the developing germ cells (Kato et al., 2005a). Additionally, these transporters have been shown to interact with some nucleoside-based drugs used for HIV treatment and chemotherapy agents indicating that these transporters may be clinically relevant. ENTs transport substrates bidirectionally based on concentration gradient (Kiss et al., 2000; Ward, 2000). These characteristics are particularly interesting regarding testicular function since ENT1 is on the basolateral membrane of Sertoli cells and ENT2 is on the apical membrane (see chapter 3). If concentration of a substrate is high outside the seminiferous tubule, ENT1 will transport the xenobiotic into the Sertoli cell and, as intracellular concentration rises, ENT2 will transport the substrate from inside the cell to the lumen of the seminiferous tubule. This represents a transepithelial pathway by which nucleosides can translocate from the blood to the germ cells within the tubules and possibly to the epididymis. A useful feature of ENT transporters is the sensitivity to the pharmacological inhibitor NBMPR. NBMPR is only known to inhibit ENT transporters at micromolar concentrations. There is also a large difference in sensitivity between ENT1 (Km 0.6nM) and ENT2 (Km ~10 µM) (Griffiths et al., 1997; Kong et al., 2004; Rodríguez-Mulero et al., 2005).
Closely related to the ENT family is the concentrative nucleoside transporter (CNT) family. These transporters also interact with nucleoside substrates and are typically thought to be involved in nucleoside salvaging. However, unlike ENT proteins, CNT transport utilizes Na\(^+\) exchange as an energy source. This means that under normal physiological conditions which feature high concentrations of extracellular Na\(^+\), CNTs will act only as an uptake transporter allowing nucleosides and nucleoside based drugs access to the cells and will not function as an efflux transporter (Pastor-Anglada et al., 2005). Another interesting feature of the CNTs is that different members of this family will interact with differently with purines and pyrimidines. CNT1 will only transport pyrimidine nucleosides and drugs that resemble pyrimidines whereas CNT2 is purine and purine analog specific (Leung et al., 2001; Li et al., 2001; Gray et al., 2004; Rodríguez-Mulero et al., 2005; Fernández-Calotti et al., 2011). CNT transporters are also not sensitive to NBMPR inhibition (Kong et al., 2004).

**OATP, carnitine, and glucose transporters.**

OATPs are bidirectional transporters that interact with a wide variety of substrates, including thyroid hormones, organic dyes, bile salts, anionic oligopeptides and many other xenobiotics including clinical drugs (Su, Mruk, Lee, et al., 2011). At least five homologs of Oatp (Oatp1a5, -3a1, -6b1, -6c1, and -6d1) have been shown to be expressed by spermatogonia (Zhou et al., 2012). Oatp6a1 has also been found in the testis, specifically in Sertoli cells, spermatogonia, and Leydig cells, although the endogenous substrate for this protein is currently unknown (Suzuki et al., 2003; Fietz et al., 2013). There has
been some interest in the transport of sulfated steroids (Ruokonen et al., 1972). It is speculated that these biologically inactive compounds may become de-sulfated (and thus active) following uptake into the testis by an unknown transport mechanism (Ruokonen et al., 1972; Fietz et al., 2013). Sodium-dependent organic anion transporter (SOAT) was found to transport sulfated steroids but it is expressed primarily by germ cells (Fietz et al., 2013). OAT2 has been found in several tissues other than the testis, most prominently in the liver and kidney (Rizwan and Burckhardt, 2007; Burckhardt, 2012). OAT2 transports small anionic compounds via exchange with succinate or fumarate (Burckhardt, 2012). There are many endogenous and clinically relevant drugs that are substrates for OAT2 including cGMP, nucleobases, prostaglandins, urate, methotrexate, zidovudine, pravastatin and cimetidine. The location for OAT2 in the testis is currently unknown. There is also evidence that organic cation transporters (OCT) 1 and 3 are expressed by Sertoli cells on the basolateral and apical membranes respectively (Augustine et al., 2005; Maeda et al., 2007).

It has been known for quite some time that L-carnitine within the MGT is important for male fertility. Concentrations of L-carnitine within the epididymis is 1000 fold higher than plasma concentrations (Kobayashi et al., 2005; Aliabadi et al., 2013). L-carnitine aids in the fatty acid oxidation and has systemic antioxidant activity. It has been speculated that L-carnitine is beneficial for fertility by supplying maturing sperm with energy through increase in lactate dehydrogenase C4 (LDH-C4), stabilizing plasma membranes, and protecting sperm from reactive oxygen species(Enomoto et al., 2002; Kobayashi et al., 2005; S Kato et al., 2009; Aliabadi et al., 2013). Since the BTB would prevent L-
carnitine from passively diffusing into the seminiferous tubules, this nutrient must be transported through Sertoli cells. One L-carnitine transporter known to be expressed in the basolateral membrane of Sertoli cells is OCTN2, an uptake transporter that requires sodium (Augustine et al., 2005; Kobayashi et al., 2005; Klaassen and Aleksunes, 2010). In addition to carnitine, OCTN2 has also been shown to transport clinical drugs such as verapemil and quinidine (Kobayashi et al., 2005; S Kato et al., 2009; Pochini et al., 2013). Other L-carnitine sodium-independent transporters like OCTN3 and carnitine transporter 2 (CT2) have also been shown to be expressed in mouse and human testis respectively and likely participate in carnitine uptake as well (Maeda et al., 2005; Pochini et al., 2013).

It is not surprising that glucose metabolism is known be critical for proper sperm maturation (Alves et al., 2013). One of the functions of the Sertoli cells is to import glucose from the blood and metabolize it to lactate which is the primary energy source of the germ cells. This requires glucose gaining entry into the Sertoli cells via the glucose transporter (GLUT). In the context of testis, glucose transport is accomplished by GLUT1, GLUT3, and GLUT8 (Alves et al., 2013). GLUT1 and GLUT3 have been shown to be sensitive to regulation from hormones and growth factors and have been localized to endothelial cells, basal membrane of Sertoli cells, and myoid cells (Kokk et al., 2004).

**Inorganic Solute Transporters**

Many inorganic solute transporters of the testis have been studied with the goal of establishing the physiological processes of the testes. Water and closely
related ion transport is also important for proper fertility (Zhou et al., 2001; Lu et al., 2007; Moretti et al., 2012). Water is crucial for sperm to move from the lumen of the seminiferous tubules all the way to the vas deferens (ductus deferens) (Hermo and Smith, 2011). Water is able to penetrate the BTB due to Sertoli cell expression of specialized water transporters known as aquaporins (AQP). AQP expression can change depending on the stages of the Sertoli cells, however, AQP0, 7, 8, and 11 have been shown to be expressed in seminiferous tubules (Hermo and Smith, 2011). Additionally, germ cells express AQP8 and 7 (Hermo and Smith, 2011; Moretti et al., 2012). Water reabsorption in the epididymis is also important for fertility as vast amounts of water (90-99%) are removed from the epididymal duct (Wong and Yeung, 1976; Byers et al., 1988; Cornwall, 2009). This water reabsorption is required for proper sperm development (Moretti et al., 2012). Within the epididymis, AQP1, 9, and 10 are known to be expressed and sodium hydrogen exchanger 3 (NHE3) is also known to be important for water reabsorption and maintenance of luminal pH (Zhou et al., 2001; Lu et al., 2007).

Many of these inorganic solute transporters aid in the physiological processes of the testis, but they may also contribute to toxicity. Examples include transporters that allow the entry of metal ions into Sertoli cells. These can be especially problematic since several metals, including cadmium (Cd) and arsenic (As) are known to be reproductive toxicants (Mruk and Cheng, 2011b). The primary transporter known for Cd, manganese (Mn) and zinc (Zn) for Sertoli cells is Zrt-, Irt-related protein 8 (ZIP8) (Himeno et al., 2009; Mruk and Cheng, 2011b). Iron (Fe) is an essential nutrient for developing sperm but can easily
reach cytotoxic concentrations if not carefully regulated (Griffin, 2004; Leichtmann-Bardoogo et al., 2012). The main transporters involved in Fe transport for germ cells are transferrin receptor (TfR1) and divalent metal transporter 1 (DMT1) (Leichtmann-Bardoogo et al., 2012). Small amounts of TfR1 located at the basolateral membrane of Sertoli cells are likely responsible for Fe uptake. TfR1 is also expressed by primary spermatocytes indicating that it is important for early germ cell development. DMT1 is localized to the apical membrane near elongating spermatids which suggests that it is vital for Fe transport later in sperm development (Leichtmann-Bardoogo et al., 2012).

While many other body systems (liver and kidney) have classically been the focal point of transporter research, the testis is gaining attention due to the importance of the BTB on drug (Fietz et al., 2013) disposition and growing interest in fertility. The testis, especially Sertoli cells, expresses a complex array of transporters that perform various functions and potentially have several clinical effects. Figure 1.6 summarizes the transporter localization and directionality within the testis discussed in this review. Research regarding the impact transporters on the testis is still in its early stages. Further understanding of transport processes of the testis can provide a basis for the physiology of the testis.
Figure 1.6: Transporter Expression at the Blood-Testis Barrier. A representative figure depicting various cell types of the testis and the location/directionality of known transporters including work presented in this dissertation. Transporters are known for rodent BTB, with the exception of MRP as noted in the legend.
Current Study

The blood-testis barrier (BTB) is critical to limiting germ cell exposure to potential reproductive toxicants (Bart et al., 2002; Hedger, 2011; Liu et al., 2011; Mruk and Cheng, 2011a; Su, Mruk, and Cheng, 2011; Zhou et al., 2012). The anatomical portion of the blood-testis barrier is composed of tight junctions between specialized epithelial cells called Sertoli cells that line the seminiferous tubules (Su et al., 2010; Mital et al., 2011; Su, Mruk, Lee, et al., 2011). Due to the BTB, most hydrophilic compounds are not able to diffuse past Sertoli cells. While this is usually beneficial for developing germ cells, the BTB can be an obstacle for therapeutic agents, including many chemotherapeutics and HIV drugs, that require access to the seminiferous tubules for full therapeutic effect. Sertoli cells express several transporters that allow nutrients to bypass the BTB via transepithelial transport. Transepithelial transport is a two-step process; first substrates are taken into the cells via uptake mechanisms, and second, substrates exit the cells across the opposite membrane via efflux. One of the standard techniques to study functional transport at the BTB is by culturing a monolayer of primary Sertoli cells in a transwell insert (Kato et al., 2005; Mruk and Cheng, 2011). Information regarding these transepithelial pathways is important for understanding the distribution of drugs into the testis.

The strategy used to determine transepithelial pathways in the testis was to use compounds that are known to cross the BTB and determine the transporters responsible. One class of HIV drugs called nucleoside reverse transcriptase inhibitors (NRTI) has been shown to accumulate in seminal plasma
at concentrations higher than that of blood, approximately 2-10 fold higher (Anderson et al., 2000; Taylor et al., 2000; van Praag et al., 2000; Pereira et al., 2002; Cruciani et al., 2006; Chan et al., 2008; Le Tortorec and Dejucq-Rainsford, 2010). Nucleoside analog (NSA) drugs are important for the treatment of cancer as well. For these reasons, I focused on determining the nucleoside transport pathway for the BTB.

One family of transporters that is relevant to the BTB is the ENT family, SLC29A (Ward, 2000; Pastor-Anglada et al., 2005; Macanas-Pirard et al., 2012). ENT-mediated transport is generally driven entirely by concentration gradients of the substrate due to the lack of net charge of substrates at physiological pH. These transporters are especially relevant because they transport both nucleosides, an essential nutrient for dividing germ cells, and NSA which are used clinically for the treatment of HIV infection and leukemia (Baldwin et al., 2004; Molina-Arcas et al., 2008; Fernández-Calotti et al., 2011). ENTs typically act as uptake transporters, allowing the entry of nucleosides from the blood into cells. Since these transporters have been shown to interact with NSA drugs used to treat HIV infection, it was anticipated that this pathway could also be the mechanism for drug accumulation in seminal plasma (Mann and Lutwak-Mann, 1982; van Praag et al., 2001; Cao et al., 2008; Chan et al., 2008; Anderson et al., 2010; Le Tortorec and Dejucq-Rainsford, 2010).

Another transporter family of high interest is the MRPs. Members of this family use ATP hydrolysis to efflux a wide variety of clinically relevant substrates, including many drugs used to treat leukemia and HIV infection (Bart et al., 2004a; Weiss et al., 2007; Eilers et al., 2008; Klaassen and Aleksunes, 2010).
Determining the transepithelial pathways that allow for nucleoside penetration across the BTB would have great impact on understanding the disposition of many important drugs with respect to the testis. *I hypothesized that using representative nucleoside and NRTIs in combination with pharmacological inhibitors, the transport pathways for nucleosides and NRTI could be determined.* To test this hypothesis, I developed the following aims:

**Aim 1 (Chapter 2)** – Determine the Basolateral Uptake Portion of Nucleoside Penetration at the BTB.

Using primary rat Seroli cells cultured in a transwell, the transepithelial transport of the model nucleoside uridine was measured in the presence/absence of the ENT inhibitor NBMPR. Immunohistochemistry (IHC) was also employed to determine the localization of ENT1 and ENT2 in human and rat tissue. These data are instrumental in determining the impact of ENTS, the only nucleoside transporters known to be expressed at the BTB, on transepithelial transport of nucleosides and NRTI.

**Aim 2 (Chapter 3)** - Determine the Location of Multidrug Resistance-Associated Proteins (MRP) in the Testis.

Several MRP transporters are known to be expressed in the testis but their sites of expression are unknown. IHC was used to determine the location of MPR1, MRP4, MRP5, and MRP8 in rat (mature and immature), rhesus macaques, and humans. By determining the location of the transporters, their function was then be speculated.
**Aim 3 (Chapter 4)** – Establish the mRNA expression profile for Xenobiotic Transporters within the MGT

Through the use of branched DNA analysis (bDNA), the expression of several xenobiotic transporters was determined for several tissues of the MGT (epididymis, prostate, vas deferens, and seminal vesicles). This study is foundational for determining the transporter profile for several tissues within the MGT.

**Aim 4 (Chapter 5)** – Characterize the Apical Efflux Component of Nucleoside Transepithelial Transport.

Following up on the study in Aim 1, I further characterized the apical membrane transporters of primary Sertoli cells in both rats and humans. This study solidified the mechanisms of nucleoside transepithelial transport at the BTB and characterized primary human Sertoli cells, which are a more applicable model to the human condition.

**Aim 5 (Appendix A)** - Determine the Location of Nucleoside Transporters within the Epididymis.

Previous reports have determined that the epididymis expresses a few nucleoside transporters (ENT1, ENT2 and CNT2) but their location is unknown. By using IHC, the location of these transporters was determined. Speculations regarding transepithelial transport of nucleosides within the epididymis were then provided.
CHAPTER 2: BASOLATERAL UPTAKE OF NUCLEOSIDES BY SERTOLI CELLS IS MEDIATED PRIMARILY BY EQUILIBRATIVE NUCLEOSIDE TRANSPORTER 1 (ENT1).


**Introduction**

The anatomical portion of the blood-testis barrier (BTB) is composed of tight junctions formed between the Sertoli cells that line the seminiferous tubules inside the testis (Pelletier, 2011; Mital, et al, 2011; Li et al, 2012). This barrier prevents many exogenous agents from gaining entry into the lumen of the seminiferous tubules and contacting germ cells. It is also the responsibility of the Sertoli cells to provide nutrients such as nucleosides that allow for spermatogenesis (Kato et al, 2009; Mruk and Cheng, 2011). Although this barrier is beneficial for sperm cell development, it can be an obstacle for drugs that are required to bypass the BTB to achieve full therapeutic effect. Examples of such drugs include many antiretroviral medications used to treat infection of human immunodeficiency virus (HIV). By limiting the entry of many antiretrovirals into the male genital tract (MGT), the BTB may be contributing to the testes’ serving as a sanctuary site for HIV (Byrn and Kiessling, 1998; Anderson et al, 2000;

---

1 I have completed approximately 80% of the data generation presented for this chapter (all but the intact seminiferous tubules work which was provided by KK Evans). The entire initial draft was written by me.
Olson, 2002; Dahl et al, 2010). Since the tight junctions of the BTB prevent paracellular diffusion of hydrophilic drugs, transcellular transport through the Sertoli cells is required for antiretrovirals to bypass the BTB.

One class of HIV antiretrovirals, nucleoside reverse transcriptase inhibitors (NRTIs), may be able to bypass the BTB (Augustine et al, 2005; Else et al, 2011; Pereira et al, 2002). Clinical data have shown seminal plasma concentrations of zidovudine (AZT) and didanosine (ddI) are up to 10 fold higher than blood plasma (Prins et al, 2007; Dumond et al., 2008). Understanding the transepithelial transport pathway NRTIs use to bypass the BTB could potentially be useful in designing other drugs to cross into the lumen of seminiferous tubules.

Since NRTIs are nucleoside analogs, these medications may use the same nucleoside transport pathway(s) used by endogenous nucleosides, such as uridine. Currently, Kato et al represent the field’s understanding of the physiological pathway for nucleosides crossing the BTB (Kato et al 2005). They found that uridine uptake into primary Sertoli cells is dominated by two sodium independent components which possess characteristics similar to equilibrative transporter 1 (ENT1) and equilibrative transporter 2 (ENT2). ENT proteins are bidirectional transporters that facilitate nucleosides transport according to concentration gradient (Ward et al, 2000; Baldwin et al, 2004). Function of ENT1 and ENT2 is commonly differentiated based on their relative sensitivity to NBMPR; ENT1 is very sensitive to NBMPR inhibition ($K_i= 0.1$ to $68.5$ nM), ENT2 is unaffected by NBMPR at concentrations up to $1 \mu$M, but is blocked by $100 \mu$M NBMPR (Griffiths et al, 1997; Takano et al, 2010; Yao et al, 2011; Abd-Elfattah et
al, 2012; Nishimura et al, 2012). ENT1 and ENT2 have been shown to transport AZT and are speculated to transport other NRTI drugs as well (Ward et al, 2000; Pastor-Anglada et al, 2005). It has also been shown that basolateral entry of nucleosides into Sertoli cells is ENT dependent, although it has not been clear whether ENT1, ENT2, or both are involved (Kato et al, 2005). A minor sodium dependent component was also found to contribute to uridine uptake which was ascribed to be a concentrative nucleoside transporter (CNT). CNT proteins are unidirectional uptake transporters that typically localize to the apical membrane of epithelial cells and usually play a role in nucleoside salvaging (Kato et al, 2005; Lu et al, 2004; Errasti-Murugarren et al, 2012). NBMPR does not interact with CNT transporters allowing it to be a tool for distinguishing between ENT and CNT mediated transport (Ritzel et al, 2000; Kong et al, 2004; Fernandez-Calotti et al, 2011; Nishimura et al, 2012).

Despite the work done on nucleoside transport in Sertoli cells, there are still many gaps in our current understanding. For example, previous studies have not localized nucleoside transporters to apical or basolateral membrane, nor have they demonstrated whether ENT1, ENT2, or both are responsible for basolateral nucleoside uptake. The purpose of this study is to address these questions. To accomplish this, we determined the kinetic and selectivity characteristics of transport of the representative nucleoside, uridine, in intact seminiferous tubules *ex vivo*. Primary cultured Sertoli cells were isolated from rat testis and also analyzed for their ability to transport uridine. Immunohistochemical analysis was performed on both rat and human tissue to localize ENT1 and ENT2. These results support the conclusions that (i) rENT1 and hENT1 are located on the
basolateral membrane of Sertoli cells; (ii) ENT1 is primarily responsible for basolateral nucleoside uptake into Sertoli cells; and (iii) rENT2 and hENT2 are localized to the apical membrane.

**Materials and Methods**

**Materials:** Quantigene HV Signal Amplification Kit and Quantigene Discovery Kit were purchased from Genospectra (Fremont, CA). Oligonucleotide probe sets for ENT1, ENT2, CNT1, and CNT2 were developed as published previously (Augustine et al, 2005). CNT3 sequence was obtained from GenBank and target sequences were analyzed by ProbeDesigner software version 1.0 (Genospectra, Fremont, CA). Probes were designed with a Tm of approximately 63°C, enabling hybridization conditions to be held constant at 53°C for each oligonucleotide probe set (Supplemental Table 1). Every probe developed through the ProbeDesigner software was BLAST-searched against the nucleotide database to ensure minimal or no cross-reactivity with other known rat sequences and expressed sequence tags. RNAzol B reagent was purchased from Tel-Test Inc. (Friendswood, TX) Non-radiolabeled uridine, DMEM/F12 media, tenofovir disoproxil fumarate (TDF), zidovudine (3’-azido-3’-deoxythymidine, AZT), and didanosine (2’,3’-dideoxyinosine, ddI) were purchased from Sigma-Aldrich (St. Louis, MO). NBMPR was purchased from Santa Cruz Biotechnology Inc (Santa Cruz, CA). Stock solutions of NBMPR were made with DMSO. MACH4 IHC staining kit was acquired from Biocare Medical (St. Louis, MO). [3H]uridine (specific activity: 30.1Ci/mMol) was purchased from American Radiolabeled Chemicals Inc (St. Louis, MO). ENT1 (SLC29A1) and the ENT2 (SLC29A2) rabbit
antibodies were purchased from Lifespan Biosciences and quality was determined by the manufacturer (Seattle, WA). BD Matrigel Matrix and transwell inserts used for primary Sertoli cell cultures were purchased from BD Biosciences (San Jose, CA). All other reagents were purchased from a standard scientific supplier at the highest available purity.

**Branched DNA Assay:** Specific oligonucleotide probes for ENT1, ENT2, CNT1, CNT2, and CNT3 were diluted in lysis buffer supplied by the Quantigene HV Signal Amplification Kit. Substrate solution, lysis buffer, capture hybridization buffer, amplifier, and label probe buffer used in the analysis were all obtained from the Quantigene Discovery Kit. The assay was performed in 96-well format with RNA isolated from seminiferous tubules added to the capture hybridization buffer and 50 μl of the diluted probe set. The total RNA was then allowed to hybridize to the probe set overnight at 53°C. Hybridization steps were performed per the manufacturer's protocol the following day. Luminescence of the samples was measured with a Quantiplex 320 bDNA luminometer interfaced with Quantiplex Data Management Software, version 5.02 (Bayer, Walpole, MA). Total RNA was isolated from rat seminiferous tubules or rat kidney tissue using RNAzol B reagent per the manufacturer's protocol. The integrity of the RNA was confirmed by ethidium bromide staining after agarose gel electrophoresis. Background for each transporter was determined using negative control wells which had all reagents except for RNA. The background was then subtracted to demonstrate expression above background levels.

**Ex Vivo Transport Experiments with Intact Seminiferous Tubules:** All protocols for obtaining animal tissue samples were approved by the
University of Arizona Institutional Review Board (IRB) or Institutional Animal Care and Use Committee (IACUC). Seminiferous tubules were dissected from rat or mouse testes in chilled Ringer’s solution containing (mM): 103 NaCl, 25 NaHCO$_3$, 19 sodium gluconate, 1 sodium acetate, 1.2 NaH$_2$PO$_4$, 5 KCl, 1 CaCl$_2$, 1 MgCl$_2$, and 5.5 glucose at pH 7.4. Images of the tubules were then taken for measurements of length needed to normalize the data. Uptake baths containing [3H]uridine in Ringer’s solution, alone or with varied concentrations of unlabeled uridine or NBMPR, were covered with oil to prevent evaporation and brought to a temperature of 35° C. Individual tubules were transferred by a glass needle into the appropriate bath for a given period of time, and then transferred to wells containing 1 N NaOH for extraction of accumulated radioactivity, which was subsequently measured by an LS 6000 scintillation counter. At least three individual tubules were analyzed for each condition in all experiments. For studies using NBMPR, the concentration of DMSO was equal in all uptake baths and never exceeded 1 %.

**Sertoli Isolation:** Sertoli cell isolation was performed using the protocol of Mruk and Cheng, 2011. Briefly, the tunica was separated from the seminiferous tubules. Then the tubules were cut into 1 mm pieces, incubated in 50/50 mixture of DMEM/F12 media, and resuspended in media with 0.002 % DNase and 0.1 % trypsin to release interstitial cells. After washing and resuspension, the media was replaced with a DMEM/F12 media with 1 M glycine and 2 mM EDTA to lyse interstitial cells. The cells were resuspended in DMEM/F12 media with 0.1 % collagenase and 0.005 % DNase to remove the myoid layer. After washing, the cells were given fresh DMEM/F12 media with
0.1% hyaluronidase and 0.005 % DNase to break down the extracellular matrix. Cells were then plated at a density of 0.5 x 10^6 cells/cm² onto transwell inserts previously coated with a thin layer of Matrigel (diluted 1:7 with media) as per manufacturer’s instructions (BD Biosciences). The cell media was supplemented with EGF and human transferin. After 36-48 hours at 37 °C, cells were treated with a 20 mM tris buffer (pH 7.4) for 2.5 minutes to lyse germ cells and then given fresh DMEM/F12 media supplemented with EGF and human transferin. Cells were then incubated at 37 °C and cultured for an additional 4 days (6 days total from isolation). The media was changed as needed, typically every 1 to 2 days.

**Primary Sertoli Cell Transport Experiments:** Once the cells were confluent (day 6), the media was replaced with Waymouth Buffer containing (WB; mM): 135 NaCl, 13 HEPES, 2.5 CaCl₂, 1.2 MgCl₂•6H₂O, .8 MgSO₄•7H₂O, 5 KCl, 28 D-Glucose at pH 7.4. To measure basolateral-to-apical transepithelial uridine flux, the cells were incubated at room temperature for 10 minutes before the buffer in the basolateral compartment was replaced with WB containing 1 µCi/mL of [³H]uridine (approximately 30 nM) plus additional test agent (NBMPR, unlabeled uridine or NRTI drug) as required. At selected time intervals, WB from the apical compartment removed and assessed for radioactivity via liquid scintillation spectroscopy.

To determine the rate of uridine transport across the basolateral membrane WB in the apical compartment was replaced with white paraffin oil. At 15 minutes, cells were lysed with 0.5 N NaOH, 1 % SDS solution for 20 minutes. The NaOH was neutralized using 1 N HCl. The radioactivity in the extract was
measured by liquid scintillation spectroscopy. Each point represents an average of data collected in triplicate. For studies using NBMPR, the concentration for DMSO did not exceed 1 %.

**Sample Collection**: Animal samples were collected from euthanized rats either 21 days old (immature) or at least two months old (mature). The samples were fixed in 10% neutral buffered formalin overnight. A small incision was made in the tunica the next day and the samples remained in 10% neutral buffered formalin for another night. The following day, formalin was replaced with 70% ethanol until the samples were embedded in paraffin. Paraffin embedded human samples were purchased from the National Disease Research Interchange (NDRI) or were provided from the University of Arizona Medical Center pathology department. Patients had testis removed as part of therapy for prostate cancer. Human testis tissue was evaluated by a local pathologist and determined to be normal. Sectioning of all paraffin-embedded tissue was accomplished using a microtome with sections sliced 5 microns thick with one section per slide. Protocols for obtaining samples were approved by the University of Arizona Institutional Review Board (IRB) or Institutional Animal Care and Use Committee (IACUC).

**Immunohistochemistry.** IHC staining was performed on formalin-fixed, paraffin-embedded samples. Slides were deparaffinized with xylene and rehydrated with ethanol. The samples were then heated in an antigen retrieval buffer; citrate (pH 6.0) for ENT1, tris-EGTA (pH 9.0) for ENT2. Endogenous peroxide activity was blocked by a 0.3% hydrogen peroxide/methanol solution. Staining for ENT1 and ENT2 was performed with the MACH4 kit according to the
manufacturer’s instructions (Biocare Medical). All slides were imaged with a Leica DM4000B microscope and a DFC450 camera (Leica Microsystems Inc., Buffalo Grove, IL).

**Statistics:** Data are presented as means ±SE with the sample size representing separate experiments (each typically performed in triplicate). All tests of significance of observed differences were done by one-way analysis of variance using a Tukey *post hoc* multiple comparison test with p <0.05 representing significance.

**Results**

**Basolateral Uptake of [3H]Uridine by Rodent Seminferous Tubules:** To analyze the role of ENT transporters in nucleoside transport across the BTB, we measured the ability of intact isolated single rodent seminiferous tubules to accumulate [³H]uridine, thereby providing a measure of basolateral uptake. Figure 2.1 shows a time course for [³H]uridine basolateral uptake in seminiferous tubules for rat (Figure 2.1A) and mouse (Figure 2.1B). The concentrations of [³H]uridine in the bath were 0.41 µM for rat; 1.88 µM for mouse. Transport for both rat and mouse tubules was nearly linear for the first 5 minutes. Accumulation of [³H]uridine into rat tubules was reduced by 54-83% over the first 20 minutes in the presence of 5 mM unlabeled uridine, suggesting that uridine uptake involved a saturable process.

The basolateral uptake of [³H]uridine in both rat and mouse seminiferous tubules was inhibited by increasing concentrations of unlabeled uridine in a
manner adequately described using the Michaelis-Menten equation for the competitive interaction of labeled and unlabeled substrate introduced by Malo

**Figure 2.1: Time course of basolateral transport of [3H]uridine by rodent seminiferous tubules.**
Composite graphs depicting [3H]uridine transport by rat (A) or mouse (B) intact seminiferous tubules through the basolateral surface over time. The mean concentration of [3H]uridine in the baths were 0.41 µM and 1.88 µM for rats and mice, respectively. [3H]uridine transport in the presence of 5 mM unlabeled uridine was analyzed to determine the saturable portion of uridine transport in rat seminiferous tubules. The insets demonstrate the saturable uptake of rat or mouse seminiferous tubules at 5 minutes with and without the presence of 5 mM uridine. Each point represents the mean (±standard error) of four experiments for rats and two experiments for mice (±half the range), each with a different animal. At least three tubules per time point were analyzed in each experiment.
and Berteloot (Malo and Berteloot, 1991). In five separate experiments $K_t$ values for uridine transport were $314 \pm 63 \mu M$ and $90 \pm 24 \mu M$, and $J_{\text{max}}$ values were $2.5 \pm 0.6 \text{ pmol/(min-mm)}$ and $0.55 \pm 0.2 \text{ pmol/(min-mm)}$, for rat and mouse seminiferous tubules, respectively. Figure 2.2 shows the kinetic profiles for uridine uptake into these tubules, corrected for the non-saturable component of total uridine uptake.

Since uridine is commonly used as a substrate for ENT-mediated transport, it was anticipated that this process would be inhibited by NBMPR, a potent inhibitor of ENT1 and weak inhibitor of ENT2. The interaction between NBMPR and uridine transport is shown in Figure 2.3. The $IC_{50}$ of NBMPR on $[^3H]$uridine was calculated by using the following equation (Groves et al, 1994):

$$J = \frac{J_{\text{app}} [\text{Uridine}^*]}{IC_{50} + [\text{NBMPR}]_o} + D [\text{Uridine}^*]$$

where $J$ is the rate of $[^3H]$uridine uptake; $J_{\text{app}}$ is the product of the maximum rate of $[^3H]$uridine uptake ($J_{\text{max}}$) and the ratio of the $K_i$ of NBMPR and $K_t$ for uridine transport; $IC_{50}$ is the concentration of $[\text{NBMPR}]_o$ that reduced mediated (i.e., blockable) $[^3H]$uridine transport by 50%. The concentration of NBMPR was carried out to 500 nM, but maximal inhibition was achieved by 100 nM for both rats and mice. NBMPR inhibited this transport with an $IC_{50}$ for the mediated (i.e. blockable) fraction of $[^3H]$uridine uptake of $23.6 \pm 3.1 \text{ nM}$ for rat tubules (Figure 2.3A) and $12.9 \pm 0.7 \text{ nM}$ for mouse tubules (Figure 2.3B). These $IC_{50}$ values are
similar to the range of IC$\textsubscript{50}$ values (0.1 nM to 68.5 nM) reported for ENT1 by others (Griffiths et al, 1997; Takano et al, 2010).

The 400 nM concentration of NBMPR did not appear to block uridine uptake into seminiferous tubules to the same extent as 5 mM unlabeled uridine (compare Fig 2.2 to Fig. 2.3 A, B). To compare the inhibition of NBMPR to that of unlabeled uridine, seminiferous tubules were categorized into four groups based on supplements in the media: control (no supplements), 5 mM uridine, 400 nM NBMPR, or 100 µM NBMPR (Figure 2.3C). The control group differed significantly from the other groups and there was also a significant difference between the 5 mM uridine and the 400 nM NBMPR groups. No significant difference was found between the other pairings. These data suggest that ENT2 plays no significant role in basolateral uridine transport, but that a small fraction (18.8%) of that accumulation may involve a pathway other than ENT1.

**Basolateral Uptake of [3H]Uridine by Primary Rat Sertoli Cells.** To characterize the contribution of Sertoli cells to [3H]uridine uptake by seminiferous tubules, primary Sertoli cells were isolated from rat testes and [3H]uridine basolateral uptake was characterized on Matrigel-coated transwell plates. Figure 2.4 shows a time course of basolateral-to-apical (transepithelial transport) of [3H]uridine across primary cultured Sertoli cells. By 15 minutes, radiolabel appeared in the apical compartment. This signal was completely blocked at all time points by the addition of either 5 mM uridine, 100 nM NBMPR or 100 µM NBMPR.
Figure 2.2: Ex vivo kinetic analysis of basolateral transport of [3H]uridine by rodent seminiferous tubules. Composite graphs demonstrating basolateral [3H]uridine transport by rat (A) or mouse (B) intact seminiferous tubules in the presence of increasing concentrations of unlabeled uridine. The mean concentrations of [3H]uridine in the baths were 0.45 µM and 0.37 µM for rats and mice, respectively. K_t values were calculated to be 314 µM and 90 µM for rat and mouse seminiferous tubules, respectively. Each point represents an average (±standard error) of at least five different experiments, each with a different animal. At least three separate tubules for each unlabeled uridine concentration were analyzed in each experiment.
Since ENT transporters are bidirectional, primary Sertoli cells were exposed to NBMPR and [³H]uridine in the basolateral compartment followed by light paraffin oil application on the apical side (which prevents apical efflux of the hydrophilic uridine) to determine if inhibition of the transepithelial transport of [³H]uridine was mediated by basolateral uptake or cellular efflux across the apical membrane. Figure 2.5 shows the 15 minute accumulation of [³H]uridine into Sertoli cells across the basolateral membrane. That accumulation was reduced by 95% in the presence of 100 nM NBMPR, indicating that the inhibition of transepithelial transport seen in Figure 2.4 was due to the blocking of [³H]uridine basolateral uptake into Sertoli cells.

**mRNA Expression of Nucleoside Transporters in Fresh Seminiferous Tubules:** In the light of evidence of CNT expression in primary cultured Sertoli cells (Kato et al, 2005), we determined mRNA expression of nucleoside transporters in freshly isolated rat seminiferous tubules, freshly isolated Sertoli cells, and primary Sertoli cells cultured for six days. Figure 2.6 shows branched DNA analysis of freshly isolated seminiferous tubules (6A) and primary Sertoli cells (6B). Expression of nucleoside transporters in seminiferous tubules was normalized to rat kidney tissue (which is known to express ENT1, ENT2, CNT1, CNT2, and CNT3) and expressed in relative light units (RLU) (Rodriguez-Mulero et al, 2005; Ishida et al, 2012). ENT1 expression was approximately 2.53 fold higher in seminiferous tubules than in kidney tissue. ENT2 expression was also highly expressed with RLU readings 0.94 compared to kidney. Expression of CNT1, CNT2, and CNT3 were much lower than that of ENT1 or ENT2 (0.05, 0.09, and .05 fold respectively).
Figure 2.3. Ex vivo analysis of the effects of NBMPR on basolateral transport of [3H]uridine by rodent seminiferous tubules. Composite graphs depicting [3H]uridine transport by rat (A) or mouse (B) intact seminiferous tubules in the presence of increasing concentrations of NBMPR. The mean concentrations of [3H]uridine in the baths were 0.43 µM and 1.11 µM for rats and mice, respectively. IC$_{50}$ values were calculated to be 23.6 nM and 12.9 nM for rat and mouse seminiferous tubules, respectively. Each point represents an average (±standard error) of at least five experiments, each with a different animal. At least three separate tubules for each NBMPR concentration were analyzed in each experiment.

The bar graph (C) represents ex vivo basolateral uptake of [3H]uridine in intact rat seminiferous tubules. The mean concentration of [3H]uridine in the baths was 45 µM. Control represents [3H]uridine uptake in the absence of NBMPR or unlabeled uridine. [3H]uridine transport in the presence of 5mM unlabeled uridine was analyzed to determine the saturable portion of uridine transport. The concentrations of NBMPR are enough to block just ENT1 activity (400 nM) or both ENT1 and ENT2 (100 µM). The height of each bar is 6.22 (control), 1.12 (5 mM uridine), 2.08 (400 nM NBMPR), and 1.55 (100 µM NBMPR) fmols mm$^{-1}$ min$^{-1}$. Asterisks (*) indicate significance (p < 0.05) compared to control whereas the dagger (†) indicates significance (p < 0.05) compared to 400 nM NBMPR.
In primary Sertoli cells, RLU values were normalized to GAPDH. ENT1 was found to be highly expressed (0.44 ± 0.14 and 0.35 ± 0.07 RLU for day 0 and day 6 respectively) as was ENT2 (0.75 ± 0.33 and 0.45 ± 0.12 for day 0 and day 6 respectively). Small amounts of CNT1 were detected (0.04 ± 0.01 and 0.04 ± 0.02 for both day 0 and day 6 respectively) but CNT2 and CNT3 expression were below background. No significant difference in transporter expression was observed between freshly isolated cells (day 0) and cells cultured for 6 days (day 6).

**Immunohistochemical Staining of Rat and Human Testes.** Immunohistochemical (IHC) staining for ENT1 and ENT2 was performed on rat testes to determine the subcellular distribution of these transporters. rENT1 and rENT2 were localized in Sertoli cells using IHC staining on both immature (Figure 2.7A, C) and mature (Figure 2.7B, D) rats. In both mature and immature testes, rENT1 was located on the basolateral membrane of Sertoli cells (Figure 2.7A, B). In contrast, slides stained for rENT2 expressed positive staining on the apical membrane but not on the basolateral membrane (Figure 2.7C, D).

IHC staining was also performed on adult human testes to determine if hENT1 and hENT2 share the same localization as their rat counterparts (Figure 2.8). Consistent with the expression profiles observed in rat testis, IHC staining revealed the presence of hENT1 on the basolateral membrane (Figure 2.8A), and hENT2 on the apical membrane (Figure 2.8B) of the Sertoli cells.

**NRTI Inhibition of [3H]Uridine Transport by Primary Cultured Rat Sertoli Cells.** To determine whether the transporters used by uridine could also interact with NRTIs, transepithelial transport of uridine by primary Sertoli cells
Figure 2.4. In vitro analysis of role of rENT1 and rENT2 in [3H]uridine transepithelial transport by primary cultured Sertoli cells. Confluent monolayers of primary cultured rat Sertoli cells were exposed to 40 nM [3H]uridine in the basolateral compartment. Apical media was counted to determine transepithelial transport. Cells were exposed to concentrations of NMBPR sufficient to inhibit ENT1 (100 nM), ENT2 and ENT1 (100 μM), or unlabeled uridine (5 mM). Control cells were not given NBMPR or unlabeled uridine. The lines describing the data were fit by eye. Each point represents an average (±standard error) of triplicate wells of Sertoli cells derived from a mixture of three rats.
was measured in the presence of the NRTIs zidovudine (5 mM), didanosine (5 mM), and tenofovir disoproxil fumarate (1 mM). Each of these NRTIs significantly inhibited uridine transepithelial transport (Figure 2.9). The ability of these drugs to block basolateral uridine transport supports the contention that these NRTIs interact with the same transporter(s) used by uridine and may also act as substrates.

Discussion

We present a novel ex vivo technique for determining BTB transport using freshly isolated seminiferous tubules. Using both this novel ex vivo and traditional in vitro systems that employed 6 day cultured Sertoli cells, we demonstrated that, based on pharmacologic inhibition by NBMPR, basolateral uptake of nucleosides by Sertoli cells is dominated by ENT1. We also demonstrate that ENT2 does not play a significant role in basolateral uridine uptake into Sertoli cells. NBMPR inhibited 66% of total uridine uptake in rat seminiferous tubules at concentrations that pharmacologically inhibit ENT1 but do not affect ENT2 (100 nM) (Abd-Elfattah et al, 2012; Ward et al, 2000; Nishimura et al, 2012) (Fig. 2.2A, B). Of the saturable component (i.e. the portion of transport blocked by 5 mM unlabeled uridine), 400 nM NBMPR inhibited 81% of uridine transport and increasing the NBMPR concentration to 100 μM (sufficient to block ENT2) did not result in a significant decrease in uridine uptake (Fig. 2.3C). This strongly implicates ENT1 as the primary transporter in nucleoside uptake into seminiferous tubules. The results with intact tubules were confirmed and extended by the observations in the primary cultured rat Sertoli cells. In these
Figure 2.5. Effects of NBMPR on the basolateral uptake of \[^{3}H\]uridine in primary cultured rat Sertoli cells. Confluent monolayers of primary cultured rat Sertoli cells were provided with 40 nM \[^{3}H\]uridine in the basolateral compartment. After 15 minutes, cells were lysed and the lysates were counted to determine cellular accumulation of \[^{3}H\]uridine. Cells were exposed to concentrations of NBMPR sufficient to inhibit ENT1 uptake (100 nM), ENT2 and ENT1 (100 μM), or unlabeled uridine (5 mM). Control cells were not given NBMPR or unlabeled uridine. Each point represents an average (± half the range) of duplicate wells of Sertoli cells derived from a mixture of three rats. The height of each bar is 26.81 (control), 1.33 (5 mM uridine), 0.53 (400 nM NBMPR), and 0.34 (100 μM) fmol mm\(^{-1}\) min\(^{-1}\).
Sertoli cells, 100 nM NBMPR blocked the entire saturable portion of transepithelial uridine transport (Fig. 2.4), supporting the contention that transepithelial transport of uridine past the BTB depends on functional ENT1. Importantly, this inhibition was also observed in the basolateral uptake of uridine (Fig. 2.5), strongly supporting the conclusion that ENT1 mediates the basolateral entry for the transepithelial transport of uridine across Sertoli cells.

We also discovered that ENT2 is located on the apical membrane and not on the basolateral membrane. This is based on three observations. First, IHC data located ENT2 to the apical membrane but not to the basolateral membrane (Fig. 2.8). Second, inhibition of ENT1 (via 100 nM NBMPR) virtually stops all transepithelial transport of uridine across the basolateral membrane of Sertoli cells (Fig. 2.5). Third, increasing NBMPR concentration from 100 nM to 100 µM had little further effect on inhibition of uridine uptake (Fig. 2.4, 2.5). These data demonstrate that ENT2 must play little to no role in basolateral uptake of uridine into seminiferous tubules.

Interestingly, in seminiferous tubules, there was a small portion (approximately 19%) of [3H]uridine transport that was blocked by unlabeled uridine but not by high concentrations of NBMPR. Since NBMPR can prevent transport of uridine in Sertoli cells, this unexplained accumulation may not reflect activity of Sertoli cells. Perhaps it is due to some effect of the myoid layer which is removed during Sertoli cell isolation. However, because such a large proportion of uptake into the tubules is blocked by 100 nM NBMPR (which has no effect on CNT-mediated transport), ENT1 seems to be the primary transporter responsible for nucleoside uptake into seminiferous tubules. This is in agreement
Figure 2.6. Branched DNA analysis of nucleoside transporter expression in seminiferous tubules relative to kidney. RNA extracted from freshly isolated seminiferous tubules or kidney tissue (A) from 4 rats was analyzed for expression of ENT1, ENT2, CNT1, CNT2, and CNT3. To account for variations among probe sets, expression of each transporter was compared to kidney as a positive control. Graph depicts above background expression relative to positive control. Results show that expression for ENT1 253.9%, ENT2 94.0%, CNT1 5.2%, CNT2 9.3% and CNT3 5.2% relative to kidney expression. Each point represents the mean (±standard error) of 6 wells.
with expression of ENT1 but not ENT2 on the basolateral membrane of Sertoli cells. The identical localizations of ENT1 and ENT2 in both rat and human tissues suggest that data gathered from rat Sertoli cells and seminiferous tubules may be applicable to humans. Unfortunately, fresh human seminiferous tubules were unavailable so uptake studies with human seminiferous tubules could not be performed.

We also demonstrated through branched DNA analysis that ENTs are the primary nucleoside transporters expressed in fresh seminiferous tubules, fresh Sertoli cells, and 6 day cultured primary Sertoli cells all had low to undetectable expression of CNTs (Figure 2.6). Furthermore, the complete inhibition of basolateral uridine uptake produced by 100 nM NBMPR suggests that CNTs do not exert an appreciable impact on basolateral transport of uridine in Sertoli cells (Figure 2.4, 2.5). This would indicate that any CNTs expressed by Sertoli cells, which bDNA analysis suggests being very modest, would be localized to the apical membrane.

Whereas the function of ENT1 on the basolateral membrane is evident, the role of ENT2 on the apical membrane is less clear. A common physiological function of ENT transporters is to transport nucleosides from the blood into the cell and this is how we believe ENT1 functions in Sertoli cells (Kato et al, 2005; Molina-Arcas et al, 2008). As ENT1 transports nucleosides into the cell, the intracellular concentration of these molecules could rise to approach that in the blood. Since ENT transporters move nucleosides according to their concentration gradient, ENT2 on the apical membrane would transport nucleosides from inside the cell into the lumen of the seminiferous tubule where they can be used by
Figure 2.7. Localization of rENT1 and rENT2 in the testis. Immunohistochemical staining for ENT1 (A, B) or ENT2 (C, D) in formalin-fixed paraffin-embedded immature (A, C) or mature (B, D) rat testes is shown at 40 X magnification. Arrows indicate positive (brown) staining for proteins. L indicates lumen of seminiferous tubules.
Figure 2.8. Localization of hENT1 and hENT2 in the testis
Immunohistochemical staining for ENT1 (A) or ENT2 (B) in formalin-fixed paraffin-embedded adult human testes is shown at 40 X magnification. Arrows indicate positive (brown) staining for proteins. L indicates lumen of seminiferous tubules.
developing germ cells. These dividing germ cells could act as a ‘nucleoside sink’ keeping the luminal nucleoside concentration low enough to drive unidirectional transport. Figure 2.10 provides an illustration of how this hypothesized process could be functioning to transport nucleosides into the lumen. To the extent that CNTs are expressed in the apical membrane of Sertoli cells, their activity would be expected to reabsorb some fraction of nucleoside secreted by ENT2. Since we suspect that CNTs impact on nucleoside transport into native Sertoli cells is likely to be minimal (see earlier discussion), we do not expect these transporters to have a substantial impact on nucleoside transport. Clearly, more studies involving ENT2 and CNTs on the apical membrane of Sertoli cells are required to verify this model.

Using this model, if the NRTI concentration inside the seminiferous tubules rose above that of the blood, then ENT2 and ENT1 would remove drug from the tubules and transport it back into the blood. This would suggest that the concentration of NRTI drugs would never rise above that of the blood in the seminiferous tubule. This is puzzling since clinical data has indicated that NRTI drugs are up to 10 fold more concentrated in seminal plasma than in blood plasma (Dumond et al, 2008). One possible explanation could be that NRTI drugs reach concentrations similar to blood levels in the seminiferous tubules, but are then concentrated further down the male genital tract (MGT). Fluid from the seminiferous tubule flows into the epididymis where up to 99% of the water can be reabsorbed (Lu, 2008; Cornwall, 2009). Assuming drug is not also reabsorbed, this would increase the concentration of drug approximately 100 fold. During ejaculation, drug from the epididymis would mix with secretions
Figure 2.9. Inhibition of [3H]uridine transport by NRTI in primary cultured rat Sertoli cells. Confluent monolayers of primary cultured rat Sertoli cells were exposed to media in the basolateral compartment containing 40 nM [3H]uridine and either zidovudine (5 mM), didanosine (5 mM), or tenofovir disoproxil fumarate (1 mM). Control wells were not exposed to any NRTI drugs. Media from the apical compartments were counted to assess transepithelial transport of [3H]uridine after 15 minutes. Each bar represents an average (± standard error) of triplicate wells of Sertoli cells derived from a mixture of three rats. The height of each bar is 27.52 (control), 11.52 (zidovudine), 9.51 (didanosine), and 13.79 (tenofovir disoproxil fumarate). Asterisks (*) indicate significance (p < 0.05) compared to control.
Figure 2.10. Model of ENT-Mediated Transport of Nucleosides Across the BTB. An illustration of the seminiferous tubule and surrounding cells depicting how nucleosides could be transported into the lumen via ENT transporters. ENT1 is the primary transporter responsible for uptake of nucleosides into the Sertoli cells due to the higher concentration of nucleosides in the blood. ENT2 is proposed to play a role in efflux of nucleosides into the lumen of the seminiferous tubules due to the higher concentration of intracellular nucleosides (mediated by ENT1) compared to the lumen of the seminiferous tubule.
from accessory sex organs (primarily secretions originating from the seminal vesicles and prostrate) resulting in dilution of the drug (Van Praag et al, 2001; Cao et al, 2008; Caballero et al, 2012). Since testicular fluid has been estimated to contribute up to 10% of the total volume for semen, this would dilute the drug to roughly 10 fold above blood concentration (Van Praag et al, 2001). This illustrates a possible mechanism for drugs crossing the BTB and then becoming concentrated in the MGT resulting in a higher concentration of drug in the seminal plasma than in blood plasma.

The assumption that drug is not reabsorbed in the epididymis requires further investigation. Studies investigating epididymal drug transport are limited. However, one study revealed that ENT1, ENT2, and CNT2 are present in the epididymis (Leung et al, 2001). Without knowing the localization of these transporters, it is difficult to speculate on their potential impact on drug transport. If, for example, CNT2 is the only nucleoside transporter on the apical membrane, then pyrimidine-based analogs, such as zidovudine and lamivudine, would not be reabsorbed since CNT2 transports purine-based analogs (Van Aubel et al, 2000; Gray et al, 2004;).

This ENT dominant system is a possible mechanism for NRTI transport at the BTB. We showed that ENTs are localized in Sertoli cells in a manner that would support transepithelial transport, ENT1 is necessary for basolateral nucleoside transport, and some NRTIs can inhibit transepithelial transport of uridine, which indicates that they interact with the same transporters (Figure 2.9). This is in agreement with other sources which have demonstrated that ENT transporters can transport NRTIs (Ward et al, 2000). Taken together, these data
indicate the possibility of a novel ENT-mediated pathway for the penetration of nucleosides and NRTIs past the BTB.
CHAPTER 3: LOCALIZATION OF MULTIDRUG RESISTANCE-ASSOCIATED PROTEINS ALONG THE BLOOD-TESTIS BARRIER IN RAT, MACAQUE, AND HUMAN TESTIS.


Introduction

The epithelial cells that form most of the static cellular mass in seminiferous tubules are called Sertoli cells. Sertoli cell possess a basolateral membrane that faces the outside of the tubule which is exposed to nutrients from the blood, and an apical membrane that is in contact with germ cells (Mruk and Cheng, 2011a; b; França et al., 2012). It is the primary job of Sertoli cells to nurture and protect the developing germ cells (Kato et al. 2009). Germ cell development is a dynamic process that produces several distinct morphologies, starting from the spermatagonia developing into the haploid round spermatids, and ending with release of immature spermatozoan into the lumen of the tubule (Gerton and Millette, 1986; Oliveira et al., 2009; Su, Mruk, and Cheng, 2011; Xiao, Mruk, CKC Wong, et al., 2014). During this development, the germ cells are sensitive to toxic agents that may be able damage the sperm or have genotoxic effects on the offspring. To help protect the germ cells from potential toxicants,

---

2 I have completed 100% of the data generation presented for this chapter. The entire initial draft was written by me.
the Sertoli cells form a blood-testis barrier (BTB) (Bart et al., 2004a; Chihara et al., 2013; Wang et al., 2013).

The anatomical portion of the BTB is composed of tight junctions between the Sertoli cells (Pelletier, 2011; Mital, et al, 2011; Li et al, 2012). These tight junctions are located near the outside edge of the seminiferous tubule, just apical of the spermatagonia. This barrier prevents many exogenous agents from gaining entry into the lumen of the seminiferous tubules and contacting germ cells (Mann and Lutwak-Mann, 1982; Mruk and Cheng, 2010). Although this barrier is beneficial for sperm cell development, it can be an obstacle for drugs that are required to bypass the BTB to achieve full therapeutic effect. Examples of such drugs include many antiretroviral medications used to treat infection of human immunodeficiency virus (HIV). By limiting the entry of many antiretrovirals into the seminiferous tubules, the BTB may be contributing to the testes’ serving as a sanctuary site for HIV (Byrn and Kiessling, 1998; Anderson et al., 2010; Dahl et al., 2010; Avery et al., 2011). Since the tight junctions of the BTB prevent or reduce paracellular diffusion of hydrophilic drugs, transcellular transport through the Sertoli cells is required for antiretrovirals to bypass the BTB.

In addition to the tight junctions between Sertoli cells, it has also been reported that there is a transport portion of the BTB to counteract passive diffusion (Bart et al, 2004). Many of the transporters that line the BTB belong to the ATP-binding cassette (ABC) family which uses energy from ATP hydrolysis to actively efflux a wide variety of substrates (Beringer and Slaughter, 2005; Klaassen and Aleksunes, 2010; Michaud et al., 2012). This family includes transporters such as P-glycoprotein (P-gp), breast cancer resistance protein
(BCRP) and members of the multidrug resistance-associated protein (MRP) subfamily (Bart et al., 2004). Within this family, P-gp (ABCB1), MRP1 (ABCC1), MRP4 (ABCC4), MRP5 (ABCC5), and MRP8 (ABCC11) mRNA expression has been found in rat Sertoli cells while MRP2 and MRP3 were found to be expressed at low amounts (Bart et al., 2004; Augustine et al., 2005). Additionally, BCRP and P-gp have been localized in human testis to the peritubular myoid cells (Bart et al., 2004b; Qian, Y-H Cheng, Mruk, et al., 2013). The physiological functions of MRP1, MRP4 and MRP5 are cytoprotective in many tissues and are known to efflux a wide variety of compounds (Klaassen et al., 2010). MRP8 is known to play a role in ear wax synthesis, auxiliary body odor, and breast cancer due to the transport of many pro-growth hormones and amino acids (Guo et al., 2003; Bortfeld et al., 2006; Kruh et al., 2007). In the testis, it has been speculated that these transporters contribute to keeping xenobiotic compounds out of the BTB thereby protecting dividing germ cells from potential toxicants (Kato et al., 2005).

Many drugs used to treat HIV have been shown to be transported by MRP transporters (Reid et al., 2003; Kohler et al., 2011; Yao et al., 2011; Michaud et al., 2012). This would imply that the MRPs expressed by Sertoli cells could influence the ability of HIV drugs to bypass the BTB. However, it is difficult to determine the effect MRP transport has on disposition across the BTB until the localization of each member is known. The purpose of this study is to determine the subcellular location in the testis of MRP transporters that may interact with HIV drugs using, immunohistochemical analysis of rat, macaque, and human tissue.
Materials and Methods

Materials: MACH4 IHC staining kit was acquired from Biocare Medical (St. Louis, MO). MRP1 (ABCC1), MRP5 (ABCC5) and MRP8 (ABCC11) antibodies were purchased from Abcam (Cambridge, MA) and MRP4 (ABCC4) antibodies were purchased from Lifespan Biosciences (Seattle, WA). Testis from MRP4−/− mice was a generous gift from Dr. J. Schutes (St. Jude’s Hospital). All other reagents were purchased from a standard scientific supplier at the highest available purity.

Sample Collection: Rat samples were collected from euthanized male Sprague Dawley rats either 3 weeks (immature) or at least 12 weeks old (mature). The samples were fixed in 10% neutral buffered formalin overnight. A small incision was made in the tunica the next day and the samples remained in 10% neutral buffered formalin for another night. The following day, formalin was replaced with 70% ethanol until the samples were embedded in paraffin. Paraffin embedded rhesus macaque testis from an 8 year old Macaque was purchased from Oregon National Primate Research Center (ONPRC) tissue distribution program. Paraffin embedded human samples were purchased from the National Disease Research Interchange (NDRI) or were provided from the University of Arizona Medical Center pathology department. Sectioning of all paraffin-embedded tissue was accomplished using a microtome with sections sliced 5 microns thick with one section per slide. Protocols for obtaining samples were approved by the University of Arizona Institutional Review Board (IRB) or Institutional Animal Care and Use Committee (IACUC).
Figure 3.1. MRP1 localization in the testis. Immunohistochemical staining for MRP1 in formalin-fixed paraffin-embedded immature (A) or mature (B) rat testes, mature rhesus macaque (C) or mature human (D) is shown at 40 X magnification. Black bar in panel A indicates length of 10 microns. Arrows indicate positive (brown) staining for proteins.
**Immunohistochemistry.** IHC staining was performed on formalin-fixed, paraffin-embedded samples. Slides were deparaffinized with xylene and rehydrated with ethanol. The samples were then heated in an antigen retrieval buffer: citrate (pH 6.0) for MRP1 and MRP5, or tris-EGTA (pH 9.0) for MRP4 and MRP8. Endogenous peroxide activity was blocked by a 0.3% hydrogen peroxide/methanol solution. Staining for all antibodies was performed with the MACH4 kit according to the manufacturer’s instructions (Biocare Medical). All slides were imaged with a Leica DM4000B microscope and a DFC450 camera (Leica Microsystems Inc., Buffalo Grove, IL). Each experiment also contained a negative control slide which was not exposed to any primary antibodies, but otherwise was treated the same as every other slide. The negative slides contained very little to no positive (brown) staining.

**Results**

**Immunohistochemical Staining of Rat, Macaques, and Human Testes for MRP1.** Immunohistochemical (IHC) staining for MRP1 was performed on testes tissue to determine the subcellular distribution of this transporter. MRP1 was localized in Sertoli cells using IHC staining on immature (Figure 3.1A) and mature (Figure 3.1B) rat, mature *rhesus macaques* primate (1C) and mature human (1D) testis. In all cases, MRP1 was located on the basolateral membrane of Sertoli cells (Figure 3.1). Positive staining can also be observed in Leydig cells located in the interstitial region.

**Immunohistochemical Staining of Rat, Macaques, and Human Testes for MRP4.** IHC staining for MRP4 was also performed on testicular
Figure 3.2. MRP4 localization in the testis. Immunohistochemical staining for MRP4 in formalin-fixed paraffin-embedded immature (A) or mature (B) rat testes, mature rhesus macaque (C) mature human (D) mature mouse (E) or MRP4−/− mouse (F) is shown at 40 X magnification. Black bar in panel A indicates length of 10 microns. Arrows indicate positive (brown) staining for proteins.
tissue acquired from immature (3.2A) and mature (B) rats, as well as adult primates (C) and humans (D). Interestingly the data demonstrated a species difference in MRP4 localization. Positive staining was observed on the apical membrane of both immature and mature rats. However, in macaques and human tissue, the staining appeared basolateral. Due to the unexpected nature of these results, we verified specificity of the MRP4 antibody by performing IHC on normal mouse tissue (E) and MRP4−/− mouse tissue (F). No positive staining was observed in the MRP4−/− tissue indicating that the MRP4 antibody used is specific for MRP4.

**Immunohistochemical Staining of Rat, Macaques, and Human Testes for MRP5.** Previous work performed in our lab indicated that MRP5 was expressed in testis (Augustine, et al 2005). However, in all species no positive staining was observed in Sertoli cells (Figure 3.3). There was positive staining in Leydig cells for mature and immature rats (arrows in Fig. 3.3A and B), which accounts for previous data indicating testicular expression of MRP5 (Augustine et al, 2005). Interestingly, there was no staining in the macaques tissue (3.3C) and only minimal staining observed in human tissue (3.3D). As a positive control, rat kidney, which is known to express MRP5 on the apical membrane of proximal tubule cells, was stained and apical staining was observed, indicating that the MRP5 antibody was functional (data not shown).

**Immunohistochemical Staining of Macaques, and Human Testes for MRP8.** Due to the fact that rodents do not express an MRP8 ortholog, only human and rhesus macaque tissue was stained for MRP8.
**Figure 3.3 MRP5 localization in the testis.** Immunohistochemical staining for MRP5 in formalin-fixed paraffin-embedded immature (A) or mature (B) rat testes, mature rhesus macaque (C) or mature human (D) is shown at 40 X magnification. Black bar in panel A indicates length of 10 microns. Arrows indicate positive (brown) staining for proteins.
Interestingly, both species demonstrated distinct staining on round spermatids, which are germ cells that have undergone meiotic division, but have not yet developed the characteristic sperm cell morphology. Only this stage of germ cell development seems to express MRP8 and MRP8 does not seem to be expressed by Sertoli cells.

**Discussion**

We present novel information concerning the localization of MRP transporters in the testis of three species: rats, rhesus macaques, and humans. We stained tissue isolated from immature (pre-puberty) and mature (post-puberty) rats but, due to difficulty in obtaining immature maraques and human tissue and the lack of age-dependent differences in rats, we only stained mature macaques and human tissues. MRP transport function can be difficult to study at the blood-testis due to overlapping substrate specificity, lack of specific inhibitors, obtaining enough human tissue to culture primary Sertoli cells, and technical challenges in studying efflux transport. While this information is novel for the testis, many of these transporters have been localized in other barrier tissues. In the blood-brain barrier, MRP1, MRP4, and MRP5 are expressed on the apical side of capillary endothelial cells. In choroid plexus epithelial cells, MRP1 is expressed only on the basolateral membrane while MRP4 is on both apical and basolateral membranes. In the placenta, MRP1 is on the apical side of syncytiotrophoblasts and on the basolateral side of fetal membranes, along with MRP4 and MRP5 (Klaassen and Aleksunes, 2010). Each transporter appeared to
Figure 3.4. MRP8 localization in the testis  Immunohistochemical staining for MRP8 in formalin-fixed paraffin-embedded mature rhesus macaque (A) or mature human (B) is shown at 40 X magnification. Black bar in panel A indicates length of 10 microns. Arrows indicate positive (brown) staining for proteins.
have a different staining pattern indicating different physiological functions and different effects on drug disposition past the BTB.

MRP1 staining likely represents the expected function of MRP transporters along the BTB. The basolateral localization indicates that MRP1 acts as part of the transporter portion of the BTB in effluxing xenobiotics out of the seminiferous tubule thereby representing a spermatoprotective response. This would suggest that MRP1 would likely act as an obstacle for getting those antiviral drugs or other chemotherapeutics that are substrates for MRP1 into the testes.

In humans and non-human primates, MRP4 has the same localization, and presumably function, as MRP1, i.e., acting as a spermatoprotective response to potentially toxic agents. Unexpectedly, we discovered a different localization for MRP4 in rats at both mature and immature ages. It is difficult to speculate on the potential function of MRP4 in rats. Since this transporter is known to transport a wide variety of substrates, including secondary signaling molecules such as cGMP, perhaps it is involved in paracrine signaling to the nearby germ cells (Kruh et al., 2007; Morgan et al., 2012). Nonetheless, it is clear that this represents a potential issue in using rats as a model for BTB disposition of drugs, as MRP4 could be aiding drug disposition into the seminiferous tubule in a manner not representative of the human condition. More studies are needed to assess the impact this species difference has on HIV drug transport.

In all species tested, MRP5 was not expressed along the BTB, but positive staining was observed in Leydig cells for rats. One of the primary functions of the Leydig cell is steroidogenesis (McGee and Narayan, 2013). Like MRP4, MRP5 is
known to transport a wide variety of compounds and signaling molecules (Kruh et al., 2007). It is likely that these transporters may play a role in aiding hormone signaling. Another possibility is that MRP5 is simply cytoprotective for the Leydig cells. Whatever its physiological function may be, it is apparent that MRP5 would not be expected to have a significant impact on drug disposition across the BTB.

MRP8 also displayed an interesting and unexpected localization. Instead of being localized to the Sertoli cells, it was restricted to round spermatids. These spermatids are haploid but, as the name suggests, still possess a spherical morphology. It is at this stage that the spermatids are downregulating pro-division signals so that they may begin restructuring to a more elongated morphology (Pang et al., 2006). MRP8 is known to transport steroid sulfates and neurosteroids such as DHEAS (Kruh et al., 2007). Since many MRP8 substrates are pro-growth hormones, it is possible that the spermatids are expressing MRP8 as a means of effluxing cell division signals that are no longer needed. If this is true, it may be expected that a defect in MRP8 would result in an increased incidence in germ cell tumors. A single nucleotide polymorphism (SNP) is known to exist in the human population for this gene and although it has been linked to breast cancer, no information is available regarding its correlation to germ cell tumors (Toyoda and Ishikawa, 2010). While it could be speculated that this transporter is serving a cytoprotective function in round spermatids, this seems unlikely since MRP8 is not expressed throughout germ cell development and there are no apparent reasons why round spermatids would be any more sensitive to toxic agents than any other stage of germ cell development. What our study does conclude about MRP8 is that although this transporter may limit drug
distribution to the round spermatids, it would not be expected to play a role in disposition of drugs past the BTB.

In conclusion, with the intent of furthering the field’s understanding of drug transport at the BTB this study provides novel data demonstrating the localization of MRP1, MRP4, MRP5 and MRP8 in four types of testicular tissue originating from three different species. Based on our data, we drew three major conclusions: 1) MRP1 may limit drug penetration into the seminiferous tubules; 2) MRP4 has a species-specific difference localization and may be expected to limit drug disposition in humans and non-human primates, but facilitate disposition of (selected) drugs in rat testis; 3) neither MRP5 nor MRP8 are likely to have a major effect on drug transport at the BTB. Based on our data, further research can also be performed to better understand the physiological function(s) of these MRP transporters.
CHAPTER 4: XENOBIOTIC TRANSPORTER EXPRESSION ALONG THE MALE GENITAL TRACT.


Introduction

In order for a xenobiotic compound to influence function of a target tissue, it must be present within the target at sufficient concentrations. Transporters are one of the primary determinants of xenobiotic absorption, distribution, and elimination processes for many xenobiotics (Augustine et al., 2005; Klaassen and Aleksunes, 2010). Several families of proteins have been demonstrated to transport a wide variety of xenobiotic and endogenous substrates. These transporters include multiple drug resistance (Mdr) proteins, multidrug resistance-associated proteins (Mrp), organic anion transporters (Oat) organic anion transporting polypeptides (Oatp), organic cation transporters (Oct), equilibrative nucleoside transporters (Ent) and concentrative nucleoside transporters (Cnt). These transporters have broad and sometimes overlapping substrate specificities that include many clinically used drugs in addition to environmental toxicants (Bart et al., 2002; König et al., 2013; Lozano et al., 2013). Xenobiotic transporters may play an important part in excluding these.

3 I have completed 100% of the data generation presented for this chapter. The entire initial draft was written by me.
compounds, or conversely facilitate the distribution and tissue-specific accumulation of select agents. While drug transport has been well studied in organs such as the kidney, intestine and liver, there is a paucity of information concerning transporter expression in the male reproductive system (Slitt et al., 2007; Cangoz et al., 2013; König et al., 2013).

The male genital tract (MGT) begins with the seminiferous tubules located inside the testis where spermatogenesis occurs. Spermatogenesis ends with the release of immature spermatids into the lumen of the seminiferous tubules. These tubules converge at the rete testis before connecting to the proximal region of the epididymis (caput). The spermatids mature as they move through the epididymis and become fertile in the distal portion of the epididymis (cauda) where they are stored until ejaculation. During ejaculation, the sperm travels through the vas deferens (ductus deferens) while the seminal vesicles and prostate add secretions to the tract. These secretions from the seminal vesicles and the prostate are the primary components of seminal plasma.

One of the primary functions of the MGT is to keep out potentially toxic agents that may damage the sperm. This function is especially important in the testis, the site of spermatogenesis, as well as the epididymis, where the sperm are stored, and to the vas deferens which serves as the route for sperm during ejaculation. However, since the sperm become heavily exposed to the secretions from the seminal vesicles and the prostate during ejaculation, any toxicants entering from these tissues could also adversely affect sperm, as well as the sexual partners. Since transporters play a pivotal role in determining the distribution of xenobiotic compounds, knowledge of tissue-specific expression of
xenobiotic transporters along the MGT is essential for understanding the potential for accumulation of toxicants within the MGT. The constitutive expression levels of drug transporters in the tissues of the MGT may also provide insight into how well therapeutic agents are able to penetrate the various tissues of the MGT for the purposes of treating diseases, such as cancer or HIV infection (Deleage et al., 2011; Dueregger et al., 2013).

While studies have been performed determining the expression of the major xenobiotic transporters in the testis, a comprehensive analysis of transporter expression for other tissues in the MGT is currently lacking (Augustine et al., 2005). Therefore, this study was undertaken to determine the constitutive mRNA expression levels of 30 xenobiotic transporters in rat caput and cauda regions of the epididymis, seminal vesicles, vas deferens, and prostate in relation to the liver and kidney.

**Materials and Methods**

**Materials:** Quantigene HV Signal Amplification Kit and Quantigene Discovery Kit was purchased from Genospectra (Fremont, CA). RNAzol B reagent was purchased from Tel-Test Inc. (Friendswood, TX). All other reagents were purchased from standard scientific suppliers at the highest available purity.

**Sample Collection:** Samples were collected from euthanized rats at least 10 weeks old. Protocols for obtaining samples were approved by the University of Arizona Institutional Animal Care and Use Committee (IACUC).

**Development of Specific Oligonucleotide Probe Sets:** The following probe sets were used: Mdr1a and 1b (Brady et al., 2002); Mdr2 (Leazer
and Klaassen, 2003); Mrp1, 2, and 3 (Augustine et al., 2005); Mrp4, 5, and 6 (Leazer and Klaassen, 2003); Mrp7 and 9 (Augustine et al., 2005) Oatp1, 2, 3, 4, and 5 (Li et al., 2002); Oct1, 2, 3, N1, and N2 (Slitt et al., 2002); Oat1, 2, and 3 (Buist et al., 2002); Cnt1 and 2 as well as Ent1 and 2 (Leazer and Klaassen, 2003); and Cnt3 (see chapter 2).

**Total RNA Isolation:** Total RNA was isolated using RNAzol B reagent (Tel-Test Inc., Friendswood, TX) as per manufacturer's protocol. Each RNA pellet was resuspended in 0.2 ml of 10 mM Tris-HCl buffer, pH 8.0. The concentration of total RNA in each sample was quantified spectrophotometrically at 260 nm. RNA integrity and quality were analyzed by formaldehyde agarose gel electrophoresis with ethidium bromide staining. The quality of RNA samples was determined by the integrity and relative ratio of 28S and 18S rRNA bands.

**Branched DNA Assay:** Specific oligonucleotide probes for each gene were diluted in lysis buffer. Substrate solution, lysis buffer, capture hybridization buffer, amplifier, and label probe buffer used in the analysis were all obtained from the Quantigene Discovery Kit. The assay was performed in 96-well format with 10μg of RNA isolated from various tissues and then added to the capture hybridization buffer and 50 μl of the diluted probe set. The total RNA was then allowed to hybridize to the probe set overnight at 53°C. Hybridization steps were performed per the manufacturer's protocol the following day. Luminescence of the samples was measured with a Quantiplex 320 bDNA luminometer interfaced with Quantiplex Data Management Software, version 5.02 (Bayer, Walpole, MA). Background for each transporter was determined using negative control wells
Results

**Figure 4.1 Mdr and BCRP Expression.** Expression level of Mdr and Bcrp mRNA levels in kidney, liver, *caput, cauda*, seminal vesicles (SV), prostate, and vas deferens (VD). mRNA levels are expressed as mean relative light units (RLU)/10 μg total RNA ± S.E.M. (n=3).
which had all reagents except for RNA. The background was then subtracted to
demonstrate expression above background levels. The mRNA expression levels of
the major xenobiotic transporters in liver and kidney were compared to that of
the organs in the male reproductive system; namely the caput and cauda regions
of the epididymis, seminal vesicles, prostate, and vas deferens (ductus deferens).
Expression levels for the liver and kidney were used as positive controls since
these xenobiotic transporters are expressed by at least one of these tissues
(Augustine et al., 2005). For each transporter investigated, either the kidney or
the liver was considered the positive control depending on which tissue exhibited
higher expression levels. The mRNA expression levels for the tissues along the
MGT were normalized to the control tissue. Any expression that was 90% of
control or higher was considered highly expressed, while 20% or higher was
considered moderate expression and anything below 20% was considered low
expression. Figure 4.1 shows that Mdr1a, 1b and Bcrp expression was high in both
sections of the epididymis. The prostate showed moderate expression levels for
all three transporters while seminal vesicles only showed moderate levels of Bcrp.
Expression for mRNA of Mdr2 was not found at high levels in any tissue.

All tissues highly expressed mRNA for Ent1, except for the caput of the
epididymis which had moderate mRNA expression (79%). Ent2 mRNA levels
were moderate for all MGT tissues ranging from 24-82% of control (Figure 4.2).
Cnt1 was found to be only be mildly expressed in any of the MGT tissues (0.5-
10%), but Cnt2 was found at moderate levels throughout (28-56%). The
epididymis caput had more 228% expression of Cnt3 compared to kidney, while
the cauda expression levels were slightly higher than that of the kidney (127%).
Figure 4.2 Nucleoside Expression. Expression level of nucleoside transporters mRNA levels in kidney, liver, caput, cauda, seminal vesicles (SV), prostate, and vas deferens (VD). mRNA levels are expressed as mean relative light units (RLU)/10 μg total RNA ± S.E.M. (n=3).
Figure 4.3 Mrp Expression. Expression level of Mrp mRNA levels in kidney, liver, caput, cauda, seminal vesicles (SV), prostate, and vas deferens (VD). mRNA levels are expressed as mean relative light units (RLU)/10 μg total RNA ± S.E.M. (n=3).
Interestingly, Mrp1 was found to be highly expressed in the epididymis, particularly in the caput (143%), however all tissues seemed to express Mrp1 at moderate levels ranging from 57-78% (Figure 4.3). Conversely, the epididymis had almost no detectable expression of either Mrp2 or and Mrp3. The seminal vesicles had the highest expression of all the MGT tissues for both Mrp2 and Mrp3 and the vas deferens demonstrated 42% and 74% expression compared to control for Mrp2 and Mrp3 respectively. The caput and cauda showed marked differences in expression of Mrp4 with the cauda showing 35% expression levels compared to positive control, but the caput exhibited 5% expression compared to control. Mrp5 and Mrp7 expression was strikingly high for both sections of the epididymis and the seminal vesicles. The caput expressed the highest amounts of Mrp6, with every other tissue being moderate. Mrp9 expression was only above 20% for the seminal vesicles and the prostate.

Oat2 and Oat3 were found to be moderately expressed in seminal vesicles and the caput (Figure 4.4). Other than these exceptions, Oat transporters were expressed at very low levels by tissues in the MGT. Oct1 was decently expressed in the prostate and the vas deferens (Figure 4.5). Oct2 mRNA was was not well detected in any tissues of the MGT except the seminal vesicles (23%). Seminal vesicles were found to express Oct3 at levels nearly double that of positive control and every other MGT tissue expressed Oct3 at intermediate levels. OctN1 was expressed at low levels for all the tissues of the MGT except for the caput (22%), seminal vesicles (25%) and the prostate (52%), but OctN2 expression was markedly high in the epididymis, particularly in the proximal region. All other tissues demonstrated little levels of OctN2 expression. All tissues exhibited
**Figure 4.4 Oat Expression.** Expression level of Oat mRNA mRNA levels in kidney, liver, *caput, cauda*, seminal vesicles (SV), prostate, and vas deferens (VD). mRNA levels are expressed as mean relative light units (RLU)/10 μg total RNA ± S.E.M. (n=3).
Figure 4.5 Oct Expression. Expression level of Oct mRNA levels in kidney, liver, *caput, cauda*, seminal vesicles (SV), prostate, and vas deferens (VD). mRNA levels are expressed as mean relative light units (RLU)/10 μg total RNA ± S.E.M. (n=3).
moderate amounts of OctN3 expression, except for the seminal vesicle which had very high levels of expression.

Oatp1a1 and Oatp1a6 were not found to be well expressed in the MGT with expression levels ranging from 2-6% compared to control (Figure 4.6). However, Oatp1a4 was found with moderate expression in the prostate and vas deferens (31% and 65% respectively). Particularly high expression was detected in the seminal vesicles and epididymis with 139% for seminal vesicles, 146% for the caput and 105% for the cauda. Oatp1a5 was found to be modestly expressed by seminal vesicles, but all other tissues demonstrated low expression.

**Discussion**

An important spermatoprotective function of the blood-testis barrier is to prevent the entry of potential toxicants and thus limit their exposure to the developing germ cells. Once the immature sperm leave the testis, this vital function is maintained in the epididymis where the blood-epididymal barrier separates the adluminal compartment from the rest of the body ((Mital et al., 2011; Dubé and Cyr, 2012)). The epithelia of the seminal vesicles and the blood-prostate barrier also serve to keep potentially toxic compounds out of the secretions to which the sperm will be exposed (Liu et al., 2013).

The purpose of this study was to quantitatively determine the constitutive expression of the major xenobiotic transporters in caput and cauda regions of the epididymis, vas deferens, prostate, or seminal vesicles from adult Sprague-Dawley rats. The results demonstrated a complex expression pattern across these various tissues which helps define role of transport in drug distribution within the MGT.
**Figure 4.6 Oatp Expression:** Expression level of Oatp mRNA levels in kidney, liver, *caput, cauda*, seminal vesicles (SV), prostate, and vas deferens (VD). mRNA levels are expressed as mean relative light units (RLU)/10 μg total RNA ± S.E.M. (n=3).
The epididymis has classically been divided into 3 regions, the *caput*, *corpus*, and the *cauda*, each with distinct transcriptional profiles (Johnston *et al.*, 2007). Due to the small size of the *corpus*, we were not able to harvest sufficient mRNA to investigate the expression profile of this region. Therefore, the mRNA harvested from the epididymis originated from the proximal *caput* or the distal *cauda*. There has been recent interest in the influence of the epididymis in male fertility, especially with the secretions’ impact on sperm development (Sullivan and Saez, 2013). A potential transepithelial pathway using xenobiotic transporters, such as Ent1, Ent2,Oat3, Oatp1a4, OctN2 and OctN3, which were determined to be expressed in the epididymis could represent a mechanism for toxicants to accumulate in epididymal secretions. The toxicant α-chlorohydrin (also known as 3-MCPD) is an organic molecule found in soy products that is known to cause edema of the *caput* and cause epididymal sperm immotility (Creasy, 2001). Since it is a polar compound, it is likely that transporters play a key role in the disposition of the compound into the epididymal cells and thereby the epididymal secretions to which the sperm are exposed. Interestingly, α-chlorohydrin is also known to interfere with transport of other compounds indicative of another link between toxicants and transporters of the MGT (Hinton *et al.*, 1983). Carbendazim, a fungicide, is another classic epididymis toxicant that is reported to cause duct necrosis and have deleterious effects on epididymal sperm (Akbarsha *et al.*, 2001). Since it is known to affect epididymal sperm, carbendazim most likely requires penetration of the MGT in order to have a toxicological effect. Carbedazim may gain entry to the epididymal sperm through transport-mediated processes. A transport-dependent mechanism
seems relevant since carbendazim has been reported to be transported by fungal ABC transporters (Song et al., 2012).

An understanding of constitutive drug transporter expression in the epididymis may also be useful in the treatment of HIV infection. It has been speculated that the MGT acts as a sanctuary site for HIV by shielding the virus from therapy (see chapter 2). Epididymal efflux transporters such as P-gp Mrp1, Mrp5, Mrp6, and Mrp7 may be contributing to the MGT acting as a sanctuary site. One class of drugs used to treat HIV infection, nucleoside reverse transcriptase inhibitors (NRTI) have been shown to accumulate in seminal plasma at higher concentrations than that of the blood (Cruciani et al., 2006; Dumond et al., 2008). One potential mechanism for NRTI accumulation within seminal plasma involves transport via ENT1/2 into seminiferous tubules to concentrations equal to that of the blood, and subsequent concentration within MGT as the epididymis removes 99% of the water in the ducts (Cornwall, 2009; Klein et al, 2013). Knowledge of which transporters are constitutively expressed in the epididymis can help assess the impact of drug transport at the blood-testis barrier (BTB) by determining if drugs that are able to get into the seminiferous tubules will be reabsorbed distal in the epididymis.

The vas deferens is a muscular tube that allows for sperm to travel from the epididymis to the urethra during ejaculation. Since contractility of the vas deferens is important for proper ejaculation, drugs that interfere with muscular contraction (notably, α-adrenergic blockers) may cause side effects that interfere with patient compliance (Koslov and Andersson, 2013). Drug transporters expressed in the vas deferens such as Oct1, Oct3, OctN2, and Oatp1a4 can affect
the distribution of xenobiotics and thus potentiate undesirable side effects for several drugs. Secretions of the vas deferens are suspected to play an important role in male fertility (Koslov and Andersson, 2013). As with the epididymis, xenobiotic transporters of the vas deferens could add potential toxicants to the MGT via transepithelial transport which may adversely affect fertility (Yi et al., 2013).

Here we report several xenobiotic transporters expressed in the prostate that may play a role in several clinical observations and could impact a variety of drug therapies. It has been reported that a blood-prostate barrier exists with properties similar to the blood-testis barrier and may limit the distribution of therapeutic compounds (Fulmer and Turner, 2000; Liu et al., 2013). It is reasonable to expect that transporters such as P-gp, Mrp1, Mrp3, Mrp5, Mrp6, Mrp7, and Mrp9 are important for the blood-prostate barrier through the efflux of xenobiotics compounds. It is possible that uptake transporters such as Ent1, Ent2, and Oatp1a4 represent a route of bypassing the epithelial barrier and achieve therapeutic concentrations in the prostate.

Both uptake and efflux transporters in the prostate may be involved in the treatment or the development of cancer. The most well studied role for transporters in the context of cancer is on the distribution of chemotherapeutics. Many ABC transporters are known to interact with anticancer drugs (Reid et al., 2003). Cancerous cells have also been observed to upregulate many ABC transporters such as P-gp and BCRP which may contribute to resistance (Rothweiler et al., 2010). Knowledge of constitutive expression of transporters may provide a background to which prostate cancer cells can be compared in
future studies. In addition to determining the disposition of chemotherapeutics, xenobiotic transporters may also affect the exposure of carcinogens, chemopreventative agents (such as sulforaphane or catechins), and endocrine disrupting chemicals to the prostate, all of which are suspected to play a role in carcinogenesis (Clarke et al., 2011; Lubelska et al., 2012; Dankers et al., 2013; González-Sarrías et al., 2013; Mulware, 2013).

Despite being in close proximity, and thus being in a similar environment to the prostate, the seminal vesicles rarely develop cancer (Cai et al., 2013; Wang et al., 2013). The tremendously reduced incidence of cancer may be at least partially related to efflux transporters expressed higher by the epithelial cells of the seminal vesicles than the prostate. We found several possible transporters including Mrp2, Mrp5, Mrp7, and Mrp9. There is also a paucity of information concerning the xenobiotic toxicity in seminal vesicles. The main toxicant reported in the literature is flutamide which is an antiandrogen drug used for the treatment of prostate cancer which is a known substrate for MRP1 (Dianne M. Creasy, 2001; Grzywacz et al., 2003). Drug transport in seminal vesicles could also be important for the treatment of HIV infection. Macrophages infected with HIV have been detected in seminal vesicles and may contribute to the MGT acting as a sanctuary site for the virus (Deleage et al, 2011). Increasing the bioavailability of antiretroviral drugs will be essential in an effort to eliminate the virus from a patient. Additionally, an understanding of constitutive expression of transporters in the seminal vesicles is valuable information because the seminal vesicle secretions represent the primary component of ejaculate (Schramm et al., 1988; Simbini et al., 1998). It is conceivable that xenobiotic transporters could
form a transepithelial pathway though epithelial cells of the seminal vesicles which would lead to xenobiotic exposure in the seminal vesicle secretions. This indicates that the seminal vesicles may be a major contributor to the secretion of drugs or toxicants into the ejaculate.

In conclusion, this study presents a comprehensive analysis of the mRNA expression of the 30 major xenobiotic transporters located in tissues along the MGT. The findings from this study can serve as a valuable reference to a variety of interests in medical research including male fertility, toxicology of the MGT, HIV infection and cancer chemotherapy. As such, the constitutive expression of these xenobiotic transporters may provide insight into the molecular mechanisms associated with the transport of drugs into and out of the MGT.
CHAPTER 5: MECHANISM OF NUCLEOSIDE AND NRTI TRANSEPITHELIAL TRANSPORT BY HUMAN AND RAT SERTOLI CELLS IN VITRO.

Text and figures derived from; David M. Klein, Michael J. Kerins, Esau Calderon, Stephen H. Wright, and Nathan J. Cherrington submitted to Drug Metabolism and Disposition for publication.

Introduction

The blood-testis barrier (BTB) is critical to limiting germ cell exposure to potential reproductive toxicants (Bart et al., 2002; Hedger, 2011; Liu et al., 2011; Mruk and Cheng, 2011a; Su, Mruk, and Cheng, 2011; Zhou et al., 2012). The anatomical portion of the blood-testis barrier is composed of tight junctions between specialized epithelial cells called Sertoli cells that line the seminiferous tubules (Su et al., 2010; Mital et al., 2011; Su, Mruk, Lee, et al., 2011)). These sustentacular cells are important for the secretion of many substances including nucleosides (Griffin, 2004; Maeda et al., 2005; Kato et al., 2006; R Kato et al., 2009). Due to the BTB, most hydrophilic compounds are not able to diffuse past Sertoli cells. To overcome this, Sertoli cells express several transporters that allow nutrients to bypass the BTB via transepithelial transport. Transepithelial transport is a two-step process; first substrates are taken into the cells via uptake.

4 I have completed approximately 100% of the data generation presented for this chapter. The entire initial draft was written by me.
mechanisms, and second, substrates exit the cells on the opposite membrane via efflux. One of the standard techniques to study functional transport at the BTB is by culturing a monolayer of primary Sertoli cells in a transwell insert (Kato et al., 2005; Mruk and Cheng, 2011; Klein et al., 2013). Many transporters are also able to interact with therapeutic drugs (Kong et al., 2004; Klein et al., 2013). Information regarding these transepithelial pathways is important for understanding the distribution of drugs into the testis. Currently, there is a paucity of information regarding xenobiotic transepithelial transport pathways at the BTB.

One family of transporters that is relevant to the BTB is the equilibrative nucleoside transporter (ENT) family, SLC29A (Ward, 2000; Pastor-Anglada et al., 2005; Macanas-Pirard et al., 2012). ENT-mediated transport is generally driven entirely by concentration gradients of the substrate due to the lack of net charge of substrates at physiological pH. These transporters are especially clinically relevant because they transport both nucleosides, an essential nutrient for dividing germ cells, and nucleoside analog drugs (NSA) that are used clinically for the treatment of HIV infection and leukemia (Yao et al.; Baldwin et al., 2004; Molina-Arcas et al., 2008; Kulkarni et al., 2010; Fernández-Calotti et al., 2011; Klein et al., 2013). Under normal physiological conditions, ENTs typically act as uptake transporters, allowing the entry of nucleosides from the blood into cells. This is likely to be the function of ENT1 (SLC29A1) in the testis, based on its basolateral localization in Sertoli cells (see chapter 2). However, immunohistochemical (IHC) data has provided evidence of ENT2 (SLC29a2) expression on the apical membrane of Sertoli cells (see chapter 2). Due to the
higher concentrations of substrates in the blood than inside the seminiferous tubules, we speculated that ENT1 and ENT2 could be working in concert to form a transepithelial pathway to allow for the access of nucleosides into the seminiferous compartment (see chapter 2). Since these transporters have been shown to interact with NSA drugs used to treat HIV infection, it was anticipated that this pathway could also be the mechanism for drug accumulation in seminal plasma (Mann and Lutwak-Mann, 1982; van Praag et al., 2001; Cao et al., 2008; Chan et al., 2008; Anderson et al., 2010; Le Tortorec and Dejucq-Rainsford, 2010).

The multidrug resistance-associated proteins (MRP) are also of high interest regarding BTB transport. Members of this family use ATP hydrolysis to remove a wide variety of clinically relevant substrates, including many drugs used to treat leukemia and HIV infection, from cells (Bart et al., 2004; Weiss et al., 2007; Eilers et al., 2008; Klaassen and Aleksunes, 2010; Klein et al., 2014b). Previous work using IHC provided evidence that MRP4 (ABCC4) is expressed in Leydig cells and the apical membrane of rodent Sertoli cells (Morgan et al., 2012; Klein et al., 2014a).

Determining the transepithelial pathways that allow for nucleoside penetration across the BTB would have great impact on understanding the disposition of many important drugs with respect to the testis. Using monolayer cultures of primary rat Sertoli cells, we previously determined that ENT1 is largely responsible for the uptake of endogenous nucleosides like uridine (see chapter 2). The purpose of this study is to extend these findings to NRTI drugs such as zidovudine (AZT) and to determine their efflux mechanisms at the apical
membrane of Sertoli cells. To accomplish this, we used cultured monolayers of both rat and human Sertoli cells and investigated transepithelial transport of uridine (a representative nucleoside) and the NRTI drugs AZT and didanosine (ddI).

**Materials and Methods**

**Materials:** Non-radiolabeled uridine, DMEM/F12 media, MK571, zidovudine (3’-azido-3’-deoxythymidine, AZT), and didanosine (2’,3’-dideoxyinosine, ddI) were purchased from Sigma-Aldrich (St. Louis, MO). 6-S-[(4-Nitrophenyl)methyl]-6-thioinosinezidovudine (NBMPR) was purchased from Santa Cruz Biotechnology Inc (Santa Cruz, CA). Human Sertoli cells were purchased from Applied Biological Materials (ABM; Richmond, BC). Stock solutions of NBMPR were made with DMSO and experimental solutions contained ≤1% DMSO as did control solutions. [3H]uridine (specific activity: 30.1Ci/mMol), [3H]AZT (12.2 Ci/mMol) and [3H]ddI (29.0 Ci/mMol) were purchased from American Radiolabeled Chemicals Inc (St. Louis, MO). BD Matrigel Matrix and transwell inserts used for primary Sertoli cell cultures were purchased from BD Biosciences (San Jose, CA). All other reagents were purchased from a standard scientific supplier at the highest available purity.

**Sertoli Isolation:** Sertoli cell isolation was performed using the protocol of Mruk and Cheng, 2011. Briefly, the tunica was separated from the seminiferous tubules. Then the tubules were cut into 1mm pieces, incubated in a 50/50 mixture of DMEM/F12 media, and resuspended in media with 0.002 % DNase and 0.1 % trypsin to release interstitial cells. After washing and resuspension, the
media was replaced with a DMEM/F12 media with 1M glycine and 2 mM EDTA to lyse interstitial cells. The cells were resuspended in DMEM/F12 media with 0.1 % collagenase and 0.005 % DNase for 30 minutes to remove the myoid layer. After washing, the cells were given fresh DMEM/F12 media with 0.1% hyaluronidase and 0.005 % DNase for 30 minutes to break down the extracellular matrix. Cells were then plated at a density of 0.5 x 10^6 cells/cm² onto transwell inserts (or bottom of a well for apical uptake studies) previously coated with a thin layer of Matrigel (diluted 1:7 with media) as per manufacturer’s instructions (BD Biosciences). The cell media was supplemented with EGF and human transferrin. After 36-48 hours at 37 °C, cells were treated with a 20 mM tris buffer (pH 7.4) for 2.5 minutes to lyse germ cells and then given fresh DMEM/F12 media supplemented with EGF and human transferrin. Cells from either rat or humans were then incubated at 37 °C and cultured for an additional 4 days (6 days total from isolation). The media was changed as needed, typically every 1 to 2 days. Rat tissue was collected from euthanized male Sprague Dawley rats at least 12 weeks old (mature). Protocols for obtaining samples were approved by the University of Arizona Institutional Review Boards (IRB) and Institutional Animal Care and Use Committee (IACUC). Transepithelial electrical resistance (TEER) was measured once a day in 12 wells using chopstick electrodes with a Millicell-ERS voltohmmeter (Millipore Billerica, MA). The measurements were then subtracted from a negative control (a well coated with matrigel, but no cells, and incubated on the same plate with no) and are reported as the average ±SE.
Primary Sertoli Cell Transport Experiments: Once the cells were confluent (day 6), the media was replaced with Waymouth Buffer containing (WB; mM): 135 NaCl, 13 HEPES, 2.5 CaCl₂, 1.2 MgCl₂•6H₂O, .8 MgSO₄•7H₂O, 5 KCl, 28 D-Glucose at pH 7.4. To measure basolateral-to-apical transepithelial flux, the cells were incubated at room temperature for 10 minutes and then the buffer in the basolateral compartment (or the entire media in the case of apical uptake studies) was replaced with WB containing 1 µCi/mL of [³H]substrate (approximately 30 nM for uridine and didanosine, 10nM for AZT) plus additional test agent (NBMPR, unlabeled substrate or MK571) as required. At selected time intervals, WB from the apical compartment was removed and assessed for radioactivity via liquid scintillation spectroscopy.

Statistics: Transport data are presented as means ±SE performed in triplicate wells representative experiments. Rodent studies were repeated at least 3 times while due to their scarcity, human studies were performed once. All tests of significance of observed differences were done by one-way analysis of variance using a Tukey post hoc multiple comparison test with p <0.05 representing significance.

Results

Transepithelial Transport of Nucleosides through Primary Rat Sertoli Cells. Transepithelial transport of uridine (Figure 5.1A), zidovudine (B) and didanosine (C) was assessed in the basolateral to apical direction (B-A) through a monolayer of rat Sertoli cells for 20 minutes. This directionality is representative of a substrate being transported from the blood to inside the seminiferous tubule.
Figure 5.1: Transepithelial Transport of Nucleosides through Primary Rat Sertoli Cells. The transepithelial transport of \[^3H\]uridine (A), \[^3H\]AZY (B), and \[^3H\]ddI (C) were measured using monolayers of cultured rat Sertoli cells from a single representative experiment. Cells were exposed to buffer containing labeled substrate (control), labeled substrate and 1mM concentration of unlabeled substrate (block), or labeled substrate and NBMPR at concentrations to inhibit ENT1 only (100nM) or to inhibit ENT1 and ENT2 (100µM). NBMPR was either applied to the basolateral compartment (noted as (B)) or the apical compartment (noted as (A)). Each point represents the mean (±standard error) of at least 3 wells.
This time point was selected based on time course experiments that demonstrated near-linear transepithelial transport at 20 minutes (data not shown). Uridine is classically used as a representative endogenous nucleoside, and AZT and ddI are, respectively, pyrimidine and purine NRTI drugs used to treat HIV infection (Leung et al., 2001; Yao, et al., 2011). Cells that were not exposed to any inhibitors are labeled as control. Sertoli cells in the block group were exposed to 5mM unlabeled substrate to saturate transport processes. Signal detected in the block group reflected a non-saturable effect, including paracellular flux between the cells and diffusion through the transwell membranes. The difference between the control and the block groups represents the saturable component of the signal and is generally interpreted as transport mediated. For all the substrates investigated, at least 50% of the control signal was saturable and, therefore, indicative of functional transport.

To investigate the role of ENTs in the transepithelial transport of NRTIs, the ENT inhibitor NBMPR was used (Griffiths et al., 1997; Takano et al., 2010; Tomi et al., 2011). This compound is known to selectively inhibit ENT1 at low concentrations ($K_i = 0.1$ to $68.5\text{nM}$) but will also inhibit ENT2 at the high micromolar range ($>10\mu\text{M}$) (Griffiths et al., 1997; Kong et al., 2004; Takano et al., 2010; Tomi et al., 2011). Cells were exposed to one of two concentrations of NBMPR in order to differentiate the role of ENT1 versus ENT2; a low ($100\text{nM}$) concentration of NBMPR to inhibit ENT1 and a high ($100\mu\text{M}$) concentration to inhibit both ENT1 and ENT2. Since a cell layer is impermeable to NBMPR for this time frame (Ward, 2000), ENT activity was selectively determined on each
membrane by adding NBMPR to either the basolateral (labeled as B) or the apical (labeled as A) compartments.

For uridine transport, ENT1 inhibition on the basolateral membrane was sufficient to eliminate the saturable portion of signal; increasing the concentration of NBMPR had no further effect. These data suggest that ENT1 is required for transepithelial transport of uridine (Figure 5.1A) and agree with our own previous experiments (see chapter 2). The dependence on basolateral ENT1 for transepithelial transport was also observed for AZT and ddI (Figure 5.1B and C). The absence of ENT2 activity on the basolateral membrane is consistent with previous reports that ENT2 is only expressed on the apical membrane of Sertoli cells (see chapter 2). Application of low and high doses of NBMPR to the apical compartment demonstrated no effect of ENT1 inhibition for any of the test substrates and varied responses for ENT2 inhibition. ENT2 inhibition on the apical membrane eliminated all transepithelial transport of uridine, but for the NRTI drugs, ENT2 inhibition was not significantly different than control group (Figure 5.1). The influence of apical application of NBMPR indicates that, in rat Sertoli cells, ENT2 efflux is a critical part of uridine transepithelial transport, but not for AZT or ddI.

**MRP4 Inhibition of Nucleoside and NRTI Transepithelial Transport Through Rat Sertoli Cells.** While the basolateral entry of NRTI drugs appears to be mediated by ENT1, the apical efflux mechanisms were still unknown. We suspected that MRP4 could be responsible for the apical efflux of NRTI drugs from rat Sertoli cells based on 3 observations: 1) MRP4 is known to interact with NRTI drugs such as AZT and ddI, 2) it is not thought to interact with uridine, and
Figure 5.2 MRP4 Inhibition on Nucleoside and NRTI Transepithelial Transport Through Rat Sertoli Cells.
The transepithelial transport of $[^3\text{H}]$uridine (A) and $[^3\text{H}]$AZT (B) was measured using monolayers of cultured rat Sertoli cells. Cells were exposed to buffer containing labeled substrate (control), labeled substrate and $1\text{mM}$ concentration of unlabeled substrate (block), labeled substrate and NBMPR at concentrations to inhibit ENT1 only ($100\text{nM}$) or to inhibit ENT1 and ENT2 ($100\mu\text{M}$), or labeled substrate and $1\text{mM}$ MK571 applied to the apical compartment. NBMPR was either applied to the basolateral compartment (noted as (B)) or the apical compartment (noted as (A)). Each point represents the mean ($\pm$standard error) of at least 3 wells.
3) it is expressed on the apical membrane of rat Sertoli cells (Reid et al., 2003; Kruh et al., 2007; Klaassen and Aleksunes, 2010; Toyoda and Ishikawa, 2010). To test the importance of MRP4 on transport of NRTI drugs in rat Sertoli cells, we measured the impact of MRP4 inhibition via MK571 on AZT transepithelial transport. In addition to being a leukotriene LTD4 receptor antagonist, MK571 is known to inhibit many ABC transporters, including MRP1 and MRP4 (Reid et al., 2003). To control for potential confounding factors, we limited MK571 exposure to the apical membrane of Sertoli cells. To our knowledge, MRP4 is the only NRTI transporter sensitive to MK571 inhibition that is also expressed on the apical membrane of rat Sertoli cells (see chapter 3). Therefore, it is reasonable to expect that apical MK571 would selectively inhibit MRP4 in this system.

The profile of uridine transport was qualitatively similar to what was reported in Figure 5.2 A: there was dependence on basolateral ENT1 and apical ENT2 function. Importantly, however, uridine transepithelial transport was not affected by 1mM MK571 in the apical compartment. This was expected since (i) uridine is not known to be a substrate for MRP4, and (ii) selective inhibition of ENTs effectively eliminated all transepithelial uridine flux. The profile of AZT flux differed. Whereas, AZT uptake via the basolateral membrane was sensitive to ENT1 inhibition, and apical ENT2 inhibition had little effect (Figure 5.2B), apical MRP4 inhibition completely eliminated transepithelial AZT flux. This indicates that MRP4, and not ENT2, is responsible for the apical efflux component of AZT transepithelial transport for rat Sertoli cells.
Figure 5.3: Validation of Human Sertoli Cells with TEER and Apical Uptake. Human Sertoli cells assessed for tight junctions via transepithelial electrical resistance (TEER) (A) and transporter expression via apical uptake (B) in a single representative experiment. TEER measurements are reported as the resistance (after subtraction of the blank) divided by the growth area (0.33 cm²). Apical uptake was assessed using human Sertoli cells cultured at the bottom of a well with apical membranes predominantly interacting with the media. Uptake of [³H]uridine was assessed in the presence of 1mM uridine (block), NBMPR concentration sufficient to inhibit ENT1 (100nM), NBMPR concentration sufficient to inhibit ENT1 and ENT2 (100µM) or with only transport buffer (control).
Validation of Human Sertoli Cells with TEER and Apical Uptake. Previous studies indicate that the site of expression for MRP4 is species dependent; apical in rodents and basolateral in humans. Thus the MRP4 mediated apical efflux observed (Figure 5.3) in a rodent model should not be representative of the human condition. To more closely reflect the human condition, functional NRTI transport in human primary Sertoli cells (hSCs) was investigated. There were several concerns that needed to be addressed before relying on hSCs as a suitable model for BTB transport; namely that the cells 1) form tight junctions, 2) maintain polarity, and 3) maintain a nucleoside expression profile similar to what can be expected in human tissue based on previous IHC. To address the first concern, transepithelial electrical resistance (TEER) was measured in hSCs cultured on transwell inserts (Figure 5.3A). TEER is a common way to assess tight junction formation and our data shows that the hSCs demonstrated resistance consistent with that observed in primary rat Sertoli cells, that is, peak Ohms/cm² at around 80 (Mruk and Cheng, 2011a).

The next two concerns were addressed by investigating functional uridine uptake through the apical membrane of hSC. In this system, the cell’s basolateral membrane is facing the bottom on the well, thereby reducing/eliminating exposure to experimental media and effectively restricting uridine transport to the apical membrane. Since ENT transporters are bidirectional, this configuration allowed us to detect the functional presence of ENT1 or ENT2 on only the apical membrane. As shown in Figure 5.3B, the low concentration of NBMPR had no effect on uridine transport, whereas the high concentration eliminated uridine flux. Thus these data support the conclusion that the hSCs had
The transepithelial transport of $[^3H]$uridine (A) and $[^3H]$AZT (B) was measured using monolayers of cultured human Sertoli cells. Cells were exposed to buffer containing labeled substrate (control), labeled substrate and 1mM concentration of unlabeled substrate (block), labeled substrate and NBMPR at concentrations to inhibit ENT1 only (100nM) or to inhibit ENT1 and ENT2 (100µM), or labeled substrate and 1mM MK571 applied to the apical compartment. NBMPR was either applied to the basolateral compartment (noted as (B)) or the apical compartment (noted as (A)). Each point represents the mean (±standard error) of at least 3 wells in a sample experiments.
functional ENT2 on the apical membrane, but not ENT1. Since hSCs are known to express ENT1 (see chapter 2), it seems likely that ENT1 is restricted to the basolateral membrane, which is consistent with the location of ENT1 expression shown in intact human tissue. In summary, the data provided sufficient evidence that the hSCs form tight junctions in vitro, maintain polarity, and have a nucleoside transporter expression profile qualitatively similar to in vivo conditions.

**Transepithelial Transport of Uridine and AZT in Primary Human Sertoli Cells.** The transepithelial transport of uridine and AZT through hSCs was measured using conditions similar to those used in the experiments with rodent Sertoli cells (Figure 5.4). The profile for uridine transepithelial transport was qualitatively similar to what was observed using rat Sertoli cells: there was dependence on basolateral ENT1, apical ENT2, and no effect from MRP4 inhibition (Figure 5.4A).

For transepithelial AZT transport, the hSCs demonstrated sensitivity to the low concentration of NBMPR, similar to the rat counterparts. Importantly, and in contrast to the results obtained with rat Sertoli cells, the transport of AZT through the hSCs monolayer was not affected by apical application of MK571, but instead, was significantly reduced by the high concentration of NBMPR in the apical compartment (Figure 5.4B). This indicates that, unlike rats, MRP4 is not responsible for the apical efflux portion of AZT transport in primary human Sertoli cells. Instead, the data suggest that ENT2 plays a larger role in AZT efflux in humans. This is consistent with previous reports that MRP4 is not expressed in
the apical membrane in humans, but is in rodents. Interestingly, basolateral exposure to the inhibitor MK571 may have caused a slight stimulation of transepithelial transport, which is consistent with a basolateral expression of MRP4, although the increase was not significantly different from control.

Discussion

The BTB is well known to limit the disposition within the male urogenital tract of most hydrophilic drugs in use (Bart et al., 2002; Maeda et al., 2007). While this is generally beneficial for the protection of developing germ cells, the BTB can create a sanctuary site for viruses and malignant cancer cells by shielding them from therapeutics (Shehu-Xhilaga et al., 2005; Kulkarni et al., 2010; Else et al., 2011; Palmer et al., 2011; Locatelli et al., 2012). There are numerous reports supporting the notion that HIV can replicate within the male genital tract (MGT) despite being undetectable in the blood (Chan et al., 2008; Anderson et al., 2010; Dahl et al., 2010). It is also known that male patients suffering from acute lymphoblastic leukemia (ALL) are at risk of developing testicular relapse, which refers to relapse after chemotherapy due to leukemia cells surviving in the testis (Arya et al., 2010). Improving drug disposition of antivirals and chemotherapeutics would be critical in treating these life threatening diseases.

Many drug treatment regimens in both antiviral and chemotherapy treatments include nucleoside based drugs (referred to as nucleoside analogs, NSA) or, specifically for HIV treatment, nucleoside reverse transcriptase inhibitors (NRTI). There are several reports that note many NRTI drugs are able
Figure 5.5 Models for Nucleoside and NRTI Transport at the BTB in Rats and Humans. An illustration of a seminiferous tubule and surrounding cells modified from figure 2.10 depicting how nucleosides could be transported into the lumen via ENT/MRP transporters in humans (A) and rats (B). ENT1 is the primary transporter responsible for uptake of nucleosides and NRTI drugs into the Sertoli cells due to the higher concentration of nucleosides in the blood. ENT2 is the predominant apical efflux transporter for nucleosides. In humans, ENT2 plays a role in efflux of NRTI drugs into the lumen of the seminiferous tubules due to the higher concentration of intracellular nucleosides compared to the lumen of the seminiferous tubule. In rodents, MRP4 plays a bigger role in the apical efflux of NRTI drugs than ENT2.
to accumulate in seminal plasma at concentrations higher than that of blood, which implies the necessity of a transepithelial drug transport mechanism (Taylor and Pereira, 2001; Chan et al., 2008; Dumond et al., 2008). This is especially important since the disposition of drugs into the MGT is generally very poor and few transepithelial pathways for the BTB are known. Understanding the transepithelial transport pathways at the BTB, especially those for nucleosides, provides a crucial step in predicting the disposition of drugs into the testis. This is the first study to report transepithelial transport of NRTI drugs through monolayers of Sertoli cells of both human and rat origin. These studies also identified the predominant transporters responsible for transepithelial pathway through the use of classic pharmacological inhibitors.

The data support the conclusion that ENT1 mediates the basolateral uptake of nucleosides and NRTI drugs in both rat and human Sertoli Cells (Figure 5.1, 5.2, and 5.4). In vivo, the concentration of substrates in the blood is likely to be the driving force for this uptake into Sertoli cells. The transporter responsible for efflux into the apical compartment was revealed to vary based upon substrate and species. In both human and rat Sertoli cells, ENT2 mediates the apical efflux of intracellular nucleosides, thereby allowing these important nutrients to be accessible to developing germ cells. This also seems to be the dominant mechanism by which NRTI drugs are able to gain entry into the MGT for humans (Figure 5.4A). However, rat Sertoli cells were not shown to depend on ENT2 for transepithelial transport of AZT. Instead, MRP4 appeared to be the dominant means for apical efflux (Figure 5.2). This provides not only the functional data to validate previous IHC findings, but also serves as a cautionary
note to the interpretation of results obtained using rodent Sertoli cells for characterizing the BTB and illustrates the value of hSCs when investigating transport of potential MRP4 substrates across the BTB for clinical use.

An interesting aspect of a bidirectional transepithelial pathway is the situation in which substrate concentration inside the MGT reaches a higher level than that of the blood. In this ENT-based pathway, ENT2 and ENT1 should to remove substrate from the tubules back into the blood and suggests that the concentration of NRTI drugs should never rise in the MGT above that of the blood. However, clinical data has indicated that several NRTI drugs can reach up to a 10 fold higher concentration in seminal plasma when compared to blood plasma (Pereira et al., 1999, 2002; Dumond et al., 2008). A possible explanation is that NRTI drugs are transported into the seminiferous tubules and reach concentrations equal to that in the blood, but are then concentrated further down the MGT. As fluid from the seminiferous tubule flows through the rete testis and the epididymis, up to 99% of the water is reabsorbed back into the blood (Lu, 2008; Cornwall, 2009). Assuming drug is not also reabsorbed, this would be expected to increase the concentration of drug approximately 100 fold. During ejaculation, drug from the epididymis would mix with secretions from accessory sex organs (primarily the seminal vesicles and prostate) resulting in dilution of the drug (van Praag et al., 2001; Cao et al., 2008; Caballero et al., 2012). Since epididymal fluid has been estimated to contribute 2-10% of the total volume for semen, this would dilute the drug to roughly 2-10 fold above blood concentration, which is close to the clinical observations of plasma:semen concentrations (van Praag et al., 2001). This explanation provides a possible mechanism for drugs
crossing the BTB through an equilibrative pathway and then becoming concentrated in the MGT due to water reabsorption resulting in a higher concentration of drug in the seminal plasma than in blood plasma.

Herein we report the novel use of commercially available hSCs for functional transepithelial transport. Since primary cells of any origin are known to commonly change levels of protein expression once isolated from the body and placed in culture, our conclusions focused on the qualitative aspects of transport (i.e. location of transporter expression and whether or not inhibition of specific pathways affected transepithelial transport) rather than the quantitative, since the absolute rates of transport supported by these processes may change substantially from those occurring in vivo, for both humans and rat. While there is always potential complications associated with using primary cells, the data suggest that hSCs are a useful model for assessing transepithelial transport at the BTB, particularly when focusing on nucleoside transport. The hSCs are able to maintain polarity, reform tight junctions in vitro, and preserve qualitative transport profiles similar to what would be expected in vivo for all the transporters investigated. Nevertheless, more studies are required to determine how suitable hSCs may be for studying other aspects of Sertoli cell function.

Based on these data, we have provided models for the dominant transport pathways of nucleosides and NRTI drugs at the BTB in humans and rats (Figure 5.5). The primary known species difference is the opposite location of MRP4 (apical in rats; basolateral in humans). The distinct site of expression suggests that strong MRP4 substrates may appear to penetrate well in a rodent model, but may actually have greater difficulty accessing the MGT in humans. It’s worth
noting that inhibiting MRP4 on the basolateral membrane of hSCs caused a slight increase in transepithelial transport (Figure 5.4). This is suspected to be reflective of MRP4 effluxing AZT out of the Sertoli cells and back into the basolateral compartment. When MRP4 is inhibited on the basolateral membrane of hSC, the lack of backwards efflux would cause intracellular concentrations to rise and therefore stimulate transepithelial transport. The relatively modest effect of MRP4 observed in hSCs could be due to other factors such as a lower human MRP4 affinity for AZT than rat mrp4, or human cells having lower expression of MRP4 compared to the rat. Whether a lower expression/affinity is due to the isolation and culturing procedure or reflective of native hSCs remains to be determined.

These data not only provide strong evidence that NRTI drugs such as AZT and ddI undergo transepithelial transport through Sertoli cells, but also demonstrate the mechanism by which this is accomplished in both humans and rats (Figure 5.5). Knowing these pathways provides a first and crucial step in understanding the dynamics of drug disposition to this important tissue. The work using hSCs suggests that NSA penetration of the BTB is dependent upon higher affinity for ENT transporters and lower affinity for MRP4. We also present a potential weakness of using rodent models to investigate transport of MRP4 substrates. Taken together, these data demonstrate a transepithelial pathway that is used by both endogenous nucleosides and clinically relevant drugs to bypass the BTB.
CHAPTER 6: SUMMARY AND FUTURE DIRECTIONS

Summary

Taken together, these studies provide foundational knowledge for the transport profile at the blood-testis barrier (BTB), especially regarding nucleoside transport. The primary objective of these studies was to determine the transport mechanism by which nucleoside reverse transcriptase (NRTI) drugs were able to accumulate within seminal plasma and overall, these studies were successful in determining a large part of how these compounds appear in seminal plasma. These results have repeatedly shown that ENT1 is responsible for the uptake of nucleosides and NRTI into the Sertoli cell while ENT2 is responsible for the efflux of nucleosides out through the apical membrane. These two transporters appear to work in concert to provide a gradient driven transepithelial pathway that can allow for the penetration of nucleosides and nucleoside based drugs within the seminiferous tubule. This mechanism may help develop a predictive model for drug permeability through the BTB based on a novel compound’s interaction with ENT1 and ENT2.

Additionally, my studies extended the characterization of BTB transport profile to many members of the ABC transport family, primarily focused on the sites of expression of MRP either through the localization of proteins (Chapter 3, Appendix A) or profile of mRNA within various tissues (Chapter 4). The localization data for MRP4 was extended to functional analysis (Chapter 5) because of the greater implications it held for the differences in handling NRTI and NSA drugs between human and rat. These studies provided functional
evidence using primary Sertoli cells and the pharmacological inhibitor MK571 that supported what was observed in formalin fixed paraffin-embedded fixed tissue, i.e. a species dependent subcellular location. Specifically, MRP4 was located in the basolateral membrane of human Sertoli cells, versus the apical membrane in rodents. My data provides a cautionary flag with respect to relying solely on rodent models for understanding BTB transport dynamics, a practice that is fairly common in the field right now. The studies in chapter 5 also represent the novel use of human Sertoli cells for functional transport data. According to all the characteristics we investigated, hSCs seem suitable for studying nucleoside transport. It is highly unlikely that primary human Sertoli cells will recapitulate the BTB transport dynamics completely and so more thorough analysis is needed to determine how well they represent other aspects of the human BTB in vivo.

The last novel idea put forth by these studies is the concept that the epididymis can cause drugs penetrating the BTB to concentrate about 100 fold due to water reabsorption. As far as I can tell, this has not been suggested in the realm of reproductive toxicology/pharmacology. When this concept was discussed with leading experts in the field, they seem to agree that the epididymal concentration of compounds accessed to the MGT through the testis is a reasonable mechanism and could possibly explain some epididymal specific toxicants (by being concentrated to above toxic threshold). This concept was first thought of in response to how nucleoside reverse transcriptase (NRTI) drugs were able to concentrate within seminal plasma while accessing the MGT through an ENT-dependent transport pathway. An equilibrative pathway would only
allow for compounds within the MGT to reach concentrations equal to that of blood before working “backwards” (i.e. out of the seminiferous tubule and back to the blood). The hypothesis was then put forward that NRTI drugs could reach blood concentration within the testis, but when moved downstream towards the epididymis, water reabsorption would cause drug concentrations to increase 100 fold due to 99% of the water being removed from the MGT (figure 6.1). During ejaculation, the high concentration of drug would mix with seminal vesicle and prostate secretions that would dilute the drug as it mixes to form seminal plasma. The epididymis is thought to contribute 2-5% of the volume of seminal plasma, which would represent a final drug concentration of around 2-5 fold above blood which is what clinical studies observe (Besançon et al., 1985; Simbini et al., 1998; Liuzzi et al., 2004; Cao et al., 2008; Dumond et al., 2008). This organ is especially relevant to HIV infection because immune cells infected with simian immunodeficiency virus (SIV) are most commonly found in the epithelium of the epididymis (Le Tortorec and Dejucq-Rainsford, 2010), which indicates the epididymis may be a prominent site of HIV infection in the MGT. While this mechanism appears reasonable, it would be interesting to see studies that able to establish that the epididymal water reabsorption is indeed primarily responsible for drug concentration in seminal plasma.

These concepts were generally investigated through the lens of understand the pharmacology of NRTI drugs, and they have many potential implications, one of which is HIV drug treatment. Improving the disposition of drugs to the MGT would undoubtedly be critical for the eradication of HIV from the patient’s body. Being able to better predict novel nucleoside drug penetration of the BTB through
Figure 6.1: A model demonstrating how NRTIs may bypass the BTB via ENT1 and ENT2 (left) and then become concentrated in the epididymis due to reabsorption of water (right)
interaction of ENT transporters would be very valuable. Additionally, these findings also have implications to cancer therapy, especially acute lymphoblastic leukemia (ALL). This childhood cancer is well known to occasionally penetrate the BTB where it is shielded from chemotherapy and upon cessation of treatment, cause a relapse of leukemia. This event is referred to as testicular relapse. Oncologists will typically sample the testis if a patient suffers from a relapse and if tumor is detected, the young patients (mean age 5) will undergo orchiectomy or testicular irradiation, both of which are functionally the same for the patient and will result in lifelong treatment and side effects (Locatelli et al., 2012). Finally, these studies also impact our understanding of the physiology of the testis. Many of the carefully orchestrated process of the testis are not well understood, including the mechanism for how endogenous compounds gain access to the developing germ cells. Currently, the causes of about 30% of cases of male infertility are of unknown (Gudeloglu et al., 2014). Knowing the location of transporter gives great insight into how they function endogenously (Chapter 3 and 4) and extends the field’s understanding of the processes involved for male fertility. Despite all the work that has been done, future studies would be instrumental in solidifying the field’s knowledge of the physiological and pharmacological properties of the testis and MGT.

These studies have some very interesting potential applications in toxicology and pharmacology. Not only do these studies further the field’s understanding on drug disposition (which has been thoroughly discussed in this work) but also provide some potential mechanisms for reproductive toxicology. For example, the lack of apical nucleoside transporters implies that NSA drugs,
which are known to be spermatotoxic, would not be able to access the epithelial
cells from within the duct. This would mean that once these drugs become
concentrated, they may still not be very toxic to the epididymal epithelium due to
not being exposed to the DNA polymerase within Principal cell. Several toxicants
are also known to be toxic to the epididymis specifically despite not having a
known mechanism of action (Dianne M. Creasy, 2001). It could very well be that
these compounds access the MGT within the seminiferous tubules at
concentrations below toxicity. As the toxicants move down the tubule, water
reabsorption causes the toxicants to become concentrated to toxic threshold
causes damage to the epithelium. This could be a mechanism of toxicity for
compounds using the ENT-dominant pathway or through a separate pathway.
The toxicity could be especially damaging if the compound damages the DNA
within the sperm without killing the cell. This deleterious effect could be passed
on to offspring if the sperm participates in conception causing a
transgenerational effect (a parent if exposed to a toxicant that has an effect on the
offspring).

The epididymal concentration has several potential applications within
pharmacology. There has been an interest in developing a male contraceptive but
there are issues in creating a drug that is spermatotoxic without damaging the
spermatogonia (germ cell precursors). A possible solution would be to administer
the drug at a concentration lower than spermatotoxic threshold within the testis,
but as the drug concentrates within the epididymis it could reach toxic
concentration specifically within the epididymis. This would require a compound
to be able to access a transepithelial pathway within the testis, and not be reabsorbed by the epididymis.

Finally, knowing the ENT pathway gives insight into the physiology of the testis. The endogenous function of the nucleoside pathway is likely to provide developing germ cells with nucleosides needed for DNA synthesis. An interesting feature of the equilibrative nature of ENTs is they are relatively insensitive to overexpression since intracellular concentrations cannot rise above blood levels. However, reduction in expression could be harmful for spermatogenesis since the ENTs could become saturated with substrates and thus fail to provide the numerous germ cells with sufficient nucleosides. Additionally, compounds that compete with nucleosides could competitively inhibit ENT transporters and become spermatotoxic in that way. Indeed, it is possible that this is the mechanism of NRTI spermatotoxicity.

Future Studies

The following is a grant that I wrote which I feel best indicates the future direction of my work to apply the knowledge of BTB transporters to the clinical field. The testis and epididymis mark the beginning of the male genital tract (MGT) and are responsible for the generation and storage of sperm. Due to anatomical features such as tight junctions and polarized epithelial cells, these reproductive tissues are impermeable to most hydrophilic compounds. These barriers can act as obstacles for therapies requiring entry into the MGT. For example, the blood-testis barrier (BTB) contributes to testicular relapse in acute lymphoblastic leukemia (ALL), which is why many of these young patients are
subjected to severe testicular irradiation or orchiectomy, leading to infertility and significant hormone disruption. However, these epithelial cells express xenobiotic transporters that allow certain agents to reach therapeutic concentrations within the MGT. Developing new therapies that access these privileged sites through carrier-mediated transport systems is identified as the top priority by the NIH Therapeutic Targeting, Blood-Brain Barrier, Gene Therapy and Vascular Biology review group. This particular strategy is the focus of the current application. Rather than studying why drugs fail to cross the BTB, the major focus of our studies is to establish the endogenous transport processes that could allow drugs to access the MGT. Understanding these pathways will be foundational for the development of therapies, such as antiviral, chemotherapy, contraception, and fertility agents, that require access to the MGT in order to achieve full therapeutic effect.

Previous data showed that BTB penetration of nucleosides occurs via transepithelial transport through Sertoli cells, thus bypassing the tight junctions. The process relies on basolateral nucleoside uptake via ENT1 (equilibrative nucleoside transporter 1) and apical efflux via ENT2. Since these transporters are “equilibrative” in nature and interact with nucleoside-based drugs like nucleoside reverse transcriptase inhibitors (NRTI) and nucleoside analogs (NSA), they allow these drugs to bypass the BTB and reach seminiferous concentrations equal to that of the blood. Being able to predict a drug’s ability to access the MGT could greatly improve drug delivery outcomes, especially as it concerns chemotherapy and antiviral treatment. Given the combined expertise of our research team and
our collaborators, we expect to create a computational model that can predict a
drug’s ability to circumvent the BTB. Importantly, this computational model and
any drugs identified as able to penetrate the BTB will be publically available and
directly used by our collaborators to investigate the potential to reposition their
current drugs for additional use in treatment of leukemia or any of the other
indications mentioned. We also suggest that once inside the MGT, these drugs
are then concentrated downstream in the epididymis through water reabsorption
inherent in sperm maturation. The concept that the testis and epididymis work in
concert to transport and concentrate xenobiotics within the MGT provides novel
insight into how these two organs synergistically influence xenobiotic disposition.
Therefore, we hypothesize that the penetration of MGT relevant drugs into the
BTB occurs through transport processes that can be modeled computationally,
and that therapeutic concentration of these drugs is greatly affected by
epididymal transporters and water reabsorption. The following aims have been
developed to test this hypothesis:

**Aim 1: Establish the molecular mechanisms by which MGT relevant
drugs can cross the BTB for clinical use.** We will employ a high-
throughput transepithelial transport assay with human Sertoli cell
monolayers to screen a library of drugs used as antiviral or chemotherapy
treatments. Using these data, we will apply computational methods to
produce models that predict the ability of a compound to penetrate the BTB.

**Aim 2: Determine the impact of epididymal water reabsorption on
drug concentrations within the MGT in vivo.** Water reabsorption
within the epididymis will be inhibited genetically using NHE3\(^{-/-}\) mice, and pharmacologically using amiloride. Steady-state tissue drug concentrations of NRTI and NSA drugs (NRTI: abacavir, indinavir, zalcitabine, zidovudine, didanosine, tenofovir and lamivudine; NSA: cytarabine, clofarabine, and nelarabine) will be determined in C57BL6 and NHE3\(^{-/-}\) mice in testis, epididymis, seminal vesicles, and prostate tissues. Additionally, steady state drug concentrations will also be determined in Mdr\(^{-/-}\), Mrp1\(^{-/-}\), Mrp4 \(^{-/-}\), Ent1\(^{-/-}\) or Ent2\(^{-/-}\) mice to establish the role of these Sertoli cell transporter processes.

Aim 3: Determine the role of nucleoside transport in the epididymis and the impact on nucleoside-based drug disposition. Although our hypothesis states that equilibrative transport in Sertoli cells and subsequent concentration in the epididymis is responsible for MGT drug concentrations, epididymis transport could unexpectedly contribute. This possibility will be determined using immunohistochemistry to localize the ENT1, ENT2, MRP4, MRP1, MDR, and CNT2 transporters and transport assays using epididymal cells from Mdr\(^{-/-}\), Mrp1\(^{-/-}\), Mrp4 \(^{-/-}\), Ent1\(^{-/-}\) and Ent2\(^{-/-}\) mice.

To investigate this hypothesis, we would identify the transport processes that influence NRTI distribution on three different levels: (i) computational analysis based on isolated primary Sertoli cells, (ii) selected tissues from whole animals, and (iii) isolated primary epididymal cells. These studies will use tissues from transporter knockout animals, and cells from wild-type animals from which selected transporters are knocked-down using lentiviral technology.
Specific Aim 1: Establish the molecular mechanisms by which MGT relevant drugs can cross the BTB for clinical use.

Rationale: There is a great need to be able to treat certain diseases within the MGT including leukemia, HIV, Ebola and novel methods of male contraception. In building on our previous work that determined the mechanisms by which some drugs are able to bypass the BTB and accumulate within the MGT, this aim will further the treatment of testis-related diseases (including ALL) in two ways: 1) developing a model that will assist predicting the ability of drugs to bypass the BTB; and 2) providing information on which drugs used to treat ALL and HIV infection would be able to penetrate the BTB. The goals for this aim would allow for the information gained concerning transporters in the MGT to be accessible and applicable to the clinic. In order to thoroughly characterize this system, we would need to assess transepithelial transport for several compounds. To accomplish this, we have compiled a library of accessible drugs used to treat viral infections and ALL that have already been assessed for safety in human use. This library will be compiled based on the National Cancer Institute (www.cancer.gov) and the NIH (aidsinfo.nih.gov) list of drugs approved to treat ALL and HIV. These two lists provide 43 and 38 drugs approved to treat ALL and HIV respectively. Based on this list, we will eliminate redundant drugs and enzyme or antibody based therapies as they will not be testable in our system. Not only will these drugs allow us to better understand the transport processes of the BTB, but will also add value to the medication by characterizing their ability to penetrate the BTB. Since many of these drugs are nucleoside analogs, it’s reasonable to expect some
proportion of them may be effective at treating leukemia in addition to antiviral activity. The drugs that are shown to enter the seminiferous tubules can be investigated for the purpose of being retasked for treating leukemia thereby reducing the risk of testicular relapse. This will greatly reduce the need for orchiectomy or testicular irradiation for young ALL patients.

**Experimental Approach:** Traditionally, rat models represent a gold standard for studying male reproductive tissues. However, we previously determined that the localization of multidrug resistance-associated protein 4 (MRP4) is the complete opposite between rodents and primates which can complicate studies regarding xenobiotic transport and make rodent data irrelevant for this issue (see chapter 3). Immortalized lines of cells of Sertoli cell origin do exist, however none of these are human in origin, and none express the full panoply of transporters observed in either native Sertoli cells or in primary cultures of Sertoli cells(Kato et al., 2005a). Although primary cells carry risks of changing expression profiles once placed into culture, they nevertheless provide a first-order approximation of the transport profile of the BTB(Mruk and Cheng, 2011a; Su, Mruk, Lee, et al., 2011; Klein et al., 2013). For these reasons, we are using primary human Sertoli cells for our experiments. Human Sertoli cells will be purchased from ABM. In our initial studies with human cells, we have placed commercially ordered human cells on permeable supports using the standard ‘mouse protocol’. These cells have been shown in our hands to develop transepithelial electrical resistance (TEER) over time in culture (Figure 5.3), confirming the formation of a functional epithelial barrier by these cells. We
have also shown that these cells maintain ENT2 function (inhibition by ‘High NBMPR’) on the apical membrane, with no evidence of apical ENT1 function (no inhibition by ‘Low NBMPR’), both of which are consistent with maintenance of polarized Sertoli cell function (Figure 5.3). We also have demonstrated that B-A transport of uridine and the NRTI drugs AZT and ddI by human Sertoli cells are sensitive to low concentrations (100nM) NBMPR on the basolateral membrane, suggesting basolateral ENT1 localization consistent with the rat model (see chapter 3). Taken together, the preliminary data suggest that these cultured human Sertoli cells display the same polarized distribution of ENT transport as that evident in Sertoli cells isolated from rats and in native human Sertoli cells.

**Aim 1.1-Determine the Transport of Nucleoside-Based Drugs Across a Monolayer of Human Sertoli Cells.** The cells will be cultured on a transwell insert coated with matrigel to aid in adherence and align for polarization. Culturing the cells with this system allows for transepithelial transport in the basolateral to apical (B-A) direction to be assessed using previously published methods (see chapter 2). We would first start with a time course experiment that would measure the transport of a drug over time (1 hour) in both the B-A and A-B direction. The proposed time course experiments will (i) show if Sertoli cells are able to transport NSA or NRTI drugs; (ii) show if the epithelium supports net drug secretion (basolateral-to-apical; B-A) or absorption (apical-to-basolateral; A-B); and (iii) establish the time point to be used in subsequent studies assessing the transport mechanism involved in NRTI transport in Sertoli cells. One of the drugs of interest will be added to the basolateral or apical compartment of
transwell inserts plated to confluence with Sertoli cells. At periodic intervals, a sample of the opposite compartment will be collected (the volume replaced) and assessed using mass spectrometry. The procedure will be repeated with separate inserts and media in cold (4 degrees Celsius) to slow down transport function. At the end of each experiment, the permeable transwell support will be rinsed with ice cold buffer, cut from the insert and the steady-state level of accumulated labeled substrate determined.

**Aim 1.2- Develop a Model (pharmacophores and Bayesian models) for Penetration of Nucleoside Drugs into the Blood-Testis Barrier.**

**Approach:** Using the data generated in Aim 1.1 we will construct Bayesian machine learning models for the interaction of ligands with the ENT-dominated transepithelial transport processes in human Sertoli cells. We will attempt to combine data into multi-event Bayesian and other machine learning models (e.g., support vector machine and random forest) as a way to predict compounds of most interest for potential clinical interaction(s) based on the inhibitor data generated with multiple substrates. We will also develop and validate models using open modeling and descriptor technologies that will enable provision of models online or as a mobile app thereby facilitating sharing of information created in this project.

**Experimental strategy: Pharmacophore and Bayesian modeling:** We will use open source (i.e., the Chemistry Development Toolkit (CDK)) and open algorithms (R, LIBSVM, Weka, etc.) and commercially available tools we have
used previously (e.g., Discovery Studio 4.1, Biovia, San Diego, CA) and CDD Models and CDD Vault, (Collaborative Drug Discovery, Inc, Burlingame CA) (16, 38) to produce predictive transporter models. These models will enable us to input a molecule's structure and predict interaction with each transporter substrate (33). The output of the predictions by both pharmacophore and Bayesian models will create a profile of predicted interactions.

**Specific aim 2: Determine the impact of epididymal water reabsorption on drug concentration within the MGT in vivo.**

**Rationale:** Based on our preliminary data of transepithelial transport of radiolabeled NRTI drugs through primary Sertoli cells (Figure 5.1, 5.4), we are confident that these drugs do penetrate the BTB and that the testis is at least a contributor to the appearance of high concentrations of NRTIs in seminal plasma. The data in hand (chapter 2) suggest that ENTs contribute significantly to transepithelial NRTI flux across Sertoli cells, and this raises an important issue: ENTs provide an ‘equilibrating’ rather than a ‘concentrating’ pathway. With this in mind, we propose that the high concentration of NRTIs in human seminal plasma (Taylor et al., 2000; Pereira et al., 2002; Liuzzi et al., 2004; Chan et al., 2008) reflects a two-step process. The first step is the ‘entry’ of NRTIs into the MGT, which we propose occurs in the testis. The second step is the concentration of NRTI within the MGT, which we suggest occurs in the epididymis as a secondary response to the reabsorption of water by the epididymis. Here we propose two sets of experiments to test these ideas. First, we will determine the distribution of NSA and NRTI within the several tissues of
the MGT and the influence of selected transporters on this distribution profile. Second, we will directly determine the impact of epididymal water reabsorption on NRTI concentration within the MGT. The first set of experiments will determine the \textit{in vivo} distribution of NSA and NRTI drugs while the second set will determine if epididymal water reabsorption is the mechanism responsible for NRTI concentration. Additionally, since SIV has been shown to penetrate all MGT tissues and HIV is speculated to also be present in the seminal vesicles, there is great value in understanding NRTI penetration of other MGT tissues as well (Deleage \textit{et al.}, 2011; The effect of efavirenz versus nevirapine-containing regimens on immunologic, virologic and clinical outcomes in a prospective observational study., 2012).

\textbf{Experimental Approach:} Epididymal water reabsorption is dependent upon ion reabsorption (particularly \textit{Na}$^+$ reabsorption) and is largely inhibited by amiloride (Wong and Yeung, 1976; Zuo \textit{et al.}, 2010). One of the primary transporters responsible for \textit{Na}$^+$ reabsorption in the epididymis is sodium/hydrogen exchanger-3 (NHE3) which is responsible for \textasciitilde75\% of the water reabsorption (Bagnis \textit{et al.}, 2001; Zhou \textit{et al.}, 2001). To test the effect epididymal water reabsorption has on drug concentration, we will inhibit NHE3 activity and then measure drug concentration in the epididymis. Since the lumen of the ducts in the \textit{cauda} is relatively large (diameter of \textasciitilde270 microns in Korean field mice) (Kang \textit{et al.}, 1997) detection of differences in drug concentration in experimental vs. control tissues should be readily evident from samples of whole \textit{cauda} tissue. We have developed two approaches for inhibiting NHE3, both of
which have been shown to decrease epididymal water reabsorption by more than 70%: 1) Pharmacological inhibition using the drug amiloride; and 2) genetic knockout mice that lack functional NHE3 (locally available from Dr. Fayez Ghishan; see letter). These knockout mice have been shown to have severely dilated epididymal ducts (due to decrease of water reabsorption) and problems with fertility (Zhou et al., 2001).

We suggest that two issues underscore the relevance of including both the pharmacological inhibition described and studies using Sertoli cells isolated from knock-out animals. First, there is evidence that genetic elimination of transport proteins can be accompanied by ‘compensatory’ changes in the amount and/or expression profile of other transport proteins. For this reason, we will compare mRNA levels of the remaining candidate transporters in fresh and cultured Sertoli cells to those in cells from wild-type mice (using our standard methods) (Augustine et al., 2005; Klein et al., 2014b). For example, comparison of data obtained using Ent1 in wild type mice to that obtained using Ent2 ‘knockout’ will provide a means for assessing the physiological impact of a target transporter on NRTI transport. Second pharmacological inhibition always carries the risk of having off target effects; for example inhibiting transporters in other tissues that can have unforeseen consequences that may complicate the interpretation of our results. Using both approaches will increase our confidence that the anticipated outcome is due to inhibition of NHE3 and not because of potential confounding variables inherent in both pharmacologic (non-specific inhibition) and genetic (compensation of other transporters) approaches alone.
Aim 2.1 - Determine the steady state accumulation of NRTIs within the tissues of the MGT. C57BL6 or knock-out mice (Mrp1⁻/⁻, Mdr1⁻/⁻, Mrp4⁻/⁻, Mrp5⁻/⁻, Ent1⁻/⁻, and Ent2⁻/⁻) will be dosed through osmotic infusion pump containing one of four NRTIs (zidovudine 15/mg/kg/day, didanosine 10mg/kg/day, tenofovir 20mg/kg/day, or lamivudine 15mg/kg/day) plus inulin 10mg/kg/day, the latter acting as an extracellular space marker. On days 1, 2, and 4, mice will be euthanized and organs from the MGT (testis, caput and cauda of the epididymis, seminal vesicles and prostate) will be extracted, weighed and analyzed for drug/inulin content via mass spectrometry (LC/MS/MS) using previously published methods (Dumond et al., 2008). Blood plasma will also be collected and analyzed for drug concentration. Data will be expressed in terms of ‘tissue-to-plasma’ ratios for drug content (corrected for extracellular space, with the assumption that drug concentration in ECS is equal to that in the plasma). These experiments will establish a time frame for steady state accumulation in the MGT for each drug and will (i) quantitatively determine the profile of drug distribution within the MGT; and (ii) establish the influence of the candidate transporters on this distribution.

Aim 2.2 – Test the influence of epididymal water reabsorption on the steady-state distribution of NRTIs within the MGT. Na/H⁺ exchange is a major contributor to epididymal water reabsorption (Wong and Yeung, 1976; Bagnis et al., 2001; Zuo et al., 2010) Therefore, we will determine the effect of reducing/eliminating NHE activity on the steady-state accumulation of NRTIs. One set of studies will determine the influence on NRTI accumulation of the
global inhibition of NHE activity (using the NHE-specific inhibitor, 5-(N-ethyl-n-isopropyl)-amiloride (EIPA), 10mg/kg/day). A second set of studies will use NHE3\(^{-/-}\) mice, which have reduced fertility and epididymal water reabsorption (Zhou et al., 2001). C57BL6 and NHE3\(^{-/-}\) mice will be dosed through osmotic infusion pumps containing one of four NRTIs (zidovudine, didanosine, tenofovir and lamivudine) and inulin which will act as a paracellular marker at doses described above until steady state which will be determined by studies in aim 3.1. After steady-state has been established mice will be euthanized and tissue from the testis and cauda will be extracted and analyzed for drug concentration via mass spectrometry (LC/MS/MS) using previously published methods (Dumond et al., 2008).

**Specific Aim 3: Determine the processes that support transepithelial NRTI transport in mouse epididymal cells in vitro.**

**Rationale:** The fluid inside the seminiferous tubules pools, containing immature spermatozoa, within an anastomosing network of tubules known as the rete testis (Hess, 1999) which then travels via the efferent ducts to the epididymis, a crescent-shaped organ around the outside of the testis. The epididymis has 3 primary roles in male reproduction: 1) storage of sperm prior to ejaculation 2) support of sperm maturation 3) reabsorption of water (Leung et al., 2001; Cornwall, 2009). The latter role is particularly significant in the context of toxicity. Between the rete testis and epididymis, up to 98% of the water is reabsorbed from the MGT. This allows the epididymis to maintain a high concentration of proteins which are secreted to aid in sperm development (Besançon et al., 1985; Cornwall, 2009). We suggest that this
process also concentrates compounds secreted ‘upstream’ of the epididymis in the testis; the testing of this hypothesis is the central focus of Aim 2. In Aim 3, we will determine the identity of transport processes in epididymal cells that can directly influence the concentration of NRTI in the stored seminal fluid by mediating transport into or out of the lumen of the epididymal ducts. Primary cultures of rat epididymal cells possess Ent1, Ent2 and Cnt2-mediated nucleoside transport (Leung et al., 2001; Klein et al., 2014b). However, there is virtually no information on the function of multidrug transporters in the epididymal epithelium. Our preliminary work has identified the presence of 11 multidrug transporters in rat epididymal tissue, including 8 transporters known to interact with one or more NRTIs (Table 1), but the profile of expression within the epididymal epithelium, i.e., apical vs. basolateral, is currently not known. Based on this information our initial survey of transporter location and function will target: P-gp, Mrp1, Mrp4, Mrp5, Ent1, Ent2, and Cnt2. Since the purpose of determining NSA transport focuses on testicular relapse, we do not plan to investigate the ability of these drugs to be transported in the epididymis.

**Aim 3.1. Determine the location of nucleoside and ABC transporters in mouse and human epididymis.** Localization of transporters in the epididymis will be determined using immunohistochemistry (IHC) and the methods we used in our recent report on the location of multidrug transporters in Sertoli cells (see chapter 2). Briefly, epididymides from mice will be fixed with formalin and embedded in paraffin. Paraffin blocks of human epididymis will be obtained from Pathology as described in the Human Subjects Section. Slides of epididymal
tissue will be blocked with 0.5% H$_2$O$_2$ and then exposed overnight to an antibody against the mouse proteins Ent1, Ent2, Cnt2, Mrp1, Mrp4 and Mrp5, all of which are commercially available and verified for use in mouse and human IHC by us (Ent1, Ent2, Mrp1, Mrp4, Mrp5) or by the vendor (Cnt2). Following exposure to the appropriate secondary antibody, diaminobenzidine (DAB), and counterstain, the location of the marker brown stain will be assessed microscopically as in our previous work (see chapter 3).

**Aim 3.2. Determine the transport of NRTI drugs in epididymal cells isolated from mice using knock-down technology to reduce levels of various transporters.** Cell transport can be studied by culturing primary cells onto transwell inserts (Mruk and Cheng, 2011a; Klein et al., 2013). The resulting cell layer provides the means to assess both cellular transport (apical vs. basolateral) and transepithelial transport. Although primary cells carry risks of changing expression profiles once placed into culture, they nevertheless provide a first-order approximation of the transport profile of reproductive tissues (Mruk and Cheng, 2011a; Su, Mruk, Lee, et al., 2011; Klein et al., 2013). Isolation of primary epididymal cells will be performed as previously described (Leung et al., 2001). These cells will be cultured on a matrigel transwell for transport studies for four to six days. If matrigel coated transwells are not found to support adequate growth and/or adherence to the inserts, one of the several other commercially available epithelial growth supports and techniques (collagen, adhesion factor etc.) will be used.
**Gene knock-down experiments.** Initial studies will characterize the extent of carrier-mediated NRTI transport using the same experimental approach described for establishing the time course and polarity of NRTI transport in Sertoli cells. Mouse epididymal cells will be harvested and cultured on transwell inserts. Three days after isolation, cells will have transporter mRNA levels knocked down for Ent1, Ent2, Cnt2, Bcrp, P-gp, Mrp1, Mrp4, or Mrp5 using a lentiviral vector to introduce an DNA sequence to silence target gene expression for the selected transport protein (preliminary studies will identify the hairpin sequence that reduces mRNA and protein for each candidate transporter by at least 70% compared to that in control cells transfected with a nonsense DNA sequence). We will determine A-B flux, B-A flux, and cell accumulation of each of the radiolabeled NRTIs (using the time point determined in the initial studies described above). These experiments will allow us to determine the contribution of each candidate transporter to the total flux of each NRTI in mouse epididymal cells.

**Aim 3.3. Determine the transport of NRTI drugs in epididymal cells isolated from mice from which selected transporters have genetically eliminated.** Epididymal cells isolated from C57BL6, Ent1−/−, Ent2−/−, Abcg2−/−, Mrp4−/−, Mrp5−/−, and Mdr1a−/− will be cultured on transwell inserts until they reach confluence. Three wells from each strain will be exposed to one of the four NRTI drugs for the time determined in Aim 3.2. We will determine A-B flux, B-A flux, and cell accumulation of each of the radiolabeled NRTIs. These experiments will be interpreted in the context of information obtained in the knockdown studies to
determine the contribution of each candidate transporter to the total flux of each NRTI in mouse epididymal cells.
Appendix A: LOCALIZATION OF NUCLEOSIDE TRANSPORTERS IN THE EPIDIDYMIS

Text and figures derived from; David M. Klein, Marcus C. Harding, Hui Li, Erica L Toth, Anika L Dzierlenga, Meghan K Crowther, John D. Clarke, and Nathan J. Cherrington submitted to Drug Metabolism and Disposition for short communication publication.

Introduction

The epididymis is an organ that contains a single convoluted tube that connects the testis to the ductus deferens (or vas deferens). This tubule is classically divided into three sections: the caput (head), corpus (body), and cauda (tail) (Byers et al., 1988; D M Creasy, 2001; Klein et al., 2014b). The two primary functions of the epididymis are sperm maturation and storage. There has been a growing interest in the mechanisms of sperm maturation within the epididymis. It is suspected that the luminal secretions within the convoluted tubule are critical for proper sperm development (Bagnis et al., 2001; Hermo and Smith, 2011; Mital et al., 2011). Understanding the transporter expression profile of the epididymis is foundational for knowing how endogenous and xenobiotic compounds move into or out of the epididymal duct.

The epididymis is also important for understanding drug disposition within the male genital tract (MGT) (see chapter 4). Any xenobiotics that are able to cross the blood-testis barrier (BTB) and accumulate in the seminiferous

---

5 All of the work presented was done by me including the writing of the initial draft.
tubules would likely flow into the epididymal duct. This can have profound effects on reproductive toxicology since nearly 99% of water within the duct is reabsorbed, which results in a 100 fold increase in concentration of any drug within the ducts (Wong and Yeung, 1976; Zhou et al., 2001; Lu et al., 2007; Cornwall, 2009; Hermo and Smith, 2011; Moretti et al., 2012). Therefore, it is important to understand which drugs the epididymis is capable of reabsorbing.

One class of drugs that has great implications regarding the MGT is the nucleoside analog drugs (NSA). These drugs are used to treat HIV infection and various cancers such as acute lymphoblastic leukemia (ALL), which is known to have a significant testicular relapse rate (Arya et al., 2010; Grundy et al., 1997; Klein et al., 2013). Many of these drugs are known to be substrates of nucleoside transporters due to their structural similarity to endogenous nucleosides (Yao et al.; Pastor-Anglada et al., 2005). There are two families of nucleoside transporters; the equilibrative nucleoside transporters (ENT, SLC29) and the concentrative nucleoside transporters (CNT, SLC28) (Kong et al., 2004; Li, Boado, & Pardridge, 2001; Mangravite et al., 2003; Rodríguez-Mulero et al., 2005). Previous data have determined that the epididymis expresses mRNA and protein for three nucleoside transporters: ENT1, ENT2, and CNT2 (Klein et al., 2014; Leung, et al., 2001). ENT1 and ENT2 are known to handle all the endogenous nucleosides with relatively equal affinity, but CNT2 only transports purine nucleosides (Gray et al., 2004; Kong et al., 2004; Ward, 2000). Another key difference is that ENT transporters are bidirectional; they move substrate according to concentration gradient (from higher concentration to lower concentration) while CNT transporters are uptake transporters driven by Na+ co-
transport. The previous studies were valuable in determining expression of nucleoside transporters, however they did not determine the subcellular location of the transporters, which has implications on their capacity to support transepithelial transport. Knowing the location of transporters is critical to determining their function within a tissue. For drug disposition, expression of bidirectional transporters in both sides of the cell could reveal transepithelial pathways through the epithelium. This would indicate that nucleoside drugs that bypass the BTB could be reabsorbed downstream in the epididymis. The purpose of this study is to determine the localization of the three nucleoside transporters shown to be expressed in the epididymis (ENT1, ENT2, and CNT2) via immunohistochemistry (IHC) to test whether transepithelial transport of these xenobiotics beyond the blood-testis barrier is possible.

**Materials and Methods**

**Materials**: MACH4 IHC staining kit was acquired from Biocare Medical (St. Louis, MO). ENT1 (SLC29A1) and ENT2 (SLC29A2) antibodies were purchased from Lifespan Biosciences (Seattle, WA). CNT2 (SLC28A2) and Na+/K+ ATPase were purchased from Abcam (Cambridge, MA). All other reagents were purchased from a standard scientific supplier at the highest available purity.

**Sample Collection**: Rat samples were collected from euthanized male Sprague Dawley rats at least 12 weeks old (mature). The samples were fixed in 10% neutral buffered formalin overnight. The following day, formalin was replaced with 70% ethanol until the samples were embedded in paraffin.
Sectioning of all paraffin-embedded tissue was accomplished using a microtome with sections sliced 5 microns thick with one section per slide. Protocols for obtaining samples were approved by the University of Arizona Institutional Animal Care and Use Committee (IACUC).

**Immunohistochemistry.** IHC staining was performed on formalin-fixed, paraffin-embedded samples. Slides were deparaffinized with xylene and rehydrated with ethanol. The samples were then heated in an antigen retrieval buffer: citrate (pH 6.0). Endogenous peroxide activity was blocked by a 0.3% hydrogen peroxide/methanol solution. Staining for all antibodies was performed with the MACH4 kit according to the manufacturer's instructions (Biocare Medical). All slides were imaged with a Leica DM4000B microscope and a DFC450 camera (Leica Microsystems Inc., Buffalo Grove, IL). Each experiment also contained a negative control slide which was not exposed to any primary antibodies, but otherwise was treated the same as every other slide. The negative slides contained very little to no positive (brown) staining. For all the tissues examined, the localization was consistent throughout the different sections of the epididymis, therefore only one representative image was taken for each protein.

**Results and Discussion**

Figure A.1 shows the localization for Ent1 (A), Ent2 (B), Cnt2 (C) and sodium/potassium ATPase (Na⁺/K⁺ ATPase) for rat epididymis. The Na⁺/K⁺ ATPase serves as a basolateral membrane marker to demonstrate the staining
pattern for basolateral localization in the principal cells (Wen et al., 2014). Since the gap junctions (which typically differentiate between the apical and basolateral

**Figure A.1. Localization of Nucleoside Transporters in the Epididymis.** Immunohistochemical staining for ENT1 (A), ENT2 (B), CNT2 (C) and Na+/K+ ATPase (D) in formalin-fixed paraffin-embedded mature rat epididymis shown at 40 X magnification. Arrows indicate positive (brown) staining for proteins.
membranes) in the epithelium are near the luminal side, the basolateral membrane is a characteristic U shape when viewing a cross section of the tubule (Cai et al., 2013; Cyr, 2011). The IHC slides show an identical staining pattern between Ent1 and Na+/K+ ATPase, which indicates that Ent1 is located on the basolateral membrane of principal cells. This is the expected localization for Ent1 in most cell types. Since Ent transporters are driven purely by concentration gradient, the typical physiological function is to aid in the uptake of nucleosides to cells that are building DNA and this is likely to be the case for the epididymis. This indicates that xenobiotic substrates for Ent1 would be able to access the principal cells from the blood. However, Ent1 would not interact with substrates already within the lumen of the epididymal duct.

Ent2 showed positive staining for the nuclei of the principal cells that line the duct. While this localization is not typical, there have been reports of Ent2 expression in the nucleus of various cell types (Hansen et al., 2007). It is difficult to speculate on the function of nuclear Ent2 within the context of drug disposition. While the apparent function may be allowing nucleosides to enter the nucleus, this function seems to be unnecessary since the nuclear pores is expected to be sufficient to allow for the diffusion of nucleosides into the nucleus. Regarding drug disposition, the impact of Ent2 would be expected to be minimal because it does not access any extracellular compartments. It may be that nuclear localization of Ent2 may increase access to the nucleus for substrates that may be able to be taken into cells via Ent1 but are not able to diffuse through nuclear pores to appreciable concentrations.
Cnt2 was found to be expressed primarily by the basal cells. Basal cells are thought to play an important role in nutrient salvaging (Arrighi, 2014). The primary physiological function of Cnt2 is thought to be nucleoside salvaging of the intestine and kidney (absorption) and so, Cnt2 expression of the basal cells is consistent with basal cell nutrient salvaging (Rodríguez-Mulero et al., 2005). This would indicate that purine nucleosides and purine analog drugs would be able to affect the epididymal epithelium. It’s difficult to speculate why only Cnt2 is expressed instead of both Cnt1 and Cnt2, however evidence from multiple groups investigating mRNA and protein indicate that this is the case (Leung et al., 2001; Klein et al., 2014b).

Overall, it was found that nucleoside transporters, particularly for purine nucleosides, are able to grant access for their substrates to the epithelium of the epididymis. It is interesting that none of the nucleoside transporters investigated forms a transepithelial pathway that connects nucleosides in the lumen with the blood. This would indicate that NSA drugs or other xenobiotics that accumulate into the MGT through transepithelial nucleoside transport in the testis would not be reabsorbed by the epididymis and therefore could be concentrated roughly 100 fold within the epididymal duct. This can potentially have significant implications in male reproductive toxicology by providing a mechanism for potential nucleoside toxicants to concentrate to toxic thresholds within the epididymis. This could also explain the clinical observation that many NSA drugs used to treat HIV can accumulate within seminal plasma to concentrations 2-10 fold above that of blood (Anderson et al., 2000; Taylor et al., 2000; Liuzzi et al., 2004).
APPENDIX B: FUNDING ACKNOWLEDGEMENTS

This work was supported by the National Institutes of Health [Grants NIAID AI083927, ES006694, and HD062489] and the National Institute of Environmental Health Science Toxicology Training Grant [ES007091].
REFERENCES


Meyts ER-D, Skakkebaek NE, and Toppari J (2013) Testicular Cancer Pathogenesis, Diagnosis and Endocrine Aspects, MDText.com, Inc.


Sibler SJ (St. LH (1991) Effect of Age on Male Fertility.


Tomi M, Nishimura T, and Nakashima EMI (2011) Mother-to-Fetus Transfer of Antiviral Drugs and the Involvement of Transporters at the Placental Barrier. 100:3708–3718.


