Characterizing the Expression and Localization of Drug Transporters and Metabolic Enzymes in the testes of Uninfected and HIV-1 Infected, Treated Subjects – Potential Contribution to an HIV-1 Sanctuary

By

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

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Master of Science

Department of Pharmaceutical Sciences – Leslie Dan Faculty of Pharmacy
University of Toronto

2015

Abstract

Previous studies have reported that HIV-1 is capable of both acute and persistent infection in the testes. The naturally restrictive environment in the testes, due in part to the expression of drug transporters at the blood-testes-barrier (BTB), could limit antiretroviral (ARV) penetration into this tissue and contribute to the formation of a viral sanctuary. Therefore, we conducted a comprehensive characterization of gene and protein expression and localization of 11 major drug transporters and metabolic enzymes relevant to ARV therapy in testicular tissue isolated from both uninfected and HIV-1 infected, treated individuals. We also quantified drug concentration levels in testicular tissue versus plasma to gain additional insight on ARV drug penetration into the testes. Overall, our findings indicate that the testes express many key drug transporters and metabolic enzymes relevant to ARV therapy, which could limit the penetration of certain ARV compounds, and contribute to persistent HIV-1 infection and formation of a viral sanctuary site.
Acknowledgments

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<tr>
<td>3TC</td>
<td>Lamivudine</td>
</tr>
<tr>
<td>3TC-TP</td>
<td>Lamivudine Triphosphate</td>
</tr>
<tr>
<td>ABC</td>
<td>ATP-binding Cassette Transporter</td>
</tr>
<tr>
<td>ARV</td>
<td>Antiretroviral</td>
</tr>
<tr>
<td>ATV</td>
<td>Atazanavir</td>
</tr>
<tr>
<td>BBB</td>
<td>Blood-Brain Barrier</td>
</tr>
<tr>
<td>BCRP</td>
<td>Breast Cancer Resistance Protein</td>
</tr>
<tr>
<td>BCSFB</td>
<td>Blood-Cerebrospinal Fluid</td>
</tr>
<tr>
<td>BTB</td>
<td>Blood-Testes Barrier</td>
</tr>
<tr>
<td>CAR</td>
<td>Constitutive Androstane Receptor</td>
</tr>
<tr>
<td>CNS</td>
<td>Central Nervous System</td>
</tr>
<tr>
<td>CNT</td>
<td>Concentrative Nucleoside Transporter</td>
</tr>
<tr>
<td>CSF</td>
<td>Cerebrospinal Fluid</td>
</tr>
<tr>
<td>CYP450</td>
<td>Cytochrome P450</td>
</tr>
<tr>
<td>DCs</td>
<td>Dendritic Cells</td>
</tr>
<tr>
<td>DDI</td>
<td>Drug-drug Interactions</td>
</tr>
<tr>
<td>DRV</td>
<td>Darunavir</td>
</tr>
<tr>
<td>DTG</td>
<td>Dolutegravir</td>
</tr>
<tr>
<td>EFV</td>
<td>Efavirenz</td>
</tr>
<tr>
<td>ENT</td>
<td>Equilibrative Nucleoside Transporter</td>
</tr>
<tr>
<td>ETR</td>
<td>Etravirine</td>
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</table>
EVG – Elvitegravir
FTC – Emtricitabine
FTC-TP – Emtricitabine Triphosphate
GR – Glucocorticoid Receptor
HAART – Highly Active Antiretroviral Therapy
HBMECs – Human Brain Microvessel Endothelial Cells
HIV – Human Immunodeficiency Virus
IDV – Indinavir
LPV – Lopinavir
LXR – Liver-X Receptor
MGT – Male Genital Tract
MRP – Multidrug Resistance Protein
MVC – Maraviroc
NFV – Nelfinavir
NNRTI – Non-Nucleoside Reverse Transcriptase Inhibitor
N(t)RTIs – Nucleos(t)ide Reverse Transcriptase Inhibitor
OATP – Organic Anion Transporting Polypeptide
OAT – Organic Anion Transporter
OCT – Organic Cation Transporter
PBMCs – Peripheral Blood Mononuclear Cells
P-gp – Permeability Glycoprotein
PI – Protease Inhibitor
PPAR – Peroxisome Proliferator Activated Receptor
PXR – Pregnane-X Receptor
RAL – Raltegravir
RT – Reverse Transcriptase
RTV – Ritonavir
SIV – Simian Immunodeficiency Virus
SLC – Solute Carrier Transporter
SQV - Saquinavir
T-20 – Enfurvitide
TDF – Tenofovir DF
TFV – Tenofovir
TFV-DP – Tenofovir Diphosphate
UGT - UDP glucuronosyltransferase
List of Appendices

**APPENDIX A** – Supplemental Tables & Figures

**APPENDIX B** – Methods not included in Chapter 5

**APPENDIX C** – Data not included in Chapter 5
1 Introduction/background

1.1 HIV-1 Epidemiology and Transmission

Since its first discovery in 1981, Human Immunodeficiency Virus-1 (HIV-1) has caused more than 30 million deaths and is currently estimated to infect another 35 million people (UNAIDS, 2012). In 2012 alone, the virus has killed approximately 1.6 million people and is estimated to have newly infected 2.3 million people (UNAIDS, 2012). HIV-1 is transmitted via physiological fluids primarily through sexual contact or through contaminated needle drug use.

![Graph showing stages of HIV-1 infection](image)

**Figure 1-1.** Stages of HIV-1 infection and associated clinical characteristics (2012)
HIV-1 Infection is divided into two main stages (Fig. 1-1). During the acute stage, the virus quickly multiplies in host cells but the immune system is able to respond effectively and decreases viral load (Simon, Ho, & Karim, 2006). This is followed by the clinical latency stage, where HIV-1 can persist at low levels in cellular and tissue reservoirs (Chan, Dietrich, Hosie, & Willett, 2013; Douek, Picker, & Koup, 2003) and a gradual decrease in the number of naïve and memory CD4 T-cells occurs until the counts falls below a critical level and AIDS is triggered (Douek et al., 2003; Simon et al., 2006).

### 1.2 Current Pharmacological Treatment

Within the last couple of decades there have been significant advances in the treatment of HIV-1. Current antiretroviral (ARV) treatment can be extremely effective at targeting and suppressing plasma viral replication during the chronic stage of infection. These ARV drugs are classified into six categories (Kis, Robillard, Chan, & Bendayan, 2010; Panel on Antiretroviral Guidelines for Adults and Adolescents, 2015) and target different stages of the HIV-1 life cycle (Fig. 1-2).
Figure 1-2. Different stages of the HIV-1 viral life cycle targeted by ARV compounds
1.2.1 Protease Inhibitors

Protease inhibitors (PIs) target the HIV viral protease and block the virus from assembling new viral particles. HIV protease is critical to the viral life cycle as it is responsible for the cleavage of the viral Gag and GagPol precursor proteins into their mature constituents (Kohl et al., 1988), which include major structural proteins such as the matrix, capsid and nucleocapsid as well as functional proteins such as the reverse transcriptase and integrase (Wensing, van Maarseveen, & Nijhuis, 2010). The structure of HIV protease is well established and consists of two symmetrically assembled homodimers made up of 99 amino acids, which combine to form a central substrate-binding cleft responsible for enzyme activity (Navia et al., 1989). Currently, ten PIs are licensed for clinical use in treating HIV infections. These agents act as competitive inhibitors of HIV protease, binding with high affinity to the enzyme’s active site and preventing it from performing its normal proteolytic processing activity (Wensing et al., 2010). The pharmacokinetics of PIs are generally unfavourable due in part to extensive protein binding (Warnke, Barreto, & Temesgen, 2007) and interactions with drug efflux transporters such as P-gp (Kim et al., 1998; Michaud et al., 2012), therefore most PIs are administered with a boosting agent to achieve effective serum concentrations. Drug interactions are also a major concern in PI-containing regimens due to their extensive interactions with the cytochrome P450 (CYP450) enzymes. For example, ritonavir (RTV) is a potent inhibitor of CYP3A4 activity (Koudriakov et al., 1998), whereas darunavir (DRV) induces CYP3A4 activity (Brown, Paul, & Kashuba, 2009).
1.2.2 Non-nucleoside and Nucleoside/nucleotide Reverse Transcriptase Inhibitors

Non-nucleoside reverse transcriptase inhibitors (NNRTIs) and nucleoside/nucleotide reverse transcriptase inhibitors (N(t)RTIs) prevent the virus from converting its RNA genome into DNA by inhibiting the activity of viral reverse transcriptase (RT). Crystal structures of the unbound HIV RT indicate that it is an asymmetric heterodimer made up of a functional p66 subunit responsible for catalytic activity, and a p51 structural subunit (de Béthune, 2010; Kohlstaedt, Wang, Friedman, Rice, & Steitz, 1992). NNRTIs act as non-competitive inhibitors of HIV RT and bind to a hydrophobic pocket distant from the catalytic site, inducing a conformational change that inhibits RT activity (de Béthune, 2010; Hsiou et al., 1996). On the other hand, N(t)RTIs are structural analogues of the deoxyribonucleosides that are the natural substrates for HIV RT, and act as competitive inhibitors by occupying the catalytic site and terminating DNA chain elongation through their lack of a 3’-hydroxyl group on their sugar moiety (Mitsuya & Broder, 1986; Warnke et al., 2007). Unlike NNRTIs, which bind to RT directly, N(t)RTIs need to be converted to their active 5’-triphosphorylated form by intracellular kinases before they can be incorporated into the nascent DNA chain by HIV RT (Gao, Agbaria, Driscoll, & Mitsuya, 1994; Warnke et al., 2007). NNRTIs display variable oral bioavailability between drugs as a result of different protein binding characteristics and poor gastrointestinal tract solubility due to their non-polar chemical structure (Crauwels et al., 2013; Grennan & Walmsley, 2009; Ma et al., 2005). Drug interactions are the primary concern when using NNRTIs as it has been demonstrated that this class of ARVs are extensively metabolized by CYP450s, and in particular efavirenz (EFV) and nevirapine (NVP) can also induce CYP3A4 activity (Ma et al., 2005).
N(t)RTIs generally display favourable pharmacokinetics due in part to their low protein binding, ability to utilize endogenous nucleoside transport pathways, and the long intracellular half-lives of the active triphosphate metabolites (Cihlar & Ray, 2010). However, tissue penetration and plasma half-life of the parent molecule can be affected by the expression pattern and localization of drug efflux transporters and drug influx transporters. For example, the combined effect of organic anion transporter 1 (OAT1) expression on the basolateral membrane and multidrug resistance protein 4 (MRP4) expression on the apical membrane of the kidney proximal tubules contribute to tenofovir (TFV) uptake from the blood, and excretion into urine, respectively (Cihlar, Ho, Lin, & Mulato, 2001; Imaoka et al., 2007; Ray et al., 2006). Intermediate monophosphate metabolites of several NRTIs have also been demonstrated as substrates of efflux transporters MRP4, MRP5 and MRP8 (Borst, de Wolf, & van de Wetering, 2007; Guo et al., 2003).

1.2.3 Integrase Strand Transferase Inhibitors

Integrase strand transfer inhibitors (INSTIs) prevent HIV integrase from inserting viral DNA into the host genomic DNA. The active integrase enzyme is comprised of two monomers associating with each other to form a homo-dimer, that may further arrange itself into a functional tetramer or higher order tertiary structure (Ellison, Gerton, Vincent, & Brown, 1995; Engelman, Bushman, & Craigie, 1993; McColl & Chen, 2010). Each integrase monomer contains three functional domains, the N-terminal domain (NTD) responsible for higher-order multimeric structure formation (Zheng, Jenkins, & Craigie, 1996), the catalytic core domain (CCD) containing the conserved DDE motif responsible for coordinating divalent metal ions critical to catalytic activity (Engelman & Craigie, 1992; Goldgur et al., 1999), and the C-terminal domain...
(CTD) that is believed to contribute to non-specific DNA binding activity (McColl & Chen, 2010). Structure-activity studies have indicated that HIV integrase undergoes a conformational change upon initial binding of viral DNA that only then allows INSTIs to bind to the active site (Alian et al., 2009). INSTIs are a structurally diverse group of molecules, but share a common mode of action that involves the chelation of the divalent ions coordinated by the DDE motif within the CCD (Grobler et al., 2002), preventing the critical strand transfer reaction necessary for successful integration as well as to a smaller degree the 3’-end processing reaction (McColl & Chen, 2010). Three INSTIs (raltegravir (RAL), elvitegravir (EVG), and dolutegravir (DTG)) are currently approved for use in treating HIV infections. Pharmacokinetic profiles of INSTIs are generally favourable and well above the protein-corrected in vitro IC95 values, with the exception of EVG, which needs to be administered with a pharmacoenhancer (colbicistat or RTV) to prevent excessive CYP3A4 metabolism (Adams, Greener, & Kashuba, 2012). Drug interactions are minimal for RAL and DTG, but UDP glucuronosyltransferase 1A1 (UGT1A1) mediated interactions can occur when administering EVG with boosted lopinavir (LPV) or atazanavir (ATV) in addition to potential CYP3A4 interactions (Adams et al., 2012).

1.2.4 Fusion/entry Inhibitors

Fusion/entry inhibitors block the initial binding, fusion and entry of the virus across the cell membrane. There are currently two drugs approved for use in HIV treatment, the fusion inhibitor enfuviritide (T-20), and the CCR5 receptor antagonist maraviroc (MVC), each blocking a different stage of the HIV entry process. Briefly, HIV entry into a host cell is mediated by both the surface glycoprotein gp120, and the transmembrane glycoprotein gp41 (Haqqani & Tilton, 2013). Upon gp120 recognition of CD4 on the host cell, it undergoes an initial conformation
change that creates and exposes the coreceptor-binding site. HIV gp120 can utilize either the CCR5 or CXCR4 chemokine receptors as a co-receptor depending on the viral tropism (R5 or X4 respectively). Once the co-receptor binds to gp120, it undergoes a second conformation change that exposes the transmembrane gp41 fusion peptide, which inserts into the host cell membrane to create a fusion pore that allows the entry of the viral capsid into the host cytoplasm. The CCR5 receptor antagonist MVC prevents the gp120-CCR5 interaction by binding to a hydrophobic pocket in the transmembrane domains, causing a conformation change that renders it unable to interact with gp120 (Dorr et al., 2005; Haqqani & Tilton, 2013). The pharmacokinetics of MVC are favorable with rapid absorption taking place shortly after administration (MacArthur & Novak, 2008). It is primarily metabolized by CYP3A4 and therefore is susceptible to drug-drug interactions with CYP3A4 inhibitors such as ritonavir, or inducers such as EFV and ETR (Brown et al., 2009; Mugundu, Hariparsad, & Desai, 2010). MVC is also a substrate for P-gp (Michaud et al., 2012), which may affect its penetration intracellularly or into certain tissue compartments. The fusion inhibitor enfurvitide is a small, linear synthetic peptide that mimics the HR2 domain on gp41 and interferes with the critical HR2-HR1 interaction that occurs during viral-host membrane fusion (Haqqani & Tilton, 2013). T-20 is not orally bioavailable and must be given as a twice daily subcutaneous injection, limiting its clinical utility to use primarily as salvage therapy (Warnke et al., 2007).

1.2.5 Combination Therapy

Current HIV treatment strategy utilizes a combination of ARV compounds from multiple functional classes to achieve durable viral suppression (<50 RNA copies/ml in plasma) (Esté & Cihlar, 2010). Using multiple compounds simultaneously takes advantage of synergistic effects
of different compounds to target different viral targets, lowers the dosage necessary of each individual drug to avoid toxicity, and decreases the likelihood of viral drug resistance developing. There are now 25 approved ARV compounds on the market available to physicians, and in recent years, drug manufacturers have focused on developing simple fixed-dose combinations, such as Atripla® (efavirenz (EFV)/emtricitabine (FTC)/tenofovir DF (TDF)), that have significantly reduced pill burden and contributed to improvements in therapy adherence (Langebeek et al., 2014). Based on numerous long-term clinical studies, most combination regimens revolve around the use of TDF/FTC due to its potent and durable efficacy and safety, and this dual N(t)RTI backbone combined with either a boosted PI, or an INSTI is currently recommended as the preferred regimen for treatment-naïve patients (Table 1-1). However, treatment failure can occur due to a variety of causes that range from lack of efficacy due to drug resistance, inadequate drug exposure, or non-adherence, to toxicity, and/or incompatibility with other essential medications (Esté & Cihlar, 2010). Furthermore, individual patient factors such as genotypic resistance profile, and other comorbid conditions can preclude them from first-line regimen options (Camacho & Teófilo, 2011). For these reasons, there are no universal recommendations for treatment-experienced patients and/or patients with a complex medical history other than the avoidance of drug combinations that have proven to be ineffective, and treatment regimens become highly individualized (Tables 1-2 and 1-3).
Table 1-1. First line HAART regimens recommended by the NIH.

<table>
<thead>
<tr>
<th>Preferred Regimens</th>
<th>PI-Based Regimen</th>
<th>INSTI-Based Regimen</th>
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<tr>
<td>DRV/r + TDF/FTC</td>
<td>DTG/ABC/(3TC or FTC)(^a)</td>
<td>DTG + TDF/(3TC or FTC)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>EVG/c/TDF/FTC(^b)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>RAL + TDF/(3TC or FTC)</td>
</tr>
</tbody>
</table>

\(^a\) only for patients who are HLA-B*5701 negative  
\(^b\) only for patients with pre-treatment estimated CrCl ≥70 mL/min  


Table 1-2. Alternative HAART regimens recommended by the NIH for patients failing first-line regimens.

<table>
<thead>
<tr>
<th>Alternative Regimens</th>
<th>NNRTI-Based Regimens</th>
<th>PI-Based Regimens</th>
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</thead>
<tbody>
<tr>
<td>EFV + ABC/(3TC or FTC)</td>
<td>ATV/c + TDF/(3TC or FTC)(^b)</td>
<td></td>
</tr>
<tr>
<td>RPV/TDF/(3TC or FTC)(^a)</td>
<td>ATV/r + TDF/(3TC or FTC)</td>
<td>(DRV/c or DRV/r) + ABC/(3TC or FTC)(^c)</td>
</tr>
<tr>
<td></td>
<td>DRV/c + TDF/(3TC or FTC)(^b)</td>
<td></td>
</tr>
</tbody>
</table>

\(^a\) only for patients with pre-treatment HIV RNA <100,000 copies/mL and CD4 cell count >200 cells/mm³  
\(^b\) only for patients with pre-treatment estimated CrCl ≥70 mL/min  
\(^c\) only for patients who are HLA-B*5701 negative  

Table 1-3. Other HAART regimens recommended by the NIH for patients who are pregnant, and/or display severe drug resistance, and/or suffer from comorbidities.

<table>
<thead>
<tr>
<th>Other Regimens</th>
<th>NNRTI-Based Regimen</th>
<th>PI-Based Regimen</th>
<th>INSTI-Based Regimen</th>
<th>Other regimens when TDF or ABC cannot be used</th>
</tr>
</thead>
<tbody>
<tr>
<td>EFV + ABC/(3TC or FTC)(^a)</td>
<td>(ATV/c or ATV/r) + ABC/(3TC or FTC)(^b)</td>
<td>RAL + ABC/(3TC or FTC)(^b)</td>
<td>DRV/r + RAL(^c)</td>
<td></td>
</tr>
<tr>
<td>LPV/r + ABC/(3TC or FTC)(^b)</td>
<td></td>
<td></td>
<td>LPV/r + 3TC</td>
<td></td>
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<tr>
<td>LPV/r + TDF/(3TC or FTC)</td>
<td></td>
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<td></td>
</tr>
</tbody>
</table>

\(^a\) only for patients who are HLA-B*5701 negative and with pre-treatment HIV RNA < 100,000 copies/mL
\(^b\) only for patients who are HLA-B*5701 negative
\(^c\) only for patients with pre-treatment HIV RNA < 100,000 copies/mL and CD4 cell count > 200 cells/mm\(^3\)

1.3 Current Treatment Challenges

Despite the success of HAART to suppress HIV replication long-term and substantially increase the life expectancy of patients infected with HIV, there remain significant challenges that need to be addressed in order to optimize treatment. Most critically, HAART is unable to completely eradicate the virus from an infected individual as demonstrated in numerous studies that observed a rapid plasma viral rebound upon cessation of ARV therapy (Davey et al., 1999; García et al., 1999; Harrigan, Whaley, & Montaner, 1999; Neumann et al., 1999), which for now means that HIV-infected individuals need to remain on treatment for the remainder of their lives. Such long-term ARV treatment is not an ideal solution and poses critical issues including the possible development of drug resistant viral variants that necessitate potentially more aggressive treatment regimens, drug interactions with other comedications, as well as the cumulative toxicity effects associated with long-term pharmacological treatment.

1.3.1 Cellular Reservoirs

The main barrier to a cure for HIV-1 infections is the presence of a viral reservoir that is established early during the first few weeks of acute infection, and acts as a source of new viral particles upon treatment failure or cessation. The first evidence that a latent and inducible viral reservoir was present in HIV-infected patients was proposed in several independent studies that demonstrated CD4+ T cells carried replication-competent proviral DNA that could be induced to produce infectious viral particles in vitro (Chun et al., 1997; Finzi et al., 1997; Wong et al., 1997). Studies since then have indicated that the primary cellular reservoir for latent HIV-1 infections are the resting CD4+ T cells, which can be further subdivided into naïve T-cells that
have yet to undergo antigen-stimulated expansion, and memory T-cells, which are activated T-cells that have returned to a quiescent state to preserve immunological memory (Berard & Tough, 2002). Direct infection of these resting T-cells can occur, with the frequency of direct memory T-cell infection occurring at a higher rate than naïve T-cell infection (Pierson et al., 2000). However, direct infection of resting T-cells by free viral particles is generally an inefficient process owing to defects in reverse transcription and delays in the integration event (Vatakis, Bristol, Wilkinson, Chow, & Zack, 2007; Vatakis, Kim, Kim, Chow, & Zack, 2009). Due to these difficulties, it is likely that latently infected memory CD4+ T-cells were infected while they were in an activated state, which is more permissive for viral replication, and reverted back to their resting memory state carrying an integrated provirus (Han, Wind-Rotolo, Yang, Siliciano, & Siliciano, 2007). Resting memory T-cells form an ideal long-term reservoir for latent HIV-1 infection due to their suppressed levels of transcription activity, their extremely long half-life, and capability to undergo homeostatic proliferation in the presence of IL-7 (Chomont et al., 2009; Han et al., 2007). However, despite the comparatively lower number of infected naïve T-cell subset compared to memory T-cells, there is some evidence that suggest the naïve T-cell subset may play an important role in persistent HIV-1 infection. Naïve T-cells also have a long half-life, proliferate in number after treatment with ARV therapy, and appear to be capable of harboring integrated proviral DNA more stably compared to memory T-cells (Chan et al., 2013; Wightman et al., 2010). Overall, further work is ongoing to characterize and delineate the varying roles that specific T-cell subsets play in persistent infection.

Although memory CD4+ T cells are recognized as the primary reservoir for latent HIV-1 infections, there is now evidence to suggest that CD4+ T cells may not be the only source of
rebounding virus. Genotype comparisons between replication-competent virus isolated from CD4+ T cells to virus isolated from the population that emerged after treatment cessation indicate that other cell types and even anatomical compartments can act as viral reservoirs (Chun et al., 2000). Indeed, recent studies have implicated cells from the monocyte/macrophage lineage, resident macrophages and astrocytes of the CNS, as well as dendritic cells and follicular dendritic cells as capable of harboring latent virus, or periodically reseeding peripheral infection (Alexaki, Liu, & Wigdahl, 2008).

Monocytes circulate for days through blood before migrating into various tissues and differentiating into macrophages, which are a key component of the innate immune response initiated shortly after HIV-1 infection. Although infection of macrophages occurs at a relatively low frequency, the HIV-1 infection of macrophages is less cytotoxic and more productive in nature (Swingler et al., 1999; Swingler, Mann, Zhou, Swingler, & Stevenson, 2007), possibly contributing to persistence through low-level virus replication. It is also believed that macrophages can carry a wide range of HIV-1 viral variants, and that they may make up more than 10% of productively infected cells during the acute stage of infection, with that percentage increasing as the disease progresses due to CD4 T cell depletion (Smith, Meng, Salazar-Gonzalez, & Shaw, 2003; Xu et al., 2008). Specialized resident macrophages of the CNS such as the perivascular macrophages and microglia are also capable of being infected by HIV-1 (He et al., 1997; Lavi et al., 1997), and are believed to be major contributors to HIV-associated neuroinflammation and neurocognitive disorders (Lindl, Marks, Kolson, & Jordan-Sciutto, 2010). Perivascular macrophages are believed to be the critical interface between virus in the peripheral compartment and the CNS due to their continuous repopulation from cells of a bone-
marrow origin, and their capability to leave the CNS (Alexaki et al., 2008; Elbirt et al., 2015; Kida, Steart, Zhang, & Weller, 1993; Williams, Alvarez, & Lackner, 2001). On the other hand, microglia have demonstrated limited repopulation, and it is suspected that they can form long-term reservoirs for latent virus seeded during initial acute infection (Gray et al., 2014).

Similar to the other non-traditional HIV-1 reservoirs, astrocytes have also been demonstrated to be carriers of latent infection despite their low infection rates. HIV-1 infections of astrocytes generally result in non-productive infection as evidenced by a lack of viral protein production detected (Churchill et al., 2006; Gorry et al., 1999). However, it has been demonstrated in vitro that latent HIV-1 in astrocytes can be transmitted to monocytic cell lines and T-cell lines (Chiodi, Fuerstenberg, Gidlund, Asjö, & Fenyö, 1987; Sabri et al., 1999), and eventually result in productive infection from these cells, suggesting a role for astrocytes in reseeding infection during instances of viral rebound.

Finally, increasing attention has now been focused on characterizing interactions of HIV-1 with dendritic cells, which have long been known to play a crucial role in the initial systemic dissemination of HIV-1 during acute infection (Ahmed, Kawamura, Shimada, & Piguet, 2015). Although dendritic cells can be productively infected by HIV-1, or facilitate infections by transporting viral particles on its surface, it remains unclear whether they can contribute as a long-term reservoir for latent infections due to their relatively short half-life. However, recent studies on follicular dendritic cells that reside in B-cell follicles of secondary lymphoid organs demonstrate an intriguing new modality for dendritic cells (DCs) as a long-term reservoir, wherein they sequester the viral particle in a stable, infectious state for approximately 2 months (Keele et al., 2008). More work is needed to better understand this phenomenon and determine
whether it may play a role in increasing the effective half-life of infectious free viral particles, and whether this may continue to occur during the clinical latency stages of infection.

Overall, it remains a debate whether persistent HIV-1 infections are perpetuated by periodic reactivation of quiescent virus from long-lived cellular reservoirs such as the memory CD4 T-cells, or whether it is perpetuated by constant low-level replication mediated by certain alternative reservoirs mentioned above. Recent studies have provided evidence supporting both theories. For example, treatment intensification does not reduce residual viraemia in patients who are already successfully suppressed, suggesting a lack of viral replication (Dinoso et al., 2009; Gandhi et al., 2010; McMahon et al., 2010). On the other hand, treatment intensification with RAL in virally suppressed subjects has also been shown to increase the number of 2-long terminal repeat circles (Buzón et al., 2010), which suggests that RAL is interfering with viral replication. It is likely that persistent HIV-1 infection utilizes both of these pathways in varying degrees, and indeed research on improving HIV-1 treatment has focused on either attacking the latent cellular reservoir (Barouch & Deeks, 2014), or targeting low-level viral replication by improving pharmacological therapy effectiveness, which is the main focus of this project.

1.3.2 Viral Sanctuary Sites

ARV compounds are generally very effective at suppressing viral replication in plasma, and are capable of reaching and exceeding therapeutic concentration ranges in this compartment. However, there is now mounting evidence that persistent HIV-1 infection are perpetuated in part by low-level viral replication, oftentimes in viral sanctuary sites located in certain anatomical compartments such as the CNS, the lymph nodes, and the genitourinary tract. Drug disposition
into these tissue sanctuary sites are dependent on a combination of the intrinsic physicochemical properties of the ARV compounds themselves such as lipophilicity, molecular weight, polarity, degree of ionization, and degree of protein binding, as well as the tissue-specific expression patterns of drug transporters and metabolic enzymes known to interact with many commonly prescribed ARVs. Together, these factors can result in subtherapeutic concentrations of certain ARV compounds in anatomical compartments, thereby creating viral sanctuary sites where inadequate viral suppression may lead to the development of drug resistant viral variants, or facilitate persistent infections.

The most well understood viral sanctuary site is the CNS, which is protected by the blood-brain barrier (BBB) and the blood-cerebrospinal fluid (CSF) barrier (BCSFB) in order to prevent harmful xenobiotics from entering and damaging the nervous system. The BBB is made up of both a physical barrier created by tight junctions between neighboring brain microvessel endothelial cells, as well as a biochemical barrier made up of specialized drug transporters expressed on the surface of these cells, whereas the BCSFB is represented mainly by the choroid plexuses. The presence of the BBB and the BCSFB makes it difficult for simple diffusion of ARV compounds into the nervous system, and only drugs that are of a low molecular weight, uncharged, and have a high lipid solubility are capable of crossing the barrier by passive diffusion. In order to better understand the link between ARV CNS penetration and virological response, the CNS-penetration-effectiveness (CPE) parameter was created and calculated for common ARV compounds based on a combination of extensive literature reviews, measured CSF drug concentrations, and physicochemical properties of the drug (Letendre et al., 2008). The CPE parameter classifies ARV compounds into either low-CNS penetration (rank 0),
intermediate CNS penetration (rank 0.5), or high-CNS penetration (rank 1), and found that lower CPE rankings were associated with an 88% increase in the likelihood of viral RNA detection in the CSF, suggesting that inadequate ARV penetration into the CNS may be responsible for continued viral replication in this compartment despite effective suppression in plasma. Furthermore, phylogenetic studies comparing viral variants isolated from the CSF and those isolated from plasma indicate compartmentalization occurring in the CNS is likely due in part to poor penetration of ARV compounds creating a distinct pharmacological compartment and independently selecting for drug resistant viral variants (Canestri et al., 2010; Cunningham, Smith, Satchell, Cooper, & Brew, 2000; Smit et al., 2004). Lastly, studies assessing viral RNA levels in post-mortem CNS tissue samples isolated from patients with an undetectable plasma viral load indicate that there is continued viral replication in the CNS compartment compared to plasma (Langford et al., 2006).

The lymphatic organs are also believed to be a major sanctuary site for persistent HIV-1 infections. Only 2% of lymphocytes are in circulation at any given time, while the majority remain associated with the spleen, the lymph nodes, and the gut-associated lymphatic tissues, making the lymphatic organs a major site of viral infection and persistence. Recently, follicular dendritic cells present in the follicles of secondary lymphoid tissues have been implicated as mediators of persistent infections, stably sequestering and archiving viral particles on their surface and facilitating infection of CD4 T-cells. Studies investigating ARV-treated macaques have revealed that simian immunodeficiency virus (SIV) RNA can be detected in the spleen and lymph nodes even though the animals had undetectable plasma viral RNA (Horiike et al., 2012). Furthermore, detectable viral DNA and RNA have been found in multiple lymph nodes in
macaques infected with a chimeric SIV carrying HIV reverse transcriptase, despite successful plasma viral suppression in these animals using EFV, TFV, and FTC (Kline et al., 2013). These persistent infections are likely to be caused in part due to low drug penetration into the lymph nodes. It has been demonstrated previously that PIs display variable penetration into the lymph nodes of HIV-1 infected patients, with indinavir (IDV) demonstrating a two-fold increase in lymph node concentration compared to plasma, whereas nelfinavir (NFV) and LPV displayed lymph node to plasma concentration ratios of 0.58 and 0.21 respectively (Solas et al., 2003). Studies in macaques infected with SIV and treated with AZT/3TC/IDV found that 3TC concentrations in lymph nodes were significantly lower than that found in peripheral blood mononuclear cells (PBMCs) and was associated with persistent viral replication (Bourry et al., 2010). Lastly, recent studies assessing ARV penetration in the lymph nodes of HIV-1 infected subjects found a large decrease in TFV-DP, FTC-TP, ATV, DRV and EFV concentrations in lymph node tissues compared to PBMCs, and that this decrease correlate with continued viral replication, corroborating results seen in macaque models (Fletcher et al., 2014).

Together, it is clear that low drug penetration into key anatomical compartments where HIV-1 resides is a major contributing factor to persistent infections. In terms of optimizing ARV therapy, attention should not only be focused on drug penetration and viral suppression in the blood, but it is also necessary to understand the extent to which ARV compounds distribute into tissues and the factors that govern this process.
1.4 Role of drug transporters and drug metabolic enzymes in ARV tissue disposition

Tissue and cellular drug concentrations depend on a dynamic interplay between passive transport via diffusion, active and facilitated transport mediated via drug efflux and uptake transporters, and drug metabolism by metabolic enzymes (Fig. 1-3). Drug transporter proteins and drug metabolic enzymes are widely expressed throughout the body and play a crucial role in regulating drug disposition into tissues. For example, the expression of the efflux transporter P-gp on the apical surface of enterocytes is a likely contributing factor to the poor oral bioavailability observed in patients given ATV (Kis, Zastre, Hoque, Walmsley, & Bendayan, 2013). Generally, drug transport proteins are categorized as primary active transporters dependent on ATP hydrolysis, and secondary or tertiary active transporters, which rely on pre-established ionic gradients to drive transport activity (Su, Mruk, & Cheng, 2011). There are two main groups of drug transporters that interact with ARV drugs, the ATP-binding cassette (ABC) transporters, which act primarily as efflux transporters moving substrates against their concentration gradient via ATP hydrolysis, and the solute-carrier (SLC) transporters, which act primarily as secondary or tertiary uptake transporters. In regards to drug metabolism, most ARV drugs are oxidatively metabolized through the cytochrome P450 (CYP450) pathway, while a few are conjugated through the UDP-glucuronosyltransferase (UGT) family.
Figure 1-3. The balance of passive and active drug influx and efflux activity combined with drug metabolism processes determines the degree of drug permeability across the cell membrane.
1.4.1 ATP-binding cassette transporters

The ABC transporter superfamily are ATP dependent membrane-associated transport proteins and function primarily to efflux substrates extracellularly against a concentration gradient (Klaassen & Aleksunes, 2010). Members of this superfamily are known to be expressed at key tissue barriers such as the blood-brain barrier, blood-intestine barrier, and the blood-testes barrier and are believed to play a significant role in regulating the pharmacokinetics of their substrates (Schinkel & Jonker, 2003; Weiss & Haefeli, 2010).

The most well studied ABC transporter is permeability glycoprotein (P-gp), which is encoded by the \textit{MDRI} gene in humans and is widely distributed with high expression levels in the small intestine, liver, kidney and brain (Kis et al., 2010; Schinkel & Jonker, 2003). Substrates for P-gp are diverse but are generally amphipathic organic molecules ranging from less than 200Da to 1900Da (Schinkel & Jonker, 2003). \textit{In vitro} studies with P-gp overexpressing cell lines have established that NRTIs and PIs can act as both substrates and inhibitors of P-gp (Fujimoto, Higuchi, & Watanabe, 2009; Kim et al., 1998; Lee et al., 1998; Michaud et al., 2012; Storch, Theile, Lindenmaier, Haefeli, & Weiss, 2007), NNRTIs generally only inhibit P-gp activity (Lade, Avery, & Bumpus, 2013; Michaud et al., 2012; Moss et al., 2013; Storch et al., 2007; Weiss & Haefeli, 2013), whereas FIs and INSTIs act as P-gp substrates (Brown et al., 2009; Michaud et al., 2012; Reese et al., 2013) (Table 1-4). In the brain, P-gp is expressed at high levels in the brain microvessel endothelial cells (Ronaldson, Persidsky, & Bendayan, 2008; Weiss & Haefeli, 2010), which are a major component of the blood-brain barrier. \textit{In vivo} studies in P-gp knockout mice have demonstrated increased accumulation of ARVs in the brain (Kim et al., 1998; Robillard et al., 2014; Ronaldson et al., 2008), indicating the critical role that P-gp can
play in determining how well certain ARV penetrate into the CNS. Furthermore, there has been some evidence that also suggest P-gp is expressed on the surface of astrocytes and microglia, where they are involved ARV efflux from these cells (Lee, Schlichter, Bendayan, & Bendayan, 2001; Ronaldson & Bendayan, 2006; Ronaldson, Dallas, & Bendayan, 2004), potentially contributing to their role as alternative reservoirs for persistent HIV-1 infection. Expression of P-gp has also been demonstrated in PBMCs (Chaudhary, Mechetner, & Roninson, 1992; Klimecki, Futscher, Grogan, & Dalton, 1994), and its expression appears to relate to the activation status of these cells (Köck et al., 2007). Functional studies investigating P-gp expression on the surface of PBMCs have also demonstrated that they are capable of limiting intracellular penetration of PIs in vitro (Ford et al., 2004; Jones et al., 2001), providing some evidence that intracellular accumulation of certain ARV compounds may not reflect that measured in plasma. Lastly, P-gp expression has been demonstrated on the placental trophoblast (Camus et al., 2006), and could play a role in limiting fetal exposure to ARVs. Studies have demonstrated that inhibiting P-gp activity in human placenta resulted in increased movement of saquinavir (SQV) across the placenta (Mölsä et al., 2005). In vivo studies in P-gp knockout mice have also demonstrated that fetuses in knockout animals experienced higher exposure to SQV compared to wild-type animals (Smit, Huisman, van Tellingen, Wiltshire, & Schinkel, 1999).
Table 1-4. Relevant ABC transporter interactions with ARV drugs

<table>
<thead>
<tr>
<th>Transporter Name</th>
<th>ARV Class</th>
<th>Substrate</th>
<th>Inhibitor</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>P-gp</td>
<td>NRTIs</td>
<td>ABC, TDF, 3TC, ZDV</td>
<td>ABC, TDF</td>
<td>Kim et al, 1998; Bleasby et al, 2006; Storch et al, 2007; Michaud et al, 2012</td>
</tr>
<tr>
<td></td>
<td>NNRTIs</td>
<td>DLV, ETV, NVP, RPV</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>PIs</td>
<td>ATV, DRV, IDV, LPV, NFV, RTV, SQV, TPV</td>
<td>ATV, DRV, LPV, NFV, RTV, SQV, TPV</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Fls</td>
<td>MVC</td>
<td></td>
<td></td>
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<tr>
<td></td>
<td>INSTIs</td>
<td>RAL, DTG</td>
<td></td>
<td></td>
</tr>
<tr>
<td>MRP1</td>
<td>NRTIs</td>
<td>FTC</td>
<td>FTC, TFV, ABC, 3TC,</td>
<td>Bleasby et al, 2006; Weiss et al, 2007; Michaud et al, 2012</td>
</tr>
<tr>
<td></td>
<td>NNRTIs</td>
<td>EFV, NVP</td>
<td></td>
<td></td>
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<tr>
<td></td>
<td>PIs</td>
<td>ATV, IDV, LPV, NFV, RTV, SQV</td>
<td>RTV</td>
<td></td>
</tr>
<tr>
<td>MRP2</td>
<td>NRTIs</td>
<td>FTC, ABC, TFV, 3T,</td>
<td>Huisman et al, 2002; Bleasby et al, 2006; Weiss et al, 2007; Michaud et al, 2012</td>
<td></td>
</tr>
<tr>
<td></td>
<td>NNRTIs</td>
<td>EFV, NVP</td>
<td></td>
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<tr>
<td></td>
<td>PIs</td>
<td>ATV, RTV, SQV, IDV</td>
<td></td>
<td></td>
</tr>
<tr>
<td>MRP4</td>
<td>NRTIs</td>
<td>ABC, 3TC, TFV, ZDV</td>
<td></td>
<td>Schuetz et al, 1999; Bleasby et al, 2006; Ray et al, 2006; Michaud et al, 2012</td>
</tr>
<tr>
<td>BCRP</td>
<td>NRTIs</td>
<td>ABC, DDI, 3TC, d4T, ZDV,</td>
<td>ABC</td>
<td>Wang et al, 2003; Bleasby et al, 2006; Weiss et al, 2007; Pan et al, 2007; Michaud et al, 2012</td>
</tr>
<tr>
<td></td>
<td>NNRTIs</td>
<td>EFV</td>
<td>EFV, RPV</td>
<td></td>
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<tr>
<td></td>
<td>PIs</td>
<td>ATV, LPV, NFV, RTV, SQV</td>
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<td></td>
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<tr>
<td></td>
<td>INSTIs</td>
<td>DTG, RAL</td>
<td>EVG</td>
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</table>
Another key ABC transporter subfamily relevant to ARV therapy is the multidrug-resistance proteins (MRPs). In general, substrates for the MRPs include amphiphilic organic anions between 300 and 1000Da in molecular mass, although substrate specificity could be broadened to organic cations, or other anionic compounds in the presence of reduced glutathione (Keppler, 2011). In terms of ARV therapy, MRP1, 2, and 4 are the most well understood and are believed to be the primary MRPs involved in ARV transport (Table 1-4). MRP1 protein expression has been detected in a variety of human tissues and cell types, with the highest expression levels found in the lungs, testis, kidney, skeletal and cardiac muscles, placenta, as well as macrophages (Keppler, 2011; Kis et al., 2010). MRP2 expression has also been extensively characterized and it consistently demonstrates localization to the apical membrane of polarized cells such as the kidney proximal tubules, small intestines, colon, and placenta (Keppler, 2011). Lastly, MRP4 expression has been characterized in different tissues with varying membrane localization depending on cell type (Russel, Koenderink, & Masereeuw, 2008). For example, MRP4 has been found on the basolateral membrane of the glandular epithelial cells of the prostate gland (Lee, Klein-Szanto, & Kruh, 2000), whereas in the proximal tubule epithelial cells of the kidneys, it localizes to the apical membrane (van Aubel, Smeets, Peters, Bindels, & Russel, 2002). In vitro studies using MRP overexpressing cell lines have demonstrated interactions between MRP isoforms and commonly used PIs and NNRTIs, and N(t)RTIs (Table 1-4). MRP 1 and MRP2 are involved predominantly in the transport of PIs, but can also be inhibited in a concentration dependent manner by N(t)RTIs and NNRTIs (Huisman et al., 2002; König & Herzog, 2010; Michaud et al., 2012; Van Der Sandt et al., 2001). On the other hand, nucleoside analogues are the primary substrates for MRP4 (Michaud et al., 2012; Ray et al., 2006; Reid et al., 2003; Schuetz et al., 1999), and some studies have also indicated that MRP4 along with MRP2 may be
involved in the transport of their phosphorylated metabolites (Anderson et al., 2011; Anderson, Lamba, Aquilante, Schuetz, & Fletcher, 2006; Paintsil et al., 2011). However, the lack of an MRP specific inhibitor, combined with overlapping substrate specificity has made it difficult to delineate the precise role of each isoform. Nevertheless, in vitro studies using primary cultures of human brain microvessel endothelial cells (HBMECs) co-cultured with primary astrocytes have indicated a potential role for MRPs in limiting ATV penetration across the BBB (Bousquet et al., 2008). Furthermore, inhibition of MRP activity in an in vitro lymphocyte cell line has been demonstrated to increase intracellular accumulation of LPV (Janneh, Jones, Chandler, Owen, & Khoo, 2007). Altogether, there is strong evidence to suggest that MRP isoforms could play a critical role in modulating ARV drug penetration in tissues and cell types where they are expressed and more work is necessary to elucidate their effect on ARV therapy.

BCRP is a drug transport protein originally discovered based on its overexpression in a chemotherapy resistant breast cancer cell line (Doyle et al., 1998; Schinkel & Jonker, 2003) and it displays high expression levels in the small intestines and liver, as well as key tissue and cell types important in HIV-1 infection such as the HBMECs, astrocytes, microglia and PBMCs (Kis et al., 2010; Schinkel & Jonker, 2003; Weiss & Haefeli, 2010). BCRP is unique to other ABC transporters because its protein product only forms half of the functional transporter and it needs to homodimerize for activity (Kage et al., 2002). Like P-gp, in vitro studies have demonstrated that BCRP interacts with multiple commonly used ARV compounds (Table 1-4). Its primary substrates include several NRTIs and INSTIs, and recently has been shown to be capable of transporting EFV (Michaud et al., 2012; Pan, Giri, & Elmquist, 2007; Peroni et al., 2011; Wang et al., 2003), while PIs act as inhibitors (Weiss et al., 2007). In vivo studies in a Bcrp-knockout
mouse model demonstrated a moderate increase in brain penetration of abacavir compared to the wild-type mice and BCRP likely acts in concert with P-gp and MRPs also expressed at the BBB to limit ARV penetration (Giri et al., 2008). In the placenta, studies using an *in situ* rat placenta model also demonstrated that BCRP plays a role in limiting maternal-to-fetal transport of TDF (Neumanova, Cerveny, Ceckova, & Staud, 2014). Lastly, BCRP expression has been demonstrated on the surface of PBMCs isolated from healthy volunteers, and incubation with non-specific inhibitors of BCRP indicates that its functional expression contributes in part to decreased SQV accumulation in PBMCs (Janneh et al., 2005). Altogether, it is clear that BCRP acts in synergy with other major drug efflux transporters in modulating ARV penetration into key tissues and cell types relevant to HIV-1 infection. However, more work is necessary utilizing either BCRP-specific substrates, or BCRP-specific inhibitors to evaluate its contribution to ARV permeability.

### 1.4.2 Solute-carrier transporters

The SLC transporter superfamily consists primarily constituted of secondary or tertiary active transporters that rely on pre-established ionic gradients to mediate the influx and efflux of a wide variety of substrates (Klaassen & Aleksunes, 2010). Members of this superfamily relevant to ARV therapy include the organic anion transporting polypeptides (OATPs), the organic anion transporters (OATs), the organic cation transporters (OCTs), the concentrative nucleoside transporters (CNTs), and the equilibrative nucleoside transporters (ENTs).

The OATP subfamily is the largest of the SLC transporters, and 11 members have been identified in humans so far (Kalliokoski & Niemi, 2009). In general, OATPs are responsible for...
the influx of a variety of substrates that include many common therapeutic compounds such as statins, ARVs, and multiple anti-cancer drugs, but also many endogenous compounds such as bile acids, thyroid hormones, prostaglandins, and bilirubin glucuronide (Shitara et al., 2013). The most well understood and ARV therapy relevant OATP isoforms are OATP1A2, 1B1, 1B3, and 2B1. These isoforms are primarily expressed in the intestine, liver and kidneys, where they play a major role in absorption and clearance of their substrates, but have also been shown to be present at key tissues and cell types relevant to HIV-1 infection such as the BBB and on the surface of immune cells (Kis et al., 2010; Minuesa et al., 2011; Shitara et al., 2013). In vitro studies with OATPs indicate that they primarily interact with PIs (Table 1-5). In particular, PIs are substrates for OATP1A2, 1B1, and 1B3, but can also inhibit the activities of OATP 1B1, 1B3, and 2B1 (Annaert, Ye, Stieger, & Augustijns, 2010; Hartkoorn & Kwan, 2010; Michaud et al., 2012). More recent studies with newly approved ARV compounds have also indicated that MVC is a substrate OATP1B1, while elvitegravir (EVG) and RPV act as inhibitors (Custodio et al., 2014; Weiss & Haefeli, 2013), suggesting that substrate specificity for the OATPs may extend beyond PIs. Most studies examining functional expression of OATPs thus far have focused on characterizing its role in intestinal absorption and hepatobiliary excretion of xenobiotics. For example, a study investigating the absorption of celiprolol (an OATP2B1 substrate) in healthy volunteers indicated that an OATP2B1 polymorphism associated with decreased transport activity resulted in a decrease in celiprolol plasma AUC (Ieiri et al., 2012), and inhibition of OATP1A2 in HepG2 cells resulted in decreased accumulation of SQV (Su, Zhang, & Sinko, 2004). Studies investigating the role of OATPs in mediating ARV penetration into HIV-1 sanctuary sites and cellular reservoirs are limited. A non-significant trend towards a higher DRV CSF concentration was observed in patients carrying an OATP1A2 decreased
function polymorphism (Calcagno et al., 2012), suggesting OATP1A2 expression at the BBB could have an effect on PI penetration into the CNS. Lastly, inhibition of OATP activity in a CD4+ T-cell culture system was able to decrease the intracellular accumulation of SQV and LPV (Janneh et al., 2008). Overall, there is evidence to suggest that expression of OATP isoforms relevant to ARV therapy has the potential to influence ARV disposition into key tissue sanctuaries and cellular reservoirs for HIV-1 infection, but more work is necessary to demonstrate this effect directly and in in vivo systems.
### Table 1-5. Relevant SLC transporter interactions with ARV drugs

<table>
<thead>
<tr>
<th>Transporter Name</th>
<th>ARV Class</th>
<th>Substrate</th>
<th>Inhibitor</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>OATP1A2</td>
<td>PIs</td>
<td>DRV, LPV, SQV</td>
<td></td>
<td>Hartkoorn et al, 2010; Michaud et al, 2012</td>
</tr>
<tr>
<td></td>
<td>NNRTIs</td>
<td>PV</td>
<td>RPV</td>
<td>Campbell et al, 2004; Annaert et al, 2010; Hartkoorn et al, 2010; Michaud et al, 2012</td>
</tr>
<tr>
<td></td>
<td>PIs</td>
<td>DRV, LPV, SQV</td>
<td>DRV, LPV, SQV, IDV, ATV, RTV</td>
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<tr>
<td></td>
<td>Fls</td>
<td>MVC</td>
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<tr>
<td></td>
<td>INSTIs</td>
<td>EVG</td>
<td></td>
<td></td>
</tr>
<tr>
<td>OATP1B1</td>
<td>PIs</td>
<td>ATV, IDV, RTV, SQV</td>
<td></td>
<td>Kis et al 2010; Annaert et al, 2010; Michaud et al, 2012</td>
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<tr>
<td>OATP2B1</td>
<td>PIs</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>OAT1</td>
<td>NRTIs</td>
<td>TFV, AZT, 3TC, DDI, ddC</td>
<td>TFV, ZDV, 3TC, DDI, d4T</td>
<td>Cihlar et al, 2007; Burckhardt, 2012; Michaud et al, 2012; Nagle et al, 2013</td>
</tr>
<tr>
<td></td>
<td>INSTIS</td>
<td>RAL</td>
<td>RAL</td>
<td></td>
</tr>
<tr>
<td>OCT1</td>
<td>NRTIS</td>
<td>3TC, TFV/TDF, ddC, AZT</td>
<td>ABC, FTC, 3TC, TDF, ddC, ZDV</td>
<td>Zhang et al, 2000; Jung et al, 2008; Minuesa et al, 2009; Michaud et al, 2012</td>
</tr>
<tr>
<td></td>
<td>Fls</td>
<td></td>
<td>IDV, NFV, RTV, SQV</td>
<td></td>
</tr>
<tr>
<td>CNT1</td>
<td>NRTIS</td>
<td>ddC, AZT, d4T</td>
<td></td>
<td>Yao et al, 2001; Minuesa et al, 2011</td>
</tr>
<tr>
<td>ENT2</td>
<td>NRTIS</td>
<td>AZT, DDI, ddC</td>
<td>AZT, DDI, TFV</td>
<td>Yao et al, 2001; Minuesa et al, 2011; Klein et al, 2013</td>
</tr>
</tbody>
</table>
Organic anion transporters (OATs) belong to the \textit{SLC22} subfamily and are primarily expressed in the renal proximal tubules, where they function primarily in the renal elimination of both endogenous and exogenous compounds, although some isoforms are also expressed in the liver, placenta, and choroid plexus (Burckhardt, 2012; Roth, Obaidat, & Hagenbuch, 2012). There are six OAT isoforms presently found in humans, although only the OAT1, and OAT3 isoforms have been demonstrated to be substantially involved in the transport of N(t)RTIs. N(t)RTIs can also inhibit transport activity (Table 1-5) (Burckhardt, 2012; Michaud et al., 2012; Nagle, Wu, Eraly, & Nigam, 2013; Ray et al., 2006). Organic cation transporters (OCTs) also belong to the \textit{SLC22} subfamily and unlike the OATs, are primarily a liver specific transporter, but can also be found in the kidneys, brain, and placenta (Nies, Koepsell, Damme, & Schwab, 2011; Roth et al., 2012). Three isoforms of OCTs have been identified in humans so far. N(t)RTIs act as both substrates and inhibitors of OCT1, 2, and 3 (Leung & Bendayan, 2001; Michaud et al., 2012; Minuesa et al., 2009), whereas PIs have been demonstrated to inhibit the activity of OCT1 and 2 (Jung et al., 2008; Michaud et al., 2012; Zhang et al., 2000). Data regarding the role that OATs and OCTs play in tissue and intracellular disposition of ARV compounds are limited. Nevertheless, the expression of OAT1 and three isoforms have been demonstrated in the choroid plexus (Alebouyeh et al., 2003), and studies using an \textit{in vitro} model of the blood-CSF barrier have indicated that OAT expression at the choroid plexus likely contributes to efflux of ZDV from the CSF by mediating uptake of ZDV into the choroid epithelium (Strazielle, Belin, & Ghersi-Egea, 2003). In CD4+ T cells isolated from HIV-infected patients, a strong correlation was found between intracellular 3TC and 3TC-TP concentrations and mRNA expression of OCT1 and 2, while co-incubation with PIs known to inhibit OCT activity resulted in a decrease in 3TC and 3TC-TP concentrations (Jung et al., 2013).
The concentrative nucleoside transporter (CNTs) and equilibrative nucleoside transporters (ENTs) belong to the SLC28 and SLC29 subfamilies respectively (Young, Yao, Baldwin, Cass, & Baldwin, 2013). CNTs are Na\(^+\)-dependent transporters with three known isoforms expressed in humans, primarily in the kidneys, liver and intestines (Young et al., 2013). CNT1 and 3 are the most well characterized isoforms in terms of their role in ARV drug transport. *In vitro* studies characterizing CNT transport activity have demonstrated that nucleoside analogues are substrates for both CNT1 and 3 (Minuesa et al., 2011; Yao et al., 2001). ENTs are Na\(^+\)-independent transporters and there are four known isoforms identified in humans. ENT1 and 2 are the isoforms most relevant to ARV therapy and can be found expressed ubiquitously throughout the body (Kato, Maeda, Akaike, & Tamai, 2005; Klein, Evans, et al., 2013; Young et al., 2013). Like the CNTs, several nucleoside analogues have been demonstrated as substrates for ENT1 and 2, and can also act as inhibitors of ENT transport activity (Klein, Evans, et al., 2013; Minuesa et al., 2011; Yao et al., 2001). Early evidence that nucleoside transporters could potentially play a role in ARV penetration across the BBB was demonstrated in a guinea pig brain perfusion model, where inhibition of ENT1 activity resulted in decreased uptake of didanosine into the brain (Gibbs, Jayabalan, & Thomas, 2003). More recently, a study examining nucleoside transporter expression in lateral ventricle choroid plexus tissue isolated from humans observed low-level gene expression of ENT1, 2, and CNT3, and found that uptake of inosine, a nucleoside transporter substrate, into the choroid plexus is mediated by ENT2 and CNT3 (Redzic, Malatiali, Grujicic, & Isakovic, 2010), suggesting that these transporters are functionally expressed and could contribute to ARV uptake into the CNS. In PBMCs isolated from healthy volunteers, intracellular accumulation assays using the model substrate uridine have revealed that ENT1 is functionally expressed on PBMCs (Minuesa et al., 2008). However, previous studies examining
ZDV uptake in PBMCs isolated from healthy volunteers and the Molt-4 T-lymphoblastic human cell line indicate that nucleoside transporter mediated uptake may represent only a small percentage of nucleoside uptake into lymphocytes (Purcet et al., 2006).

1.4.3 Metabolic Enzymes

Metabolic enzymes play a critical role in the metabolism, elimination, and detoxification of both endogenous and xenobiotic compounds. Most ARV compounds undergo phase I oxidative metabolism via the cytochrome P450 (CYP450) superfamily of metabolic enzymes. The CYP450 superfamily represents a diverse array of isoforms spread across 18 families and 44 subfamilies, with the CYP1, 2 and 3 families responsible for metabolizing most xenobiotic compounds (Zanger & Schwab, 2013). CYP450 isoforms are primarily expressed in the liver, intestines and to a smaller extent in the kidneys (Michaud et al., 2012). As seen in table 1-6, CYP3A4 and 3A5 are isoforms responsible for the majority of ARV drug metabolism and in vitro studies have demonstrated that they are capable of metabolizing nearly all PIs, as well as some NNRTIs, INSTIs, and FIs (Back, Sekar, & Hoetelmans, 2008; Brown et al., 2009; Chiba, Hensleigh, & Lin, 1997; Erickson et al., 1999; Hochman, Chiba, Yamazaki, Tang, & Lin, 2001; Koudriakova et al., 1998; Le Tiec, Barrail, Goujard, & Taburet, 2005; Michaud et al., 2012). Other isoforms such as CYP2B6, 2C9, 2C19 and 2D6 can also play a smaller role metabolizing PIs and or NNRTIs (Erickson et al., 1999; Koudriakova et al., 1998; Le Tiec et al., 2005; Li, Wang, Guo, & Ma, 2010; Siccardi et al., 2010; Ward, Gorski, Jones, Hall, & Flockhart, 2003; Xu & Desta, 2013). Of note, some ARV compounds are also known to undergo phase II conjugation reactions via the UDP-glucuronosyltransferase (UGT) pathway (Michaud et al., 2012). UGT pathway enzymes are divided into two main families, UGT1 and 2, and like the CYP450
enzymes are primarily expressed in the liver (Xu, Li, & Kong, 2005). So far, only the UGT1A1 and 2B7 isoforms are known to be involved in the metabolism of certain NRTIs, INSTIs, and NNRTIs (Barbier et al., 2000; Bélanger, Caron, & Harvey, 2009; Brown et al., 2009; Cottrell, Hadzic, & Kashuba, 2013; Le Tiec et al., 2005; Michaud et al., 2012). Extensive work has been carried out to understand the role of hepatic drug metabolic enzymes in modulating ARV pharmacokinetics in the blood plasma, especially how drug-drug interactions can both be advantageous (using ritonavir and colbicistat as pharmacoenhancers through their inhibitory effects on CYP450 isoforms (Larson, Wang, Delille, Otofokun, & Acosta, 2014)) and detrimental to ARV therapy (Kis et al., 2010). It is only in recent years that evidence has been found that extrahepatic CYP450 drug metabolism may play a role in determining tissue concentrations of xenobiotics, despite much lower metabolic enzyme expression outside of the liver. For example, using a radiolabelled inhibitor of CYP2B1 that inhibits the enzyme upon its metabolism, Miksys and Tyndale were able to demonstrate in an in situ rat model that the CYP2B1 isoform is metabolically active in the brain (Miksys & Tyndale, 2009). Furthermore, a recent study using a reserpinized rat model that was capable of producing dopamine only through a tyramine (CYP2D substrate) dependent pathway, found that inhibition of CYP2D resulted in a decrease in CNS dopamine levels and that the administration of exogenous tyramine increased CNS dopamine levels, suggesting that CYP2D is metabolically active in the CNS (Bromek, Haduch, Gołembiowska, & Daniel, 2011). These studies suggest that while extrahepatic drug metabolism may have a negligible effect on plasma drug concentrations, it could potentially play a role in altering tissue drug levels.
Table 1-6. Relevant CYP450 drug metabolic enzyme interactions with ARV drugs

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>ARV Class</th>
<th>Substrate</th>
<th>Inhibitor</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>PIs</td>
<td>ATV, DRV, IDV, LPV, NFV, RTV, SQV, TPV</td>
<td>DRV, RTV</td>
<td></td>
</tr>
<tr>
<td></td>
<td>FIs</td>
<td>MVC</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>INSTIs</td>
<td>DTG</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CYP2D6</td>
<td>PIs</td>
<td>NFV, IDV, RTV</td>
<td>DRV, RTV</td>
<td>Koudriakova et al, 1998; Li et al 2005; Brown et al 2009; Li et al, 2010</td>
</tr>
</tbody>
</table>

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1.5 Testis as an HIV-1 Sanctuary Site

1.5.1 HIV-1 infection in the testis

Detection of HIV-1 RNA in the seminal secretions isolated from HIV-1 infected subjects with undetectable viral RNA in plasma (Sheth et al., 2009), combined with the identification of seminal viral variants that are phylogenetically distinct from those in plasma (Anderson et al., 2010; Ghosn, Viard, et al., 2004) suggest that the male genital tract and its associated accessory organs could contribute to both HIV-1 transmission and/or persistent infection. In particular, several recent studies have focused on characterizing the nature of HIV-1 infections in the testicular compartment.

Due to the difficulties of obtaining testicular samples from HIV-1 infected human subjects, most studies on the testicular compartment have made use of an established SIV-infected cynomolgus macaque model that displays many of the same immunological disease progression characteristics as HIV-1 infections in humans, including the development of end-stage AIDS-like symptoms (Haigwood, 2004). Using this in vivo model, Le Tortorec et al. demonstrated that CD4 T-cells and macrophages in the testicular interstitium are infected shortly after intravenous inoculation during the acute stage of infection and remain infected throughout the asymptomatic stage (Le Tortorec et al., 2008). Similar studies using immunofluorescence imaging in pig-tailed macaques testes revealed colocalization of both αβTCR and CD68+ cells with SIV p27 (Shehu-Xhilaga et al., 2007), while in-situ real-time qPCR in asymptomatic HIV-1 infected subjects have also indicated that CD4 T-cells and macrophages are the primary targets for HIV-1 infection in the testes (Paranjpe et al., 2002). Furthermore, using an ex vivo organotypic model of
human testes cultured from HIV-1 infected individuals, HIV-1 infection was able to produce low levels of infectious virus particles, with the main virus-producing cells being resident testicular macrophages (Roulet et al., 2006). These data suggest that HIV-1 can productively infect the testes early during the acute stage of infection. However, infection in this compartment is unlikely to contribute to viral shedding in semen for several reasons. The testes harbor fewer numbers of HIV-1 target cells relative to other male genital tract (MGT) accessory organs such as the epididymis, prostate and seminal vesicles, which likely is a contributing factor to the low levels of infection observed in this compartment (Le Tortorec et al., 2008). Also, infected cells in the testes localize primarily to the testicular interstitium and likely are not able to cross into the seminiferous lumen (Le Tortorec et al., 2008; Roulet et al., 2006). Lastly, testicular secretions only account for approximately 10% of seminal volume as opposed to the 20 - 40% contributed by the prostate and 50 - 80% contributed by the seminal vesicles (Cao & Hendrix, 2007). Indeed, in studies examining vasectomized men, there was no difference observed in semen viral RNA levels between the vasectomized and unvasectomized men (Krieger et al., 1998). Nevertheless, viral DNA has been detected in the testis and recently viral DNA levels have been demonstrated to remain consistent despite effective ARV therapy in macaques infected with SIV (Matusali et al., 2015), suggesting that the testes are potentially capable of harboring long-term cellular reservoirs such as memory CD4 T-cells and contribute to persistent infection.

### 1.5.2 Structure of the testis

The testicular anatomical structure can be broken down into the seminiferous tubules, which are surrounded by the seminiferous epithelium and are the site for spermatogenesis, and the interstitial space between the tubules, where steroidogenesis occurs (Fig. 1-4) (Bronson, 2011).
The seminiferous epithelium is made up of columnar epithelial Sertoli cells that rise towards the center of the seminiferous lumen from the basal layer of myoid cells that form a ring around the outer circumference of each seminiferous tubule (Bronson, 2011; Dym & Fawcett, 1970). Immature spermatogonia start their maturation process at the outermost edge of the seminiferous epithelium, and as they mature, will move up in between the Sertoli cells towards the center of the seminiferous tubule until they detach from the Sertoli cells as mature spermatozoa (Russell, 1977). The Sertoli cells are a critical component of the seminiferous epithelium and function to create the blood-testes barrier (BTB), one of the tightest blood-tissue interfaces in the body (Cheng & Mruk, 2012). Unlike other blood-tissue barriers such as the blood-brain barrier (BBB) and the blood-retinal barrier (BRB), which are formed primarily by tight-junctions between neighbouring endothelial cells, the BTB is made up of specialized cell-cell junctions such as gap junctions, desmosomes, apical and basolateral ectoplasmic specialization protein complexes, in addition to tight-junctions between Sertoli cells (Cheng & Mruk, 2002, 2010, 2012; Su, Mruk, & Cheng, 2011), which are located at a distance from the capillaries that penetrate through the testicular interstitium. Furthermore, the BTB does not remain static like the BBB or the BRB, it is a dynamic barrier that needs to continuously form and reform as maturing spermatogonia pass in between neighbouring Sertoli cells (Mital, Hinton, & Dufour, 2011), all without breaking barrier integrity.
Figure 1-4. Cross-section of seminiferous tubule showing the major structural elements
The peritubular myoid cells as mentioned above, are smooth muscle cells that form a ring around the outside of each seminiferous tubule. These cells are primarily responsible for providing structural support to the Sertoli cells and the seminiferous epithelium and are capable of contractile action that generates a peristaltic wave propagating along the length of the seminiferous tubule (Maekawa, Kamimura, & Nagano, 1996). The myoid cells do not form a physical barrier to the seminiferous tubule, but can participate in cytokine signaling and express a number of growth and differentiation factors that are capable of modulating Sertoli cell activity and spermatogenesis, and also secrete extracellular matrix components to support seminiferous epithelium structural integrity (Fijak & Meinhardt, 2006; Maekawa et al., 1996; Skinner, McLachlan, & Bremner, 1989; Verhoeven, Hoeben, & De Gendt, 2000).

Lastly, the interstitial space is made up of capillaries and extracellular matrices, and primarily populated by the Leydig cells, as well as macrophages and lymphocytes (Fawcett, Neaves, & Flores, 1973). Leydig cells are endocrine cells that play a major role in the production of testosterone, which is critical to ensure normal spermatogenesis (Bartlett, Weinbauer, & Nieschlag, 1989; Lipsett et al., 1966). In addition to androgen production, Leydig cells also play a role in regulating the testicular immune response by influencing the number of testicular macrophages and lymphocytes circulating within the interstitium (Hedger & Meinhardt, 2000; Wang, Wreford, Lan, Atkins, & Hedger, 1994).

1.5.3 ARV penetration in the testis

The presence of the BTB creates a highly regulated microenvironment in the testes meant to protect developing germ cells from harmful xenobiotics, which could likely affect ARV
penetration into this tissue compartment and create a sanctuary site for HIV-1 infection. Expression of several ABC drug efflux transporters relevant to ARV therapy has been demonstrated throughout the testicular tissue. In particular, P-gp expression has been detected in Sertoli cells, Leydig cells, and peritubular myoid cells (Bart et al., 2004; Mruk, Su, & Cheng, 2011), MRP1 in both Sertoli cells and Leydig cells (Bart et al., 2004; Klein, Wright, & Cherrington, 2013), MRP4 in Sertoli cells (Klein et al., 2013), and BCRP has been detected in Sertoli cells, peritubular myoid cells, and in the testicular endothelial cells (Bart et al., 2004).

Several uptake transporters have also been detected in the testes, but it remains unknown whether SLC transporters relevant to ARV therapy are also present.

Studies examining functional expression of drug transporters in relation to ARV therapy are limited. However, recent work published by our laboratory using a triple knockout mice model lacking P-gp and BCRP (MDR1a<sup>−/−</sup>, and MDR1b<sup>−/−</sup>) demonstrated that the presence of these two drug efflux transporters was capable of limiting ATV penetration into the testicular tissue (Robillard et al., 2014). Another recent study investigating nucleoside uptake at the BTB also demonstrated that ENT1 was functionally expressed and capable of mediating the uptake of uridine in primary cultures of rat Sertoli cells, suggesting a potential pathway for nucleoside analogue uptake in the testes (Klein et al., 2013). Functional expression of MRP1 was demonstrated using a MRP1 knockout mouse model, which was unable to efflux the anticancer drug etoposide phosphate (a MRP1 substrate) from the seminiferous lumen, and resulted in the destruction of nearly all germ cells (Wijnholds et al., 1998). Together, these data suggest that drug transporters play a crucial role in modulating xenobiotic distribution throughout the
testicular tissue, and could have a significant impact on ARV drug penetration and HIV-1 treatment efficacy.
2 Rationale

The testicular compartment is believed to be a sanctuary site for persistent HIV-1 infection, due in part to poor ARV drug permeability. ARV drugs commonly used as part of HAART have shown a decreased seminal plasma concentration when compared with blood (Else, Taylor, Back, & Khoo, 2011). However, drug permeability in the testes cannot be accurately extrapolated from the seminal plasma drug concentration and no studies have quantified ARV concentrations in human testicular tissues. Furthermore, drug permeability into the testicular tissue depends on interactions between drug efflux transporters, drug influx transporters and drug metabolic enzymes expressed at key epithelial sites. Our laboratory and others have demonstrated the functional expression of ABC drug efflux transporters in the testes and at the blood-testes barrier in both rodent in-vitro and in-vivo models, as well as a primary cultures of human Sertoli cells (Robillard et al., 2014; Su, Cheng, & Mruk, 2009). However, few studies have examined the protein expression of SLC transporters and CYP450 metabolic enzymes relevant to ARV therapy in the testes of uninfected individuals and no studies have been performed to examine expression of these proteins in the testes of HIV-1 infected subjects.

The presence of drug transporters and metabolic enzymes both in the testes and at the blood-testis barrier could contribute to poor ARV drug penetration and provide a sanctuary site for HIV-1 in this tissue, where it could play a role in persistent infection. Therefore, it is critical to investigate the expression and localization of key drug transporters and metabolic enzymes in the testes and at the blood-testis barrier while simultaneously assessing ARV drug concentrations in testicular tissue to better understand ARV permeability in this complex but crucial compartment.
3  Goal

To determine the expression and localization pattern of drug transporters and drug metabolic enzymes in both uninfected and HIV-infected, treated subjects and examine whether their expression is associated with differences in ARV drug concentrations in the testes relative to blood.

4  Hypothesis

The expression of drug transporters and drug metabolic enzymes in the testes of HIV-1 infected subjects is associated with a decrease in the concentration of ARV drugs in the testes relative to blood.

4.1  Objectives

1. To quantify the mRNA and protein expression levels of drug transporters and drug metabolic enzymes in testicular tissue samples from uninfected and HIV-1 infected, treated subjects.
2. To determine the localization of both drug transporters and drug metabolic enzymes in testicular tissue samples from uninfected and HIV-1 infected, treated subjects.
3. To quantify ARV drug concentrations in plasma and testicular tissue extracts from HIV-1 infected, treated subjects.
5 Antiretroviral Drug Transporters and Metabolic Enzymes in the Human Testicular Tissue – Potential Contribution to HIV-1 Sanctuary Site

This manuscript has been submitted for publication.

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Writing of the Manuscript: Yiying Huang (preparation of draft, submission), Md. Tozammel Hoque (editorial review of the manuscript), Dr. Jenabian (editorial review of the manuscript), Dr. Fletcher (editorial review of the manuscript), Reina Bendayan (guidance for the preparation of the manuscript, overall editorial review of several drafts of the manuscript, manuscript submission)

Provided LCMS/MS equipment and technical expertise: Dr. Courtney Fletcher
5.1 Abstract

HIV-1 is capable of persistent infection in the testes, a proposed viral sanctuary site with potentially limited antiretroviral (ARV) penetration due to a naturally restrictive environment caused in part by the blood-testis barrier (BTB). We characterized 12 representative drug transporters and two metabolic enzymes in testicular tissue samples obtained from uninfected (N=8) and virally suppressed HIV-1 infected subjects on ARV therapy (N=5) undergoing elective orchiectomy for gender reassignment, and quantified ARV concentrations in plasma and testicular tissues from HIV-1 infected subjects. Our data demonstrate that key drug transporters and metabolic enzymes relevant to ARV therapy are present in the testes of both groups and localize primarily at the BTB. Furthermore, protease inhibitors that are substrates for ATP binding cassette membrane transporters display low testicular tissue penetration. Together, these data suggest the testes are a complex pharmacological compartment that can restrict the distribution of ARVs and potentially contribute to HIV-1 persistence.
5.2 Introduction

Current antiretroviral therapy (ART) utilizes a combination of different classes of antiretroviral drugs (ARV) simultaneously to treat HIV-1 infection and has been very effective at suppressing plasma viral replication (Maartens, Celum, & Lewin, 2014). However, ART remains unable to completely eradicate the virus as several studies have shown that viral rebound occurs following treatment cessation (Davey et al., 1999). The source of this persistent infection remains unclear, but it is believed that sub-therapeutic ARV concentrations in both cellular reservoirs such as CD4 memory T-cells, and tissue sanctuary sites such as the central nervous system (CNS), gastrointestinal and genitourinary tracts, can result in inadequate viral suppression and facilitate the evolution of drug resistant virus (Smith, Wightman, & Lewin, 2012).

The extent of ARV penetration into viral reservoirs and sanctuary sites depends on the physicochemical properties of the compound and a dynamic interplay between drug efflux, influx and metabolic processes (Cory, Schacker, Stevenson, & Fletcher, 2013). ARVs can be effluxed by ATP-binding cassette (ABC) membrane-associated transporters, influxed by solute-carrier (SLC) transporters and metabolized by cytochrome P450 (CYP450) enzymes (Kis et al., 2010; Walubo, 2007). Expression of these transporters and metabolic enzymes in cellular reservoirs such as CD4+ T-cells has been shown to alter intracellular ARV concentrations relative to plasma (Janneh et al., 2008; Janneh et al., 2005). Similarly, the presence of these proteins at key blood-tissue barriers such as the blood-brain barrier (BBB) has been shown to limit ARV penetration into the CNS (Kim et al., 1998; Robillard et al., 2014).
Several studies have suggested that the testes can be infected by HIV-1 early during acute infection, and remain infected during the asymptomatic stage (Le Tortorec et al., 2008; Roulet et al., 2006). Furthermore, the blood-testis barrier (BTB), formed by columnar epithelial Sertoli cells that line the seminiferous tubules, creates one of the tightest cellular barriers in the body (Cheng & Mruk, 2012). Previous studies performed by our group as well as others have demonstrated the functional expression of ABC transporters i.e., permeability glycoprotein (P-gp), multidrug-resistance protein 1 (MRP1), and breast cancer resistance protein (BCRP) in rodent and human Sertoli cell culture models (Bart et al., 2004; Klein, Wright, et al., 2013; Qian, Mruk, Wong, & Cheng, 2013; Robillard, Hoque, & Bendayan, 2012), that can result in low penetration of atazanavir (ATV), a protease inhibitor (PI), into rodent testicular tissues (Robillard et al., 2014). Together, these studies suggest that the testes could limit ARV penetration and act as a viral sanctuary site for HIV-1, and possibly contribute to persistent HIV-1 infection. However, few studies have thoroughly characterized the expression of drug transporters and metabolic enzymes in human testes and to the best of our knowledge, none have examined this expression in HIV-1 infected subjects. Herein, we have investigated the expression of ABC transporters (P-gp, MRP1, MRP2, MRP4, and BCRP), and SLC transporters (organic anion transporting polypeptides (OATPs) 1A2, 1B1, 2B1, organic anion transporter 1 (OAT1), organic cation transporter 1 (OCT1), concentrative nucleoside transporter 1 (CNT1), and equilibrative nucleoside transporter 2 (ENT2)) known to be involved in the disposition of several ARVs. In addition, we have also included in our study two major CYP450 metabolic enzymes (CYP3A4, and CYP2D6), which play a major role in the metabolism of ARV compounds. Our study provides rare insight into the expression and localization patterns of these key drug transporters and metabolic enzymes in testicular tissues obtained from uninfected
(N=8) and HIV-1 infected individuals (N=5) undergoing elective orchiectomy for gender reassignment. Furthermore, by combining the expression and localization data with ARV quantification data in plasma versus testicular tissues, we aimed to determine if there is an association between drug transporter and metabolic enzyme expression in human testes, and ARV penetration in this tissue.
5.3 Materials and Methods

5.3.1 Sample Population

Study participants undergoing elective orchiectomy for gender modification were voluntarily recruited at the Metropolitan Centre of Plastic Surgery in Montréal, Canada. Study participants were eligible for the study if they were males 18 years or older and willing to give informed consent; undergoing a transgender sex change after having obtained legal agreement; with HIV-1 infection confirmed by immunoblotting and receiving effective ARV therapy with a plasma viral load below level of detection (<50 copies/mL) for at least 6 months (no restriction in CD4 T-cell count). Participants were excluded if they had recent acute illness (within the past three months); recent sexually transmitted infection; active cancer or an uncontrolled coagulation disorder. This study obtained ethical approval from the McGill University Health Centre Ethical Review Board. All study subjects provided written informed consent for participation in the study.

5.3.2 Tissue Processing

Blood and testicular samples were collected from all enrolled subjects. Two 8 mL blood samples were collected in EDTA tubes for ARV quantification from HIV-1 infected subjects prior to their next ARV dose post-surgery. Testicular tissue samples for both biochemical assays and ARV quantification were cut into small pieces, snap frozen in liquid nitrogen and stored at -80°C until study assessments.

5.3.3 Cell Culture

The HepG2 cell line was purchased from the American Type Culture Collection (ATCC, Manassas, VA). Transient transfection of the HEK293T cell line with pEF/Amp-OATP1B1 and -OATP1A2 vectors provided by Dr. Richard Kim (University of Western Ontario, London,
Canada) was completed using Lipofectamine (Invitrogen, Carlsbad, USA) according to manufacturer’s protocol.

All cell culture systems were maintained according to previously published protocols (Banerjee, Allen, & Bendayan, 2012; Robillard et al., 2012) at 37°C humidified 5% CO₂-95% air with fresh media replaced every 2-3 days. Cells were subcultured with 0.25% trypsin-EDTA upon reaching 80 – 90% confluency.

5.3.4 Total RNA Extraction, cDNA Synthesis and qPCR

Total RNA was isolated from testicular tissue samples using Trizol reagent (Invitrogen) according to the manufacturer’s protocols and treated with 1U/mL DNaseI to remove contaminating genomic DNA. RNA concentration (absorbance at 260nm), and purity (absorbance ratio 260/280nm) was assessed using a DU Series 700 Scanning UV/VIS Spectrophotometer (Beckman Coulter, Brea, USA). We then used 2µg of total RNA for reverse transcription to cDNA using a high-capacity reverse transcription cDNA kit (Applied Biosystems, Waltham, USA) according to manufacturer’s instructions. The reverse transcription reaction was initiated at 25°C for 10 min, followed by 37°C for 120 min and then 85°C for 5 min.

The mRNA expression levels of drug transporters and metabolic enzymes were assessed using TaqMan primers designed and validated by Life Technologies (supplemental Table A1) and analysed by quantitative real-time PCR on a Mastercycler ep Realplex 2S thermal cycler (Eppendorf, Eppendorf, Germany) using TaqMan qPCR chemistry. All reactions were performed in triplicate with each 20µL reaction containing 200ng of cDNA, 1µL of 20X primer mix and
10μL of TaqMan qPCR mastermix. The expression level of each gene of interest was presented as normalized RNA expression levels (arbitrary units) relative to the human glyceraldehyde 3-phosphate dehydrogenase (GAPDH) housekeeping gene using the ΔCt method, where ΔCt is equal to the Ct value of the gene of interest minus the Ct value of GAPDH and the normalized value is equal to $2^{-\Delta Ct}$.

5.3.5 Immunoblot analysis

Immunoblotting was performed as described previously (Robillard et al., 2012). Briefly, whole tissue lysates were extracted from frozen testicular samples using a modified radioimmunoprecipitation lysis buffer and manually homogenized by passing it repeatedly through 20G needles in a microfuge tube, followed by a 1-hour incubation on ice. The samples were then centrifuged at 20,000X g for 10 minutes at 4°C, and the supernatant protein concentration was quantified via a Bradford assay (Bio-rad, Hercules, USA) before being aliquoted, and snap frozen in liquid nitrogen until ready for immunoblot analysis.

Proteins from the tissue lysate samples were separated on a 10% SDS-polyacrylamide gel and transferred onto a polyvinylidene fluoride membrane. The membranes were blocked for two hours at room temperature in 5% skim milk–Tris-buffered saline containing 0.1% Tween 20 and incubated overnight with primary antibody (supplemental Table A2). The blots were then incubated with corresponding horseradish peroxidase-conjugated secondary antibodies (1:5000 dilution). Signals were enhanced using a SuperSignal West Pico chemiluminescence system (Thermo-Fisher Scientific, South San Francisco, USA) and detected by exposure to X-ray film. Quantitative comparisons were made using densitometric analysis with the AlphaDigiDoc RT2 software.
5.3.6 Localization of Selected Drug Transporters and Metabolic Enzymes

Tissue sectioning and staining was performed by the Pathology Research Program at the University Health Network (Toronto, ON). Briefly, a testicular tissue sample from an uninfected and an HIV-1 infected, treated individual were fixed in 10% neutral buffered formalin for 48 hours and fixed in paraffin wax before it was sectioned (8µm) and mounted on to glass slides. Primary antibodies for the proteins of interest (see supplemental Table A2) were used to probe the mounted sections along with the appropriate corresponding fluorescently labelled secondary antibody (see Fig. 5-5 legend). Standard DAPI staining was used to identify the cell nuclei. Negative control slides (supplemental Figs. A2A-C) stained with only secondary antibodies were used to verify primary antibody signal specificity. Confocal microscopy work was completed at the Advanced Optical Microscopy Facility at the Toronto MARS Discovery Tower using a LSM700 confocal microscope (Zeiss, Oberkochen, Germany). Acquired images were processed using the Zen LE browser (Zeiss).

5.3.7 ARV Quantification in Plasma and Testicular Tissue

Testicular tissue lysates and plasma samples from HIV-infected, treated subjects were analysed in the Antiviral Pharmacology Laboratory, University of Nebraska Medical Center according to previously validated methods (Fletcher et al., 2014). Briefly, ARV concentrations in plasma were quantified directly, and testicular tissue concentrations were quantified from tissue homogenates. ATV, DRV, RTV, EFV, TFV, 3TC, and FTC were extracted from testicular tissue and plasma using 70:30 methanol/water, mixed with 13C internal standards (IS), and proteins were precipitated using an acetonitrile preparation step. To analyse tenofovir-diphosphate (TFV-DP), emtricitabine-triphosphate (FTC-TP), and lamivudine-triphosphate (3TC-TP) in testicular tissue,
the phosphorylated drug was isolated from interferences, metabolites, and unphosphorylated drug using an ion-exchange solid-phase extraction. Phosphorylated drug was then desalted with a reversed-phase extraction, dried and reconstituted before analysis.

Final sample extracts were separated and quantified using a Shimadzu Nexera ultra high-performance liquid chromatograph attached to an AB Sciex 5500 qTrap mass spectrometer. Ion pairs (TFV/FTC/3TC/ATV/DRV/RTV, positive; EFV, negative) were monitored in multiple reaction-monitoring mode. Analyte peaks were normalized to the corresponding IS peak, with the exception of EFV, for which no IS was available and was instead normalized to the FTC-IS. The linear range of quantification for each analyte was 2 – 400 fmol total mass on column, with the exception of ATV, which was 0.2 – 40 fmol total mass on column. To compare between plasma and testicular tissue concentrations in ng/mL, we assumed that 1g of tissue was equivalent to 0.96mL (Mardirossian et al., 1999) and that the volume of one cell was equivalent to 239.6fL (Fletcher et al., 2014).

5.3.8 Statistical Analyses

Statistical analysis was performed using GraphPad Prism 6 (GraphPad Software, Inc., San Diego, USA). Comparisons between groups were performed applying the Mann–Whitney U non-parametric test. A value of $p < 0.05$ was considered to be statistically significant.
5.4 Results

5.4.1 Demographics of Study Participants

A total of thirteen participants were enrolled in this study (Table 5-1). All of the HIV-1 infected, treated subjects were virally suppressed with a plasma viral load <50 copies/mL. All study subjects were on hormone replacement therapy that was ceased six weeks prior to orchiectomy.
**Table 5-1. Patient Characteristics**

<table>
<thead>
<tr>
<th>Subject ID</th>
<th>Age</th>
<th>Viral load (copies/mL)</th>
<th>Antiretroviral Compounds</th>
<th>Other Medications</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>NNRTIs</td>
<td>NRTIs</td>
</tr>
<tr>
<td>HIV (-) Men N=8</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>40</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>4</td>
<td>44</td>
<td>-</td>
<td>-</td>
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<tr>
<td>5</td>
<td>40</td>
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<td>-</td>
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<tr>
<td>11</td>
<td>21</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td><strong>Median:</strong></td>
<td>40</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Range:</strong></td>
<td>(21-59)</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

| HIV (+) Men N=5 | | | | | | |
| 7              | 46  | <50                    | -            | -     | DRV, RTV | DTG | MVC | citalopram |
| 12             | 27  | <50                    | RPV          | FTC, TFV | - | - | - | cyproterone acetate*, folic acid*, estradiol*, levothyroxine*, estradiol* |
| 25             | n/a | <50                    | EFV          | ABC, 3TC | - | - | - | |
| 31             | 24  | <50                    | -            | 3TC, ZDV | DRV, RTV | RAL | - | |
| 39             | 47  | <50                    | -            | ABC, 3TC, TFV | ATV, RTV | - | - | |
| **Median:**    | 36.5 |                        |              |       |     |        |     |             |
| **Range:**     | (24-47) |                     |              |       |     |        |     |             |

**NNRTIs**: Non-nucleoside reverse transcriptase inhibitors; **NRTIs**: Nucleoside/nucleotide reverse transcriptase inhibitors; **PIs**: Protease inhibitors; **INSTIs**: Integrase strand transferase inhibitors; **FIs**: Fusion inhibitors; **DRV**: Darunavir; **RTV**: Ritonavir; **DTG**: Dolutegravir; **MVC**: Maraviroc; **RPV**: Rilpivirine; **FTC**: Emtricitabine; **TFV**: Tenofovir; **EFV**: Efavirenz; **ABC**: Abacavir; **3TC**: Lamivudine; **ZDV**: Zidovudine; **RAL**: Raltegravir; **ATV**: Atazanavir. ARV compounds listed in bold were included in our drug quantification studies. *Medications stopped at least 6 weeks prior to orchiectomy.*
5.4.2 Relative mRNA Expression of Drug Transporters and Metabolic Enzymes in the Testes of Uninfected and HIV-1 infected, Treated Subjects

We used TaqMan qPCR to analyse the expression of ABC transporters \( ABCB1 \) (P-gp), \( ABCC1 \) (MRP1), \( ABCC2 \) (MRP2), \( ABCC4 \) (MRP4), and \( ABCG2 \) (BCRP), SLC transporters \( SLCO1A2 \) (OATP1A2), \( SLCO1B1 \) (OATP1B1), \( SLCO2B1 \) (OATP2B1), \( SLC22A6 \) (OAT1), \( SLC22A1 \) (OCT1), \( SLC28A1 \) (CNT1), and \( SLC29A2 \) (ENT2) and metabolic enzymes \( CYP3A4 \) and \( CYP2D6 \) in the testes of both the uninfected and HIV-1 infected, treated subject groups. All genes analysed displayed interindividual differences in expression levels with no significant differences in the average gene expression levels between the two study groups (Fig. 5-1).

For the ABC transporters, \( ABCC1 \) demonstrated the highest expression levels relative to \( GAPDH \), \( ABCB1/MDR1 \), \( ABCC4 \) and \( ABCG2 \) showed moderate expression levels, while \( ABCC2 \) showed nearly undetectable expression levels (Fig. 5-1A). For the SLC transporters, we observed nearly undetectable mRNA expression levels for \( SLCO1B1 \), \( SLC22A6 \), and \( SLC29A2 \) relative to \( GAPDH \), low-level expression for \( SLCO1A2 \), and \( SLC22A1 \), and high expression of \( SLCO2B1 \) (Fig. 5-1B). We also observed low expression of \( CYP3A4 \) and nearly undetectable expression levels for \( CYP2D6 \) (Fig. 5-1C).
Figure 5-1. Relative mRNA expression of (A) ABC transporters, (B) SLC transporters and (C) drug metabolic enzymes in seven uninfected individuals and five HIV-1 infected, treated individuals assessed using quantitative real-time PCR. The results are expressed as mean relative mRNA expression +/- S.E.M. normalized to the housekeeping gene GAPDH. Differences between the two experimental groups were not statistically significant ($p > 0.05$).
5.4.3 Protein Expression of Drug Transporters and Metabolic Enzymes in the Testes of Uninfected and HIV-1 Infected, Treated Subjects

Our results demonstrate that ABC transporters P-gp (Fig. 5-2A), MRP1 (Fig. 5-2B), BCRP (Fig. 5-2D) were expressed at the protein level in testes with interindividual variability observed in the two study groups, while MRP4 (Fig. 5-2C) showed more consistent expression. Although ABCC2 gene expression was low, robust bands corresponding to MRP2 (supplemental Fig. A1A) were detected in both study groups.
Figure 5-2. Immunoblot analysis of ABC transporters P-gp (A), MRP1 (B), MRP4 (C), BCRP (D) in testicular tissue lysates from five uninfected and five HIV-1 infected, treated subjects. Protein lysates from cell lines overexpressing MDR1, MRP1, MRP4 were loaded in the first lane and used as positive controls (+) for their respective immunoblots while the breast cancer cell line MX100, known to overexpress BCRP, was used as the positive control for BCRP. β-actin was used as the loading control for each immunoblot. Differences between the two experimental groups were not statistically significant ($p > 0.05$).
SLC transporters OATP2B1 (Fig. 5-3A), OAT1 (Fig. 5-3B), CNT1 (Fig. 5-3C) and ENT2 (Fig. 5-3D) were expressed at the protein level in the testicular tissue and displayed interindividual variations in both study groups, whereas OATP1B1 (supplemental Fig. A1C) expression was more consistent. We did not detect protein expression of OATP1A2 (supplemental Fig. A1B), nor OCT1 (supplemental Fig. A1D) in the testicular tissues of either study groups, which correspond with our gene expression results indicating low expression of both transporters.

Lastly, despite nearly undetectable gene expression of both metabolic enzymes, we were able to detect protein expression of CYP3A4 (Fig. 5-4A) and CYP2D6 (Fig. 5-4B) in the testes and also observed interindividual variability for both enzymes in the study groups.

Overall, densitometric analysis comparisons between the uninfected and HIV-1 infected, treated patient groups revealed no significant differences in protein expression levels relative to actin for any of the studied drug transporters or metabolic enzymes (data not shown).
Figure 5-3. Immunoblot analysis of SLC transporters OATP2B1 (A), OAT1 (B), CNT1 (C), and ENT2 (D) in testicular tissue lysates from five uninfected and five HIV-1 infected, treated subjects. Protein lysates isolated from the MDCK-OATP2B1 cell line were used as the positive control (+) for OATP2B1, whereas HepG2 cell protein lysate was used as the positive control (+) for OAT1, CNT1, and ENT2. β-actin was used as the loading control for each immunoblot. Differences between the two experimental groups were not statistically significant (p > 0.05).

Figure 5-4. Immunoblot analysis of metabolic enzymes CYP3A4 (A) and CYP2D6 (B) in testicular tissue lysates from five uninfected and five HIV-1 infected, treated subjects. HepG2 cell protein lysate was used as the positive control (+) for both CYP3A4 and CYP2D6. β-actin was used as the loading control for each immunoblot. Differences between the two experimental groups were not statistically significant (p > 0.05).
5.4.4 Localization of Drug Transporters and Metabolic Enzymes in Testes of Uninfected and HIV-1 Infected, Treated Subjects

To gain further insight into how drug transporters and metabolic enzymes could modulate ARV disposition in this tissue, we used immunofluorescence confocal microscopy to assess drug transporter localization in the testis of uninfected and HIV-1 infected, treated subjects. Overall, we did not observe a difference in localization pattern for the drug transporters and metabolic enzymes included in this study between the two subject groups.

We found that P-gp (Fig. 5-5A), MRP1 (Fig. 5-5A), and MRP4 (supplemental Figs. A3, A4) localized near the basolateral side of the seminiferous epithelium and in the microvessel endothelial cells, similar to previous immunofluorescence and immunohistochemistry results in rat and human testes (Bart et al., 2004; Klein, Wright, et al., 2013). However, we identified MRP2 (supplemental Figs. A3, A4) localization in the testicular interstitium contrary to previous studies indicating the absence of MRP2 in human testicular tissue (Klein, Wright, et al., 2013). We also found that BCRP (Fig. 5-5A) localized towards the apical side of the seminiferous epithelium and also throughout the testicular interstitium and in endothelial cells, similar to the BCRP localization previously reported in rat testes (Qian et al., 2013).

For the SLC transporters, we found that OATP2B1 (Fig. 5-5B), OATP1B1, OAT1 (supplemental Figs. A3, A4), ENT2 (Fig. 5-5B), and CNT1 (Fig. 5-5B) localized at the seminiferous epithelium and throughout the testicular interstitium. However, we did not detect fluorescence from OATP1A2, and OCT1 (supplemental Figs. A3, A4), which reflects our immunoblotting results showing undetectable protein expression.
For CYP450 drug metabolic enzymes, we found that both CYP3A4 (Fig. 5-5C) and CYP2D6 (Fig. 5-5C) localized primarily in the seminiferous epithelium and testicular endothelium.
Figure 5-5. Immunofluorescence imaging of selected transporters in testicular fixed tissue sections from HIV-1 infected, treated subjects with representative images showing localization pattern of (A) ABC transporters, (B) SLC transporters, and (C) CYP450 metabolic enzymes. Tissue sections were stained with DNA dye, DAPI (blue), and examined by immunofluorescence using respective primary antibodies corresponding to our proteins of interest (green). Anti-Na+/K+ATPase-α1 (red) antibody was used as a marker for the plasma membrane. Cells were stained with Alexa Fluor-conjugated secondary antibodies 488/555 alone to verify the signal specificity of the primary antibodies (Supplemental Figs. S2A-C). Scale bar, 20µm.
5.4.5 ARV Quantification in Plasma and Testicular Tissue

We performed drug quantification analysis in both the plasma and testicular tissues of HIV-infected, treated subjects (Table 5-2). Overall, we observed similar plasma versus testicular concentrations for the nucleoside/nucleotide reverse transcriptase inhibitors (NRTIs) tenofovir (TFV), lamivudine (3TC) and emtricitabine (FTC), higher testicular concentrations for the protease inhibitor (PI) ritonavir (RTV), and lower testicular concentrations for the non-nucleoside reverse transcriptase inhibitor (NNRTI) efavirenz (EFV), the PIs atazanavir (ATV), and darunavir (DRV). Notably, we observed DRV concentrations in the testicular tissue were consistently lower than that observed in plasma, and fall below previously reported therapeutic levels (Panel on Antiretroviral Guidelines for Adults and Adolescents, 2015). NRTI concentrations in most HIV-1 infected, treated subjects reached plasma and testicular tissue concentrations approximating those previously reported in pharmacokinetic studies in plasma (Bazzoli et al., 2010). However, we observed large interindividual variability in 3TC plasma and testicular tissue concentrations, with one subject’s testicular 3TC concentration falling below its target therapeutic range. We also quantified the active phosphorylated metabolite of the NRTIs included in this study, which displayed testicular tissue concentrations above those previously reported in PBMCs isolated from virally suppressed, HIV-1 infected patients on a tenofovir disoproxil fumarate (TDF)/FTC-containing regimen (Adams et al., 2013), and in health subjects given 300mg of 3TC (Yuen et al., 2004).
### Table 5-2. Plasma and Tissue ARV Quantification Data

<table>
<thead>
<tr>
<th>Patient ID</th>
<th>Drug</th>
<th>Plasma (ng/mL)</th>
<th>Testes (ng/mL)</th>
<th>Testes:Plasma Ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>7</td>
<td>DRV</td>
<td>2043.70</td>
<td>396.59</td>
<td>0.19</td>
</tr>
<tr>
<td></td>
<td>RTV</td>
<td>48.90</td>
<td>389.29</td>
<td>7.96</td>
</tr>
<tr>
<td>12</td>
<td>TFV</td>
<td>61.70</td>
<td>44.71</td>
<td>0.72</td>
</tr>
<tr>
<td></td>
<td>TFV-DP</td>
<td>-</td>
<td>5.83</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>FTC</td>
<td>214.40</td>
<td>251.79</td>
<td>1.17</td>
</tr>
<tr>
<td></td>
<td>FTC-TP</td>
<td>-</td>
<td>691.63</td>
<td>-</td>
</tr>
<tr>
<td>25</td>
<td>3TC</td>
<td>207.80</td>
<td>147.10</td>
<td>0.71</td>
</tr>
<tr>
<td></td>
<td>3TC-TP</td>
<td>-</td>
<td>174.69</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>EFV</td>
<td>7448.90</td>
<td>1856.45</td>
<td>0.25</td>
</tr>
<tr>
<td>31</td>
<td>3TC</td>
<td>125.80</td>
<td>140.36</td>
<td>1.12</td>
</tr>
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<td></td>
<td>3TC-TP</td>
<td>-</td>
<td>108.80</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>DRV</td>
<td>2375.80</td>
<td>523.11</td>
<td>0.22</td>
</tr>
<tr>
<td></td>
<td>RTV</td>
<td>236.00</td>
<td>683.82</td>
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</tr>
<tr>
<td>39</td>
<td>TFV</td>
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<td></td>
<td>TFV-DP</td>
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<td>13.94</td>
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<tr>
<td></td>
<td>3TC</td>
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<td>3227.47</td>
<td>-</td>
</tr>
<tr>
<td></td>
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<td>1029.76</td>
<td>0.79</td>
</tr>
<tr>
<td></td>
<td>RTV</td>
<td>271.80</td>
<td>523.81</td>
<td>1.93</td>
</tr>
</tbody>
</table>

DRV: Darunavir; RTV: Ritonavir; TFV: Tenofovir; TFV-DP: Tenofovir-diphosphate; FTC: Emtricitabine; FTC-TP: Emtricitabine-triphosphate; 3TC: Lamivudine; 3TC-TP: Lamivudine-triphosphate; EFV: Efavirenz; ATV: Atazanavir.
5.5 Discussion

A major obstacle in the pharmacological treatment of HIV-1 infection is the presence of viral sanctuary sites, which are characterized by poor ARV permeability and could contribute to ineffective viral suppression, development of viral drug resistance, and persistent infection (Cory et al., 2013; Smith et al., 2012). The extent of ARV distribution into tissues is dependent on the drug’s physicochemical properties as well as the presence of drug transporters and metabolic enzymes as demonstrated in studies published by our lab and others at the BBB (Kim et al., 1998; Robillard et al., 2014). However, very limited data are available on the expression of drug transporters and metabolic enzymes relevant to ARV therapy in human testicular tissues and to the best of our knowledge, no one has investigated their expression in the context of HIV-1 infection.

In this study, we detected gene expression of ABC and SLC drug transporters in both uninfected and HIV-1 infected, treated subjects that generally reflected trends seen previously using pooled human testicular samples (Nishimura & Naito, 2005). We also detected protein expression and localization patterns of the ABC transporters P-gp, MRP1, MRP4, and BCRP in both of our experimental groups that largely paralleled our gene expression data as well as previously published data in uninfected human testes (Bart et al., 2004; Klein et al., 2013). Our study is also the first to provide a comprehensive survey of protein expression and localization data for SLC transporters relevant to ARV therapy (OATP1B1, OATP2B1, OAT1, CNT1, and ENT2) in the testes of both uninfected and HIV-1 infected, treated subjects, which could have a significant impact on ARV penetration into this compartment. For example, TFV is a known substrate of OAT1 (Ray et al., 2006), and its uptake by OAT1 at the basolateral membrane of the renal
proximal tubule cell has been proposed as a key step in TFV excretion (Nagle et al., 2011), demonstrating the key role that SLC transporters can play in mediating ARV transport. Although there have yet to be studies demonstrating the functional expression of SLC transporters in relation to ARV therapy in human testicular tissues, studies examining nucleoside transport in rat Sertoli cells have indicated that ENT1 and ENT2 can actively transport the pyrimidine nucleoside analogue uridine across the BTB (Klein et al., 2013), and studies examining the penetration of the contraceptive adjudin into rodent testes have shown direct evidence that drug influx transporters are required for its transport across the BTB (Su, Mruk, Lee, & Cheng, 2011).

We showed drug metabolic enzymes CYP3A4 and CYP2D6 are expressed at the protein level in the testicular tissues, and may contribute to local drug metabolism within the testes similar to that observed in the brain (Ferguson & Tyndale, 2011). Interestingly, we were able to detect protein expression for MRP2, OATP1B1, OAT1, CNT1, ENT2, CYP3A4, and CYP2D6 despite low to undetectable levels of gene expression for these drug transporters and metabolic enzymes, which suggest possible post-transcriptional modifications negatively affecting mRNA stability of these genes (Vogel & Marcotte, 2012).

Overall, we did not observe a significant difference between gene or protein expression levels of drug transporters and metabolic enzymes in our two experimental groups. There has been evidence that HIV-associated inflammation is likely responsible for the downregulation of efflux transporter expression via NFκB activation by proinflammatory cytokines in the brain (Ashraf, Ronaldson, Persidsky, & Bendayan, 2011). However, HIV-associated inflammation is unlikely to affect transporter and metabolic enzyme expression levels in the testes due to the immuno-privileged status of this tissue (Meinhardt & Hedger, 2011). The testes possess a naturally
attenuated inflammatory response as evidenced by the lack of an IL-1b and TNF response to LPS stimulation in rodents (O’Bryan et al., 2005). Recent studies in primate models of HIV-1 infection have also revealed a lack of leukocyte and neutrophil influx into the testicular tissue and observed little damage to the testicular architecture (Winnall et al., 2015), suggesting an attenuated inflammatory response to HIV-1 infection in the testes. Furthermore, the HIV-1 infected subjects included in our study were all on ART and virally suppressed, which should reduce HIV-associated inflammation. Indeed, in our previous study in the sigmoid colon, we observed a return to basal levels of protein expression of the ABC transporters P-gp, MRP1, MRP2 and MRP4 when comparing the uninfected to the HIV-1 infected, treated subjects (De Rosa et al., 2013).

Although we did not observe a significant difference between subject groups, we detected large interindividual variability for certain transporters and metabolic enzymes. Long-term ARV therapy has been reported to have the potential to modulate transporter expression in HIV-1 infected subjects via transcription factor activation (i.e. nuclear receptors) (Chan, Hoque, & Bendayan, 2013). ARV compounds are known to induce drug transporter and metabolic enzyme regulation by acting as ligands of nuclear receptors such as the pregnane-X receptor (PXR) (Zastre et al., 2009) and the constitutive androstane receptor (CAR) (Chan, Patel, Cummins, & Bendayan, 2013). However, gene expression studies on uninfected human testicular tissue samples have indicated that PXR and CAR display very low expression levels in the testicular tissue, and that the dominant nuclear receptors in the testes are the liver-X Receptors (LXRs) α and β, peroxisome proliferator-activated receptors (PPARs) α and γ, and glucocorticoid receptor (GR) (Nishimura, Naito, & Yokoi, 2004). Although these nuclear receptors have the potential to
modulate transporter and metabolic enzyme expression, it remains unknown whether ARV treatment can induce their activation and whether they are functionally expressed in the testicular tissue. In addition, factors unique to each individual subject such as smoking, diet, environmental toxin exposure (Chang & Waxman, 2006; Lamba et al., 2004; Molina-Molina et al., 2013), as well as ARV regimens, which can also contribute to nuclear receptor regulation of drug transporter and metabolic enzyme expression, are likely responsible for the variability we observed.

Our ARV quantification studies in the testicular tissues of HIV-1 infected, treated individuals revealed for the first time that nucleoside analogues, FTC, 3TC, and TFV penetrated effectively into the testicular tissue, while their phosphorylated metabolites displayed lower but still virally effective concentrations when compared to previous data in PBMCs (Adams et al., 2013; Yuen et al., 2004). This may reflect compartment differences in kinase activity and cellular activation states (Bazzoli et al., 2010). In addition, we observed low accumulation of EFV, as well as concentrations below therapeutic range for the PI, DRV, which may lead to selection and perpetuation of drug resistant virus (Ghosn et al., 2004), particularly for NNRTIs such as EFV that display a low genetic barrier to resistance. Despite the variability we observed between tissue and plasma concentrations of the PIs, RTV and ATV, concentrations of both compounds were within the therapeutic range (D’Avolio et al., 2014). However, DRV appeared to consistently display a testicular concentration below its target therapeutic range (Panel on Antiretroviral Guidelines for Adults and Adolescents, 2015). The low accumulation of EFV and sub-therapeutic DRV testicular tissue concentration may result in inadequate viral suppression, and lead to the selection and perpetuation of drug resistant virus (Ghosn et al., 2004).
Previous studies quantifying ARV concentrations in human seminal plasma found high accumulation of FTC (440%), 3TC (316% - 420%), and TFV (510%) and poor accumulation of EFV (undetectable – 9%) and DRV (9 – 11%) relative to blood plasma (Else et al., 2011), which reflect the concentration patterns we observed in testicular tissue. Favourable penetration of NRTIs into tissues and genital tract secretions are likely a result of their small molecular weight and low protein binding, which may be able to counteract their interactions with efflux transporters. Penetration of nucleoside analogues may also be enhanced by the expression of OAT1 we observed in the testes, which is a known SLC uptake transporter for TFV and 3TC (Burckhardt, 2012). On the other hand, the unfavourable physicochemical properties of certain PIs and NNRTIs such as DRV and EFV combined with their affinity for efflux transporters such as P-gp and BCRP can drastically limit their accumulation in the testes. Interestingly, we observed that RTV accumulation in testicular tissue was higher than in plasma, contrary to data from seminal plasma (Else et al., 2011), suggesting a possibility for local drug-drug interactions preventing efflux of RTV from the testicular tissue through inhibition of P-gp, MRPI or MRP2. Although our data suggest a potential association between decreased concentrations of certain ARVs and drug transporter expression in the testes, due to the relatively small number of subjects included in this study, our data should be interpreted as preliminary, and further studies are necessary to confirm these observations. Interestingly, studies on chemotherapy for childhood lymphoma relapse in the testes have indicated that methotrexate, which is a substrate for P-gp, MRPI, MRP4, and BCRP (Chan, Lowes, & Hirst, 2004), accumulates at 2- to 4-fold lower concentrations in the testicular interstitial space, and at 15- to 20-fold lower concentrations in the seminiferous tubule lumen compared to plasma (Riccardi, Vigersky, Barnes, Bleyer, &
Poplack, 1982). This provides further evidence of the potential role that ABC drug transporters can play in restricting drug penetration into the testes.

Overall, our data suggest the testes are a complex pharmacological compartment that has the potential to limit the entry of ARVs known to be substrates for ABC transporters. This knowledge could assist clinicians in selecting ARV treatment regimens that are more effective in suppressing viral replication in this tissue. Further work is necessary to better understand what role the testicular compartment plays in both ARV disposition and HIV-1 persistence.
Conclusions and Summary

A major obstacle in the pharmacological treatment of HIV-1 infections is the presence of viral sanctuary sites, which are characterized by poor ARV permeability and could contribute to ineffective viral suppression, development of viral drug resistance, and persistent infection (Cory et al., 2013; Dahl, Josefsson, & Palmer, 2010; Fletcher et al., 2014). The degree of ARV permeability into tissues is dependent on the drug’s physicochemical properties as well as the presence of drug transporters and drug metabolic enzymes. Our lab and others have studied extensively how the expression of drug efflux transporters pertaining to the ATP-binding cassette (ABC) superfamily at the blood-brain barrier can have a significant impact on the extent of ARV penetration into the brain (Ashraf, Robillard, Chan, & Bendayan, 2014; Chan, Patel, et al., 2013; Varatharajan & Thomas, 2009). However, few studies have examined the expression of drug transporters and drug metabolic enzymes relevant to ARV therapy in human testicular tissues and whether they are capable of altering drug penetration into the testes.

The first objective of this thesis was to determine both the gene and protein expression levels of ABC transporters, SLC transporters, and CYP450 metabolic enzymes relevant to ARV therapy in the testicular tissue of both uninfected and HIV-1 infected, treated subjects. We found that the testicular tissues express many of the drug transporters and metabolic enzymes known to play a role in modulating the absorption and elimination of common ARV compounds, and that there were no significant differences in expression levels between the uninfected and HIV-1 infected, treated subjects.

The second objective of this study was to determine the localization pattern of drug transporters and metabolic enzymes relevant to ARV therapy in the testicular tissue of both uninfected and
HIV-1 infected, treated subjects using immunofluorescence imaging. Our data revealed a distinctive localization pattern of drug transporters and metabolic enzymes primarily at the seminiferous epithelium, where the BTB is, but we also detected localization in the peritubular myoid cells, and the testicular endothelium. Overall, based on visual inspection, there were no significant differences in the localization pattern between testicular tissue sections from uninfected and HIV-1 infected, treated subjects.

Our last objective for this study was to quantify the ARV concentrations in testicular tissue and plasma to determine whether there is a difference in ARV tissue penetration. Most studies examining ARV penetration in the MGT have focused on seminal plasma drug quantification. However, seminal fluid is composed of secretions from several accessory glands along the MGT, and it is difficult to extrapolate tissue ARV concentrations simply from seminal plasma concentrations. In our study, we report for the first time data in humans indicating that nucleoside analogues penetrate effectively into the testicular tissue, whereas certain PIs and NNRTIs demonstrated decreased accumulation in the testes compared to plasma. Altogether, the data from our study and others suggest that ARV compounds with favorable physicochemical properties (unpolarized, low molecular weight) and that are not high-affinity substrates for efflux transporters will generally penetrate more effectively into the testicular tissue, reaching tissue concentrations approximating that observed in plasma.

In summary, the data we have presented in this study support our hypothesis that drug transporter and metabolic enzyme expression in the testes is associated with a decrease in ARV drug penetration into this compartment relative to blood. We have demonstrated that many of the ABC drug efflux transporters, SLC drug uptake transporters, and CYP450 metabolic enzymes
relevant to ARV therapy are expressed and localize to key functional areas in the testicular tissue. Furthermore, our drug quantification data suggests that PIs and NNRTIs that are substrates for efflux transporters display lower testicular tissue accumulation. Our findings confirm that the testes are a complex pharmacological compartment, which certainly has the capability of limiting ARV drug penetration and support the broader consensus that the testes could act as a sanctuary site for HIV-1 infection.
7 Study limitations and future directions

One of the key limitations in our study is the low number of samples we could include, since we were sampling from a very unique patient population. This means that the results we observed should be interpreted with caution, as they may not be as representative when extrapolated to a larger population. Furthermore, we were not able to account for environmental variables such as exposure to toxins, diet, and smoking, which could all have an impact on drug transporter and metabolic enzyme expression as discussed in Chapter 6.

For the immunofluorescence localization studies, we were able to clearly determine visually from the microscopy images where the testicular macrostructures are (spermatogenic tubules, seminiferous epithelium, interstitial space, testicular capillaries). However, we were unable to conclude with certainty exactly what cell type each drug transporter and metabolic enzyme localized to. This could potentially be an interesting area of exploration in the future as results from several other studies have also indicated that drug transporters are expressed at the level of the peritubular myoid cells, the Leydig cells, and the testicular endothelium (Bart et al., 2004; Cheng & Mruk, 2012; Klein et al., 2013). Specific markers for these cells are available and further immunofluorescence imaging studies using these cell-type specific markers would provide evidence that perhaps the biochemical barrier function of the BTB extends beyond the level of the Sertoli cells at the seminiferous epithelium. Further insight into the nature of xenobiotic transport at the seminiferous epithelium can also be gained by examining basolateral versus apical localization of drug transporters. Membrane localization of drug transporters on polarized cells such as the Sertoli cells can have a profound impact on the directionality of transport. For example, OATP2B1 is expressed on the basolateral side of hepatocytes, where it
serves to uptake substrates from circulation (Kullak-Ublick et al., 2001). Whereas it is expressed on the apical side of intestinal epithelial cells, where it serves to uptake its substrates from the intestinal lumen (Kobayashi et al., 2003). However, due to the complexity of the BTB and its dynamic nature, more work is required to first identify markers that are specific to the basolateral and the apical membranes of the Sertoli cell.

In terms of the drug quantification studies, it is important to keep in mind that the concentrations we observed likely represent slight overestimates of true testicular tissue ARV concentrations since we were not able to isolate and remove the testicular capillaries from the bulk tissue. Therefore, it is possible that trace amounts of drugs from blood plasma were included in the quantification studies when we prepared whole tissue homogenates.

Furthermore, our drug quantification studies reported total drug concentrations in plasma and testicular tissues, which includes both unbound and protein-bound drug. Only the free, unbound fraction of drug is capable of therapeutic activity, whereas the protein-bound fraction is rendered inert. Therefore, the degree of protein binding of each ARV compound we quantified in this study needs to be taken into account when evaluating virological efficacy in both plasma and testicular tissues. Generally, the N(t)RTIs TFV, FTC, and 3TC display favorable protein-binding characteristics (10%, 5%, and 5% protein-bound respectively) (Bazzoli et al., 2010), which indicates that the concentrations we report in our study likely represent a close approximation of the unbound fraction of these ARV compounds. On the other hand, EFV, ATV, DRV, and RTV display unfavourable protein-binding characteristics (99.5%, 86%, 94%, and 99% protein-bound respectively) (Bazzoli et al., 2010), which suggests that the unbound fraction of these compounds may be much lower than that we reported and may fall below therapeutic levels in the testicular
tissue. Given that this is a significant issue in many pharmacological studies assessing therapeutic concentrations of ARV compounds, several studies have been carried out to determine a protein-binding corrected therapeutic concentration for key ARV compounds such as EFV, DRV, and ATV (Boffito et al., 2011; Reddy et al., 2002). However, data remains lacking on other commonly used ARVs that display a high degree of protein binding.

In addition, since the nature of our study was observational, we could only conclude a correlation between drug transporter and metabolic enzyme expression and the degree of testicular ARV accumulation. Other factors such as passive transport processes, which depend in large part on the physicochemical properties of each ARV compound, as well as drug-drug interactions mediated by drug transporters and metabolic enzymes (Kis, Walmsley, & Bendayan, 2014), could both modulate how well a given ARV compound accumulates in the testicular tissue. Further functional studies are needed to delineate the contribution of drug transporters and metabolic enzymes to ARV penetration in the testes. Indeed, several studies have demonstrated functional expression of P-gp, MRP1, and BCRP in knockout animal models (Enokizono, Kusuhara, Ose, Schinkel, & Sugiyama, 2008; Uhr, Holsboer, & Müller, 2002; Wijnholds et al., 1998), and our lab has also published in vivo studies indicating that mice lacking P-gp and BCRP accumulate ATV to a higher level in the testicular tissue than wild type animals (Robillard et al., 2014). Yet, few studies have examined the functional expression of SLC transporters relevant to ARV therapy in Sertoli cells. If these transporters are functionally active in the testes, the data we have presented in this study suggests that SLC transporters could play a major role as uptake transporters or modulate ARV testicular penetration via drug-drug interactions.
Lastly, due to our limited sample size, we were unable to investigate whether the testicular ARV accumulation pattern we observed had any effect on viral dynamics in this compartment. To this end, quantifying viral RNA levels in the testicular tissues of HIV-1 infected subjects that are virally suppressed would indicate whether ARV treatment is truly effective in the testes. A similar study has been recently published using an \textit{in vivo} chronically infected, ARV-treated cynomolgus macaques model that indicates ARV treatment is capable of decreasing viral RNA load in the testicular tissue (Matusali et al., 2015). However, this study only included a single treatment regimen, and it remains unclear whether the complex treatment combinations used in the clinic will be equally as suppressive in the testicular tissue. In addition to concerns that low drug permeability in the testes could lead to ineffective viral suppression, there is also a concern that altered ARV permeability in the testes could also lead to the development of drug-resistant viral variants. Therefore, future studies could also be conducted to examine resistance characteristics in viral populations isolated from the testicular tissue to see if they are reflective of tissue ARV concentrations.
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9 Appendices

9.1 Appendix A - Supplemental Tables & Figures

Table A1. Primers used for TaqMan qPCR gene expression analysis.

<table>
<thead>
<tr>
<th>Protein Name</th>
<th>Gene of Interest</th>
<th>Assay ID</th>
</tr>
</thead>
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<tr>
<td>P-gp</td>
<td>ABCB1</td>
<td>Hs00184500_m1</td>
</tr>
<tr>
<td>MRP1</td>
<td>ABCC1</td>
<td>Hs01561502_m1</td>
</tr>
<tr>
<td>MRP2</td>
<td>ABCC2</td>
<td>Hs00166123_m1</td>
</tr>
<tr>
<td>BCRP</td>
<td>ABCG2</td>
<td>Hs01053790_m1</td>
</tr>
<tr>
<td>OATP1A2</td>
<td>SLCO1A2</td>
<td>Hs00366488_m1</td>
</tr>
<tr>
<td>OATP1B1</td>
<td>SLCO1B1</td>
<td>Hs00272374_m1</td>
</tr>
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<td>OATP2B1</td>
<td>SLCO2B1</td>
<td>Hs01030343_m1</td>
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<td>OAT1</td>
<td>SLC22A6</td>
<td>Hs00537914_m1</td>
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<tr>
<td>OCT1</td>
<td>SLC22A1</td>
<td>Hs00427552_m1</td>
</tr>
<tr>
<td>CNT1</td>
<td>SLC28A1</td>
<td>Hs00984403_m1</td>
</tr>
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<td>ENT2</td>
<td>SLC29A2</td>
<td>Hs00155426_m1</td>
</tr>
<tr>
<td>CYP3A4</td>
<td>CYP3A4</td>
<td>Hs02576167_m1</td>
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<tr>
<td>CYP2D6</td>
<td>CYP2D6</td>
<td>Hs02576167_m1</td>
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Table A2. Antibodies used in western blot and immunofluorescence imaging experiments

<table>
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<th>Antibody Name</th>
<th>Antibody Type (Clone)</th>
<th>Experiments Used</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anti-P-gp</td>
<td>Mouse Monoclonal (C219)</td>
<td>WB</td>
</tr>
<tr>
<td>Anti-P-gp</td>
<td>Mouse Monoclonal (D11)</td>
<td>IFC</td>
</tr>
<tr>
<td>Anti-MRP1</td>
<td>Rat Monoclonal (MRPr1)</td>
<td>WB, IFC</td>
</tr>
<tr>
<td>Anti-MRP2</td>
<td>Mouse Monoclonal (M2III-6)</td>
<td>WB, IFC</td>
</tr>
<tr>
<td>Anti-MRP4</td>
<td>Rat Monoclonal (M4I-80)</td>
<td>WB, IFC</td>
</tr>
<tr>
<td>Anti-BCRP</td>
<td>Rat Monoclonal (BXP-53)</td>
<td>WB, IFC</td>
</tr>
<tr>
<td>Anti-SLC01A2</td>
<td>Rabbit Polyclonal</td>
<td>WB, IFC</td>
</tr>
<tr>
<td>Anti-SLC01B1</td>
<td>Rabbit Polyclonal</td>
<td>WB, IFC</td>
</tr>
<tr>
<td>Anti-SLC02B1</td>
<td>Rabbit Polyclonal</td>
<td>WB, IFC</td>
</tr>
<tr>
<td>Anti-SLC22A6</td>
<td>Rabbit Polyclonal</td>
<td>WB, IFC</td>
</tr>
<tr>
<td>Anti-SLC22A1</td>
<td>Rabbit Polyclonal</td>
<td>WB, IFC</td>
</tr>
<tr>
<td>Anti-CNT1</td>
<td>Rabbit Polyclonal (H-70)</td>
<td>WB, IFC</td>
</tr>
<tr>
<td>Anti-SLC29A2</td>
<td>Rabbit Polyclonal</td>
<td>WB, IFC</td>
</tr>
<tr>
<td>Anti-CYP3A4</td>
<td>Mouse Polyclonal</td>
<td>WB, IFC</td>
</tr>
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<td>Anti-CYP2D6</td>
<td>Rabbit Polyclonal</td>
<td>WB, IFC</td>
</tr>
<tr>
<td>Anti-β-Actin</td>
<td>Mouse Monoclonal (C4)</td>
<td>WB</td>
</tr>
<tr>
<td>Anti-Na+/K+-ATPase</td>
<td>Rabbit Polyclonal (H-300)</td>
<td>IFC</td>
</tr>
<tr>
<td>Anti-Na+/K+-ATPase</td>
<td>Mouse Monoclonal (M7-PB-E9)</td>
<td>IFC</td>
</tr>
</tbody>
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Supplemental Figure A1. Immunoblot analysis of ABC transporter MRP2 (A) and SLC transporters OATP1A2 (B), OATP1B1 (C), and OCT1 (D) in testicular tissue lysates from five uninfected and five HIV-1 infected, treated subjects. Protein lysates isolated from the transfected MDCKII-MRP2, HEK-OATP1A2, and HEK-OATP1B1 cell lines were used as the positive controls (+) for MRP2, OATP1A2 and OATP1B1 respectively, whereas HepG2 protein lysate was used as the positive control (+) for OCT1. B-actin was used as the loading control for each immunoblot.
Supplemental Figure A2. Representative negative control imaging slides from both an uninfected and an HIV-infected, treated individual demonstrating staining with only DAPI and (A) Alexa Fluor-conjugated mouse 488 and rabbit 555 secondary antibodies (B) Alexa Fluor-conjugated rabbit 488 and mouse 555 secondary antibodies and (C) Alexa Fluor-conjugated rat 488 and rabbit 555 secondary antibodies.
**Supplemental Figure A3.** Immunofluorescence imaging of drug transporters and metabolic enzymes in testicular tissue sections from uninfected subjects with representative images showing (A) P-gp, (B) MRP1, (C) MRP2, (D) MRP4, (E) BCRP, (F) OATP1A2, (G) OATP1B1, (H) OATP2B1, (I) OAT1, (J) OCT1, (K) CNT1, (L) ENT2, (M) CYP3A4, and (N) CYP2D6 localization. The tissue sections were stained with DNA dye, DAPI (blue), and examined by immunofluorescence using respective primary antibodies corresponding to our proteins of
interest (green). Anti-Na+/K+ATPase-α1 (rabbit polyclonal red) antibody was used as a marker for the plasma membrane. Cells were stained with Alexa Fluor-conjugated secondary antibodies 488/555 alone to verify the signal specificity of the primary antibodies (Supplemental Figure A2A-C). Scale bar, 20μm.
Supplemental Figure A4. Immunofluorescence imaging of drug transporters and metabolic enzymes in testicular tissue sections from HIV-1 infected, treated subjects with representative images showing (A) MRP2, (B) MRP4, (C) OATP1A2, (D) OATP1B1, (E) OAT1, and (F) OCT1 localization. The tissue sections were stained with DNA dye, DAPI (blue), and examined by immunofluorescence using primary antibodies corresponding to our proteins of interest (green). Anti-Na+/K+ATPase-α1 (rabbit polyclonal red) antibody was used as a marker for the plasma membrane. Cells were stained with Alexa Fluor-conjugated secondary antibodies 488/555 alone to verify the signal specificity of the primary antibodies (Supplemental Figure A2A-C). Scale bar, 20µm.
9.2 Appendix B - Methods not included in Chapter 5

9.2.1 Laser Microdissection of Testicular Tissues

Laser microdissection experiments were performed on an MMI Cell Cut microscope at the Advanced Optical Microscopy Facility at the Toronto MARS Discovery Tower. A sample of frozen, uninfected testicular tissue was sectioned at 8µm thickness and mounted onto MMI membrane slides. Tissue sections were fixed in 70% ethanol and stained with hematoxylin and eosin for enhanced contrast. Fixed and stained slides were kept frozen at -80°C until ready for microdissection. RNA isolation was completed using first a Trizol (Invitrogen) method following manufacturer’s protocol, or a RNA prep column purification kit (PicoPure RNA Isolation Kit, ABI).

9.3 Appendix C – Data not included in Chapter 5

9.3.1 mRNA and Protein Expression of ABC Transporters, SLC Transporters and Drug Metabolic Enzymes in PBMCs from Uninfected Men

We also attempted to quantify both mRNA and protein expression of selected drug transporters and drug metabolic enzymes in PBMCs isolated from uninfected individuals. We found that \textit{ABCC1}(MRP1) displayed the highest expression levels in PBMCs, whereas \textit{MDR1}(P-gp), \textit{ABCC4}(MRP4), and \textit{CYP3A4} displayed moderate expression, and \textit{ABCC2}(MRP2), \textit{ABCG2}(BCRP), \textit{SLCO1A2}(OATP1A2), \textit{SLCO1B1}(SLCO1B1), \textit{SLCO2B1}(SLCO2B1), \textit{SLC22A6}(OAT1), \textit{SLC22A1}(OCT1), and \textit{SLC28A1}(CNT1) displayed low to undetectable gene mRNA expression (Fig. C1).
**Figure C1.** Relative mRNA expression of ABC transporters, SLC transporters, and drug metabolic enzymes in PBMC samples from four uninfected subjects assessed using quantitative real-time PCR. The results are expressed as mean relative mRNA expression +/- S.E.M. normalized to the housekeeping gene GAPDH. n.d.: not detected.
Due the limited amount of PBMC samples, we were not able to analyze gene expression for *SLC29A2* (ENT2) and *CYP2D6*. Furthermore, we were not able to increase the amount of cDNA used in the qPCR reaction from 10ng to the more optimized 200ng we used to generate our tissue gene expression results due to sample quantity limitations. However, our results follow the general trend reported in previous gene expression studies of ABC and SLC transporters in pooled peripheral lymphocytes (Nishimura & Naito, 2005).

On the other hand, our immunoblotting experiments were neither able to detect any corresponding expression of P-gp nor MRP4, and we did not detect protein expression of BCRP (Fig. C2). We suspect that immunoblotting may not be sensitive enough to detect drug transporter expression, as seen in a similar study investigating P-gp protein expression in lymphocytes (Manceau et al., 2010). Most studies publishing data on drug transporter protein expression in lymphocytes report the use of fluorescence activated cell sorting (FACS), which is likely a more sensitive detection method better suited to PBMCs (Liptrott, Khoo, Back, & Owen, 2008).
Figure C2. Western blot analyses of ABC transporter protein expression in the PBMCs of seven uninfected subjects. Positive controls corresponding to each protein of interest (+) can be found in the first lane of each blot.
9.3.2 Laser microdissection of testicular tissue

To support our immunofluorescence imaging data and further validate the localization patterns we observed, we conducted pilot experiments using laser microdissection on an uninfected testicular tissue sample to see if we can isolate specific testicular anatomic structures for downstream gene expression analysis. In order to best preserve mRNA stability, we prepared tissue sections from snap frozen testicular samples and proceeded with a simple 70% ethanol fixation and H&E staining procedure for visualization. Figure C3 shows representative images taken from the LMD microscope both before cutting (Fig. C3A) and after (Fig. C3B). We could clearly visualize the testicular macrostructure, primarily the seminiferous tubules, but were not able to resolve individual cell types. Therefore, we chose to isolate the basolateral side of the seminiferous tubules, as this is the key functional area where the Sertoli cells localize.

We attempted two different procedures to optimize RNA yield and quality. In the first experimental procedure (Fig. C4A), we tried to collect as much tissue as possible onto a single adhesive cap, collecting a total of 389135µm² of tissue. Subsequently, we tried to isolate RNA using our standard Trizol RNA isolation procedure but were not able to yield anything usable. We suspected that by trying to maximize tissue collection, we were leaving the tissue sample at room temperature for nearly 30 minutes and allowing RNA to degrade excessively. Therefore, our second experimental procedure (Fig. C4B) emphasized sample preservation as we limited dissection time to no more than 10 minutes before removing the dissected tissue (collected on adhesive microfuge caps) and placing them back on dry ice. Afterwards, we pooled the dissected tissue samples together and used a column RNA purification and extraction kit referenced in
previously published methods (Espina et al., 2006). However, we were still unable to gather any RNA yield.
Figure C3. Representative LMD images before (A) and after (B) laser dissection.
Figure C4. Schematic of experimental setup using single sample collection and Trizol RNA isolation (A), and pooled sample collection and PicoPure RNA isolation kit (B).