Role of P-glycoprotein in the distribution of the HIV-protease inhibitor, atazanavir, in the brain and male genital tract.

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Abstract

The blood-testis barrier and blood-brain barrier are responsible for protecting male genital tract and central nervous system from xenobiotic exposure. In HIV-infected patients, low concentrations of antiretroviral drugs in cerebrospinal fluid and seminal fluid have been reported. One mechanism that may contribute to reduced concentrations is the expression of ATP-binding cassette drug efflux transporters such as P-glycoprotein (P-gp). The objective of this study was to investigate in vivo, tissue distribution of the HIV-protease inhibitor, atazanavir, in wild-type mice (WT-mice), P-gp/breast cancer resistance protein (Bcrp) knockout (Mdr1a\(^{-/-}\), Mdr1b\(^{-/-}\), Abcg2\(^{-/-}\)) mice (TKO-mice) and Cyp3a\(^{-/-}\) knockout mice (Cyp-mice).

WT-mice and Cyp-mice were pre-treated with a P-gp/Bcrp inhibitor elacridar (5 mg/kg) and the HIV-protease inhibitor and boosting agent, ritonavir (2 mg/kg i.V.), respectively. Atazanavir (10 mg/kg) was administered i.V. Plasma (C_{plasma}), brain (C_{brain}) and testes (C_{testes}) atazanavir concentrations were quantified at various times by LC-MS/MS.

In TKO-mice, we demonstrated a significant increase in atazanavir C_{brain}:C_{plasma} (5.4-fold) and C_{testes}:C_{plasma} (4.6-fold) ratios compared to WT-mice (p<0.05). Elacridar-treated WT-mice showed a significant increase in atazanavir C_{brain}:C_{plasma} (12.3-fold) and C_{testes}:C_{plasma} (13.5-fold) ratios compared to vehicle-treated WT-mice, respectively. In Cyp-mice pre-treated with ritonavir, significant (p<0.05) increases in atazanavir C_{brain}:C_{plasma} (1.8-fold) and C_{testes}:C_{plasma} (9.5-fold) ratios compared to vehicle-treated WT-mice were observed.

These data suggest that drug efflux transporters i.e., P-gp, are involved in limiting atazanavir permeability in rodent brain and genital tract. Since these transporters are known to...
be expressed in humans, they could contribute to the low cerebrospinal and seminal fluid antiretroviral concentrations reported in the clinic.

Introduction

The use of human immunodeficiency virus (HIV) protease inhibitors for the treatment of HIV infection remains a primary and effective component of combination antiretroviral therapy (1,2). Patients on antiretroviral therapy must maintain a high level of adherence in order to suppress viral replication and reduce the opportunity for HIV to develop drug resistance (3). Although the current use of combination antiretroviral therapy is capable of suppressing HIV replication in plasma, it is unable to completely eradicate the virus from the host (4). One of the challenges to achieve total HIV eradication is the existence of viral sanctuaries such as the brain and the male genital tract that are protected by the blood-brain barrier (BBB) (5) and blood-testis barrier (BTB), respectively (6). These sanctuaries sites allow HIV to evade eradication even if effective antiretroviral drug concentrations are achieved in plasma (7), and could contribute to the development of HIV-associated neurocognitive disorders (8), increased risk of viral shedding into seminal fluid of HIV infected men (9), and ultimately, serve as a potential source for systemic viral repopulation if a patient interrupts drug therapy (7).

The BBB constitutes a physical and biochemical barrier between the brain parenchyma and systemic circulation and is primarily composed of brain microvessel endothelial cells that form tight cell-cell junctions which significantly restricts paracellular permeability of xenobiotics into and out of the brain (10). The BTB is primarily composed of Sertoli cells that are epithelial
cells that form tight junctions near the basement membrane. These cells establish a specialized 
immune-privileged environment by physically and immunologically separating the blood from 
the seminiferous tubule compartment (11).

Atazanavir, a HIV protease inhibitor that is currently part of first-line combination 
antiretroviral therapy, is currently recommended to be administered with other classes of 
antiretroviral drugs such as the reverse transcriptase inhibitors to treat HIV infection in therapy-
naive patients (1). Since many HIV protease inhibitors are known to be extensively metabolised 
by the cytochrome P450 3A (CYP3A) enzyme family, they are generally co-administered with a 
the HIV protease inhibitor, ritonavir, which is also a potent inhibitor of CYP3A and serves as a 
pharmacoenhancer (12). Interestingly, studies conducted by Lorello et al. and Harrington et al. 
have demonstrated the presence of HIV RNA in semen and cerebrospinal fluid samples from 
HIV-infected patients on long-term (>3 years) combination antiretroviral therapy, respectively, 
despite undetectable viral loads in plasma (13,14). Clinical studies examining atazanavir 
pharmacokinetics in HIV-infected patients have reported a very poor permeability of atazanavir 
into the brain and testes compartments, as reflected by low median CSF: plasma ratios in the 
range of 0.008-0.02 (15) and semen: plasma ratios of 0.1 (16).

One mechanism that may contribute to the poor tissue permeability of HIV protease 
inhibitors observed clinically is the functional expression of ATP-binding cassette (ABC) 
membrane-associated drug efflux transporters i.e., P-glycoprotein (P-gp), multidrug resistance 
associated protein-1 (MRP1/Mrp1) and breast cancer resistance protein (BCRP/Bcrp) (17,18).
These transporters have been recognized to play an important role in protecting the brain and 
the testes from xenobiotic exposure (19-21). Our group and others, have previously
demonstrated using several cell culture systems of the BBB, the BTB, the gastrointestinal tract, as well as ABC transporter-overexpressing cell culture models, that atazanavir is a substrate of P-gp, a much weaker substrate of MRP1 and an inhibitor of BCRP (21-23).

A limited number of in vivo studies have investigated the role of P-gp in reducing the oral absorption and tissue distribution of antiretroviral drugs such as saquinavir, indinavir and nelfinavir (24); however, little is known on the role of P-gp and BCRP/Bcrp in the tissue distribution of atazanavir. The objective of this study was to investigate, in vivo, the role of P-gp and Bcrp on atazanavir tissue distribution in the brain and testes of wild-type mice (WT-mice) and P-gp/Bcrp (Mdr1a\(^{-/}\), Mdr1b\(^{-/}\), Abcg2\(^{-/}\)) triple knockout mice (TKO-mice). We also examined the effect of elacridar, a selective inhibitor of P-gp and Bcrp, on the accumulation of atazanavir in the brain and testes compartments of WT-mice. Finally, a Cyp3a knockout mouse model (Cyp-mice), which has eight Cyp3a genes deleted (Cyp3a11, Cyp3a13, Cyp3a16, Cyp3a25, Cyp3a41, Cyp3a44, Cyp3a57 and Cyp3a59), was used to further clarify if ritonavir (HIV protease inhibitor and CYP3A inhibitor) could inhibit P-gp and/or Bcrp and lead to an increase in atazanavir tissue accumulation.

Materials and Methods

Chemicals and Reagents

Atazanavir and ritonavir were a gift from the National Institutes of Health AIDS Research and Reference Reagent Program (Bethesda, MD). N-(4-[2-(1,2,3,4-tetrahydro-6,7-dimethyl-2-isoquinolinyl)ethyl]-phenyl)-9,10-dihydro-5-methoxy-9-oxo-4-acridine carboxamide (elacridar) was purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). 6, 7-Dimethyl-2,3-di(2-
pyridyl)-quinoxaline (DMDPQ), quantitative polymerase chain reaction primers, protease inhibitor cocktail and all LC-MS/MS and standard laboratory chemicals were purchased from Sigma-Aldrich (St.Louis, MO) and were of the highest purity. All cell culture reagents and media were purchased from Life Technologies (Grand Island, NY). ABI high-capacity reverse-transcriptase cDNA kit was purchased from Applied Biosystems (Foster City, CA). PerfeCTa SYBR Green FastMix was obtained from Quanta Biosciences Inc. (Gaithersburg, MD). The anti-P-gp antibody, C219, was purchased from ID Labs Inc. (London, ON, Canada). Anti-Mrp1 (Mrpr1) antibody was purchased from Kamiya Biomedical Company (Seattle, WA). The rat anti-Bcrp antibody (BXP-53) and mouse anti-actin antibodies were obtained from Abcam Inc. (Boston, MA). The goat anti-mouse and mouse anti-rat horseradish peroxidase-conjugated secondary antibodies were purchased through Jackson Immuno-Research Laboratories Inc. (West Grove, PA). Chemiluminescence SuperSignal West Pico System was obtained from Thermo Fisher Scientific (Waltham, MA).

Animal Models

WT-mice (FVB/NTac strain), TKO-mice (FVB/N7 strain) and Cyp-mice (FVB/192P2 strain) used in each experiment were adult male mice between 9 – 12 weeks of age purchased from Taconic Inc. (Hudson, NY). The TKO-mice model was selected to investigate the potential role of P-gp and Bcrp in the tissue distribution of atazanavir. The Cyp-mice model was chosen to examine the role of ritonavir as a P-gp/Bcrp inhibitor in the absence of Cyp3a-metabolism. All experiments, procedures and animal care were conducted in accordance to the Canadian Council on Animal Care guidelines and approved by the University of Toronto Animal Care.
Committee. Animals were housed under a 12-hr light/dark cycle at room temperature with free access to food and water.

Mouse Seminiferous Tubule Isolation

Seminiferous tubules were isolated from WT-mice or TKO-mice as previously described (25) with minor modifications. In brief, adult mouse testes were decapsulated and incubated in collagenase (0.5 mg/ml in Dulbecco’s Modified Eagle Media/Ham’s F12 nutrient mixture (1:1) at 35°C for 30 minutes. Seminiferous tubules were then washed five times by sedimentation under gravity to remove Leydig cells. All samples were frozen – 80°C for future analysis.

Total RNA extraction, cDNA synthesis and Quantitative Real-time Polymerase Chain Reaction (qPCR)

Total RNA was isolated from brain tissue, liver tissue and seminiferous tubules isolated from WT- and TKO-mice using TRIzol reagent (Invitrogen) according to manufacturer’s instructions. The concentration (absorbance at 260 nm) and purity (absorbance 260 nm / absorbance 280 nm ratio) of RNA samples were assessed using DU Series 700 UV/Vis Scanning Spectrophotometer (Beckman Coulter, Mississauga, ON, Canada). Isolated total RNA was subjected to DNAse I digestion (0.1 U/mL) to remove genomic DNA. Reverse transcription was then performed with DNAse-treated total RNA (2 µg) in a final reaction volume of 40 µl using an ABI high-capacity reverse transcription cDNA kit according to the manufacturer’s instructions (Applied Biosystems). All sample reactions were initiated at 25°C for 10 minutes, followed by 37°C for 120 minutes then 85°C for 5 minutes using Mastercycler ep Realplex 2S thermal cycler (Eppendorf Canada, Mississauga, ON, Canada).
The expression of genes encoding metabolic enzymes (Cyp3a11, Cyp2d22, Cyp2e1 and Ugt1a1) and the housekeeping gene peptidyl isomerase B (cyclophilin B) were analyzed by qPCR on a Mastercycler ep Realplex 2S thermal cycler using SYBR Green fluorescence detection as previously described by our group (Chan et al., 2011). The 10 µl final reaction volume contained 1.25 µL of diluted cDNA, 5 µl of PerfeCTa SYBR Green Fastmix (Quanta Biosciences), 0.6 µL of 1.25 µM concentrations of each primer (final concentration of each primer 150 nM), and 2.55 µL of nuclease-free water. Primers for each of the genes of interest and the housekeeping gene are listed in table 1 and primer specificity was determined using BLAST and dissociation curve analysis. For these experiments, threshold cycle (C₀) values for the gene of interest mRNA are normalized to the housekeeping gene cyclophilin B mRNA. Results of qPCR analysis of metabolic enzymes are presented as RNA level (arbitrary units) of each gene of interest normalized to the housekeeping gene Cyclophilin B, calculated using the efficiency-corrected ΔC₀ method, where C₀ is equal to the C₀ value of the sample minus the C₀ value of cyclophilin B, where the normalized RNA level is equal to 2−ΔΔC₀. Fold-change ± standard deviation (S.D.) between mRNA levels in TKO-mice and WT-mice tissues were calculated using the comparative C₀ method (ΔΔC₀) (26, 27).

Immunoblot Analysis

Immunoblotting was performed as described previously (21, 28) with minor modifications. In brief, whole tissue or isolated seminiferous tubules lysates were prepared by homogenization in modified radioimmunoprecipitation lysis buffer containing 50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM ethylene glycol tetraacetic acid, 1 mM sodium ortho-vanadate, 0.5 mM phenylmethylsulfonyl fluoride, 1% (v/v) Nonidet-P40, and 0.1% (v/v) protease inhibitor.
cocktail. Homogenates were subjected to brief sonication, followed by centrifugation for 10 minutes at 20,000 g at 4°C, and the supernatants were isolated as tissue lysates. Protein content of each lysate was determined using Bio-Rad protein assay kit. Lysates were incubated with Laemmli buffer (20 mM Tris-HCl, 2 mM EDTA, 2% SDS, 20% glycerol and 0.2% bromophenol blue) and 10% β-mercaptoethanol for 10 minutes at room temperature. Proteins were separated on 10% SDS-polyacrylamide gel and transferred onto polyvinylidene fluoride membrane. The membranes were blocked overnight in 5% skim milk Tris-buffered saline containing 0.1% Tween 20 and incubated overnight with primary antibody: mouse anti-P-gp (C219, 1:500 dilution), which recognizes an internal epitope of human and rodent P-gp; rat anti-Bcrp (BXP-53, 1:250 dilution), which recognizes an epitope corresponding to amino acid 221 to 394 of human and mouse Bcrp, or mouse anti-actin (1:2000 dilution), which recognizes an epitope on the carboxyl-terminal end of human and rodent actin. The blots were then incubated with corresponding horseradish peroxidase-conjugated secondary anti-mouse (1:10,000 dilution) or anti-rat (1:10,000 dilution) antibodies. Signals were enhanced using the chemiluminescence SuperSignal West Pico System (Thermo-Fisher Scientific, Waltham, MA) and detected by exposure to X-ray film.

Intravenous Drug and Standard LC-MS/MS Solutions

For intravenous administration, atazanavir was dissolved in a mixture of ethanol, polyethylene glycol 200 and 5% glucose (2:6:2) to obtain a concentration of 2.5 mg/mL. Ritonavir and elacridar were first dissolved in DMSO to obtain 100 mg/mL stocks and were subsequently dissolved in mixture of ethanol, polyethylene glycol 200 and 5% glucose (2:6:2) to produce 2.5 mg/mL solution for injection.
A standard stock solution of atazanavir (0.5 mg/L) was prepared using HPLC grade methanol, and further diluted 1:10 in methanol: water (1:1) mix to produce an atazanavir working solution (50 µg/mL). Similarly, the internal standard DMDPQ (0.2 µg/mL) stock solution was made and further diluted 1:10 in methanol: water (1:1) mix to create the internal standard working solution (2 µg/ml). All stock and working solutions were stored at 4°C. The standard working solutions were diluted with drug free mouse blood plasma, brain homogenate, and testes homogenate to generate concentrations of 5, 25, 50, 125, 500, 1250, 2500, 5000 ng/mL in plasma, 1, 5, 25, 75, 100, 250, 500, 1000 ng/mL in brain, and 2.4, 6, 24, 60, 120, 240, 900, 1800, 3000 ng/mL in testes. For the preparation of internal quality controls (QC) used for the validation of the assay, independent atazanavir stock and working solutions were prepared and further diluted in drug-free plasma, brain homogenate, and testes homogenate to achieve concentrations of 125, 1250 and 5000 ng/mL in plasma; 5, 75, 500 ng/mL in brain and 24, 240, 1800 ng/mL in testes. Calibration standard, quality control samples, and analysis samples were stored at -20°C for further analysis by LC-MS/MS.

**Distribution of Atazanavir in Mouse Brain, Testes and Plasma**

Tissues and plasma distribution of atazanavir were determined following an intravenous injection (10 mg/kg) via the tail vein. For studies examining the inhibition of P-gp by selective P-gp and Bcrp inhibitor i.e., elacridar (5 mg/kg) or ritonavir (2 mg/kg), WT-mice were pre-administered elacridar or ritonavir intravenously via tail-vein injection 15 minutes before receiving the tail-vein injection of atazanavir (10 mg/kg). At specific time points following atazanavir administration, mice were anaesthetized with isoflurane (IsoFlo; Abbott...
Laboratories, Abbott Park, IL) and whole blood was collected by cardiac puncture. Blood was mixed with 5 µL EDTA (5 mM), as anticoagulant, and plasma was isolated following centrifugation at 7,500 x g for 10 minutes at 4°C. Following exsanguination, brain, liver and testes tissues were collected, washed and weighed before being flash-frozen in liquid nitrogen. All samples were stored at -80°C until further analysis by LC-MS/MS.

**Tissue Preparation and Sample Extraction for LC-MS/MS**

Isolated mouse brain and testes were homogenized in distilled RNase / DNase free water (6 mL/g of tissue). 200 µL of each standard, quality control and analysis samples were pipetted into separated snap-cap microcentrifuge vials containing 100 µL of working internal standard solution. Each tube was mixed with 800 µL methanol to precipitate proteins at room temperature for 15 minutes and then was centrifuged at 5000 rpm for 5 minutes at 4°C. After centrifugation, 300-µL of each supernatant was transferred to the 2 mL autosampler vials containing 100 µL 0.1% acetic acid. The injection vials were vortex-mixed and 10 µL mixture was injected for analysis.

**Quantification of Tissue and Plasma Atazanavir Concentrations by LC-MS/MS**

Chromatographic separation was carried out at 40°C with a gradient mobile phase run. The mobile phase consisted of mobile phase A (0.1% acetic acid in water) and mobile phase B (100% methanol). The gradient profile was as follows (all solvent percentages are volume fractions): 0-1 min, 35:65 A:B; 1-5 min, linear gradient from 35:65 A:B to 10:90 A:B; 5-7 min, back to 35:65 A:B; 7-8 hold at 35:65 A:B (column equilibrating). The flow rate used was...
1mL/min, and the injection volumes were 10 µL for all the analyses. The total run time was 8 minutes and a 1:10 splitter was used to introduce effluent to MS/MS.

Mass spectral analyses were performed on an API 2000 triple quadrupole mass spectrometer, fitted with an API-Turbo Ion spray source and operated in the positive ionization mode. Lower and upper limits of quantification were arbitrarily set as the bottom and top points of the standard curve, respectively. Both inter-day and intra-sample precision varied less than 15% (CV %).

**Pharmacokinetic Analysis**

Using results from the LC-MS/MS analysis, atazanavir concentrations (ng/ml) in brain and testes tissues were normalized to the tissue mass (g) and presented as atazanavir tissue concentrations (ng/g of tissue). Atazanavir tissue: plasma ratios were calculated at each time point as the ratio of atazanavir concentration in brain (C\text{brain}) or testes (C\text{testes}) to the plasma concentration of atazanavir (C\text{plasma}). The area under the concentration (AUC)-time curves extrapolated to infinity (AUC\text{plasma}, AUC\text{brain} and AUC\text{testes}) were estimated using the linear trapezoidal method. Cumulative AUC\text{brain}:AUC\text{plasma} and AUC\text{testes}:AUC\text{plasma} ratios were calculated by summing the AUC of each tissue at each time period (0-1hr, 1-2hr, 2-3hr and 3-4hr) and dividing by the respective plasma AUC\text{plasma} for a given time period. The plasma terminal elimination half-lives were determined by linear regression of the respective natural log-transformed concentration-time plots. Clearance and volume of distribution were calculated from CL=dose/AUC\text{[0,∞]} and V=total amount of drug in body dose(mg)/C\text{plasma}, respectively.
The compartmental analyses of plasma atazanavir concentration-time profiles from WT- and TKO-mice were performed using Micromath Scientist 2.0 software (St.Louis, MO). Compartmental parameter estimates for atazanavir were determined for WT- and TKO-mice after fitting concentration data to one- and two-compartment models. Final model selection was based on goodness of fit comparisons among the models using visual inspection of the data, the coefficient of variation of parameter estimates and the weighted sum of standard differences (WSSD). Various weighting schemes were used (actual, estimated, 1/concentration and 1/concentration^2) and 1/concentration^2 weighting yielded the highest model selection criterion and the lowest coefficient of variation (S.D./parameter).

### Statistical Analyses

All results are shown as mean ± S.D. or as mean ± S.E.M. as appropriate. Comparisons between animal groups were performed using the unpaired two-tailed Student’s t test. Data were analyzed using Instat 3.0 software (Graphpad Software Inc., San Diego, CA) and a value of \( p < 0.05 \) was considered statistically significant.

### Results

#### ABC Transporter Protein Expression in Brain and Testes of WT- and TKO-mice

Since atazanavir is a known substrate of P-gp, a weak substrate of Mrp1, and an inhibitor of BCRP (23,29-31), we performed immunoblot analysis to examine the protein expression of these transporters in whole brain tissue lysates and isolated seminiferous tubules from both WT and TKO mice (mdr1a/1b-/-, abcg2-/-). We detected robust bands for P-gp (~170 kDa), Bcrp (~72 kDa) and Mrp1 (~190 kDa) in WT-mice isolated seminiferous tubules lysates and...
whole brain tissue lysates. As expected, no bands were identified for P-gp or Bcrp in either whole brain or seminiferous tubules tissues isolated from TKO-mice (data not shown). We also did not observe any significant differences in Mrp1 expression in either whole brain tissue lysates or isolated seminiferous tubules between TKO-mice and WT-mice (data not shown).

**mRNA expression of metabolic enzymes in WT-mice and TKO-mice brains, seminiferous tubules and liver**

In order to address any potential changes in drug metabolizing enzyme expression in TKO-mice, we examined the mRNA expression of several enzymes in mouse liver, testes (seminiferous tubules) and brain. In seminiferous tubules and brain, we detected very low mRNA expression of all metabolic enzymes relative to their expression in liver tissue in both WT and TKO mice. Interestingly, we observed a significant ($p<0.05$) 12-fold increase in brain mRNA expression of *Cyp3a11*, the mouse equivalent of *CYP3A4* enzyme in humans (32), in TKO-mice compared to WT-mice brain samples. No significant changes were detected in the other metabolic enzymes in TKO-mice brain, seminiferous tubules or liver tissues compared to WT-mice (Table 2).

**Pharmacokinetic parameters of atazanavir in WT- and TKO-mice plasma**

We determined the concentration of atazanavir in plasma ($C_{\text{plasma}}$) in WT- and TKO-mice (Figure 1). Non-compartmental analysis yielded terminal half-lives of 1.19 hr and 1.29 hr in WT-mice and TKO-mice, respectively. The total body clearance in plasma of WT-mice was 53 ml/hr/kg and 56ml/hr/kg in TKO-mice. The volumes of distribution based on the plasma atazanavir concentration-time profiles were $0.68 \pm 0.06$ ml and $0.75 \pm 0.26$ ml in the WT-mice and TKO-mice, respectively (Table 3).
Atazanavir plasma concentration data were then fitted to a two-compartment I.V. bolus model, which provided distribution micro-rate constants. We observed a significant increase \( (p<0.05) \) in \( k_{12} \), which represents the rate at which atazanavir distributes into the tissue compartments from plasma, in TKO-mice \( (2.92 \pm 1.80 \, \text{hr}^{-1}) \) compared to WT-mice \( (0.21 \pm 0.06 \, \text{hr}^{-1}) \). We did not observe any significant changes in the rate constants \( k_{21} \), which represents the rate at which atazanavir leaves the tissue compartment into plasma, or \( k_{10} \), which represents the rate of elimination of atazanavir from the plasma compartment (Table 3).

**Atazanavir Brain and Testes Concentrations in WT-mice and TKO-mice**

We examined the concentration of atazanavir in brain \( (C_{\text{brain}}) \) (Figure 2A) and testes \( (C_{\text{testes}}) \) (Figure 2B) in WT-mice and TKO-mice and compared tissue to plasma concentration ratios of atazanavir \( (C_{\text{brain}}: C_{\text{plasma}} \text{ and } C_{\text{testes}}: C_{\text{plasma}}) \) in both groups. We observed significant increases \( (p<0.05) \) in \( C_{\text{brain}}: C_{\text{plasma}} \) (Figure 3A) and \( C_{\text{testes}}: C_{\text{plasma}} \) (Figure 3B) ratios in TKO-mice compared to WT-mice as early as 0.5 hr post-injection. At 0.5 hr post-atazanavir injection, we detected a 5.4-fold increase in TKO-mice \( C_{\text{brain}}: C_{\text{plasma}} \) \( (0.43 \pm 0.07, \text{ Figure 3A}) \) and 4.6-fold increase in \( C_{\text{testes}}: C_{\text{plasma}} \) \( (0.73 \pm 0.26, \text{ Figure 3B}) \) compared to WT-mice \( C_{\text{brain}}: C_{\text{plasma}} \) \( (0.08 \pm 0.07) \) and \( C_{\text{testes}}: C_{\text{plasma}} \) \( (0.16 \pm 0.1) \) ratios (Figure 3A and 3B, respectively).

**Inhibition of P-gp-mediated efflux of Atazanavir by Elacridar in the Brain and Testes of WT-mice**

To determine if P-gp could be pharmacologically inhibited in WT-mice leading to an increase in atazanavir concentrations in brain and testes, we administered via tail-vein injection the selective P-gp/Bcrp inhibitor, elacridar (5 mg/kg), or vehicle to WT-mice 15 minutes prior to a tail-vein injection of atazanavir (10 mg/kg). In the elacridar-treated WT-mice, we observed a
significant increase (p<0.05) in the atazanavir C<sub>brain</sub>:C<sub>plasma</sub> ratio at 15 minutes (12.3-fold, 0.37 ± 0.1) and a more modest although significant increase at 60 minutes (3.5-fold, 0.07 ± 0.01) compared to vehicle-treated WT-mice (0.03 ± 0.01 and 0.02 ± 0.01, respectively) (Figure 4A).

We observed a modest increase in atazanavir C<sub>testes</sub>:C<sub>plasma</sub> ratio at 15 minutes in elacridar-treated WT-mice (1.7-fold, 0.16 ±0.02) compared to vehicle-treated WT-mice C<sub>testes</sub>:C<sub>plasma</sub> ratio (0.09 ± 0.02), although the increase did not reach statistical significance (Figure 4B).

Interestingly, at 60 minutes we observed a significant and substantial increase (p<0.05) in the elacridar-treated WT-mice C<sub>testes</sub>:C<sub>plasma</sub> ratio (13.5-fold, 1.08 ± 0.21) compared to vehicle-treated WT-mice C<sub>testes</sub>:C<sub>plasma</sub> ratio (0.08 ± 0.01) (Figure 4B).

Inhibition of P-gp/Bcrp-mediated efflux of Atazanavir by ritonavir in the brain and testes of Cyp-mice

To determine if the HIV protease inhibitor ritonavir could inhibit P-gp and Bcrp efflux of atazanavir, we used a Cyp-mice model that lacks expression of eight major Cyp3a enzymes. We administered ritonavir (2 mg/kg) or vehicle to Cyp-mice 15 minutes prior to a tail-vein injection of atazanavir (10 mg/kg). We observed a significant (p<0.05) but modest increase (1.8-fold) in atazanavir C<sub>brain</sub>:C<sub>plasma</sub> ratio in ritonavir-treated Cyp-mice at 15 minutes (0.024 ± 0.003) compared to vehicle-treated Cyp-mice (0.013 ± 0.002) (Figure 5A). At 60 minutes, ritonavir did not increase C<sub>brain</sub>:C<sub>plasma</sub> ratio in Cyp-mice compared to vehicle-treated Cyp-mice (0.015 ± 0.006 versus 0.015 ± 0.001, respectively) (Figure 5A). In testes, pre-treatment with ritonavir significantly increased C<sub>testes</sub>:C<sub>plasma</sub> ratio in ritonavir-treated Cyp-mice at both 15 minutes (9.5-fold, 0.181 ± 0.03) and 1 hr (2.7-fold, 0.132 ± 0.02) compared to vehicle-treated Cyp-mice (0.019 ± 0.01 and 0.049 ± 0.01, respectively) (Figure 5B).
Discussion

Since the introduction of combination antiretroviral therapy, several clinical studies examining antiretroviral drug concentrations in HIV-infected men have reported low concentrations of HIV protease inhibitors in both the cerebrospinal fluid and seminal fluid of the male genital tract (33,34). In addition to the low concentrations of antiretroviral drugs, both of these tissues have been shown to be sanctuary sites for HIV, allowing the virus to persist despite adherence to pharmacotherapy and a significant reduction in viral plasma load (5,9). These sanctuary sites of replication-competent virus are thought to contribute to the rapid viral repopulation observed in patients during interruption of therapy (7,35,36). One potential mechanism that could contribute to the low concentrations of HIV protease inhibitors at these sites is the functional expression of active drug efflux transporters such as P-gp. In this study, we have used drug transporters and metabolic enzymes knockout mice models to examine the role of efflux transporters in the distribution of the HIV protease inhibitor atazanavir in the brain and testes.

Since atazanavir is known to be highly metabolized in humans, we investigated the mRNA expression of several drug-metabolizing enzymes to ensure the relative expression was similar between TKO- and WT-mice. We confirmed similar mRNA expression of several metabolic enzymes in the TKO-mice compared to WT-mice and found that the mRNA expression of Cyp3a11, the mouse functional ortholog of human CYP3A4 (32), was significantly higher in TKO-mice brain. Although Cyp3a11 mRNA expression in TKO-mice brain was significantly increased, the relative mRNA expression of Cyp3a11 detected in TKO-mice liver
tissue was 65-fold greater than the one measured in TKO-mice brain tissue, suggesting that it may play a minor role in atazanavir metabolism at the level of the brain. A previous study examining drug-metabolizing enzymes expression in human BBB and brain cortex reported detectable levels of \textit{CYP2D6} and \textit{CYP2E1}, which is in agreement with our findings demonstrating the expression of \textit{Cyp2d22}, functional ortholog of \textit{Cyp2d6} and \textit{Cyp2e1}; however, they were unable to detect \textit{CYP3A4} within the BBB or the brain cortex (37). Interestingly, studies by Hagemeyer \textit{et al.} and Rosenbrock \textit{et al.} have reported \textit{Cyp3a11} and \textit{Cyp3a13} mRNA expression in mouse brain, suggesting that there may be species difference between humans and mice (38,39). In addition, we provide the first evidence of \textit{Cyp3a11}, \textit{Cyp2d22}, \textit{Cyp2e1} and \textit{Ugt1a1} mRNA expression in isolated seminiferous tubules of TKO-mice and WT-mice; however, since the relative expression of these enzymes in the tubules was very low, they most likely play a limited role in atazanavir metabolism at the BTB. Taken together these findings suggest that extra-hepatic drug metabolism is minor, and would most likely not play a role in the tissue distribution of atazanavir. The relative expression of \textit{CYP3A4} and \textit{UGT1A1} in human seminiferous tubules is currently unknown, further studies examining the role of CYP-mediated metabolism at the human BTB are needed.

Since little is known about the role of drug efflux transporters in atazanavir distribution \textit{in vivo}, we used TKO-mice rather than Mdr1a^{-/-}1b^{-/-} mice to avoid any potential compensatory up-regulation of other transporters such as Bcrp that has been previously reported in P-gp (mdr1a^{-/-}) knockout mice (40). In the TKO-mice, we did not observe any significant differences in the half-life, volume of distribution, AUC (0→∞) and clearance of atazanavir in the plasma compared to WT-mice, suggesting that the two animal models exhibit similar atazanavir plasma...
pharmacokinetic properties. These findings are in agreement with a study conducted by Giri et al. where the absence of Bcrp in Bcrp<sup>-/-</sup> knockout mice did not have significant effect on the plasma concentrations of the Bcrp substrate, zidovudine, and Bcrp/P-gp substrate, abacavir (41). Applying a two-compartment I.V. bolus model, using curve-fitting software, we observed a significant increase in $K_{12}$ rate constant, suggesting the distribution rate of atazanavir into tissues is enhanced in the absence of drug transporters. Since atazanavir is not known to be a substrate for Bcrp, we propose that any changes in the pharmacokinetics of atazanavir are most likely due to the absence P-gp in the TKO-mice (29,31).

Within the TKO-mice brain and testes, we observed a significant increase the $C_{\text{brain}}:C_{\text{plasma}}$ and $C_{\text{testes}}:C_{\text{plasma}}$ concentration ratios over time compared to WT-mice. These data provide the first evidence that P-gp plays an important role in limiting the distribution of atazanavir into brain and testes. Previous studies by our group have demonstrated, in vitro, that atazanavir is a substrate for P-gp at BBB (22) and BTB (21), and that the accumulation of atazanavir can be significantly enhanced using selective inhibitors of P-gp (21-23). Furthermore, in vivo studies examining the tissue distribution of the first generation of HIV protease inhibitors i.e., indinavir and saquinavir, have reported that both the oral bioavailability and the tissue distribution of these agents is altered in a similar manner through the inhibition of P-gp using a selective inhibitor and/or through the use of a P-gp (Mdr1a<sup>-/-</sup>1b<sup>-/-</sup>) knockout mouse model (24,42-44). These findings provide a potential mechanism for the low atazanavir concentrations observed in CSF and seminal fluid of HIV-infected patients (15,16).

In addition to examining atazanavir distribution in the TKO-mice model, we also used elacridar, a selective P-gp/Bcrp inhibitor, to inhibit pharmacologically the efflux of atazanavir...
from WT-mice brain and testes compartments. Following the administration of elacridar, we observed significant increases in atazanavir $C_{\text{brain}}:C_{\text{plasma}}$ (12.3 fold, at 15 minutes) and atazanavir $C_{\text{testes}}:C_{\text{plasma}}$ ratios (13.5-fold, at 60 minutes) post-atazanavir injection, suggesting that elacridar can inhibit the P-gp-mediated efflux of atazanavir from the brain and testes. Previous positron emission tomography studies investigating the function of P-gp at the level of the BBB in vivo, have demonstrated that co-infusion of cyclosporine A, an immunomodulator and known P-gp inhibitor, significantly increased the brain permeability of $^{11}$C-verapamil in both mice (45,46) and humans (47,48). Taken together, these findings combined with the data we obtained, suggest that selective inhibition of ABC membrane associated drug efflux transporter i.e., P-gp, may increase HIV-protease inhibitor concentrations at target organs of the infection such as brain and testes.

The HIV protease inhibitor ritonavir is a potent inhibitor of the Cyp3A-mediated metabolism of other HIV protease inhibitors. In the clinic, ritonavir is used as a boosting agent, known as a pharmaco-enhancer, to increase the plasma concentrations and half-life of co-administered HIV protease inhibitors such as darunavir and atazanavir (1). It is noteworthy, that previous in vitro studies have shown that ritonavir can act as both a substrate and inhibitor of P-gp, and an inhibitor of Bcrp (29-31). In order to investigate if the HIV protease inhibitor ritonavir inhibits atazanavir mediated transport by P-gp, we used a Cyp-mice model, which lacks eight members of the Cyp3a enzyme subfamily. We observed that ritonavir could modestly, but significantly enhance the accumulation of atazanavir in the brain and testes. A previous in situ perfusion intestinal study by Holmstock et al. demonstrated in mice that co-perfusion of the HIV protease inhibitor darunavir with ritonavir increased the intestinal absorption of darunavir.
through an inhibitory effect of P-gp (49). In contrast, several in vivo studies have reported that ritonavir is a poor inhibitor of P-gp, and have suggested that ritonavir effects in vivo are primarily due to inhibition of CYP3A-mediated metabolism of HIV PIs (43,44,50). Taken together our data suggest that ritonavir can modestly inhibit P-gp mediated efflux of atazanavir in vivo. However, it is clear that in the clinic, the interaction of ritonavir with atazanavir is primarily mediated through the inhibition of a CYP3A metabolism pathway (51).

In summary, using a TKO mouse model, we have demonstrated for the first time the in vivo role of P-gp in limiting the tissue distribution of atazanavir in the brain and testes. Our data also suggest that atazanavir efflux from the brain and testes can be inhibited by elacridar, a selective P-gp/Bcrp inhibitor and that the HIV protease inhibitor ritonavir, which is commonly used as an inhibitor of CYP3A4, may also play a minor role in inhibiting P-gp in vivo. These findings highlight the importance of understanding the role of drug efflux transporters in limiting tissue distribution of HIV protease inhibitors into sanctuary sites of HIV infection and could guide clinicians towards selecting antiretroviral drugs with better tissue distribution properties. Further studies are needed to elucidate fully the clinical role of drug-efflux transporters and their ability to limit the concentration of antiretroviral drugs in the brain and testes of HIV-infected patients.
Acknowledgements

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References


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Table 1: Mouse primers used for quantitative real-time PCR

<table>
<thead>
<tr>
<th>Gene</th>
<th>GeneBank #</th>
<th>Primer Sequence (5’ – 3’)</th>
<th>Reference</th>
</tr>
</thead>
</table>
| Cyp3a11  | NM_007818    | F - GACAAACAAGCAGGGATGGAC
                R - CCAAGCTGATTGCTAGGAGCA   | (52)      |
| Cyp2d22  | NM_0011634721| F - CAGTGGTTGTACTAAATGGGCT
                R - GCTAGGACTATACTTTGAGACG   | (52)      |
| Cyp2e1   | NM_021282    | F - TCCCTAAGTATCCTCCGTGA
                R - GCTACGACTATATCCAGAGCG     | (52)      |
| Ugt1a1   | NM_201645    | F - CCAGCAAGGCGACGAAGTTG
                R - ACCACGCAGCAAGAAAGAAT     | (53)      |
| Cyclophilin B | NM_011149 | F - GGAGATGCCACAGGAGGAA
                R - GCCCGTAGTGCTTCAGGTT     | (21)      |
**Table 2:** qPCR analysis of metabolic enzymes in WT- and TKO-mice brains, seminiferous tubules and liver.

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Gene</th>
<th>Relative Expression (normalized to cyclophilin B)</th>
<th>Fold Change (WT vs. TKO)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Brain</td>
<td>Cyp3a11</td>
<td>0.0005 ± 0.001</td>
<td>0.007 ± 0.001*</td>
</tr>
<tr>
<td></td>
<td>Ugt1a1</td>
<td>0.006 ± 0.001</td>
<td>0.008 ± 0.002</td>
</tr>
<tr>
<td></td>
<td>Cyp2d22</td>
<td>0.13 ± 0.03</td>
<td>0.12 ± 0.02</td>
</tr>
<tr>
<td></td>
<td>Cyp2e1</td>
<td>0.0014 ± 0.0003</td>
<td>0.0005 ± 0.0001*</td>
</tr>
<tr>
<td>Seminiferous Tubules</td>
<td>Cyp3a11</td>
<td>0.02 ± 0.01</td>
<td>0.008 ± 0.003</td>
</tr>
<tr>
<td></td>
<td>Ugt1a1</td>
<td>0.08 ± 0.02</td>
<td>0.09 ± 0.03</td>
</tr>
<tr>
<td></td>
<td>Cyp2d22</td>
<td>0.009 ± 0.005</td>
<td>0.02 ± 0.01</td>
</tr>
<tr>
<td></td>
<td>Cyp2e1</td>
<td>0.003 ± 0.003</td>
<td>0.002 ± 0.001</td>
</tr>
<tr>
<td>Liver</td>
<td>Cyp3a11</td>
<td>5.45 ± 0.67</td>
<td>6.59 ± 2.67</td>
</tr>
<tr>
<td></td>
<td>Ugt1a1</td>
<td>1.11 ± 0.28</td>
<td>1.34 ± 0.56</td>
</tr>
<tr>
<td></td>
<td>Cyp2d22</td>
<td>0.35 ± 0.05</td>
<td>0.50 ± 0.11</td>
</tr>
<tr>
<td></td>
<td>Cyp2e1</td>
<td>6.45 ± 0.86</td>
<td>11.63 ± 3.65</td>
</tr>
</tbody>
</table>

*p < 0.05 TKO-mice compared to WT-mice (n=4 mice per group)
Table 3: Atazanavir pharmacokinetic parameters in WT- and TKO-mice estimated from plasma concentration data fitted to a two-compartment single I.V. bolus model

<table>
<thead>
<tr>
<th>Parameter</th>
<th>WT-mice</th>
<th>TKO-mice</th>
</tr>
</thead>
<tbody>
<tr>
<td>$K_{12}$ ($hr^{-1}$)</td>
<td>0.21 ± 0.06</td>
<td>2.92 ± 1.60*</td>
</tr>
<tr>
<td>$K_{21}$ ($hr^{-1}$)</td>
<td>0.63 ± 0.18</td>
<td>1.59 ± 0.63</td>
</tr>
<tr>
<td>$K_{10}$ ($hr^{-1}$)</td>
<td>2.65 ± 0.19</td>
<td>3.99 ± 1.26</td>
</tr>
<tr>
<td>Volume of Distribution (ml)</td>
<td>0.68 ± 0.06</td>
<td>0.75 ± 0.26</td>
</tr>
<tr>
<td>Clearance (ml/hr/kg)</td>
<td>53</td>
<td>56</td>
</tr>
<tr>
<td>Half-life ($T_{1/2}$, hr)</td>
<td>1.19</td>
<td>1.29</td>
</tr>
<tr>
<td>AUC(0-∞)(ng·hr/ml)</td>
<td>1955</td>
<td>1722</td>
</tr>
</tbody>
</table>

Analysis of Model Fit

<table>
<thead>
<tr>
<th></th>
<th>WT-mice</th>
<th>TKO-mice</th>
</tr>
</thead>
<tbody>
<tr>
<td>WSSD</td>
<td>0.12</td>
<td>0.76</td>
</tr>
<tr>
<td>$R^2$</td>
<td>0.98</td>
<td>0.97</td>
</tr>
</tbody>
</table>

* p < 0.05, TKO-mice compared to WT-mice (n=4 mice per time per group)
Figure 1 – Atazanavir plasma concentrations over time in WT- and TKO-mice. Atazanavir plasma concentrations (ng/ml) were measured over time following an I.V. bolus administration of 10 mg/kg atazanavir to WT-mice (square) and TKO-mice (Mdr1a<sup>−/−</sup>1b<sup>−/−</sup>, Abcg2<sup>−/−</sup>) (triangle). Results are shown as mean ± S.E.M. for each time point (n=4 animals per time point per group). WT-mice and TKO-mice (Mdr1a<sup>−/−</sup>1b<sup>−/−</sup>, Abcg2<sup>−/−</sup>) atazanavir plasma concentration (C<sub>plasma</sub>) data fitted to a two-compartment I.V. bolus pharmacokinetic model are shown as solid black line and dashed black line, respectively.
Figure 2 – Atazanavir tissue concentrations over time in WT- and TKO-mice. Atazanavir (A) brain ($C_{\text{brain}}$) and (B) testes ($C_{\text{testes}}$) concentrations (ng/g of tissue) were measured over time following an I.V. bolus administration of 10 mg/kg atazanavir to WT-mice (square/solid line) and TKO-mice (triangle/dashed-line). Results are shown as mean ± S.E.M. for each time point (n=4 animals per time point per group).
Figure 3 – Atazanavir tissue to plasma concentration ratios in WT- and TKO-mice. Comparison of atazanavir tissue:plasma concentration ratios in WT-mice (square/solid line) and TKO-mice (triangle/dashed-line). Atazanavir tissue: plasma concentration ratios (A) $C_{\text{brain}}/C_{\text{plasma}}$ and (B) $C_{\text{testes}}/C_{\text{plasma}}$ are shown as mean ± S.E.M. (n=4 animals per time point per group). Statistical significant differences are indicated by an asterisk (*p < 0.05).
Figure 4 – Effect of elacridar on atazanavir tissue to plasma concentration ratios in WT- and TKO-mice. Atazanavir tissue: plasma concentration ratios (A) $C_{\text{brain}}/C_{\text{plasma}}$ and (B) $C_{\text{testes}}/C_{\text{plasma}}$ at 15 and 60-minute time points in WT-mice in the absence (vehicle, black) or presence of elacridar (5mg/kg; grey). Results are expressed as mean tissue: plasma concentration ratio ± S.E.M. ($n=4$ mice per time point per group). Statistical significant differences are indicated by an asterisk (*$p < 0.05$).
Figure 5 – Effect of ritonavir on atazanavir tissue to plasma concentration ratios in WT- and TKO-mice. Atazanavir concentration ratios (A) $C_{\text{brain}}/C_{\text{plasma}}$ and (B) $C_{\text{testes}}/C_{\text{plasma}}$ at 60 minutes in Cyp-mice in the absence (vehicle, black) or presence of ritonavir (2mg/kg; grey). Results are expressed as mean tissue: plasma concentration ratio ± S.E.M. (n=4 mice per time point per group). Statistical significant differences are indicated by an asterisk (*$p < 0.05$).