Expression of ATP-Binding Cassette Membrane Transporters in Rodent and Human Sertoli Cells: Relevance to the Permeability of Antiretroviral Therapy at the Blood-Testis Barrier

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ABSTRACT

The blood-testis barrier (BTB), composed primarily of Sertoli cells, is responsible for protecting developing germ cells from xenobiotic exposure. ATP-binding cassette (ABC) membrane-associated drug efflux transporters, P-glycoprotein (P-gp), breast cancer resistance protein (BCRP), and the multidrug resistance-associated proteins (Mrps), have been shown to restrict antiretroviral drug permeability at blood-tissue barriers such as the blood-brain barrier. However, it remains unclear whether these transporters are functional at the level of Sertoli cells and can regulate anti-HIV drug permeability at the BTB. This study investigated the functional expression of ABC transporters in a mouse Sertoli cell line system (TM4) and in primary cultures of human Sertoli cells (HSECs). Expression of multi-drug resistance Mdr1a/1b/MDR1/P-gp, Mrp1/MRP1, and Mrp4/MPR4 is confirmed by quantitative polymerase chain reaction and immunoblotting analysis in TM4 cells and HSECs.

Immunofluorescence studies revealed plasma membrane localization of P-gp, Mrp1/MRP1, and Mrp4/MPR4 in both cell systems. However, Bcrp expression and localization was only detected in rodent cells. Accumulation of 1) rhodamine-6G (R-6G), a fluorescent P-gp substrate, 2) \[^{3}H\]atazanavir, a HIV protease inhibitor and known P-gp substrate, 3) 2',7'-bis-(2-carboxyethyl)-5-(and-6)carboxyfluorescein (BCECF), a fluorescent Mrp substrate, and 4) \[^{3}H\]mitoxantrone, a BCRP substrate, by TM4 monolayer cells in the presence of established inhibitors demonstrates that these transporters are functional. In addition, several anti-HIV drugs significantly enhance the accumulation of R-6G, \[^{3}H\]atazanavir, BCECF, and \[^{3}H\]mitoxantrone by TM4 cells. This study provides the first evidence of ABC transporter expression and activity in Sertoli cells and suggests that these transporters could play an important role in restricting antiretroviral drug permeability at the BTB.

Introduction

The blood-testis barrier (BTB), composed primarily of Sertoli cells, provides structural and protective support to developing germ cells (Su et al., 2011). These cells are epithelial in origin and form tight junction complexes near the basement membrane, which physically divides the seminiferous epithelium into luminal (apical) and basolateral compartments. This allows the development of postmeiotic spermatids to occur in a specialized microenvironment within the seminiferous tubules. In addition to acting as a physical barrier, Sertoli cells also contribute to the formation of an immunological barrier by preventing the production of anti-sperm antibodies toward developing sperm as well as by preventing peripheral immune cells from entering the seminiferous tubule microenvironment (Su et al., 2011).

Despite the use of highly active antiretroviral therapy, the male genital tract remains a sanctuary site of HIV infection (Dahl et al., 2010). The most recent guidelines for antiretroviral therapy of HIV-infected, treatment-naive patients, recommend the administration of a protease, integrase, or non-nucleoside reverse transcriptase inhibitor in combination...
with two nucleoside reverse transcriptase inhibitors (Panel on Antiretroviral Guidelines for Adults and Adolescents, 2011). Data from clinical studies have suggested that one potential contributing factor to the formation of a HIV viral sanctuary in the genital tract is the low concentration of antiretroviral drugs reaching the seminal fluid in HIV-infected men (van Praag et al., 2001; Pereira et al., 2002; Ghosn et al., 2004; Cruciani et al., 2006; van Leeuwen et al., 2007). We propose that the reduced concentrations of antiretroviral drugs in seminal fluid could, in part, be due to the expression of ATP-binding cassette (ABC) membrane-associated drug efflux transporters such as P-glycoprotein (P-gp), the multidrug resistance-associated proteins (MRPs), and the breast cancer resistance protein (Bcrp) in Sertoli cells. These transporters are known to actively efflux several xenobiotics from cellular targets and significantly contribute to multidrug resistance (Kis et al., 2010a).

P-gp, a membrane-bound protein encoded by the multidrug resistance (MDR) gene, has two known isoforms in humans (MDR1 and MDR2) and three isoforms in rodents (mdr1a, mdr1b, and mdr2). MDR1 and mdr1a/b have been shown to participate in the MDR phenomenon, whereas MDR2 and mdr2 are primarily involved in phosphatidylinositol transport within the liver (Kis et al., 2010a). P-gp is expressed in many tissues such as the brain (Bendayan et al., 2006) and is capable of extruding a large number of pharmacological agents including several antiretroviral drugs such as protease inhibitors (Zastre et al., 2009) and nucleoside reverse transcriptase inhibitors (Kis et al., 2010a).

Two well studied ABC transporters of the MRP family that have demonstrated involvement in the MDR phenomenon are Mrp1 and Mrp4 (Meaden et al., 2002; Dallas et al., 2004b). Mrp1 is ubiquitously expressed in many tissues including testes, microglia, astrocytes, and kidney and liver epithelia (Haimure et al., 2004). Mrp1 preferentially transports anionic compounds and their corresponding glutathione, glucuronide-, and sulfate-based conjugates (Haimure et al., 2004; Kis et al., 2010a). Other drugs known to be substrates for Mrp1 include the HIV protease inhibitors such as saquinavir (Kis et al., 2010a). In contrast, Mrp4 has a different substrate specificity and is capable of transporting cyclic nucleotides (cAMP and cGMP), sulfate- and glucuronide-conjugated steroids and the prostaglandins E1 and E2 (Ritter et al., 2005). Mrp4 has also been shown to transport several nucleoside reverse transcriptase inhibitors such as zidovudine, adefovir, and tenofovir (Kis et al., 2010a). Mrp4 expression has been identified in the kidney, brain, and testis (Ritter et al., 2005).

The membrane drug efflux transporter, Bcrp, has similar yet more restricted substrate specificity than P-gp. It is capable of transporting several compounds including sulfated estrogens, folic acid conjugates, chlorophyll-derived photo-toxins, and chemotherapeutic drugs such as mitoxantrone and camptothecins (Lee et al., 2007). In addition, HIV-nucleoside reverse transcriptase inhibitors such as zidovudine are substrates for this transporter (Kis et al., 2010a). Bcrp expression has been confirmed in many tissues such as brain and testis (Lee et al., 2007; Su et al., 2011).

The objective of this study was to investigate the functional expression of ABC drug efflux transporters, i.e., P-gp, Mrp1, Mrp4, and Bcrp in rodent and human Sertoli cells and their potential role in limiting the permeability of antiretroviral drugs at the BTB.

### Materials and Methods

Materials. [3H]Mitoxantrone (12.7 Ci/mmol) and [3H]Ivatazanavir (3 Ci/mmol) were purchased from Moravek Biochemicals (Brea, CA). Valspodar (PSC833) was a generous gift from Novartis Pharma (Basel, Switzerland). [3S,6S,12aS]-1,2,3,4,6,7,12a-Octahydro-9-methoxy-6-(2-methylpropyl)-1,4-dioxopyrazino[1,2':1,6]pyrido[3,4-b]indole-3-propanoic acid 1,1-dimethylethyl ester (Ko 143) and 3-[[3-[[1-E]-2-(7-chloro-2-quinolinyl)ethyl]phenyl][3-[[dimethylamino]-3-oxopropyl]thio[methyl][thio]propanoic acid (MK 571) were purchased from Torcis Biosciences (Ellisville, MO). N-[4-[2-(1,2,3,4-Tetrahydro-6,7-dimethoxy-2-isouquinolinyl)ethyl]phenyl]-9,10-di(hydro-5-methylene-9-oxo-4-acridine carboxamide (GF120918, elacridar) was a generous gift from GlaxoSmithKline Inc. (Mississauga, ON, Canada). Unlabeled antiretroviral drugs were obtained through the National Institutes of Health AIDS Research and Reference Reagent Program (Bethesda, MD). An 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 (Mrp1) and anti-Mrp4 (Mr,I-80) antibodies were purchased from Kamiya Biomedical Company (Seattle, WA). The rat anti-Bcrp antibody (EXP-53) and mouse anti-GATA-4 (6H10) antibodies were obtained from Abcam Inc. (Boston, MA). The monoclonal mouse anti-MDR1 (D11) and rabbit polyclonal anti-‘Na+/K’ ATPase-α (H-300) antibodies were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). The goat anti-mouse horseradish peroxidase-conjugated secondary antibody was purchased through Jackson ImmunoResearch Laboratories Inc. (West Grove, PA). The anti-actin antibody (AC40), the mouse anti-rat horseradish peroxidase-conjugated secondary antibody, rhodamine-6G (R-6G), and all other standard laboratory chemicals were purchased through Sigma-Aldrich Canada (St. Louis, MO). Vectashield hard mounting media with 4',6-diamidino-2-phenylindole (DAPI) was obtained from Vector Laboratories, Inc. (Burlingame, CA). Tissue cell culture reagents for cell culture systems and 2',7'-bis-(carboxyethyl)-5-(and-6)-carboxyfluorescein (BCECF) free acid, BCECF-AM, and Alexa Fluor 488 and Alexa Fluor 594-conjugated secondary antibodies were purchased from Invitrogen (Carlsbad, CA). Versene (200 mg/ml), 0.25% trypsin/EDTA, trypsin-neutralizing solution was purchased from Lonza Walkersville, Inc. (Walkersville, MD).

Cell Culture. All the cell culture systems were grown and maintained at 37°C humidified 5% CO2-95% air with fresh media replaced every 2 to 3 days. Cells were subcultured with 0.25% trypsin-EDTA upon reaching 80 to 90% confluence, unless stated.

**ABBREVIATIONS:** BTB, blood-testis barrier; ABC, ATP-binding cassette; P-gp, P-glycoprotein; Mrp/MDR, multidrug resistance-associated protein; Bcrp/BCRP, breast cancer resistance protein; Mdr/MDR, multidrug resistance; PSC833, valspodar; Ko 143; [3S,6S,12aS]-1,2,3,4,6,7,12a-octahydro-9-methoxy-6-(2-methylpropyl)-1,4-dioxopyrazino[1,2':1,6]pyrido[3,4-b]indole-3-propanoic acid 1,1-dimethylethyl ester; MK 571; 3-[[3-[[1-E]-2-(7-chloro-2-quinolinyl)ethyl]phenyl][3-[[dimethylamino]-3-oxopropyl]thio[methyl][thio]propanoic acid; GF120918; N-[4-[2-(1,2,3,4-Tetrahydro-6,7-dimethoxy-2-isouquinolinyl)ethyl]phenyl]-9,10-di(hydro-5-methylene-9-oxo-4-acridine carboxamide, elacridar; R-6G, rhodamine-6G; DAPI, 4,6-diamidino-2-phenylindole; BCECF, 2',7'-bis-(carboxyethyl)-5-(and-6)-carboxyfluorescein; BCECF-AM, 2',7'-bis-(carboxyethyl)-5-(and-6)-carboxyfluorescein, acetoxymethyl ester; TM4 cell, mouse Sertoli cell; HSEC, human Sertoli cell; Hek, human embryonic kidney; qPCR, quantitative real-time polymerase chain reaction; PBS, phosphate-buffered saline; LY-335979, zosuquidar; G, cycle threshold.
Mouse Sertoli cell line. The continuous mouse Sertoli (TM4) cell line was obtained from the American Tissue Culture Collection (Manassas, VA). TM4 cells are nontumorigenic and originate from primary cultures of Sertoli cell-enriched preparations from 11- to 13-day-old BALB/c strain mice (Mather, 1980). All cell culture flasks and multiwell dishes were precoated with rat tail collagen type I (100 μg/ml) before cell plating to enhance cell adherence. Cells were cultured and maintained in 1:1 (v/v) mix of Dulbecco’s modified Eagle’s media and Ham’s F12 nutrient mix containing 5% horse serum, 2.5% fetal bovine serum, 100 U/ml penicillin, and 100 μg/ml streptomycin until reaching a state of confluence of 80 to 90%.

Primary cultures of human Sertoli cells. Primary cultures of human Sertoli cells (HSECs) were purchased from Lonza Walkersville, Inc. Cells were cultured according to the manufacturer’s instructions using Sertoli cell basal medium supplemented with Sertoli cell growth medium supplement and fetal bovine serum (12.5%). Cells were plated at approximately 4000 to 5000 cells/cm².

Human cervical carcinoma cell line overexpressing MRP1. The human cervical carcinoma cell line stably transfected with human MRP1 cDNA (HeLa-MRP1) was kindly provided by Dr. Susan Cole (Queen’s University, Kingston, ON, Canada). Cells were cultured and maintained in Dulbecco’s modified Eagle’s medium (4 mM L-glutamine and 25 mM glucose) supplemented with G418 (400 μg/ml), 10% fetal bovine serum (10%), penicillin (100 U/ml), and streptomycin (100 μg/ml).

Human breast cancer cell line overexpressing MDR1. The human breast cancer cell line MDA453/LCC6 stably transfected with human MDR1 cDNA (MDA-MDR1) was kindly provided by Dr. Robert Clarke from Georgetown University (Washington, DC), and cells were cultured in alpha minimum essential medium supplemented with fetal bovine serum (10%), penicillin (100 U/ml), and streptomycin (100 μg/ml).

Embryonic kidney cell line overexpressing MRP4. The human embryonic kidney cell line stably transfected with human MRP4 cDNA (HEK-MRP4) was kindly provided by Dr. Piet Borst (Netherlands Cancer Institute, Amsterdam, The Netherlands). Cells were maintained in Dulbecco’s modified Eagle’s medium (Glutamax) supplemented with fetal bovine serum (5%), penicillin (100 U/ml), and streptomycin (100 μg/ml).

Human breast cancer cell line overexpressing BCRP. The human breast cancer cell line MCF7/MX100 was a generous gift from Dr. Susan Bates (Bethesda, MD). The cells were cultured and maintained in RPMI 1640 media supplemented with fetal bovine serum (10%), L-glutamine (1%), penicillin (100 U/ml), streptomycin (100 μg/ml), and mitoxantrone (100 nM).

Cell Morphology. For electron microscopy, TM4 cells or HSECs were fixed in 1% (v/v) glutaraldehyde in phosphate buffer (0.1 M, pH 7.2) for 2 hr at 4°C and postfixed with 1% (v/v) osmium tetroxide in phosphate buffer for 1 hr at 4°C. Fixed cells were then dehydrated in graded ethanol and embedded in Epon. Ultrathin sections (80 nm) of Epon-embedded material were cut and mounted on Parlodion carbon-coated nickel grids. The sections were stained with uranyl acetate and lead citrate and viewed with a Philips 410 transmission electron microscope (FEI Systems Canada, St. Laurent, QC, Canada).

Total RNA Extraction, cDNA Synthesis, and qPCR. Total RNA was extracted from TM4 cells or HSECs using TRIzol reagent (Invitrogen) according to the manufacturer’s instructions. The concentration (absorbance at 260 nm) and purity (absorbance 260 nm/absorbance 280 nm ratio) of RNA samples were assessed using a 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 incubated at 25°C for 10 min, followed by 37°C for 120 min and then 85°C for 5 min using a Mastercycler ep reaIplex 2S thermal cycler (Eppendorf Canada, Mississauga, ON, Canada).

The expression of genes encoding ABC transporters (abcb1a, abcb1b, abcc1, abcc4, and abcg2) and peptidylprolyl isomerase B (cyclophilin B) were analyzed by qPCR on a Mastercycler ep reaIplex 2S thermal cycler using SYBR Green fluorescence detection. The 10-μl final reaction mixtures contained 1.25 μl of diluted cDNA, 5 μl of PerfeCTa SYBR Green FastMix, 0.6 μl of 1.25 μM concentrations of each primer (final concentration of each primer 150 nM), and 2.55 μl of nuclelease-free water. Specific primers were designed using Primer Express 3 (Applied Biosystems) and were on exon-exon junctions to avoid any potential amplification of genomic DNA. The specificity of each reaction was assessed by melting curve analysis to ensure the presence of only a single amplification product. Validated primer sequences are shown in Table 1. Results for qPCR are presented as relative mRNA abundance ± S.E.M. of each gene of interest normalized to the housekeeping gene cyclophilin B using the comparative CT method, where ΔCT is equal to CTsample - the CT cyclophilin B and relative mRNA abundance is equal to 2-ΔΔCT. The experimental variability observed in the mRNA expression of the genes of interest and the housekeeping gene cyclophilin B between TM4 cell passages as well as between sets of primary cultures of HSECs is <5%.

Immunoblot Analysis. Western blot analysis was performed as described previously by our laboratory (Zastre et al., 2009; Kis et al., 2010b; Ronaldson et al., 2010) with minor modifications. In brief, cells were harvested in lysis buffer containing 10 mM HEPES, 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride, 1% Nonidet P-40, and 0.1% protease inhibitor cocktail. The samples were sonicated on ice for 10 s and then centrifuged for 10 min at 20,000g and 4°C, and the supernatants were isolated as whole-cell lysates. The protein content of the lysates was determined using a Bio-Rad protein assay kit. Whole-cell lysates were incubated with Laemmli buffer (20 mM Tris-HCl), 2 mM (Applied Biosystems). All sample reactions were incubated at 25°C for 10 min, followed by 37°C for 120 min and then 85°C for 5 min using a Mastercycler ep reaIplex 2S thermal cycler (Eppendorf Canada, Mississauga, ON, Canada).

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h, human; m, mouse.
EDTA, 2% SDS, 20% glycerol, and 0.2% bromphenol blue) and 10% β-mercaptoethanol for 10 min at room temperature. Proteins were separated on 10% SDS-polyacrylamide gel and then 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 the primary antibody: mouse anti-P-gp (C219, 1:500 dilution), which recognizes an internal epitope of human P-gp; rat anti-Mrp1 (Mrp1, 1:500 dilution), which recognizes an internal epitope of 168 amino acids in the amino-terminal end of MRP1; rat anti-Mrp4 (M4-180, 1:500 dilution), which recognizes an epitope composed of amino acids 372 to 431 of MRP4; rat anti-Bcrp (BXP-53, 1:100 dilution), which recognizes an epitope corresponding to amino acids 221 to 394 of human and mouse Bcrp; mouse anti-GATA-4 (6H10, 1:500 dilution), which recognizes a fragment corresponding to amino acids 27 to 211 of human and mouse GATA-4; or mouse anti-actin (AC40, 1:500), which recognizes an epitope on the carboxyl-terminal end of actin. The blots were then incubated with corresponding horseradish peroxidase-conjugated secondary anti-mouse (1:5000 dilution) or anti-rat (1:10,000 dilution) antibodies. Signals were enhanced through the use of a chemiluminescence SuperSignal West Pico System (Thermo Fisher Scientific, Waltham, MA) and detected by exposure to X-ray film.

**Immunofluorescence Analysis.** To identify the cellular localization of ABC transporters P-gp, Mrp1/MRP1, Mrp4/MRP4, and Bcrp/BCRP, we performed immunofluorescence analysis. Samples were prepared by culturing TM4 cells or HSECs on glass coverslips at a cell density of approximately 5000 cells/cm². The samples were fixed in 100% methanol and permeabilized with 0.1% Triton X-100. Fixed cell samples were then incubated with mouse monoclonal anti-P-gp antibody (D11, 1:50 dilution), which recognizes amino acids 1040 to 1280 of human MDR1, or rat anti-Bcrp (BXP-53, 1:50 dilution), rat anti-Mrp1 (Mrp1, 1:50 dilution), or rat anti-Mrp4 (M4–80, 1:50 dilution) antibodies, which recognize both human and rodent proteins. Cells were also incubated with rabbit polyclonal anti-Na+/K+ ATPase-α (H-300, 1:100 dilution), which recognizes the amino acids 551 to 880 of Na+/K+ ATPase-α1 of human and mouse origin. This enzyme was used as a marker of plasma membrane localization. Fixed cells were then incubated with the respective secondary antibodies conjugated to Alexa Fluor 488 (excitation 495 nm/emission 519 nm) for Na+/K+ ATPase-α and Alexa Fluor 594 (excitation 590 nm/emission 617 nm) for P-gp, Mrp1/MRP1, Mrp4/ MRP4, and Bcrp. Samples were mounted on glass slides with Vectashield hardmount medium containing DAPI (excitation 358 nm/emission 386 nm) as a marker of DNA. For negative controls, samples were incubated only with secondary antibody. Cells were then visualized using a plan C-achromat-63x/1.4 oil differential interference contrast objective and Zeiss LSM 510 META NLO two-photon confocal laser-scanning microscope (Carl Zeiss AG, Oberkochen, Germany) equipped with argon (458, 476, 488, and 513 nm wavelengths), a helium-neon (533 nm wavelength), and a tuneable Chameleon (720–930 nm wavelengths) laser lines.

**Functional Studies.** All uptake/accumulation experiments were performed using Hanks’ balanced salt solution, containing 1.3 mM KCl, 0.44 mM KH₂PO₄, 138 mM NaCl, 0.34 mM Na₂PO₄, and 5.6 mM d-glucose, supplemented with 0.01% bovine serum albumin and 25 mM HEPES, pH 7.4. Throughout this work, supplemented Hanks’ balanced salt solution buffer is referred to as transport buffer. TM4 cells were plated at a cell density of 15,000 cells/cm² for all functional studies.

The cellular accumulation of R-6G, a rhodamine analog and fluorescent P-gp substrate (Zastre et al., 2009), and BCECF-AM, a cell-permeable ester that is cleaved by intracellular esterases to BCECF, a fluorescent Mrp1/MRP1 and Mrp4/MRP4 substrate, were determined by fluorescent transport assay as described previously (Zastre et al., 2009; Ronaldson et al., 2010). In brief, TM4 cells were washed and preincubated at 37°C for 15 min in transport buffer alone (control) or transport buffer containing standard inhibitors of P-gp (i.e., 1 μM PSC833, a nonimmunosuppressive cyclosporine analog; 10 μM GF120918, an acridonecarboxamide derivative and potent P-gp and Bcrp inhibitor; and 100 μM quinidine, an antiarrhythmic agent) and 25 μM cyclosporine. To test Bcrp function, we used the Mrp inhibitor, MK 571, a leukotriene D₄ analog and cysteinyl leukotriene-1 receptor antagonist, or antiretroviral drugs. To initiate the assay, TM4 cells were incubated in transport buffer containing specific substrates for each of the respective ABC transporters of interest (1 μM R-6G, a substrate of P-gp, and BCECF, a substrate of Mrp1 and Mrp4) in the absence or presence of transport inhibitors noted above or antiretroviral drugs. Because BCECF-AM is a known substrate of P-gp, all assays involving BCECF were performed in the presence of the P-gp inhibitor PSC833 (1 μM). At the desired time interval, the reaction was stopped by washing TM4 cells with ice-cold PBS. Cells were then dissolved in 1% Triton X-100 at 37°C for 30 min. Cell-associated fluorescence was measured at an excitation wavelength of 525 nm and emission wavelength of 560 nm for R-6G or excitation wavelength of 505 nm and emission wavelength of 560 nm for BCECF using a SpectraMax Gemini XS Fluorescent spectrophotometer (Molecular Devices, Sunnyvale, CA). Cellular accumulation was normalized to cellular protein content as determined by the Bradford colorimetric method using bovine serum albumin as a standard.

The cellular accumulation of [³H]mitoxantrone, a known substrate of Bcrp, or [³H]atazanavir, a HIV protease inhibitor and P-gp substrate, was determined applying a radioactive transport assay as described previously (Lee et al., 2007; Kis et al., 2010b). In brief, to initiate the assay, TM4 cells were incubated with transport buffer containing 1 μM atazanavir (0.1 μCi/ml [³H]atazanavir), or 20 nM mitoxantrone (0.1 μCi/ml [³H]mitoxantrone), in the absence (control) or presence of transport inhibitors of P-gp (listed in the preceding paragraph) or Bcrp, 5 μM Ro 14-423, a fumitremorgin C1 analog and potent BCRP inhibitor, or 10 μM GF120918 (Ni et al., 2010) or antiretroviral drugs. Because mitoxantrone is a substrate for P-gp, all assays involving mitoxantrone accumulation were performed in the presence of 2 μM PSC833, a P-gp inhibitor. At the desired time interval, the fluorescent substrate containing medium was aspirated, and TM4 cells were washed twice with ice-cold PBS and solubilized in 1% Triton X-100 at 37°C for 30 min. The content of each well was collected and mixed with 3 ml of Pico-Fluor 40 scintillation fluid (PerkinElmer Life and Analytical Sciences, Waltham, MA), and the total radioactivity was measured using an LS5600 liquid scintillation counter (Beckman Coulter). Background accumulation was estimated by determining the retention of radiolabeled compounds in the cells after a minimum (zero) time of exposure by removing the radiolabeled solution immediately after its addition into each well, followed by two subsequent washes with ice-cold PBS and quantified using liquid scintillation counting. Total radioactive cellular accumulation was normalized to the total cellular protein content as determined by a Bio-Rad DC Protein Assay, using bovine serum albumin as the standard.

For all the accumulation assays, data are reported as total accumulation of an established substrate at steady state in the absence or presence of known inhibitors or antiretroviral drugs. To identify the appropriate time point for the accumulation assays in the absence or presence of inhibitors or antiretroviral drugs, a time-dependent uptake study for each of the established substrates, R-6G, atazanavir, BCECF, and mitoxantrone was performed in TM4 cells. Because of the limited availability of the primary cultures of human Sertoli cells and the fact that we can only passage them once, functional assays in this cell system could not be performed.

**Data Analyses.** All experiments were repeated a minimum of three times in cells pertaining to different passages for the TM4 cell line system. Each data point within an individual experiment represents data from triplicate trials. Results from time-dependent accumulation of each of the substrates of interest are presented as the mean ± S.E.M., and all functional studies involving inhibitors or antiretroviral drugs are shown as percentage control ± S.E.M. Statistical analyses were performed using GraphPad InStat 3.0 software (GraphPad Software Inc., San Diego, CA), and significance was
Results

Cell Characterization: Morphology and Biochemical Markers. The morphology of the Sertoli cell line systems was examined using transmission electron microscopy. Both TM4 cells (Fig. 1A) and HSECs (Fig. 1B) appeared to form normal elongated epithelial cell-like monolayers with a defined large nuclei and plasma membrane. We further examined the expression of GATA-4, a zinc finger-containing transcription factor known as a marker of Sertoli cells (Imai et al., 2004). We detected GATA-4 at a band of approximately 46 kDa, a molecular mass previously reported for this marker, in TM4 cells (Fig. 1C, lane 1) and in primary cultures of HSECs (Fig. 1C, lane 2). Rat fetal heart tissue was used as a positive control of GATA-4 expression (Fig. 1C, lane 3).

Relative ABC Transporter mRNA Expression in TM4 Cells and HSECs. qPCR analysis was performed to investigate mRNA expression of several ABC drug efflux transporters, Abcb1a (Mdr1a), Abcb1b (Mdr1b), ABCB1 (MDR1), Abcg2 (Bcrp), ABCG2 (BCRP), Abcc1 (Mrp1), ABCC1 (MRP1), Abcc4 (Mrp4), and ABCC4 (MRP4). In TM4 cells, we observed that Abcb1b (Mdr1b) mRNA expression (0.039 ± 0.007) was most abundant compared with cyclophilin B, the housekeeping gene, followed by the Mrps, Abcc4 (Mrp4) (0.031 ± 0.005), and Abcc1 (Mrp1) (0.012 ± 0.004). Abcg2 (Bcrp) had the lowest relative mRNA expression (0.003 ± 0.0005) and Abcb1a mRNA was below the threshold level of detection (Fig. 2A). In HSECs, mRNA expression of ABCC1 (0.60 ± 0.09) was the most abundant compared with cyclophilin B, followed by ABCC4 (0.59 ± 0.06). Both ABCB1 (0.00072 ± 0.0002) and ABCG2 (0.00089 ± 0.0006) had the lowest relative mRNA expression in the human cells (Fig. 2B).

Protein Expression of ABC Transporters in TM4 Cells and HSECs. We performed immunoblotting experiments to investigate protein expression of P-gp, Mrp1/MRP1, Mrp4/MRP4, and Bcrp/BCRP in TM4 cells and HSECs. We detected P-gp protein expression at approximately 170 kDa, a molecular size previously reported for P-gp, in both the TM4 cells (Fig. 3A, lanes 1 and 2) and in primary cultures of HSECs (Fig. 3B, lane 1) using the mouse monoclonal antibody C219, raised against an internal epitope of human P-gp. A similar size band was detected in the P-gp-overexpressing cell line MDA-MDR1 (Fig. 3, A, lane 3, and B, lane 2), which served as our positive control.

To investigate Mrp1/MPR1 protein expression, we used the rat monoclonal antibody Mrpr1, raised against a bacterial fusion protein containing a segment of 168 amino acids of human MRP1 located in the amino-proximal half of the protein. A band at approximately 190 kDa, a size previously reported for Mrp1/MPR1, in both TM4 cells (Fig. 3C, lanes 1 and 2), primary cultures of HSECs (Fig. 3D, lane 1) and in the positive control, HeLa cells overexpressing human MRP1, was detected (Fig. 3, C, lane 3, and D, lane 2). Mrp4/MPR4 protein expression was determined using the rat monoclonal antibody, M,I-80, raised against a fusion protein that contains the Escherichia coli maltose-binding protein and a fragment of the human MRP4 protein corresponding to amino acids 372 to 431. We detected a single band at approximately 180 kDa, a size previously reported for Mrp4/MPR4, in TM4 cells (Fig. 3E, lanes 1 and 2) and primary cultures of HSECs (Fig. 3F, lane 1) as well as in HEK cells overexpressing human MRP4 cells, which served as the positive control (Fig. 3, E, lane 3, and F, lane 2).

Bcrp/BCRP expression in TM4 cells and in primary cultures of HSECs was investigated using a rat monoclonal antibody, BXP-53, known to recognize an epitope corresponding to amino acids 221 to 394 of mouse and human Bcrp/BCRP. We detected a single band at approximately 76 kDa, a size previously reported for Bcrp/BCRP, in TM4 cells (Fig. 3G, lane 1) and in the MCF7-MX100 cell line overexpressing human BCRP (Fig. 3, G, lane 2, and H, lane 2), which served as the positive control. We were unable to detect a band for BCRP in primary cultures of human Sertoli cells (Fig. 3H, lane 1).
The cellular localization of known ABC membrane-associated drug transporters in Sertoli cells of mouse origin is currently not well documented. We therefore investigated the localization of P-gp, Mrp1, Mrp4, and Bcrp by indirect immunofluorescence using specific antibodies raised against each of the transporters of interest. TM4 cells and HSECs were fixed in methanol and then stained with DAPI to visualize the DNA. In all immunofluorescence studies, anti-Na+/K+ ATPase 1 antibody was used as a marker of the plasma membrane (Figs. 4, A–D, and 5, A–C).

**Cellular Localization of ABC Transporters in Sertoli Cells.** The cellular localization of known ABC membrane-associated drug transporters in Sertoli cells of mouse origin is currently not well documented. We therefore investigated the localization of P-gp, Mrp1, Mrp4, and Bcrp by indirect immunofluorescence using specific antibodies raised against each of the transporters of interest. TM4 cells and HSECs were fixed in methanol and then stained with DAPI to visualize the DNA. In all immunofluorescence studies, anti-Na+/K+ ATPase α1 antibody was used as a marker of the plasma membrane (Figs. 4, A–D, and 5, A–C).

We first examined P-gp localization using anti-P-gp (D11), a mouse monoclonal antibody raised against amino acids 1040 to 1280 of human P-gp protein. In addition to human P-gp, this antibody also recognizes P-gp protein of rodent origin. In the TM4 cells (Fig. 4A) and HSECs (Fig. 5A), we detected P-gp predominantly at the plasma membrane. Removal of the primary antibody led to a total absence of fluorescence signal, suggesting that the signal observed was specific for P-gp (data not shown).
Likewise, we detected Mrp1 localization in TM4 cells (Fig. 4B) and HSECs (Fig. 5B) applying immunofluorescence using the rat monoclonal antibody Mrpr1, which recognizes an internal epitope of human and rodent MRP1/Mrp1. A pattern of localization similar to that of P-gp was observed, predominantly at the plasma membrane. Fluorescence was not detected in the TM4 cells in the absence of primary antibody, indicating that the signal was specific for Mrp1 protein expression (data not shown).

We also examined the localization of Mrp4/MRP4 using M4I-80 mouse monoclonal antibody, which recognizes an internal epitope of human and rodent MRP4/Mrp4. We detected Mrp4 localization predominantly at the plasma membrane in TM4 cells (Fig. 4C) and HSECs (Fig. 5C). These signals were not detected in TM4 cells when the primary antibody was removed, indicating that the signals observed were specific to Mrp4 expression (data not shown).

We also investigated Bcrp protein localization, applying indirect immunofluorescence using the mouse monoclonal antibody BXP-53, which recognizes an internal epitope of rodent and human Bcrp/BCRP. We were able to detect Bcrp predominantly at the plasma membrane of TM4 cells (Fig. 4D). Cells incubated with secondary antibody alone did not exhibit a fluorescence signal, indicating that the signals observed were specific for Bcrp (data not shown). Because we did not detect expression of BCRP protein in HSECs (Fig. 3H, lane 1), we did not perform immunofluorescence localization studies in the human cell system.

**Function of ABC Transporters in TM4 Cells.** The time course of R-6G (Fig. 6A), [3H]atazanavir (Fig. 6B), and BCECF (Fig. 6C) at 37°C showed increasing substrate uptake until a plateau was reached at approximately 60 min under control conditions. The time course for [3H]mitoxantrone (Fig. 6D) revealed that a steady state was reached later at approximately 90 min in control conditions.

To investigate whether P-gp was functional in TM4 cells, accumulation studies were conducted using an established P-gp fluorescent substrate, R-6G. The accumulation of R-6G was significantly increased in the presence of P-gp inhibitors: PSC833 (1 μM, 151 ± 5%), cyclosporine (25 μM, 130 ± 12%), quinidine (100 μM, 138 ± 7%), and GF120918 (10 μM, 126 ± 8%) compared with the control (Fig. 7A). To further charac-
terize the potential involvement of P-gp in the permeability of antiretroviral drugs, we performed an accumulation study using the HIV protease inhibitor, atazanavir, a known substrate of P-gp (Kis et al., 2010b). The accumulation of [3H]atazanavir was significantly enhanced in the presence of selective P-gp inhibitors, PSC833 (1 μM, 132 ± 7%), cyclosporine (25 μM, 123 ± 6%), quinidine (100 μM, 128 ± 8%), and GF120918 (10 μM, 124 ± 10%) compared with the control (Fig. 7B). Together these data suggest that R-6G and atazanavir accumulation is regulated by a P-gp-mediated efflux process in the rodent Sertoli cell line system.

To assess whether Mrps were active in TM4 cells, we examined the accumulation of BCECF-AM, a cell-permeable ester that is cleaved by intracellular esterases. The cleaved ester, BCECF, is a fluorescent substrate of several Mrp isoforms including Mrp1 and Mrp4 (Ronaldson et al., 2010). BCECF accumulation in TM4 cells was significantly enhanced in the presence of increasing concentrations of the Mrp established inhibitor, MK 571 (5 and 10 μM, 136 ± 3 and 158 ± 4%, respectively) compared with the control (10 μM GF120918) (Fig. 7C).

To determine whether Bcrp was functional in TM4 cells, we investigated the accumulation of mitoxantrone, a chemotherapeutic agent and substrate for Bcrp and P-gp. Mitoxantrone accumulation was significantly increased in the presence of established Bcrp inhibitors, Ko 143 (5 μM, 136 ± 9%) and GF120918 (10 μM, 127 ± 10%), compared with the control (2 μM PSC833) (Fig. 7D).

To investigate the potential role of ABC transporters, P-gp, Mrps, and Bcrp, in the disposition of antiretroviral drugs in vitro, we performed functional assays in TM4 cells in the absence or presence of different classes of antiretroviral drugs. All drugs were used at clinically relevant concentrations observed in the plasma of HIV-infected individuals. Compared with the control, the accumulation of the P-gp fluorescent substrate R-6G (1 μM) was significantly enhanced in the presence of HIV protease inhibitors, atazanavir (20 μM, 145 ± 10%) and amprenavir (20 μM, 128 ± 14%). Darunavir (20 μM), lopinavir (20 μM), saquinavir (20 μM) abacavir (10 μM), tenofovir (5 μM), emtricitabine (20 μM), maraviroc (2 μM), and ritonavir (10 μM) had no significant effect on the accumulation of R-6G in TM4 cells. Although efavirenz (50 μM) and ritonavir (20 μM) appeared to decrease (56 ± 29 and 67 ± 19%, respectively), the accumulation of R-6G in TM4 cells (Fig. 8A), we determined that these compounds were capable of quenching the R-6G fluorescence, leading to an “apparent” decrease in R-6G accumulation (data not shown).

To further understand the role of P-gp in antiretroviral drug transport in TM4 cells, we also investigated the accumulation of the HIV protease inhibitor atazanavir in the presence or absence of several antiretroviral drugs. All were used at clinically relevant concentrations observed in plasma. The accumulation of atazanavir was significantly enhanced in the presence of HIV protease inhibitors darunavir (20 μM, 154 ± 14%), lopinavir (20 μM, 136 ± 15%), ritonavir (20 μM, 143 ± 10%), and saquinavir (20 μM, 146 ± 24%). In addition, the accumulation of atazanavir was also significantly enhanced in the presence of the HIV nucleoside reverse transcriptase inhibitor, tenofovir (5 μM, 157 ± 13%) and HIV non-nucleoside transcriptase inhibitor, efavirenz (50 μM, 196 ± 23%). None of the other antiretroviral drugs

Fig. 5. Immunocytochemical localization of ABC transporters in primary cultures of HSECs. HSECs were stained with DNA dye, DAPI (blue), and examined by immunofluorescence using anti-P-gp (A; D11, red), anti-MRP1 (B; Mrpr1, red), and anti-MRP4 (C; M4–80, red) monoclonal antibodies. Anti-Na+/K+ ATPase-α1 (H300, green) rabbit polyclonal antibody was used as a marker of plasma membrane. Cells were stained with Alexa Fluor-conjugated secondary antibodies 488/594 alone to verify the signal specificity of the primary antibodies. Scale bar, 20 μm.
tested significantly altered the accumulation of atazanavir by TM4 cells compared with the control (Fig. 8B).

The accumulation of the Mrp fluorescent substrate BCECF by TM4 cells was significantly enhanced in the presence of the HIV protease inhibitor, ritonavir (20 μM, 136 ± 13%). Efavirenz was not tested, because it also quenched BCECF fluorescence similar to our findings with R-6G (data not shown). None of the other antiretroviral drugs tested significantly altered the accumulation of BCECF compared with the control (Fig. 8C).

The accumulation of the Bcrp substrate mitoxantrone by TM4 cells was significantly enhanced in the presence of the HIV protease inhibitors lopinavir (20 μM, 146 ± 14%) and saquinavir (20 μM, 132 ± 18%) and the non-nucleoside reverse transcriptase inhibitor efavirenz (50 μM, 138 ± 12%). None of the other antiretroviral drugs tested significantly affected the accumulation of mitoxantrone compared with the control (Fig. 8D).

**Discussion**

Although current antiretroviral therapy is very effective in suppressing viral replication in plasma, it does not completely eradicate HIV infection from the host (Dahl et al., 2010). Clinical studies have reported very low concentrations of anti-HIV drugs in the brain and testes (Taylor et al., 1999; van Praag et al., 2001; Pereira et al., 2002; Ghosn et al., 2004; Cruciani et al., 2006; van Leeuwen et al., 2007). This, in part, has been attributed to the expression of ABC drug efflux transporters at blood-tissue barriers such as the blood-brain barrier, which can actively extrude several drugs including antiretrovirals from the brain (Kis et al., 2010a). Reduced drug concentrations in either brain or testis could result in ineffective viral suppression with the potential risk of anti-HIV drug resistance and the formation of a HIV reservoir (Dahl et al., 2010). Although the functional expression of ABC membrane-associated transporters and their role in antiretroviral drug transport at the blood-brain barrier and in brain parenchyma have been investigated (Dallas et al., 2004a; Ronaldson et al., 2004), to the best of our knowledge, the activity of these transporters at the blood-testis barrier has not been explored.

In this study, we examined the expression of several drug efflux transporters in two models of Sertoli cells: 1) a mouse Sertoli (TM4) cell system that has previously been characterized to maintain Sertoli cell properties in response to follicle-stimulating hormone and lack of response to luteinizing hormone (Mather, 1980) and 2) primary cultures of human Sertoli cells (Chui et al., 2011). To further characterize our cell models, we performed detailed morphology studies using electron microscopy and further confirmed the expression of GATA-4, a transcription factor and known Sertoli cell marker in both TM4 cells and primary cultures of HSECs (Imai et al., 2004).

In TM4 cells, we demonstrated for the first time mRNA and protein expression as well as cellular localization of the major ABC membrane-associated drug efflux proteins, i.e.,
P-gp, Mrp1, Mrp4, and Bcrp. In particular, applying qPCR, we detected mRNA expression of Abcb1b, Abcc1, Abcc4, and Abcg2. Furthermore, we observed P-gp, Mrp1, Mrp4, and Bcrp protein expression and plasma membrane localization using Western blot and immunofluorescence analyses, respectively. Our laboratory has previously observed the localization of P-gp at the plasma membrane and cytosol of rodent and human brain microvessel endothelial cells, astrocytes, and microglia by applying immunogold cytochemistry and electron microscopy (Bendayan et al., 2002, 2006). Our data are also in agreement with previous findings reporting mRNA expression of several ABC transporters in isolated adult male Sprague-Dawley rat Sertoli cells (Augustine et al., 2005). Our data are also in agreement with previous findings reporting mRNA expression of several ABC transporters in isolated adult male Sprague-Dawley rat Sertoli cells (Augustine et al., 2005). In the primary cultures of HSECs, we confirmed for the first time mRNA, protein expression and localization of MDR1/P-gp, MRP1, and MRP4 by applying qPCR, immunoblotting, and immunofluorescence confocal microscopy techniques. Of interest, we were unable to detect BCRP protein expression in this cell system and were only able to observe very low ABCG2 mRNA expression. A study by Melaine et al. (2002) reported Abcb1a/Abcb1b/ABCB1 gene expression in the testes of human, rat, mouse, and guinea pig, confirming that at the mRNA level this transporter is expressed in the testes of several mammalian species. Although no studies have directly examined the cellular localization of these transporters in Sertoli cells, a few immunohistochemical reports have described localization of P-gp and MRP1 in human normal and tumor testicular tissue slices and in rat testes (Bart et al., 2004; Su et al., 2009). Similar to our findings, BCRP was not detected in these tissues.

To determine whether P-gp retains function, we investigated the accumulation of R-6G and atazanavir by TM4 monolayer cells in the presence of standard P-gp inhibitors, PSC833, cyclosporine, quinidine, and GF120918. The accumulation of both P-gp substrates was significantly enhanced in the presence of the inhibitors, suggesting that P-gp-mediated efflux is involved in the overall transport of the two substrates by the Sertoli monolayer cells. Our data are the
first to demonstrate that the HIV protease inhibitors, amprenavir and atazanavir, can significantly enhance R-6G accumulation by the TM4 cells. Furthermore, we also demonstrated that atazanavir accumulation is significantly enhanced by other HIV protease inhibitors, darunavir, lopinavir, ritonavir, and saquinavir, as well as the nucleoside reverse transcriptase inhibitor, tenofovir, and the non-nucleoside reverse transcriptase inhibitor, efavirenz, by TM4 cells. These results suggest that many of the anti-HIV drugs can inhibit P-gp-mediated efflux of R-6G and atazanavir by TM4 cells. A study by Storch et al. (2007) reported similar findings examining calcine efflux, another established P-gp substrate, in the presence of tenofovir and efavirenz in LLC-PK1 (porcine kidney epithelial cells) stably transfected with MDR1. Previous in vitro studies investigating the role of P-gp in antiretroviral drug transport have shown that HIV protease inhibitors serve as both substrates and inhibitors of this transporter in cell culture systems, including Caco-2 and ABCB1 gene-transfected porcine kidney LLC-PK1 cell lines systems (Fujimoto et al., 2009; Zastre et al., 2009). Furthermore, an in vivo study using \textit{Mdr1a} (+/+) and \textit{Mdr1a} (−/−) mice reported significantly higher brain concentrations (7- to 30-fold) of the HIV protease inhibitors, indinavir, saquinavir, and nelfinavir in \textit{Mdr1a} (−/−) compared with that in wild-type \textit{Mdr1a} (+/+) mice, indicating a role for P-gp in HIV protease inhibitor permeability in the brain (Kim et al., 1998). Likewise, a study by Choo et al. in 2000 demonstrated that intravenous administration of the HIV protease inhibitor \textsuperscript{[14C]}nelfinavir, in the presence of the selective P-gp inhibitor zosuquidar (LY-335979), increased brain and testes drug concentrations 37- and 4-fold, respectively, in wild-type mice, further emphasizing the significance of P-gp in ARV drug permeability at several sites.

Clinical studies investigating the permeability of antiretroviral drugs in the seminal fluid of HIV-positive patients have observed significantly reduced concentrations of efavirenz (Ghosn et al., 2004), amprenavir (Pereira et al., 2002), ritonavir (Taylor et al., 1999), and atazanavir (van Leeuwen et al., 2007) compared with drug concentrations present in plasma. In addition, a study examining the in vivo distribution of \textsuperscript{[3H]}digoxin, an established P-gp substrate, demonstrated an increase in digoxin concentration in \textit{Mdr1a} (−/−) mice testes (593 ± 169 ng/g tissue) compared with wild-type \textit{Mdr1a} (+/+) mice testes (213 ± 83 ng/g tissue) (Schinkel et al., 1995). Taken together, our data in TM4 cells, combined with previous in vivo animal studies and clinical observations in HIV-positive men, strongly suggest that P-gp plays a role in restricting the permeability of several substrates including anti-HIV drugs at the BTB.

To examine the function of Mrps in TM4 cells, BCECF was

**Fig. 8.** Accumulation of R-6G (A), atazanavir (B), BCECF (C), and mitoxantrone (D) by TM4 cell monolayers. Accumulation of 1 μM R-6G (1 h) or 1 μM atazanavir (1 h) or 5 μM BCECF (1 h) or 20 nM mitoxantrone (2 h) in the absence or presence of selective inhibitors of ABC transporters or antiretroviral drugs at 37°C is shown. All measurements involving BCECF were performed in the presence of 10 μM GF120918 (P-gp and BCRP inhibitor) to eliminate any potential efflux of the prodrug ester BCECF-AM. Likewise, all measurements involving mitoxantrone were performed in the presence of 2 μM PSC833 (P-gp inhibitor) to eliminate any potential P-gp-mediated efflux of mitoxantrone. Results are expressed as mean percentage control ± S.E.M. of three separate experiments. *, data points that are significantly different from control (p < 0.05).
chosen as the Mrp substrate. To confirm the Mrp substrate specificity of BCECF, we demonstrated that its accumulation by TM4 cells was significantly enhanced in the presence of MK 571, a potent Mrp inhibitor (Leier et al., 1994). Our laboratory has previously reported that MK 571 is capable of inhibiting Mrp1-mediated efflux of the anticanian cancer drug vincristine and the HIV protease inhibitor, saquinavir, in a breast cancer cell line that overexpresses BCRP (Lee et al., 2010). Furthermore, Kodaira et al. (2010) observed that Ko 143 and GF120918 can inhibit the BCRP-mediated efflux of mitoxantrone in MCF7-MX100, a breast cancer cell line that overexpresses BCRP (Lee et al., 2007). In addition, lopinavir, saquinavir, and efavirenz significantly enhanced the accumulation of xenobiotic compounds such as mitoxantrone (chemotherapeutic), erlotinib (chemotherapeutic), and flavopiridol (cyclin-dependent kinase inhibitor) in wild-type Mdr1a/H11002, Abcg2/H11002, and Abcg2/H11002/Apexo M) knockout mice compared with Mdr1a/H11002, Abcg2/H11002, and Abcg2/H11002). Our current findings in TM4 and primary cultures of human Sertoli cells combined with data from previous immuno-histochemical and in vivo studies suggest that ABC drug transporter, like P-gp, can regulate drug permeability in Sertoli cells. Our laboratory has previously reported that Ko 143 and GF120918, suggesting for the first time that the transporter is active in the Sertoli cell line system. Our laboratory has previously reported that Ko 143 and GF120918 can inhibit the BCRP-mediated efflux of mitoxantrone in MCF7-MX100, a breast cancer cell line that overexpresses BCRP (Lee et al., 2007). In addition, lopinavir, saquinavir, and efavirenz significantly enhanced the accumulation of mitoxantrone by TM4 cells. These data support previous findings by Weiss et al. (2007), reporting the inhibition of Bcrp-mediated efflux of phenobarbide A, an established fluorescent BCRP substrate, in Madin-Darby canine kidney I-BCRP-overexpressing cells, by lopinavir (IC50 7.66 μM), saquinavir (IC50 27.4 μM), and efavirenz (IC50 20.6 μM). Furthermore, Gupta et al. (2004) demonstrated that ritonavir, saquinavir, and nelfinavir are inhibitors but not substrates of BCRP-mediated efflux of mitoxantrone in HEK293-BCRP cells, a human embryonic kidney cell system stably transfected with BCRP. To date, in vivo studies investigating the role of Bcrp have demonstrated an enhanced accumulation of xenobiotic compounds such as mitoxantrone (chemotherapeutic), erlotinib (chemotherapeutic), and flavopiridol (cyclin-dependent kinase inhibitor) in brain and testes of Abcg2(−/−) knockout mice compared with wild-type Abcg2(+/+) mice (Enokizono et al., 2008; Kodaira et al., 2010). Furthermore, Kodaira et al. (2010) observed that the Mdr1a(−/−), Mdr1b(−/−), and Abcg2(−/−) triple knockout mice exhibited a greater testicular concentration of these substrates compared with Mdr1a(−/−) and Mdr1b(−/−) or Abcg2(−/−) knockout mice alone. Taken together, these data suggest a cooperative role of Bcrp and P-gp in preventing xenobiotics from crossing to the BTB and further support the fact that P-gp and Bcrp could contribute to restricting the permeability of antiretroviral drugs at this site.

Our current findings in TM4 and primary cultures of human Sertoli cells combined with data from previous immuno-histochemical and in vivo studies suggest that ABC drug transporters, P-gp, Mrps, and Bcrp expressed in Sertoli cells play an important role in the permeability of antiretroviral drugs at the BTB. In addition, this study provides insights in our understanding of the mechanisms of drug transport in both rodent and human Sertoli cells and contributes to the elucidation of the mechanisms by which these transporters could regulate the ability of antiviral drugs to reach therapeutical concentrations within the genital tract of HIV-infected men.

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Authorship Contributions

Participated in research design: Robillard and Bendayan. Conducted experiments: Robillard and Hoque. Contributed new reagents or analytic tools: Bendayan. Performed data analysis: Robillard, Hoque, and Bendayan. Wrote or contributed to the writing of the manuscript: Robillard, Hoque, and Bendayan.

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