Xenobiotic Nuclear Receptors Pregnan X Receptor and Constitutive Androstane Receptor Regulate Antiretroviral Drug Efflux Transporters at the Blood-Testis Barrier

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ABSTRACT

Poor antiretroviral drug (ARV) penetration in the testes could be due, in part, to the presence of ATP-binding cassette (ABC) membrane–associated drug efflux transporters such as P-glycoprotein (P-gp), breast cancer resistance protein (BCRP), and multidrug resistance–associated proteins (MRPs) expressed at the blood-testis barrier (BTB). The functional expression of these transporters is known to be regulated by ligand-activated nuclear receptors pregnane X receptor (PXR) and constitutive androstane receptor (CAR) in various tissues. This study aimed to investigate in vitro and ex vivo the role of PXR and CAR in regulating ABC transporters at the BTB. Both PXR and CAR proteins were expressed in human testicular tissue and in mouse TM4 Sertoli cells (an in vitro cell line model of the BTB). In addition, we demonstrated an upregulation of P-gp, Bcrp, and Mrp4 mRNA and protein expression, after exposure to PXR or CAR ligands in TM4 cells. Small interfering RNA downregulation of PXR or CAR attenuated the expression of these transporters, suggesting the direct involvement of these nuclear receptors in regulating P-gp, Bcrp, and Mrp4 in this system. In an ex vivo study using freshly isolated mouse seminiferous tubules, we found that exposure to PXR or CAR ligands, including ARVs, significantly increased P-gp expression and function. Together, these data suggest that ABC transporters could be regulated at the BTB during chronic treatment with ARVs that can serve as ligands for PXR and CAR, which could in turn further limit testicular ARV concentrations.

Introduction

Despite the use of highly active antiretroviral therapy, human immunodeficiency virus (HIV)-1 persistence in anatomic reservoirs and sanctuary sites such as the lymph nodes, brain, and testes can be attributed, in part, to the low antiretroviral drug (ARV) concentrations reached in these tissues (Dahl et al., 2010; Fletcher et al., 2014; Huang et al., 2016; Jenabian et al., 2016). Semen is the most common vector for HIV transmission globally and limited ARV penetration in the testis could, in part, restrict ARV concentrations in the seminal fluid of HIV-infected men (Else et al., 2011). The testis is an immunoprivileged environment where the blood-testis barrier (BTB), due to its physical and biochemical properties, can limit the penetration of exogenous agents into this tissue. This barrier is primarily composed of adjacent epithelial Sertoli cells, which form tight junction complexes near the basement membrane and physically divide the seminiferous epithelium into apical and basolateral compartments. This allows for spermatid development in a microenvironment within the seminiferous tubules where the entrance of anti-sperm antibodies and harmful xenobiotics is restricted (Su et al., 2011).

ARV disposition in rodents and humans primarily involves intracellular drug metabolism and transport by members of the ATP-binding cassette (ABC) and solute carrier (SLC) superfamilies of drug transport proteins across biologic membranes. The ABC family includes several drug efflux transporters such as P-glycoprotein (P-gp; ABCB1), multidrug resistance–associated proteins (MRPs; ABCG2), and breast...
cancer resistance protein (BCRP; ABCG2), whereas the SLC transporters comprise organic anion-transporting polypeptides, organic anion and organic cation transporters, and equilibrative and concentrative nucleoside transporters (Kis et al., 2010). Our group and others have demonstrated in vitro and in vivo that several efflux transporters are functionally expressed at the BTB and could potentially limit the penetration of ARVs into rodent and human testes (Choo et al., 2000; Robillard et al., 2012, 2014; Huang et al., 2016).

Most ARVs from the protease inhibitor (PI) class are known to be substrates of P-gp, whereas BCRP is primarily involved in the transport of several of the nucleoside and non-nucleoside reverse transcriptase inhibitors. Both P-gp and BCRP have also been implicated in the efflux of integrase strand transfer inhibitor drugs such as dolutegravir and raltegravir. The MRP isofoms are also known to be involved in the transport of ARVs. In particular, MRP4 is involved in the efflux of several nucleoside reverse transcriptase inhibitors such as lamivudine, tenofovir, and abacavir. In addition to being substrates, many ARVs also interact with these transporters as inducers and/or inhibitors (Supplemental Table 1). Recently, we characterized the expression and localization of drug transporters and metabolic enzymes involved in ARV transport in the testes of HIV-infected individuals receiving ARV therapy and we measured plasma and testicular drug concentrations. In particular, darunavir, a P-gp substrate that is currently recommended as a preferred PI regimen (DHHS Panel on Antiretroviral Guidelines for Adults and Adolescents, 2016, Available at http://www.aidsinfo.nih.gov/ContentFiles/Adul-tandAdolescentGL.pdf), displayed subtherapeutic levels in the testis that reached approximately 19% of plasma concentrations (Huang et al., 2016), thus demonstrating the role that these transporters may play in limiting ARV testicular tissue concentrations.

The functional expression of P-gp, BCRP, and MRP isofoms is known to be regulated through the activity of ligand-activated nuclear receptors pregnane X receptor (PXR) and/or constitutive androstane receptor (CAR) in tissues such as the brain, liver, and intestine as well as in peripheral blood mononuclear cells (Assem et al., 2004; Albermann et al., 2005; Burkt et al., 2005; Chan et al., 2011). These two nuclear receptors are known to interact with endogenous ligands and a wide range of pharmacological agents and hence are recognized as major xenobiotic sensors (Urquhart et al., 2007). Our group previously demonstrated that ligand-activated PXR and CAR upregulated P-gp in vitro and in vivo at the level of the blood-brain barrier (BBB), resulting in further impairment of drug permeation and distribution in the central nervous system (Chan et al., 2011, 2013b). Our group and others have also demonstrated that ARVs can directly activate PXR and CAR, causing an induction in the expression and activity of their target genes (Dussault et al., 2001; Chan et al., 2013a).

To our knowledge, the regulation of drug efflux transporter expression and function by nuclear receptors at the BTB has not been addressed. This study aimed to investigate the role of PXR and CAR in the regulation of ABC drug efflux transporters (P-gp, Bcrp, and Mrp4) at the BTB in vitro and ex vivo in mice.

**Materials and Methods**

**Chemicals and Reagents.** Unlabeled ARVs were obtained from the National Institutes of Health AIDS Research and Reference Reagent Program (Bethesda, MD). [3H]-atazanavir (0.900 Ci/mmol) was purchased from Moravek Biochemicals (Brea, CA). Valspodar (PSC833; 6–1(25,4R,6E)–4-methyl-2-(methylamino)-3-oxo-6-octenoic acid–7,1-valine-cyclosporin A) was a generous gift from Novartis Pharma (Basel, Switzerland). Cyclosporine (CsA) was obtained from Torris Biosciences (Ellisville, MO), 1,4-Bis(2-(3,5-dichlorophenyl) benzene (TCPOBOP), dexamethasone, ketonazole, and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were purchased from Sigma-Aldrich (Oakville, ON, Canada). Pregnenolone 16α carboxinile (PCN) was purchased from Cayman Chemicals (Ann Arbor, MI). Type I collagen was purchased from BD Biosciences (San Jose, CA). Small interfering RNA (siRNA) against mouse PXR (sc-4058), CAR (sc-43663), and glucocorticoid receptor (GR) (sc-35506) and nonsilencing negative control siRNA (sc-37007) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Each siRNA product contained a pool of three target-specific 19- to 25-nt siRNAs designed to knock down gene expression. The mouse anti-actin, goat anti-PXR, rabbit anti-CAR, and rabbit anti-GR antibodies were also obtained from Santa Cruz Biotechnology Inc. Please note that the rabbit anti-CAR (sc-13065) antibody obtained from Santa Cruz Biotechnology has been reported by the company to identify CAR at around 55–60 kDa. In addition, several groups including ours have detected CAR at around 50–60 kDa using this antibody (Hernandez et al., 2009; Wang et al., 2010; Chan et al., 2011), even though the predicted molecular weight of CAR has been reported at around 40–45 kDa based on its amino acid sequence. This potentially could be due to changes in the phosphorylation of the molecule (Mutoh et al., 2009) or to other post-translational modifications that could affect the migration of the protein in different tissues. Mouse anti–P-gp antibody, rat anti-Bcrp antibody, and rat anti-Mrp4 antibody were purchased from Enzo Life Sciences (Farmingdale, NY), Abcam Inc. (Boston, MA), and Kamiya Biomedical Company (Seattle, WA), respectively. Horseradish peroxidase–conjugated secondary antibodies were ordered from Jackson Immunoresearch Laboratories Inc. (West Grove, PA).

**Human Testicular Tissue.** In collaboration with Drs. Jean-Pierre Routy (McGill University Health Center, Montréal, QC, Canada) and Pierre Brassard and Maud Belanger (Metropolitan Centre of Plastic Surgery, Montréal, QC, Canada), we were able to obtain testicular tissue from individuals who were undergoing orchietomy for sex reassignment. We obtained ethics approval for this study from the McGill University Health Centre Ethical Review Board. All study subjects provided written informed consent for participation in the study. Tissue samples were stored in saline solution immediately after surgery and then processed within 1–2 hours in which they were cut into small pieces, snap frozen in liquid nitrogen, and stored at −80°C until study assessments. A small portion of each testis was sent to the pathology laboratory (Metropolitan Centre of Plastic Surgery, Montréal, QC, Canada) for routine tests to exclude any infection or malignancy, whereas the remaining portion was used in our laboratory to examine nuclear receptor expression by western blot analyses.

**Cell Culture Systems.** The continuous mouse Sertoli cell line (TM4) and the hepatocellular carcinoma cell line (HepG2) were obtained from American Type Culture Collection (Manassas, VA). TM4 cells were grown on culture flasks and Multivell dishes precoated with rat tail collagen type 1 (100 μg/ml) to enhance cell adherence. The BCRP-overexpressing human breast cancer cell line (MCF7-MX100) was a generous gift from Dr. Susan Bates (Columbia University, New York, NY). The human breast cancer cell line DA435/LCC6 stably transfected with human MDR1 cDNA (MDR1) was kindly provided by Dr. Robert Clarke (Georgetown University, Washington, DC). The human embryonic kidney (HEK) cell line stably transfected with human MRPs CDNA (HEK-MRP4) was kindly provided by Dr. Piet Borst (Netherlands Cancer Institute, Amsterdam, The Netherlands). All cell culture systems were grown and maintained at 37°C humidified 5% CO₂–95% air with fresh media replaced every 2–3 days, according to our previously published protocols (Hoque et al., 2012; Robillard et al., 2012).

**Mouse Seminiferous Tubule Isolation.** C57BL/6 male mice were a kind gift from Dr. Finnell (University of Texas, Austin, TX). All
experiments, procedures, and animal care were conducted in accordance with the Canadian Council on Animal Care guidelines and approved by the University of Toronto Animal Care Committee. Animals were housed under a 12-hour/12-hour light/dark cycle at room temperature with free access to food and water. Seminiferous tubules were isolated as previously described (Lie et al., 2010). In brief, testes from wild-type adult mice were decapsulated, washed, and incubated in collagenase (0.5 mg/ml) in a 1:1 Dulbecco’s modified Eagle’s medium (DMEM/F12 medium) containing 35 °C for 25 minutes. Seminiferous tubules were then washed five times by sedimentation under gravity to remove Leydig cells. The tubules were then incubated in DMEM/F12 media containing the desired ligands for 2 hours. Samples were then washed and stored at −80 °C for future analysis or used immediately to perform transport assays.

**Ligand Treatment.** Monolayers of TM4 cells grown on collagen-coated six-well plates were treated with the established PXR ligands dexamethasone (25–100 μM) or PCN (10–50 μM) alone or in the presence of antagonist ketoconazole (10 μM) or with the CAR ligand TCPOBOP (50–500 nM). At the beginning of each experiment, the culture medium was aspirated and fresh medium containing ligands dissolved in dimethylsulfoxide (DMSO) was added. Seminiferous tubules were exposed to PCN (100 μM), TCPOBOP (5 μM), efavirenz (50 μM), or darunavir (100 μM). Control cells and seminiferous tubules were exposed to 0.1% (v/v) DMSO (vehicle) in the absence of ligands. mRNA or protein expression of ABC transporters was examined between 6 and 72 hours in TM4 cells and between 1 and 24 hours in seminiferous tubules with data shown for time points where consistent upregulation of transporters was observed (TM4 cells: 24 hours for mRNA, 72 hours for protein; seminiferous tubules: 2 hours for mRNA and protein). All of the ligands were tested over a range of concentrations based on their EC_{50} values to study their effects on the transporters, and final concentrations used were based on their effectiveness in inducing transporter expression and cell viability. To ensure that cells would remain viable during ligand treatment, all ligand concentrations used were tested by applying the MTT assay as described previously by our laboratory with minor modifications (Mosmann, 1983; Chan et al., 2011). After treatment with ligands for up to 72 hours, cells were incubated for 2 hours at 37°C with 5 mg/ml MTX solution in phosphate-buffered saline. The formazan content, dissolved in DMSO, from each well was determined by UV analysis at 580 nm using a SpectraMax 384 microplate reader (Molecular Devices, Sunnyvale, CA). Cell viability was assessed by measuring the absorbance of cellular reduced MTT in ligand-treated cells to that of vehicle-treated cells.

**RNA Extraction, Reverse Transcription, and Quantitative Real-Time Polymerase Chain Reaction.** Total RNA was extracted from TM4 cells and mouse seminiferous tubules using TRIzol reagent (Invitrogen, Carlsbad, CA) according to the manufacturer’s instructions. RNA concentration (absorbance at 260 nm) and purity (absorbance ratio 260/280 nm) was assessed using a DU Series 700 Scanning UV-visible spectrophotometer (Beckman Coulter, Mississauga, ON, Canada). Isolated RNA was digested in DNase I (0.1 U/ml) to remove genomic DNA. Then 2 μg DNase-treated total RNA was reverse transcribed using the high-capacity cDNA reverse transcriptase kit (Applied Biosystems, Waltham, MA). Reverse transcription was performed at 25°C for 10 minutes, followed by 37°C for 120 minutes and then 85°C for 5 minutes using a Mastercycler ep Realplex 28 thermal cycler (Eppendorf, Mississauga, ON, Canada). mRNA expression of genes encoding ABC transporters (Abcb1a, Abcb1b, Abcc4, and Abcg2) and the housekeeping genes glyceraldehyde 3-phosphate dehydrogenase (GAPDH) or cyclophilin B was analyzed by quantitative real-time polymerase chain reaction (qPCR) on a Mastercycler ep Realplex 28 thermal cycler using TaqMan qPCR chemistry. Primers and probes (Supplemental Table 2) were designed and validated by Life Technologies (Burlington, ON, Canada). All reactions were performed in triplicate with each 20-μl reaction containing 100 ng cDNA, 1 μl 20× primer mix, and 10 μl TaqMan qPCR master mix. To analyze the regulation of Abcb1b, Abcg2, and Abcc4 genes in ligand-treated samples, mRNA was normalized by the Gapdh housekeeping gene. Results are expressed as the fold change in mRNA levels ± S.E.M. and were calculated using the comparative threshold cycle method in which changes in transporter mRNA expression were calibrated to vehicle-treated cells.

**Immunoblot Analysis.** Western blot analysis was performed as described previously by our group with minor modifications (Robillard et al., 2012). Briefly, lysates from human testicular tissue, mouse seminiferous tubules, or cell lines were extracted using a modified radioimmunoprecipitation lysis buffer containing 50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM EGTA, 1 mM sodium ortho-vanadate, 0.5 mM phenylmethylsulfonyl fluoride, 1% (v/v) Nonidet-P40, and 0.1% (v/v) PI cocktail. Homogenates were subjected to brief sonication, followed by centrifugation for 15 minutes at 20,000 g and 4°C, and the supernatants were isolated as whole tissue or cell lysates. The protein content of each cell lysate sample was quantified using a Bio-Rad protein assay kit (Bio-Rad, Hercules, CA). Proteins were separated on 7% or 10% SDS-polyacrylamide gel with 5% stacking gel and were then electrophoresed onto a polyvinylidene difluoride membrane. The membranes were blocked for 2 hours at room temperature in 5% skim-milk/Tris-buffered saline containing 0.1% Tween 20 and incubated overnight at 4°C with the following primary antibodies recognizing each protein: mouse anti-P-gp (C219, 1:250 dilution), rat anti-Mrp4 (M4-180, 1:500 dilution), rat anti-Bcrp (BXP-53, 1:500 dilution), goat anti-PXR (A-20, 1:250 dilution), rabbit anti-CAR (M-127, 1:250), rabbit anti-GR (M-20, 1:2000), and mouse anti-actin (A-206, 1:2000 dilution). Membranes were treated with data shown for time points where consistent upregulation of transporters was observed (TM4 cells: 24 hours for mRNA, 72 hours for protein; seminiferous tubules: 2 hours for mRNA and protein). All of the ligands were tested over a range of concentrations based on their EC_{50} values to study their effects on the transporters, and final concentrations used were based on their effectiveness in inducing transporter expression and cell viability. To ensure that cells would remain viable during ligand treatment, all ligand concentrations used were tested by applying the MTT assay as described previously by our laboratory with minor modifications (Mosmann, 1983; Chan et al., 2011). After treatment with ligands for up to 72 hours, cells were incubated for 2 hours at 37°C with 5 mg/ml MTX solution in phosphate-buffered saline. The formazan content, dissolved in DMSO, from each well was determined by UV analysis at 580 nm using a SpectraMax 384 microplate reader (Molecular Devices, Sunnyvale, CA). Cell viability was assessed by measuring the absorbance of cellular reduced MTT in ligand-treated cells to that of vehicle-treated cells.

**Western Blot Analysis.** Protein expression was analyzed using densitometry analysis with Alpha DigiDoc RT2 imaging software (Alpha Innotech, San Leandro, CA).

**siRNA Studies.** TM4 Sertoli cells were seeded in six-well plates with a density of 0.2 × 10^6 cells/well. After 24 hours, cell monolayers at approximately 80% confluence were subjected to mPXR or mCAR targeting siRNA transfection. Transfection mix was prepared in OptiMEM GlutaMax (Invitrogen) medium with siRNA and Lipofectamine 2000 (Invitrogen) according to the manufacturer’s protocol. The final concentration of siRNA and Lipofectamine added to the cells were 100 nM and 2 μl/ml, respectively, and the cells were incubated for 24 hours in the presence of transfection mixture. The following day, the transfection mixture was replaced with fresh prewarmed TM4 cell medium, and cell culture was pursued for an additional 48 hours. After 72 hours of siRNA transfection, cells were harvested to analyze P-gp, Mrp4, Bcrp, PXR, and CAR protein expression by western blot.

**Ex Vivo Transport Assay with Seminiferous Tubules.** The tubular accumulation of [1H]-atazanavir, a known ARV substrate of P-gp, was determined by applying a radioactive transport assay as described previously (Robillard et al., 2012; Klein et al., 2013). Seminiferous tubules were dissected from the mouse testis and cut into 10- to 15-mm pieces for data normalization before transfer to a 96-well cell culture plate for incubation with ligands and/or transport assay. Tubules were either isolated and incubated at 35°C in DMEM/F12 medium containing ligands prior to washing and transport experiments using transport buffer (Ringer’s solution containing 103 mM NaCl, 25 mM NaHCO_3, 19 mM sodium glutonate, 1 mM sodium acetate, 1.2 mM NaH_2PO_4, 5 mM KCl, 1 mM CaCl_2, 1 mM MgCl_2, and 5.5 mM glucose, at pH 7.4), or they were isolated and washed directly in transport buffer without ligand treatment. For transport experiments, seminiferous tubules were preincubated at 35°C for 15 minutes in transport buffer alone or in transport buffer containing standard P-gp inhibitors CsA (25 μM) or PSC833 (5 μM),
a cyclosporine derivative and specific inhibitor of P-gp (Atadja et al., 1998). To initiate transport, the preincubation buffer was replaced with transport buffer containing 1 μM [3H]-atazanavir with or without P-gp inhibitors. At the desired time points, the reaction was stopped by washing the tubules with ice-cold transport buffer. Seminiferous tubules were then dissolved in 1 N NaOH for extraction of accumulated radioactivity. The content of each well was collected and mixed with 3 ml Pico-Fluor 40 scintillation fluid (PerkinElmer Life and Analytical Sciences, Waltham, MA), and the total radioactivity was measured using an LS6500 liquid scintillation counter (Beckman Coulter). Background accumulation was estimated by determining the retention of radiolabeled compound in the tubules after a minimum (zero) time of exposure by removing the radiolabeled solution immediately after its addition into each well. Each data point within an individual experiment represents the average of triplicate values. For the accumulation experiments, data are reported as accumulation of the substrate at steady state, as determined by the time-dependent uptake assays, in the absence or presence of inhibitor.

**Statistical Analyses.** All experiments were repeated at least three times in cells pertaining to different passages, or in seminiferous tubules isolated from two to three animals per experiment. Results are expressed as the mean ± S.E.M. Statistical analyses were performed using GraphPad Prism 7 software (GraphPad Software Inc., San Diego, CA). Comparison between groups was performed as appropriate, applying either the two-tailed t test for unpaired experimental values or one-way analysis of variance with the Bonferroni multiple-comparisons post hoc test or Dunnett post hoc t test. P < 0.05 was considered statistically significant.

**Results**

**PXR and CAR Protein Expression in Human Testicular Tissue and TM4 Sertoli Cells.** To investigate the expression of PXR and CAR in human testicular tissue obtained from three noninfected individuals, as well as in the TM4 Sertoli cell line derived from mouse testis, western blot analyses were performed using whole tissue or cell lysates, respectively. HepG2 cells known to express PXR and CAR served as a positive control in these experiments (Fig. 1). CAR was robustly expressed in both the human testicular tissue samples and TM4 Sertoli cells, with bands visible at a molecular weight of 60 kDa. PXR was also detected with protein bands at the expected molecular weight of approximately 52 kDa in all samples evaluated. Considering the exceptional rarity of human testicular tissue availability, the rest of the experimental work was performed in vitro in TM4 Sertoli cells and ex vivo in seminiferous tubules freshly isolated from mice testes.

**PXR-Mediated Upregulation of Abcb1b (P-gp) and Abcg2 (Bcrp) mRNA and Protein Expression.** Applying qPCR analysis, we examined the expression of Abcb1b and Abcg2 mRNA, which encode P-gp and Bcrp, respectively, in TM4 Sertoli cells exposed to PXR ligands PCN or dexamethasone. We also investigated the expression of Abcb1a and found that of the two murine Abcb1 isoforms encoding P-gp, Abcb1b was most abundant at the BTB (Supplemental Fig. 1). This was also demonstrated previously by our group (Robillard et al., 2012); therefore, we only investigated the regulation of Abcb1b in our studies. TM4 cells treated with increased concentrations of PCN (10–50 μM) or dexamethasone (50–100 μM) had significantly higher levels of Abcb1b (up to 2-fold) and Abcg2 (up to 3-fold) mRNA expression compared with vehicle-treated control cells at 24 hours (Fig. 2, A and B). To determine whether the increases in mRNA expression would result in changes in P-gp and Bcrp protein expression, western blot analysis was performed 72 hours post-treatment with both PXR ligands (Fig. 2, C–F). Densitometric analysis revealed approximately 50% induction in P-gp expression when cells were exposed to dexamethasone or PCN, whereas Bcrp demonstrated close to 30% and 40% increases in cells exposed to dexamethasone or PCN, respectively.

**Effect of PXR Inhibitor on Abcb1b and Abcg2 Induction.** Ketoconazole is an established antagonist of PXR (Mani et al., 2013); therefore, we tested whether the observed induction of P-gp or Bcrp was primarily mediated by PXR in the TM4 Sertoli cells using this compound. Cells were exposed to ketoconazole (10 μM) in the presence of dexamethasone or PCN for 24 hours. As expected, the addition of ketoconazole with dexamethasone or PCN prevented Abcb1b and Abcg2 upregulation (Fig. 3), further suggesting that PXR is involved in the regulation of these transporters.

**CAR-Mediated Upregulation of Abcb1b (P-gp) and Abcc4 (Mrp4) mRNA and Protein Expression.** Treatment of TM4 cells with the CAR ligand TCPOBOP (50–500 nM) resulted in significant increases in both Abcb1b and Abcc4 (which encodes Mrp4) mRNA about 1.5-fold (Fig. 4, A and B). Densitometry analysis also revealed a moderate but significant increase in the protein expression of P-gp and Mrp4 after 72-hour treatment with TCPOBOP (Fig. 4, B and C). Together, these results suggest a pathway involving CAR in the regulation of these ABC transporters.

**Downregulation of P-gp, Bcrp, and Mrp4 Expression by PXR- and CAR-Targeting siRNA.** PXR- and CAR-targeting siRNA were used to further examine the direct involvement of each nuclear receptor in the regulation of the transporters in TM4 Sertoli cells. In PXR siRNA-transfected cells compared with cells treated with control nonsilencing siRNA, PXR protein expression was downregulated by approximately 50%, whereas P-gp, Bcrp, and Mrp4 expression was significantly reduced by 32%, 27%, and 33%, respectively (Fig. 5A). Experiments using CAR siRNA demonstrated an approximately 35% decrease in CAR expression as well as P-gp and Bcrp and a 25% decrease in Mrp4 expression, compared with cells treated with control siRNA (Fig. 5B). The PXR ligand dexamethasone is also known to activate GR (Bledsoe et al., 2002), and the induction of
Fig. 2. PXR-mediated upregulation of Abcb1b (A) and Abcg2 (B) mRNA expression as well as P-gp (C and E) and Bcrp (D and F) protein expression in TM4 Sertoli cells. Cells were exposed to PXR ligands dexamethasone or PCN at various concentrations for 24 hours. mRNA expression was measured by qPCR. All ligand-treated and vehicle (DMSO-treated) samples were examined in triplicates in each experiment. Threshold cycle (CT) values of each gene of interest were normalized with the housekeeping gene Gapdh mRNA and compared with the vehicle control using the comparative CT method ($\Delta\Delta$CT). Results are expressed as the mean fold change ± S.E.M. of three separate experiments. (C–F) Induction of P-gp and BCRP protein expression by PXR in ligand-treated cells compared with the vehicle control for 72 hours. The MDA-MDR1 and MCF7-MX100 cell systems were used as positive controls for P-gp and Bcrp expression, respectively. The relative protein expression was determined by densitometry analysis. Representative immunoblots (top) are shown with their corresponding densitometry analyses (bottom). For each loaded sample, the protein of interest was first normalized against β-actin. Results are expressed as the percent change of ligand-treated samples compared with vehicle-treated control samples and reported as the mean ± S.E.M. for three separate experiments. *P < 0.05; **P < 0.01; ***P < 0.001; ****P < 0.0001 (statistically significant differences between treatment and vehicle as determined by Student’s t-test).
P-gp and Bcrp in the presence of this ligand could possibly be mediated via pathways involving GR. We investigated whether knocking down GR with targeting siRNA would result in the downregulation of P-gp and Bcrp in TM4 cells. Our data demonstrated that compared with control siRNA, exposure to GR siRNA significantly decreased the levels of GR significantly; however there were no changes in the expression of the transporters. Furthermore, dexamethasone significantly upregulated the expression of P-gp and Bcrp in the presence of GR siRNA, suggesting a PXR-mediated effect (Supplemental Fig. 2).

**PXR- and CAR-Mediated Induction of P-gp Ex Vivo in Mouse Seminiferous Tubules.** Seminiferous tubules isolated from mice testes were exposed to PXR ligands (PCN, efavirenz, or darunavir) or CAR ligands (TCPOBOP or efavirenz) to determine whether these compounds, particularly the clinically relevant ARV ligands, could induce the expression of efflux pumps in a more physiologically representative BTB system. Our group has previously demonstrated that ARVs such as darunavir, efavirenz, ritonavir, abacavir, lopinavir, and nevirapine serve as ligands for PXR or CAR by performing luciferase reporter gene assays (Chan et al., 2013a). After 2-hour exposure of seminiferous tubules to PCN, darunavir, efavirenz, or TCPOBOP, we observed a significant increase in the expression of Abcb1b mRNA by approximately 1.5-fold, 1.4-fold, 1.3-fold, and 1.5-fold, respectively (Fig. 6A). We also observed significant upregulation in P-gp protein expression by 55%, 57%, 93%, and 61% 2 hours post-treatment with PCN, TCPOBOP, darunavir, and efavirenz, respectively.

**P-gp Function in Mouse Seminiferous Tubules.** To investigate whether P-gp was functional in the seminiferous tubules, substrate accumulation assays were performed using [3H]-atazanavir. Atazanavir is a known P-gp substrate and is a commonly used ARV in the clinic (atazanavir remains on the World Health Organization list of essential medicines, http://www.who.int/medicines/publications/essentialmedicines/en/). The time course of [3H]-atazanavir at 35°C showed increasing substrate uptake until a plateau was reached at approximately 1 hour under control conditions in the seminiferous tubules (Fig. 7A). The accumulation of [3H]-atazanavir was significantly increased by 30% and 76% in the presence of P-gp inhibitors PSC833 or CsA, respectively, compared with the control (Fig. 7B), suggesting that [3H]-atazanavir accumulation is mediated by P-gp at the BTB.

**Ligand-Activated PXR and CAR Increase P-gp Function in Mouse Seminiferous Tubules.** We examined whether an upregulation of P-gp expression at the BTB would...
also result in increased function. Seminiferous tubules treated with ARVs and other established ligands of PXR and CAR resulted in a moderate but significant reduction in [3H]-atazanavir accumulation (approximately 30% in the presence of PCN and 20% in the presence of TCPOBOP, darunavir, or efavirenz) compared with the vehicle control (Fig. 8). Moreover, PSC833 (a specific P-gp inhibitor) abolished the differences in [3H]-atazanavir accumulation between vehicle control and treatment groups, further confirming the involvement of P-gp in the efflux of this drug. Together, these data demonstrate that P-gp induction regulated by ligand-activated PXR and CAR including commonly prescribed ARVs can result in an increase in its function at the BTB.

**Discussion**

ARV distribution into tissues is primarily regulated by the extent of serum protein binding, drug physicochemical properties, and expression and activity of drug transporters and drug metabolic enzymes (Walubo, 2007; Kis et al., 2010; Chillistone
Most ARVs (especially PIs) display substantial protein binding (>85%) (Bazzoli et al., 2010), resulting in lower amounts of unbound drug to distribute into tissues. Numerous ARVs that are currently recommended as preferred and alternative regimens during HIV pharmacotherapy are known to be substrates of drug efflux transporters including P-gp, BCRP, and the MRPs (Kis et al., 2010; Alam et al., 2016). We previously demonstrated the potential role of ABC transporters in restricting ARV concentrations in the testis of HIV-infected men (Huang et al., 2016). Furthermore, low seminal fluid...
fluid concentrations compared with plasma levels of ARVs in HIV-infected men have also been observed in clinical studies (Else et al., 2011), suggesting a potential contribution of efflux transporters in restricting ARV permeability along the male genital tract. The nuclear receptor–mediated induction of these transport systems during chronic therapy could further limit ARV testis concentrations by modulating blood to tissue disposition across the BTB. P-gp and BCRP are known to be regulated by the nuclear receptors PXR and CAR (Bauer et al., 2004; Narang et al., 2008; Qiao and Yang, 2014), whereas the regulation of MRP4 is primarily modulated by CAR (Assem et al., 2004). In this study, we examined the role of PXR in regulating the expression of P-gp and Bcrp, whereas we examined CAR for its role in regulating both P-gp and Mrp4.

Herein, we demonstrated the protein expression of PXR and CAR in human testicular tissue and TM4 Sertoli cells. We were not able to investigate the nuclear receptor–mediated regulation of ABC transporters in the human testis due to the limitations of obtaining this tissue. However, their expression in the human testis implicates the potential for drug-receptor interactions and regulation of drug transporters at the BTB. Further experiments were performed in vitro using TM4 Sertoli cells, a nontumorigenic cell line and BTB model that was first derived from the testis of BALB/c mice (Mather, 1980). We previously characterized this model by performing morphology studies and examining the expression of the Sertoli cell marker and transcription factor GATA-binding factor 4 (GATA-4), and we also demonstrated the expression and function of ABC transporters in the efflux of ARVs in these cells (Robillard et al., 2012; Hoque et al., 2015).

The PXR-mediated induction of P-gp and Bcrp that we observed in this study agrees with previous work from our group and others. In particular, our group previously demonstrated PXR-mediated induction of P-gp in vivo at the mouse
BBB in animals administered dexamethasone (Chan et al., 2013b) and in vitro in a human BBB cell line model (Chan et al., 2011). Bauer et al. (2004) also showed the involvement of PXR in upregulating P-gp at the rat BBB after exposure to PCN or dexamethasone. The involvement of PXR in the regulation of BCRP was also demonstrated by Narang et al. (2008) at the rat BBB and by Qiao and Yang (2014), in vitro, in human breast cancer cell lines. To further assess the role of this nuclear receptor in regulating P-gp and Bcrp at the BTB, TM4 Sertoli cells were exposed to the PXR antagonist ketoconazole, an antifungal drug and well established inhibitor of the phase I metabolic enzyme CYP3A4 (Thummel and Wilkinson, 1998). This treatment resulted in the downregulation of these transporters in the presence of dexamethasone or PCN (Fig. 3). Downregulation of P-gp and Bcrp by ketoconazole could possibly involve other nuclear receptor pathways, since this drug has been demonstrated as a pan-antagonist that could inhibit the ligand-induced activation of the liver X receptor and the farnesoid X receptor (Huang et al., 2007). However, since inhibition of these nuclear receptors as well as PXR, is dependent on activation by their respective ligands, the inhibitory effect of ketoconazole demonstrated in our experiments was most likely mediated through PXR as we only observed downregulation in the presence of PXR ligands, without any downregulatory effects when cells were exposed to ketoconazole only. Activation of CAR by the established ligand TCPOBOP, a phenobarbital-like compound, in mice (Tzameli et al., 2000) also demonstrated that this nuclear receptor can upregulate P-gp and Mrp4 in TM4 Sertoli cells. These results complement previous studies by our group, which showed that ligand-activated CAR was able to induce P-gp expression at the human BBB (Chan et al., 2011).

To examine whether PXR or CAR could directly regulate the expression of ABC transporters at the BTB, we performed studies using siRNA to knockdown the nuclear receptors and measured the corresponding effects on the transporters (Fig. 5). We observed that both PXR and CAR gene silencing resulted in significant downregulation of the transporters, suggesting that these nuclear receptors are directly involved in regulating the transporters at the BTB. We also demonstrated that the upregulation of the transporters in the presence of dexamethasone (also a GR ligand) was mediated directly through PXR (Supplemental Fig. 2), which is in agreement with the work of Pascussi et al. (2000), who proposed that dexamethasone concentrations above 10 μM directly activated PXR and induced the regulation of target genes such as CYP3A4 in human hepatocytes.
increased P-gp expression, we explored the effects of these ligands on the function of P-gp in the seminiferous tubules. We conducted transport assays with atazanavir, a commonly used PI in the clinic and P-gp substrate as previously demonstrated in brain, testis, and intestine of humans or rodents (Kis et al., 2013; Robillard et al., 2014). The accumulation of atazanavir was significantly increased in the presence of standard P-gp inhibitors PSC833 or CsA, suggesting that P-gp-mediated efflux is involved in the overall transport of this drug at the BTB ex vivo (Fig. 7B). Treatment of seminiferous tubules with these ligands increased P-gp function, as was evident by the decrease in atazanavir accumulation (Fig. 8). Together, these data suggest that ARVs are capable of inducing changes in the expression and function of ABC drug transporters through their interactions with nuclear receptors, potentially leading to alterations in their pharmacokinetic properties as well as drug-drug interactions at the BTB. PXR and CAR exhibit species differences in their ligand specificity. Our studies were performed in mice and may not fully predict activation of the nuclear receptors in humans; however, our group previously demonstrated that efavirenz and darunavir activated human PXR or CAR (Chan et al., 2013a).

In summary, to our knowledge, this study is the first to reveal that the testis is capable of altering drug pharmacokinetic properties through nuclear receptor–mediated pathways. We report novel findings demonstrating upregulation of P-gp, Bcrp, and Mrp4 expression at the BTB in vitro in mice, and we observed upregulation of P-gp expression and function in mouse seminiferous tubules after exposure to nuclear receptor ligands including ARVs. Our data indicate the high complexities of ARV pharmacokinetics and the great potential for drug-drug interactions during therapy. In addition to ARVs, nuclear receptors are modulated by other drugs used to treat HIV coinfections. For example, patients coinfected with tuberculosis may receive concurrent treatment including the rifamycin class of drugs, which are potent activators of PXR (Dooley et al., 2008). Administration of these drugs could activate the nuclear receptor–mediated regulation of drug transporters and metabolic enzymes, potentially leading to reduced drug efficacy when multidrug regimens are taken chronically by patients. Further studies are needed, especially in the human testis, to fully elucidate ARV interactions with drug transporters and nuclear receptors, and their potential contribution to the restriction of drug accumulation in this tissue and formation of a HIV sanctuary.

Our group previously demonstrated the transport of ARVs, including atazanavir and raltegravir, by ABC transporters in TM4 Sertoli cells (Robillard et al., 2012; Hoque et al., 2015); however, these studies have not been performed in a more physiologically relevant system such as freshly isolated seminiferous tubules. We investigated the role of the nuclear receptors PXR and CAR in regulating the expression and function of P-gp ex vivo in mouse seminiferous tubules. As we have observed in vitro at the BTB, exposure to PXR and CAR ligands, including darunavir and efavirenz (part of the current preferred and alternative ARV regimens; DHHS Panel on Antiretroviral Guidelines for Adults and Adolescents, 2016, Available at http://www. aidsinfo.nih.gov/ContentFiles/Adult-and- AdolescentGL.pdf), also induced the expression of P-gp at the mRNA and protein level at the BTB ex vivo. It is noteworthy that the concentrations of ARVs used in our ex vivo experiments were higher than the clinically reported concentrations achieved in plasma. Previous work by our group demonstrated the activation of PXR or CAR, as well as upregulation in the expression and function of P-gp, using clinical plasma concentrations of darunavir and efavirenz, in vitro at the BBB (Chan et al., 2013a). We anticipate that plasma concentrations of these drugs could be effective at the human BTB but it was not feasible to perform these experiments in human samples. Based on experiments conducted in mouse seminiferous tubules ex vivo, lower concentrations of the drugs were less effective in inducing P-gp expression, suggesting species differences.

Since we have determined that activation of PXR and CAR by PCN, TCPOBOP, darunavir, or efavirenz resulted in...