Expression of Six Drug Transporters in Vaginal, Cervical, and Colorectal Tissues: Implications for Drug Disposition in HIV Prevention

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Abstract

Effective antiretroviral (ARV)-based HIV prevention strategies require optimizing drug exposure in mucosal tissues; yet factors influencing mucosal tissue disposition remain unknown. We hypothesized drug transporter expression in vaginal, cervical, and colorectal tissues is a contributing factor and selected three efflux (*ABCB1*/MDR1, *ABCC2*/MRP2, *ABCC4*/MRP4) and three uptake (*SLC22A6*/OAT1, *SLC22A8*/OAT3, *SLCO1B1*/OATP1B1) transporters to further investigate based on their affinity for 2 ARVs central to prevention (tenofovir, maraviroc). Tissue was collected from 98 donors. mRNA and protein expression were quantified using qPCR and immunohistochemistry (IHC). 100% of tissues expressed efflux transporter mRNA. IHC localized them to the epithelium and/or submucosa. Multivariable analysis adjusted for age, smoking, and co-medications revealed significant ($p<0.05$) differences in efflux transporter mRNA between tissue types (vaginal *ABCB1* 3.9 fold > colorectal; vaginal *ABCC2* 2.9 fold>colorectal; colorectal *ABCC4* 2.0 fold>cervical). In contrast, uptake transporter mRNA was expressed in <25% of tissues. OAT1 protein was detected in 0% of female genital tissues and in 100% of colorectal tissues, but only in rare epithelial cells. These data support clinical findings of higher maraviroc and tenofovir concentrations in rectal tissue compared to vaginal or cervical tissue after oral dosing. Quantifying mucosal transporter expression and localization can facilitate antiretroviral selection to target these tissues.

Keywords

transporters; HIV; pharmacokinetics; mucosal; prevention; antiretrovirals

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Introduction

Even in the era of highly active antiretroviral therapy, the HIV epidemic remains a significant global burden.[1] Current treatment strategies alone will not curb the epidemic andadditional options are needed to supplement the current prevention strategies of condoms, circumcision, and safe sex counseling. Antiretrovirals administered topically or orally as pre-exposure prophylaxis (PrEP) are being investigated for their ability to protect individuals at high risk of acquiring HIV.[2] To date, the results of these trials have been mixed, and demonstrate that mucosal antiretroviral pharmacology, in addition to medication adherence are important predictors of PrEP success.[3]

The sites of sexual transmission of HIV are the mucosal surfaces of the female genital tract and the colorectum. Yet penetration of antiretrovirals (ARVs) into female genital and colorectal tissue has been demonstrated to be variable and difficult to predict.[4-8] For example, following a single dose, maraviroc concentrates two-fold in female genital tract tissue compared to blood plasma[7] but nine-fold in colorectal tissue.[6] Tenofovir achieves equivalent concentrations in female genital tract tissue compared to blood plasma, but accumulates 100-fold higher in colorectal tissue.[4,8] Physiochemical properties such as protein binding, molecular weight, and lipophilicity alone do not account for this variability. [5] Understanding and predicting which ARVs have favorable characteristics for high mucosal tissue penetration is necessary for streamlined development of the next generation of prevention compounds.

Membrane drug transporters significantly impact intestinal absorption, uptake into hepatocytes, efflux into biliary canaliculi, penetration through the blood-brain barrier, and intracellular drug accumulation.[9,10] Nearly all antiretrovirals have been documented to be substrates for, or affect the activity of, efflux and/or uptake transporters.[11] Drug transporters are expressed on other epithelial tissues in the body such as the upper gastrointestinal tract, hepatocytes, the blood-brain barrier, endothelial cells, lung, and blood-testes barrier.[10-12] In many of these tissues, interindividual variability in drug transporter protein expression is high. For example, expression of the uptake transporter MDR1 varies 3-6 fold in the intestine and up to 55-fold in the liver.[13] Likewise, expression of uptake carriers such as OATP1B1 and OCT 1 can vary in hepatic tissues 20-80 fold.[13] For many drug transporters in human liver tissues, the correlation between message and protein expression is weak.[14] In lymphocytes, protein expression of MDR1 was inversely correlated with intracellular accumulation of MDR1 substrates including HIV protease inhibitors[15], indicating protein expression may serve as a surrogate for drug transporter activity. Therefore, differences in mucosal exposure to antiretrovirals may be explained, at least in part, by variable drug transporter activity. To date, the knowledge of transporter expression in lower female genital and colorectal tissues is limited to evaluations in small sample sizes with techniques that are minimally quantitative.[12,16-18]

Maraviroc and tenofovir are two lead candidates for pre-exposure prophylaxis.[2] For this focused investigation, we explored the expression of transporters known to affect the disposition of these two compounds, including three transporters from the ATP-binding cassette (ABC) family responsible for drug efflux from cells, and three transporters from the

solute carrier (SLC) family that facilitate drug uptake into cells. MPR2, MRP4, OAT1, and OAT3 have been implicated in tenofovir disposition[19-22] while MDR1 and OATP1B1 affect maraviroc.[23-25] We determined gene expression, protein localization, and estimated intersubject variability in ninety-eight mucosal tissues.

Materials and Methods

Tissue Procurement

Vaginal, cervical, and colorectal tissue samples, each from a unique donor, were procured from surgical waste specimens and from cadaver tissues from July 2009-January 2013. Surgical waste tissues were obtained through the UNC Tissue Procurement Facility (TPF) under two UNC IRB approved studies #09-0921 and #12-0368. Written informed consent was obtained from all participants. Demographic information including age, sex, race, surgical indication, smoking status, co-medications, and menopause status was collected via retrospective chart review. Prior to release from tissue procurement facility, all specimens were examined by a pathologist to ensure there were no abnormal pathologies. When tissue was collected from rectal carcinoma cases, all tissue used was >3 cm from tumor. Cadaver tissues were obtained from the National Disease Research Interchange (NDRI). Per NDRI protocol (http://ndriresource.org), all cadaveric donors signed a witnessed consent form prior to death. Tissues were procured within 3-10 hours post-mortem and shipped on ice overnight to our laboratory for processing. All tissue was collected under applicable regulations and guidelines regarding informed consent, protection of human subjects and donor confidentiality.

After harvest, all tissue was immediately placed on ice in Iscove's Modified Dulbecco's Medium until received into the laboratory. Ninety percent of samples were received within 6 hours of harvest and no difference in mRNA expression was observed for those samples that were received after 6 hours (data not shown). For mRNA expression analysis, tissues were snap frozen in liquid nitrogen and stored at -80°C or preserved in RNALater ® (Qiagen, Hilden, Germany). When possible, a portion of the specimen was also fixed in formalin to be used for immunohistochemical protein expression analysis.

mRNA expression

Total RNA was extracted from 30 mg snap frozen or RNALater preserved tissues using the RNeasy Fibrous Tissue Kit (Qiagen). Tissues were homogenized in lysis buffer using Precellys Mixed Beads Homogenization Kit (Bectin Technologies, Villeurbanne, France) and underwent a 10 minute digestion in Proteinase K prior to RNA isolation and purification. RNA was quantified and tested for purity using a NanoDrop® UV Spectrometer (Thermo-Fisher Waltham, MA). RNA integrity was tested on a subset of samples using the Agilent Bioanalzyer 2100 (Agilent Technologies, Santa Clara,CA). First strand cDNA synthesis was performed from total RNA using Superscript® Vilo™ cDNA Synthesis kit (Life Technologies,Grand Island, NY) mRNA expression was quantified using quantitative PCR (qPCR) with TaqMan® Gene Expression Assays (*ABCB1*; Hs00184500_m1, *ABCC2*; Hs00166123_m1, *ABCC4*; Hs00988717_m1, *SLC22A6*; Hs00537914_m1, *SLC22A8*; Hs00188599, *SLCO1B1;* Hs00272374) on an ABI 7300 Real-

Time PCR System (Life Technologies). Liver and kidney from healthy donors (First Choice®, Life Technologies, Grand Island, NY) were used as comparator organs. Expression was normalized to the expression of the endogenous control gene beta-actin (ACTB) (Hs99999903_m1) using the $2^{-\text{Ct}}$ method.[26] Earlier investigations demonstrated that among five candidate endogenous control genes selected, the ACTB gene, while still being expressed consistently >Ct 35, had the least variability in expression between female genital and colorectal tissues (see Supplementary Table 1).

To further investigate the role of physiologic factors in transporter expression, the gene expression of the efflux transporters in vaginal and cervical tissue was compared between premenopausal and postmenopausal women.

Protein expression

Tissues no greater than 5 mm thickness were formalin-fixed,embedded in paraffin molds, cut into 5 μm sections, and mounted on glass slides. The slides were deparaffinized in xylene and hydrated in a graded ethanol series. Slides were then stained using standard immunohistochemical techniques with primary antibodies for MDR1, clone JSB-1 (catalogue # MAB4120, Millipore, Billerica, MA; 1:400 dilution), MRP2 clone M2III-5 (catalogue #MC267, Kamiya, Seattle, WA; 1:50 dilution), MRP4 clone 1B2 (catalogue #H00010257-M03 Novus Biologicals, Littleton, CO; 1:1500 dilution), and OAT1 (catalogue #AB118346, Abcam, Cambridge, MA; 1:600 dilution) followed by staining with the secondary antibody (Leica Biosystems, ready-to-use Bond secondary antibody kit, approximately 10 minutes). For detection, DAB (3,3'-diaminobenzidine) was used as a substrate-chromogen (approximately 8 minutes). As reliable antibodies were not found for OAT3 or OATP1B1, immunohistochemical evaluations were not performed for these transporters. All stains were performed using the Leica Bond automated tissue stainer (Leica Biosystems Wetzlar, Germany). For positive and negative controls, normal human liver tissue was used for MDR1, normal human kidney tissue for MRP2 and OAT1, and normal human prostate for MRP4. Negative stains were performed using the secondary antibody only. To determine assay consistency, we initially stained 2 sections at least 30 μm apart from each of 9 cervical, 8 colorectal, and 10 vaginal donors. Since no intraindividual variability was noted the remainder of the samples was stained in singlet. Tissues stained with the secondary antibody alone were used as negative controls. Samples were evaluated for localization staining intensity as well as localization of expression.

Statistical Analysis

Association between continuous variables was evaluated with Spearman rank correlation. mRNA expression was compared between tissues types with an exact Kruskal-Wallis test, and when statistically significant ($p < 0.05$), pairwise multiple comparisons were performed with Dunn's method. Tissue effect upon gene expression was further evaluated with a general linear model (GLM) adjusted for age (years), smoking status (yes/no), inducer co-medication (yes/no), and hormone therapy (yes/no). Additional sensitivity analyses adjusted for race (Caucasian/African American), when known, in addition to the above covariates. mRNA expression was natural-log transformed to satisfy the GLM normality assumption. Pairwise tissue comparisons are presented as covariate-adjusted geometric mean ratios, and

were evaluated with the Tukey-Kramer multiple comparison method. Exact Wilcoxon rank-sum tests were used to explore the effect of menopause upon gene expression within vaginal and cervical tissue.

All analyses were conducted two-sided at a 0.05 significance level. Statistical analyses were performed with SAS 9.2 (SAS Institute Inc. Cary, NC) and SigmaPlot 11.0 (Systat Software Inc. San Jose, CA).

Results

A total of 98 tissues, each from a unique donor, were procured and analyzed in this study. Demographic information for tissue donors is summarized in Table 1. Expression of each transporter gene is reported as the ratio to ACTB expression as outlined in the methods.

ABC Efflux Transporters

mRNA expression was measured in 40 vaginal, 30 cervical, and 21 colorectal tissue specimens. *ABCB1*, *ABCC2*, and *ABCC4* were consistently expressed in all tissues analyzed. Figure 1 summarizes the expression results. mRNA expression of transporters were correlated (*ABCB1* vs. *ABCC2* r=0.73 p<0.001; *ABCB1* vs. *ABCC4* r=0.74 0. p<0.001; *ABCC2* vs. *ABCC4* r=0.66 p<0.001). The variability in raw Ct values for reference gene ACTB was 4.3, 9.4, and 4.8% for vaginal, cervical, and colorectal tissues respectively and overall variability was 6.5%. Preliminary analysis in 18 samples (5 vaginal, 6 cervical, 5 rectal) showed that RNA integrity (determined by the Agilent Bioanalyzer) was not significantly correlated with mRNA normalized expression (r=0.22, p=0.3) indicating that our results were not influenced by RNA degradation.

For protein expression, sections from 30 vaginal, 27 cervical, and 11 colorectal specimens from surgical donors were evaluable for immunohistochemical interpretation. Representative images are displayed in Figure 2. Images of positive and negative control stains are showed in Supplemental Figure 1. Images at a lower magnification are included in Supplemental Figure 2.

ABCB1/MDR1—Median (Interquartile range (IQR)) expression ratios (to *ACTB*) for *ABCB1* were 0.0402 (0.0178-0.0582), 0.0236 (0.0101-0.0524), and 0.0073 (0.0053-0.0208) for vaginal, cervical, and colorectal tissue, respectively. One colorectal sample did not have sufficient sample volume to include in the analysis. ABCB1 expression was 3-16% of comparator liver tissue and 1-5% of comparator kidney tissue. *ABCB1* expression was different between the three tissue types (p <0.001), and was significantly higher in vaginal compared to colorectal tissues (p <0.05).

In a multivariable analysis, differences in *ABCB1* expression between the tissue types remained significant after adjusting for age, smoking status, and co-medications (tissue effect, p<0.001). In the adjusted analysis, vaginal *ABCB1* expression was 3.9 fold greater than colorectal *ABCB1* expression (p<0.001). Results further adjusted for race were also significant (p<0.001). When tissues from cadaver donors were excluded, similar results were observed.

Epithelial staining for MDR1 was observed in all vaginal, cervical, and colorectal tissues (Figure 2). Although there were no quantifiable differences in epithelial staining between tissue types, the squamous mucosa of the vaginal tissue and ectocervix showed qualitatively more intense and diffuse staining than in both the glandular mucosa of the endocervix and colorectal tissue. In these latter tissues, expression was largely limited to the luminal surface of the epithelium. Many submucosal lymphocytes in all tissue types also stained positive.Lymphoid follicles, when present, showed strong positive staining for MDR1.

ABCC2/MRP2—Median (IQR) expression values for *ABCC2* were 0.0014 (0.0008-0.0025), 0.0009 (0.0004-0.0016), and 0.0003 (0.0001-0.0011) for vaginal, cervical, and colorectal tissues, respectively. This expression was 1-3% of the comparator liver tissue and <1% of the comparator kidney tissue. *ABCC2* expression was different between the three tissue types (p <0.001) and was significantly higher in vaginal compared to colorectal tissues (p<0.05). In a multivariable analysis, differences in *ABCC2* expression between the tissue types remained significant after adjusting for age, smoking status and co-medications (tissue effect, p<0.01). In the adjusted analyses, vaginal *ABCC2* expression was 2.9-fold greater than colorectal *ABCC2* expression (p<0.001). The tissue effect remained significant when further adjusted for race (p<0.01) and when cadaver tissues were excluded.

Diffuse staining was observed in the epithelia of all tissues types without any notable difference between tissue types. Similar to MDR1, positive staining was observed in submucosal lymphocytes and in lymphoid follicles of vaginal, cervical and colorectal tissues.

ABCC4/MRP4—Median (IQR) expression values for *ABCC4* were 0.0193 (0.0131-0.0291), 0.0097 (0.0061-0.0226), and 0.0237 (0.0122-0.0368) for vaginal, cervical, and colorectal tissues, respectively. This expression is 120-310% of the comparator liver and 6-14% of the comparator kidney. *ABCC4* expression was different between the three tissue types (p<0.05) with expression significantly higher in colorectal versus cervical tissues (p<0.05). In a multivariable sensitivity analysis, the differences in *ABCC4* expression remained significant after adjusting for age, smoking status, and co-medications (tissue effect, p<0.05). In the adjusted analysis, colorectal *ABCC4* expression was 2.0-fold greater than cervical *ABCC4* expression (p<0.05). The tissue effect did not reach significance when further adjusted for race (p=0.18). When tissues from cadaver donors were excluded, similar results were observed.

Epithelial staining was observed in all tissue types, with the most intense staining identified in endocervical glandular mucosa faint diffuse staining in vaginal squamous epithelium ,and very faint focal staining in colorectal columnar epithelium. While a subset of submucosal lymphocytes and monocytes were also positive in all three tissue types, the highest expression was observed in colorectal samples where staining was primarily localized to the luminal surface of the epithelium. Primary follicles in the colorectal tissue showed no definitive staining, although germinal centers of secondary follicles, present for evaluation in two samples, were moderately positive for MRP4 expression.

SLC Uptake Transporters

The SLC transporters were expressed in less than 25% of all mucosal tissues. These results are summarized in Table 2. Detecting one SLC gene did not increase the likelihood of detecting another SLC gene from the same sample. For example, there were three cervical samples with quantifiable *SLC22A6* mRNA expression and three cervical samples with quantifiable *SLC22A8* mRNA expression: only one of these samples had quantifiable levels of both genes. No tissue samples had all three SLC genes quantifiable.

SLC22A6/OAT1—*SLC22A6* was quantifiable in only 5% of samples and of those samples, expression was <0.1% of the kidney comparator tissue.

Similarly, no protein expression was observed in vaginal or cervical (Figure 2) samples by immunmohistochemistry. However, all colorectal samples had scattered cells within the epithelium that showed intense staining. Morphologically, these cells most likely represent true colorectal epithelial cells rather than intraepithelial lymphocytes, monocytes, or plasma cells. These cells comprise <1 per 500 epithelial cells at a frequency of <0.5 per 600x high power field.

SLC22A8/OAT3 and SLCO1B1/OATP1B1—*SLC22A8* was quantifiable in 8% of all samples and expression was <0.01% of the kidney comparator. *SLCO1B1* was quantifiable in 14% of all samples and was <0.1% of expression in the liver comparator. As described in methods, immunohistochemical evaluations were not performed for these 2 transporters.

Role of menopause status in efflux transporter mRNA expression

In vaginal tissue, mRNA expression was compared between 22 postmenopausal and 13 premenopausal women. Median *ABCB1* expression was decreased by 17% in postmenopausal compared to premenopausal (p<0.05). *ABCB1* expression within vaginal tissue also negatively correlated with age (r=-0.426, p<0.01). Median vaginal tissue expression of *ABCC2* and *ABCC4* were reduced in postmenopausal women by 11% and 28% respectively, but these did not reach statistical significance (p=0.26 and p=0.12). Within cervical tissue, mRNA expression was compared between 16 postmenopausal and 11 premenopausal women. There was no observed difference in cervical expression of *ABCB1, ABCC2* or *ABCC4.* Because there was only one premenopausal donor in the colorectal group, no analysis was done with this tissue type.

Discussion

To our knowledge, these data represent the most robust evaluation of drug transporter quantification and localization in female genital and colorectal tissues to date. Based on affinity for maraviroc and tenofovir, two candidates for HIV prevention that show different distribution patterns in female genital tract and colorectal tissue, we chose three efflux transporters from the ABC family (*ABCB1*/MDR1/P-glycoprotein, *ABCC2*/MRP2, and *ABCC4*/MRP4) and three uptake transporters from the SLC family (*SLC22A6*/OAT1, *SLC22A8*/OAT3, and *SLCO1B1/*OATP1B1). We found that efflux transporters MDR1, MRP2, and MRP4 are consistently expressed in both female genital and colorectal tissues,

but to different extents and with unique cellular localizations. At the mRNA level, vaginal tissue has the highest expression of *ABCB1* and *ABCC2* while *ABCC4* is most highly expressed in colorectal tissues. Uptake transporters *SLC22A6, SLC22A8, and SLCO1B1* are expressed in a minority of samples at the mRNA level, and colorectal epithelial cells express very low levels of OAT1.

The differential tissue expression profiles for each transporter observed in our study may assist with understanding how drugs are distributed to, and accumulate within, mucosal tissues (Figure 3). For drugs being considered for use in PrEP (such as a fixed dose combination tablet of two nucleoside/tide reverse transcriptase inhibitors emtricitabine and tenofovir disoproxil fumarate (FTC/TDF), and the CCR5 antagonist maraviroc) mucosal penetration is of great interest. A healthy volunteer study measuring vaginal, cervical, and colorectal penetration following a single oral dose of FTC/TDF found tenofovir and tenofovir diphosphate exposures 100-fold higher in colorectal tissue than in female genital tract tissue.[8] Both *in vitro* and clinical data have demonstrated that tenofovir is a substrate for the efflux transporter MRP4.[19] The immunohistochemical data for MRP4 displayed a distinct staining profile in the colorectal tissues with intense staining observed in the submucosal lymphocytes and faint staining observed in the epithelial layers. Less expression of efflux transporter specifically in the epithelial layer may imply less overall efflux from the tissue (Figure 3A). Tenofovir has also been implicated as an MRP2 substrate although its role in cellular disposition is less clear.[20] Both mRNA and protein expression suggest MRP2 expression is greater in vaginal compared to colorectal epithelium. Similar to MRP4, less expression of MRP2 in colorectal epithelium may contribute to less efflux of tenofovir from the tissue (Figure 3A). Tenofovir has also been shown *in vitro* to be a substrate for the uptake transporter OAT1 and to a lesser extent OAT3.[21,22] OAT1 expressing cells were not found in any of the vaginal or cervical tissues evaluated for protein. However, each of the colorectal tissues examined had isolated cells in the epithelium that clearly expressed the OAT1 uptake transporter. The overall abundance of these OAT1 expressing cells was low, although higher expression in colorectal versus female genital tissues may contribute to the greater accumulation of tenofovir in colorectal tissues[8] (Figure 3A). Additionally, tenofovir bioavailability is 25%, therefore colorectal luminal concentrations are likely high and would facilitate tissue uptake despite low transporter expression.[27]

Currently in early phase studies for PrEP, maraviroc is a known substrate of MDR1.[23-24] Higher expression of the efflux transporter MDR1 in vaginal versus colorectal tissues is consistent with clinical findings that maraviroc accumulation is greater in colorectal tissues[6] than in the female genital tract (Figure 3B).[7] Maraviroc has also been implicated as a substrate for OATP1B1[25], however this transporter was not found to be expressed in either female genital or colorectal tissues.

Although mRNA expression does not always correlate with protein expression[14,28], the use of immunohistochemical evaluations supported our mRNA expression results in these tissues. MRP4 and OAT1 antibodies for immunohistochemistry had reliable performance and low inter-batch variation. MDR1 and MRP2 antibodies were more challenging to validate and maintain. The poor quality of available antibodies limited our ability to perform quantitative analysis of protein expression. However, consistent staining of MDR1, MRP2,

and MRP4 in all tissues and sparse staining of OAT1 in colorectal tissue supported the mRNA measures of pan-detectable ABC transporters and sparsely detectable SLC transporters. MDR1 and MRP2 staining was mostly localized to the epithelium with some staining in submucosal monocytes and lymphocytes. Since the squamous epithelium of vaginal and ectocervical tissue has many more layers of epithelial cells than the single columnar layer of the colorectal tract, this may explain why the mRNA expression for these transporters was higher in vaginal than colorectal tissues. Conversely, MRP4 was only modestly detected in the epithelium in all tissues but was most remarkable in the submucosal lymphocytes and monocytes in the colorectum. Since the colorectum is highly colonized with these submucosal immune cells and lymphoid follicles[29], this may explain the significantly higher *ABCC4* mRNA expression in colorectal compared to cervical tissues.

While expression in the intestinal tract has been previously explored for certain transporters, the emphasis has been on their role in drug absorption with most studies have focusing on the small intestine.[12,30-31] Our data in 98 individuals has been confirmed with recent published reports. Hilgendorf et al. reported that *ABCB1/ABCC4* mRNA expression in colon was similar to expression in liver, kidney, and jejunum.[31] Nishimura et al , found undetectable mRNA expression of SLC22A6 and SLC22A8, low mRNA expression of *ABCC2*, moderate expression of *ABCB1*, and high expression of *ABCC4* in colonic tissues.[12] De Rosa et al. found a similar trend for mRNA expression.[18]

In the female genital tract of 12 individuals, a recent study by Zhou et al. used conventional PCR to evaluate mRNA expression of 22 drug transporters and 19 metabolizing enzymes.[17] This study found that MRP4 mRNA was highly expressed, MDR1 modestly expressed, and MRP2 and OAT1 were undetectable. This investigation only documented the presence or absence of mRNA and did not provide information on interindividual variability, protein expression, or protein localization.

Many factors contribute to the regulation of transporter expression including genotypic polymorphisms, inflammation, and xenobiotics.[32-35] A role for sex steroid regulation in drug transporter expression has been proposed based on significant differences in endometrial MDR1 expression across the menstrual cycle.[36] These findings have been supported by *in vitro* data in various cell lines.[37,38] Within vaginal tissue, we saw a 17% decrease in the expression of *ABCB1* in post-menopausal women suggesting that estrogen may play a role in the *in vivo* regulation of this gene. Since post-menopausal women are also at risk for HIV acquisition[39], future studies are needed to elucidate the effect of estrogen status on mucosal drug exposure in post-menopausal women. Expression between transporters was modestly correlated, suggesting at least some degree of co-regulation in gene expression. Despite these correlations, differences were observed between transporters in the rank order of tissue expression (i.e. vaginal>cervical>colorectal for *ABCB1* and *ABCC2;* colorectal>vaginal>cervical for *ABCC4*) which is noteworthy.

There are limitations to this investigation. From this analysis, the functionality of these transporters could not be determined. Also, our study was not performed in "healthy volunteers" but rather surgical and cadaver donors. The surgical procedures performed were

primarily for structural reasons (vaginal/rectal prolapse, 60%) and there are no data on the effect these may have on transporter expression. Additionally, our results were similar when cadaver tissues were excluded. Finally, although there are over 400 membrane transporters annotated in the human genome[10], because of the limited sample available from each donor, only six targeted membrane transporters were evaluated in this analysis Given the importance of mucosal tissue transporter localization for PrEP, we chose to use immunohistochemistry to evaluate protein expression. Now that localization of these transporters is better understood, quantifying protein concentrations using quantitative targeted proteomics may provide more information on the correlations between mRNA and protein concentrations in the future.[40]

In conclusion, in support of the development of PrEP agents, this investigation quantified the interindividual and intertissue variability of mRNA expression for six membrane drug transporters important to tenofovir and maraviroc disposition, and determined protein expression and localization in vaginal, cervical, and colorectal tissues. We found higher expression of the efflux transporter MDR1 and MRP2 and lower expression of OAT1 in the female genital tract compared to colorectal tissues. Although MRP4 expression was higher in colorectal tissues at the mRNA level, protein expression in colorectal epithelium was minimal. This may explain why the MDR1 substrate maraviroc, and MRP2/4 and OAT 1 substrate tenofovir achieve higher concentrations in colorectal tissues compared to vaginal or cervical tissue. Identifying which transporters play key roles in controlling drug distribution into female genital and/or colorectal tissues will streamline the candidate selection process for compounds requiring high mucosal exposure. Data generated from investigations such as this one can also be applied beneficially to therapeutic areas such as sexually transmitted diseases, gynecological disorders, and gastrointestinal diseases.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

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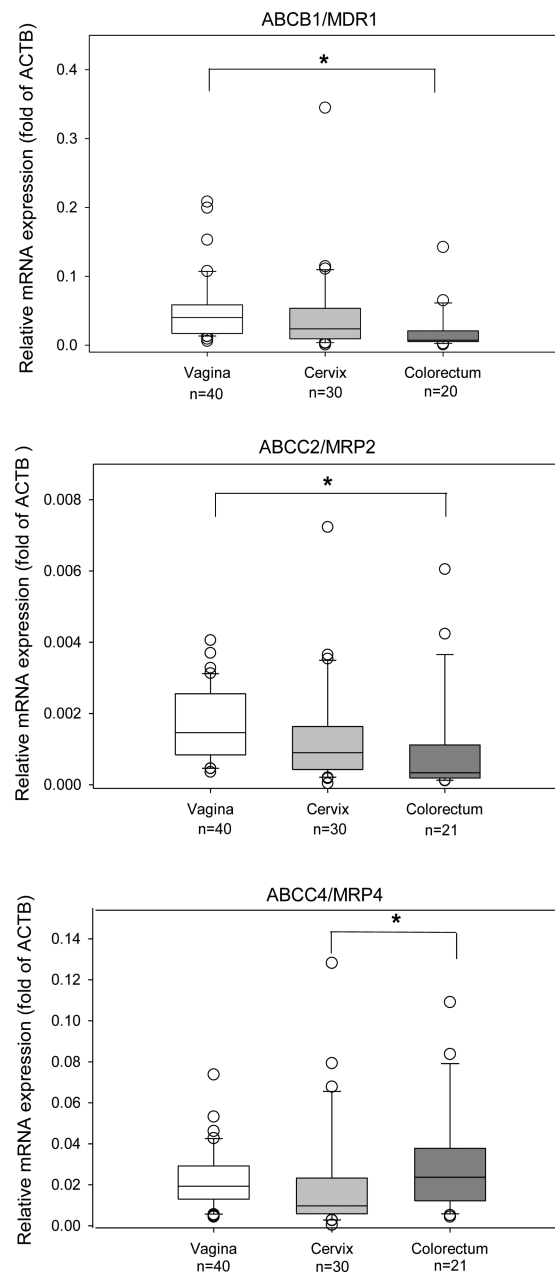
Figure 1.
mRNA expression of three ABC transporters in vaginal, cervical, and colorectal tissues. mRNA was detected by qRT-PCR and levels were normalized to ACTB Ct values using the $2^{-\Delta Ct}$ method. Box plots represent the 50th percentile (line), 25th and 75th percentile (box), 10th and 90th percentile (error bars), and outliers (circles). Tissues were compared using an exact Kruskal Wallis test and pairwise comparisons were made using the Dunn's method * p<0.05
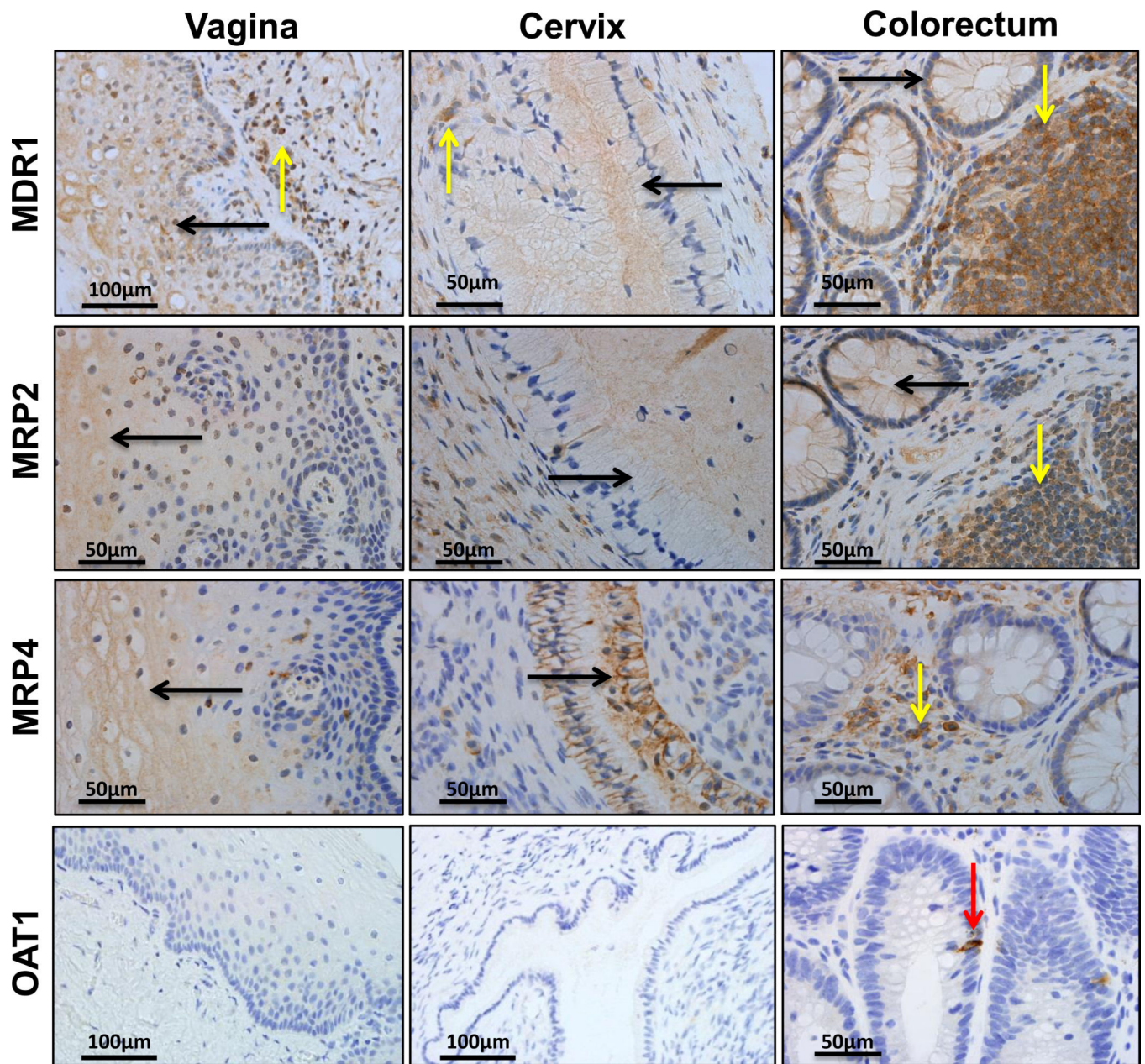
Figure 2.
Immunohistochemical staining of vaginal, cervical, and colorectal tissues. Black arrows identify expression efflux transporters expressed in the epithelial layers. Submucosal lymphocytes and/or monocytes are highlighted by the yellow arrows. The red arrow identifies a rare positive columnar epithelial cell. Images of OAT1 expression in vaginal and cervical tissue, and MDR1 in vaginal tissue were taken at an original objective magnification of 20x, and all others at an original magnification of 60x.
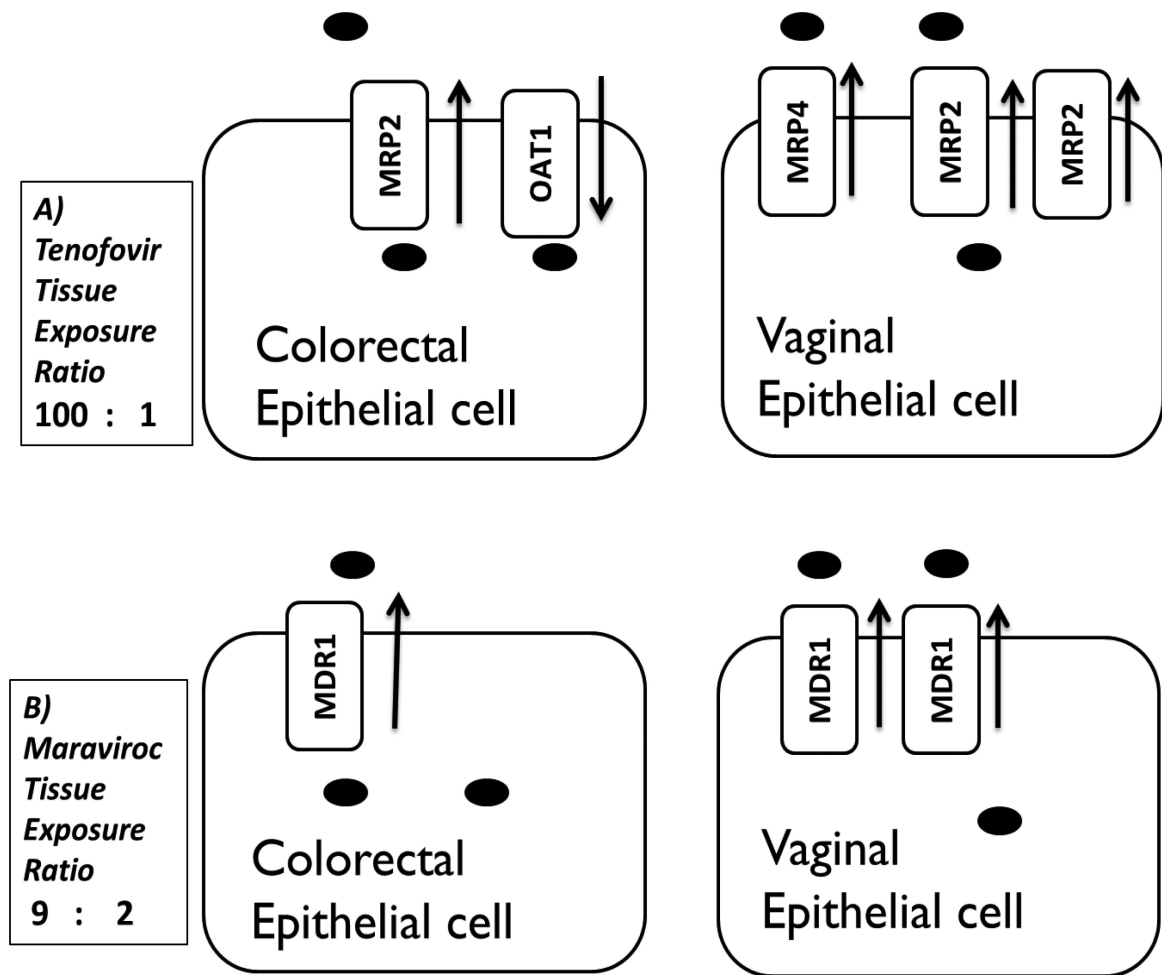
Figure 3.
Hypothesized influence of drug transporters on drug exposure in colorectal and vaginal tissues. The differential expression between colorectal and vaginal epithelial tissues for each transporter relevant to a specific substrate's drug distribution is summarized. A) Tenofovir is a substrate for MRP2, MRP4, and OAT1 B) Maraviroc is an MDR1 substrate. The black circles represent the respective drug molecules. The relative *in vivo* tissue exposures observed (colorectal:vaginal) following oral doses is summarized in the boxes to the left of the schematic.

Table 1

Demographics of Tissue Donors.

Demographic Parameters		Vagina	Cervix	Rectum
		n=47	n=31	n=21
Age median (range)		58 (36-84)	54 (39-83)	56.5 (25-92)
Males n (%)		0 (0)	0 (0)	9 (4)
Females n (%)		47 (100)	31 (100)	11 (52)
Race n (%)	Caucasian	39 (83)	19 (61)	18 (86)
	African American	4 (9)	7 (23)	1 (5)
	Asian	0 (0)	1 (3)	1 (5)
	Native American	1 (2)	0 (0)	0 (0)
	Mixed	0 (0)	1 (3)	0 (0)
	Unknown	3 (6)	3 (10)	1 (5)
Surgical Donor n (%)		46 (98)	31 (100)	14 (67)
Primary Surgical Indication	Vaginal or rectal prolapse/rectocele	38/4	3/0	1
	Stress urinary incontinence	3		
	diverticulum	1		
	Pelvic or adnexal mass		12	
	endometrial cancer or uterine fibroids		10	
	history of breast cancer or BRCA positive		3	
	abnormal pathologies		3	
	rectal carcinoma			12
Cadaver Donor n (%)		1 (2)	0	7 (33)
Cause of death	cerebrovascular accident/subarachnoid hemorrhage	1/0		1/1
	cardiopulmonary arrest/myocardial infarction			2/1
	amyotrophic lateral sclerosis			1
	anoxic encephalopathy			1
Smoker n (%)		8 (17)	7 (23)	11 (52)
Co-medications n (%)	inducers	21 (45)	19 (61)	8 (38)
	hormones	14 (30)	4 (13)	0 (0)
Menopause n (%)				
	postmenopausal	25	16	5
	Pre or peri-menopausal	14	12	1
Included in mRNA analysis n (%) [*]		40 (85)	30 (97)	21 (100)
Included in IHC analysis n (%) [*]		30 (64)	27 (87)	11 (52)

Demographic information regarding donors of tissue specimens is reported. One rectal surgical sample did not have any demographic information available. Inducers included phenytoin, simvastatin, atorvastatin, nifedipine, omeprazole, carbamazepine, lamotrigine and topiramate. Hormones included estrogen replacement therapy administered orally or vaginally as well as oral or long-active contraceptive therapy.

*The majority of tissues were evaluated for both protein and RNA expression. However, some samples (vagina n=6; cervix n=1) did not yield enough RNA to perform qRT-PCR, and some samples (vagina n=16, cervix n=4, colorectum n=10) did not have tissue available for immunohistochemistry.

Table 2

The proportion of samples for each transporter with quantifiable mRNA expression. The range of expression values (normalized to ACTB expression using $2^{-\Delta Ct}$) when detected is included in parenthesis.

Tissue	OAT1 SLC22A6 % Quantifiable (range)	OAT3 SLC22A8 % Quantifiable (range)	OATP1B1 SLC01B1 % Quantifiable (range)
Vagina (n=40)	0 (0)	$5 (3.4 \times 10^{-5} - 4.5 \times 10^{-5})$	$10 (6.0 \times 10^{-6} - 1.3 \times 10^{-4})$
Cervix (n=30)	$10 (2.2 \times 10^{-5} - 1.6 \times 10^{-4})$	$10 (1.8 \times 10^{-6} - 7.4 \times 10^{-5})$	$27 (9.4 \times 10^{-6} - 1.6 \times 10^{-4})$
Rectum (n=21)	$9.5 (8.5 \times 10^{-6} - 6.4 \times 10^{-4})$	$9.5 (2.3 \times 10^{-6} - 6.0 \times 10^{-6})$	$9.5 (1.5 \times 10^{-6} - 2.8 \times 10^{-6})$