Biochemical Pharmacology 116 (2016) 162–175

Contents lists available at ScienceDirect

Biochemical Pharmacology

journal homepage: www.elsevier.com/locate/biochempharm

Expression, regulation, and function of drug transporters in cervicovaginal tissues of a mouse model used for microbicide testing

Tian Zhou, Minlu Hu, Andrew Pearlman, Lisa C. Rohan *

Department of Pharmaceutical Sciences, School of Pharmacy, University of Pittsburgh, Pittsburgh, PA, United States
Magee-Womens Research Institute, Pittsburgh, PA, United States

ARTICLE INFO

Article history:
Received 9 May 2016
Accepted 14 July 2016
Available online 21 July 2016

Keywords:
Transporter
Cervix
Vagina
Microbicide
Tenofovir
HIV prevention

ABSTRACT

P-glycoprotein (P-gp), breast cancer resistance protein (BCRP), and multidrug resistance protein 4 (MRP4) are three efflux transporters that play key roles in the pharmacokinetics of antiretroviral drugs used in the pre-exposure prophylaxis of HIV sexual transmission. In this study, we investigated the expression, regulation, and function of these transporters in cervicovaginal tissues of a mouse model. Expression and regulation were examined using real-time RT-PCR and immunohistochemical staining, in the mouse tissues harvested at estrus and diestrus stages under natural cycling or after hormone synchronization. The three transporters were expressed at moderate to high levels compared to the liver. Transporter proteins were localized in various cell types in different tissue segments. Estrous cycle and exogenous hormone treatment affected transporter mRNA and protein expression, in a tissue- and transporter-dependent manner. Depo-Provera-synchronized mice were dosed vaginally or intraperitoneally with ^{3}H-TFV, with or without MK571 co-administration, to delineate the function of cervicovaginal Mrp4. Co-administration of MK571 significantly increased the concentration of vaginally-administered TFV in endocervix and vagina. MK571 increased the concentration of intraperitoneally-administered TFV in the cervicovaginal lavage and vagina by several fold. Overall, P-gp, Bcrp, and Mrp4 were positively expressed in mouse cervicovaginal tissues, and their expression can be regulated by the estrous cycle or by exogenous hormones. In this model, the Mrp4 transporter impacted TFV distribution in cervicovaginal tissues.

1. Introduction

The human immunodeficiency virus (HIV) pandemic continues to be a worldwide public health problem [1]. Sexual transmission is the cause of the vast majority of new HIV infections in sub-Saharan Africa, where 70% of all new infections occur [1]. Pre-exposure prophylaxis (PrEP) uses systemically or topically (vaginally or rectally) administered antiretroviral drugs to protect uninfected individuals and is considered a promising approach in preventing HIV sexual transmission. However, PrEP clinical trials have yielded inconsistent effectiveness results. For example, a 1% tenofovir (TFV) vaginal gel reduced the HIV acquisition rate by 39% in the Phase 2b CAPRISA 004 trial [2]; however, the TFV gel arm that tested the same gel product in another Phase 2b trial (VOICE) was discontinued due to futility [3]. Inconsistent results have also been observed in some other trials testing vaginally- or orally-administered TFV, alone or in combination with other antiretroviral drugs [4–8]. There is an urgent need to enhance the effectiveness of PrEP products containing TFV and other antiretrovirals to provide a practical means for HIV prevention.

In order to enhance PrEP effectiveness, we must understand the critical determinants of antiretroviral drug effectiveness. Clinical pharmacokinetic studies have revealed that cervicovaginal or colorectal tissue drug exposure is key [9,10]. The drugs used in PrEP include entry inhibitors, nucleoside/nucleotide reverse transcriptase inhibitors, non-nucleoside reverse transcriptase inhibitors, and integrase inhibitors-all of which target the early steps in the HIV life cycle before integration of viral DNA into the host genome [10–13]. A sufficient amount of these drugs must penetrate into the cervicovaginal or colorectal tissues in order to reach the submucosal immune cells and protect them from being infected by HIV particles released during sexual intercourse [9,10]. In clinical trials and preclinical studies testing of vaginal and rectal microbicides, a clear trend was observed: the higher the drug concentration, the lower the HIV acquisition rate [9,10,14,15]. Currently, the effective *in vivo* drug concentration remains unknown for

* Corresponding author at: Department of Pharmaceutical Sciences, School of Pharmacy, University of Pittsburgh, Pittsburgh, PA, United States.
E-mail address: lrohan@mwri.magee.edu (L.C. Rohan).

http://dx.doi.org/10.1016/j.bcp.2016.07.009
0006-2952/

many of the microbicide drug candidates being evaluated [14]. Therefore, it is suggested that one of the primary goals of future microbicide development is to achieve the maximally tolerated drug concentration in tissues relevant to HIV sexual transmission [14]. Many factors may account for the insufficient drug exposure in tissues after PrEP drug administration, including improper drug delivery systems or low patient adherence to the dosing regimen [10]. Long-lasting injectable formulations have been proposed to address these issues and ensure the efficiency of drug delivery to the target tissue sites, but physiological factors that determine tissue absorption and disposition in cervicovaginal and colorectal tissues must be understood to maximize the drug exposure, understand interindividual variability, reduce drug load in the products, and minimize toxicity.

Drug transporters are important regulators of antiretroviral drug pharmacokinetics and pharmacodynamics [16,17]. Transporters are transmembrane proteins that localize to the plasma membrane or the membranes of intracellular organelles such as mitochondria and control the movement of substrates in and out of the cell [17]. Among the various transporters studied, three ATP-binding cassette (ABC) transporters, P-glycoprotein (P-gp), multidrug resistance associated protein 4 (MRP4), and breast cancer resistance protein (BCRP) are highly relevant to antiretroviral drugs [17]. Their substrates span all classes of antiretroviral drugs used in AIDS treatment and/or microbicide development, such as the entry inhibitor maraviroc, most protease inhibitors, the reverse transcriptase inhibitors tenofovir and zidovudine, and the integrase inhibitor raltegravir [17–21]. Transporters can also be inhibited by antiretrovirals through competitive binding, and transporter expression can be induced by antiretrovirals by diverse mechanisms [17,18]. Besides marketed drugs, some excipients generally regarded as safe have been shown to potently inhibit ABC transporters by temporarily depleting intracellular ATP availability and/or reversibly modifying plasma membrane fluidity [17,22]. In addition, genetic polymorphisms of a number of transporters are associated with interindividual variability in antiretroviral drug pharmacokinetics [16,17,23].

There have been some published studies on cervicovaginal and colorectal tissue transporters, but more studies are needed to better understand transporter expression, regulation, and function in these tissues. We and others have reported that several efflux transporters, including P-gp, BCRP, and MRP4, were positively or even highly expressed in the cervicovaginal and colorectal tissues of human, macaque, rabbit, and mouse, as well as the cell lines derived from human cervicovaginal or colorectal tissues [24–30]. These animal models are used in PrEP microbicide testing. In addition, positive functionality of P-gp in the rabbit vagina was demonstrated, using talinolol as the substrate and verapamil as the P-gp inhibitor [25]. However, further studies are needed to establish the expression profile of important transporters under the influence of physiological factors that are commonly encountered by PrEP participants, and more evidence is needed to confirm the functional role of transporters in the pharmacokinetics of topically or systemically administered antiretroviral drugs. Sex steroid hormones such as estrogen and progesterone are known to affect the expression and activity of many drug transporters [31]. There could be two sources of hormone level variation. First, the menstrual cycle in human and estrous cycle in mammalian animals are known to cause cyclic changes of sex steroid hormones [32]. Second, the administration of exogenous hormones, such as hormonal contraceptives, may directly affect transporter expression or indirectly exert such an effect through modulating endogenous hormone levels. Depo-Provera contains medroxyprogesterone acetate (MPA) as its active ingredient and is used by many PrEP participants [33]. It is also the most widely used contraceptive in sub-Saharan Africa, where the rate of HIV sexual transmission remains highest [1]. Therefore, it is imperative to understand the effect of the menstrual cycle and exogenous hormones, including hormonal contraceptives, on transporter expression and to examine whether the positively expressed transporters in cervicovaginal and colorectal tissues are functional enough to affect the absorption/disposition of systemically or topically administered antiretroviral drugs. The information generated from such studies will enhance the understanding of the critical determinants of drug exposure in these tissues and facilitate PrEP optimization.

The aim of this study is to examine the expression, regulation, and function of important transporters in tissues relevant to HIV sexual transmission using a Depo-Provera synchronized Swiss Webster mouse model. This model has been used before in the field of microbicide research and development to evaluate the safety of vaginally administered PrEP products (microbicides) [34,35]. In this study, the mRNA and protein expression of P-gp, Bcrp, and Mrp4 in mouse tissues were examined in the estrus and diestrus stages during the natural estrous cycle and after treatment with exogenous hormones in order to understand the expression of these transporters under conditions that may be encountered by PrEP participants. Exogenous hormones included the contraceptive Depo-Provera and pregnant mare's serum gonadotropin, which are known to stimulate estrogen levels. Transporter expression in rabbit tissues and human cervicovaginal epithelial cell lines was also examined for the purpose of selecting the most appropriate model to study transporter function. Finally, using the Depo-Provera-synchronized mice, the function of the Mrp4 transporter in the cervicovaginal tissue distribution of vaginally and systemically administered TFV was investigated, with the use of MRP inhibitor MK571. To our knowledge, this is the first report showing the expression of cervicovaginal tissue P-gp, Bcrp, and Mrp4 under the influence of estrous cycle and exogenous hormones, and this is the first time that cervicovaginal tissue MRP4 function has been demonstrated in an *in vivo* model.

2. Materials and methods

2.1. Collection of tissues from naturally cycling and synchronized mice

All animal procedures were approved by the University of Pittsburgh Institutional Animal Care and Use Committee (IACUC). Female Swiss Webster mice (6 weeks old, around 23 g body weight) were used for all mouse experiments. Tissues, including uterus, endocervix, ectocervix, vagina, colorectum, and liver, were collected under four conditions: estrus and diestrus stages in the natural estrous cycle, synchronized estrus stage, and synchronized diestrus stage. For the collection of tissues from mice undergoing a natural estrous cycle, mouse estrous cycle stage was examined daily, and tissues were collected from euthanized mice when the estrus or diestrus stage was reached. The stage was determined using vaginal cytology as described by Caligioni et al. [32]. To collect the vaginal smear, the mouse vaginal lumen was injected with 20 μL of normal saline, using a pipette. The smear was evenly spread onto a glass slide and observed under a bright light microscope. For the collection of mouse tissues under synchronization, mice were treated with pregnant mare's serum gonadotropin (PMSG, Sigma-Aldrich Inc., St. Louis, MO, USA) or Depo-Provera (Pfizer Inc., New York City, NY, USA) for synchronization into estrus or diestrus stages, respectively. For PMSG treatment, each mouse was intraperitoneally (IP) injected once with 5 IU. For Depo-Provera treatment, mice were subcutaneously injected twice, on Day 1 (starting day) and Day 5, at the dose of 3 mg MPA per mouse. Synchronized estrus stage was reached 24 h after PMSG administration, and the synchronized diestrus stage was reached 7 days after first Depo-Provera administration. The stage of synchronized

mice was confirmed as described above. Harvested tissues were stored at -80 °C until further analysis. For each of the four conditions, tissues were collected from at least three mice.

2.2. Collection of rabbit tissues and cultured cells

Rabbit tissues were obtained from euthanized New Zealand White rabbits, which were a kind gift from Dr. Eric Romanowski of the Eye & Ear Institute of the University of Pittsburgh. The tissue collection was in line with IACUC regulations. The rabbits were 6–8 weeks old at the time of euthanasia and had been previously used for the testing of topical drug products for the treatment of eye infection. The infection and drug action were contained in the cornea and had no systemic effects.

End1/E6E7, Ect1/E6E7, and VK2/E6E7 cells were purchased from American Type Culture Collection (ATCC) and cultured per their protocols at 37 °C under 5% CO_2. The following cell culture media and reagents were purchased from Invitrogen Inc. (Carlsbad, CA, USA) unless otherwise specified. The culture medium was keratinocyte-serum free medium with 0.1 ng/mL human recombinant epidermal growth factor, 0.05 mg/mL bovine pituitary extract, and an additional 44.1 mg/L calcium chloride (final concentration 0.4 mM). An antibiotic combination containing 50 IU/mL penicillin and 50 μg/mL streptomycin (antibiotics purchased from Thermo Fisher Scientific Inc., Pittsburgh, PA, USA) was supplemented into the medium. The cells were initially grown in culture flasks. After 60–90% confluence was reached, the cells were detached using trypsin-EDTA and diluted in the culture medium to reach the target density (5×10^5 cells per well). This was followed by seeding onto 6-well plates. Cells were harvested from the plates using trypsin-EDTA after 60–80% confluence was reached.

2.3. RNA extraction, reverse transcription, and qRT-PCR

RNA of all the tissue and cell samples was extracted using TRIZOL (Invitrogen) per the manufacturer's instructions. RNA extraction, reverse transcription, and real-time PCR were performed as described by Zhou et al. [24]. The design and execution of quantitative PCR experiments were in line with the MIQE guidelines. The efficiency of PCR reactions was confirmed, using the relative standard curve method, with serially diluted liver or colon cDNA [24]. It should be noted that the amplification efficiency depends mainly on the G/C content of amplicon, and it was slightly different between different genes examined in this study. Nevertheless, the amplification efficiency was very close to 100% for different genes, so the mRNA level of transporters was normalized to that of GAPDH using the $2^{-\Delta Ct}$ method for plotting and comparison. The primers used in PCR are listed in Table 1.

2.4. Immunohistochemical (IHC) staining

IHC staining was conducted by the Research Histology Service of the University of Pittsburgh. The fixation and processing of mouse tissues were conducted as described by Zhou et al. [24]. The antibodies used in staining are listed in Table 2. Primary antibodies, purchased from Santa Cruz Biotechnology Inc. (Dallas, TX, USA), were applied to slides with overnight incubation at 4 °C. After washing with phosphate buffered saline solution containing Tween 20 (PBST, Santa Cruz Biotechnology), biotinylated secondary antibodies, purchased from Vector Laboratories Inc. (Burlingame, CA, USA), were applied to the slides and incubated at room temperature for 30 min. After the incubation with secondary antibodies, the slides were washed with PBST. ABC reagents (Vectastain Elite, Vector Laboratories) were applied afterward, and slides were incubated for 30 min, followed by AEC chromogen (ScyTec Laboratories Inc., Logan, UT, USA) incubation for color development. The slides were then counterstained with hematoxylin and mounted with Crystal Mount (Sigma-Aldrich). In the negative control staining for a transporter protein in a given tissue, non-immunized IgG (Santa Cruz Biotechnology) was used instead of the primary antibody. The IgG was purified from the same species in which the primary antibody was raised.

Table 2
Information on primary and secondary antibodies.

Transporter	Primary antibody	Biotinylated secondary antibody (1: 200 IgG)
P-gp (Abcb1a/Abcb1b)	H-241 (1:15)	Goat anti-rabbit
Bcrp (Abcg2)	BXP-53 (1:25)	Goat anti-rat
Mrp4 (Abcc4)	M4I-80 (1:15)	Rabbit anti-rat

2.5. Preparation of vaginal gels and solutions for mouse administration

^{3}H-TFV was purchased from Moravek Biochemicals Inc. (Brea, CA, USA). Non-radiolabeled TFV was obtained from Gilead Sciences Inc. (Foster City, CA, USA). Two batches of ^{3}H-TFV stock were used, and the specific activity of the stock solution was 13.3 and 8.8 Ci/mmol, respectively. The radioactivity of the stock solution (EtOH: H_2O = 1:1 as solvent) was 1 mCi/mL for both batches. For vaginal gel administration, 20 μL of gel was administered intravaginally to each mouse, using a 1 mL disposable syringe (BD Biosciences) sequentially capped with a 200-μL pipette tip and a 10-μL pipette tip, as shown in Fig. 8. For mouse vaginal administration, 2.7% universal placebo gel (pH4.4, components shown in Table 3) was used as a base to mix with tested agents, such as TFV or MK571. The ingredients of these gels are listed in Table 4. To prepare the universal placebo gel, sorbic acid (Sigma-Aldrich) was added to water and mixed until fully dissolved. Sodium chloride (Sigma-Aldrich) was added, and the pH was determined. If the pH was different from the target pH 4.4, then 1 M NaOH or HCl (Thermo Fisher) was added to adjust the pH. Hydroxyethyl cellulose (Ashland Inc., Covington, KY, USA) was then slowly added to the solution with a mixer, and water was added to adjust the final total weight after all the ingredients were fully dissolved. For the

Table 1
Primer information for real-time RT-PCR of transporters and Gapdh in mouse tissues.

Common Gene name (Official gene symbol)	GeneBank accession No.	Primer sequence 5′ to 3′
P-gp (Abcb1a)	NM_011076	Forward: CCATCAGCCCTGTTCTTGGAC Reverse: TCCCCAGCCTTTTAGCTTCTT
P-gp (Abcb1b)	NM_011075	Forward: CTGTTGGCGTATTTGGGATGT Reverse: CAGCATCAAGAGGGGAAGTAATG
Bcrp (Abcg2)	NM_011920	Forward: AAATGCTGTTCAGGTTATGTGGT Reverse: TCCGACCTTAGAATCTGCTACTT
Mrp4 (Abcc4)	NM_001033336	Forward: GTGCGACAGAGCGCCGAAGT Reverse: CTGGGGGTGGGCGCTTCTTG
Gapdh	NM_008084	Forward: GCCCAGAACATCATCCCTGC Reverse: CCGTTCAGCTCTGGGATGACC

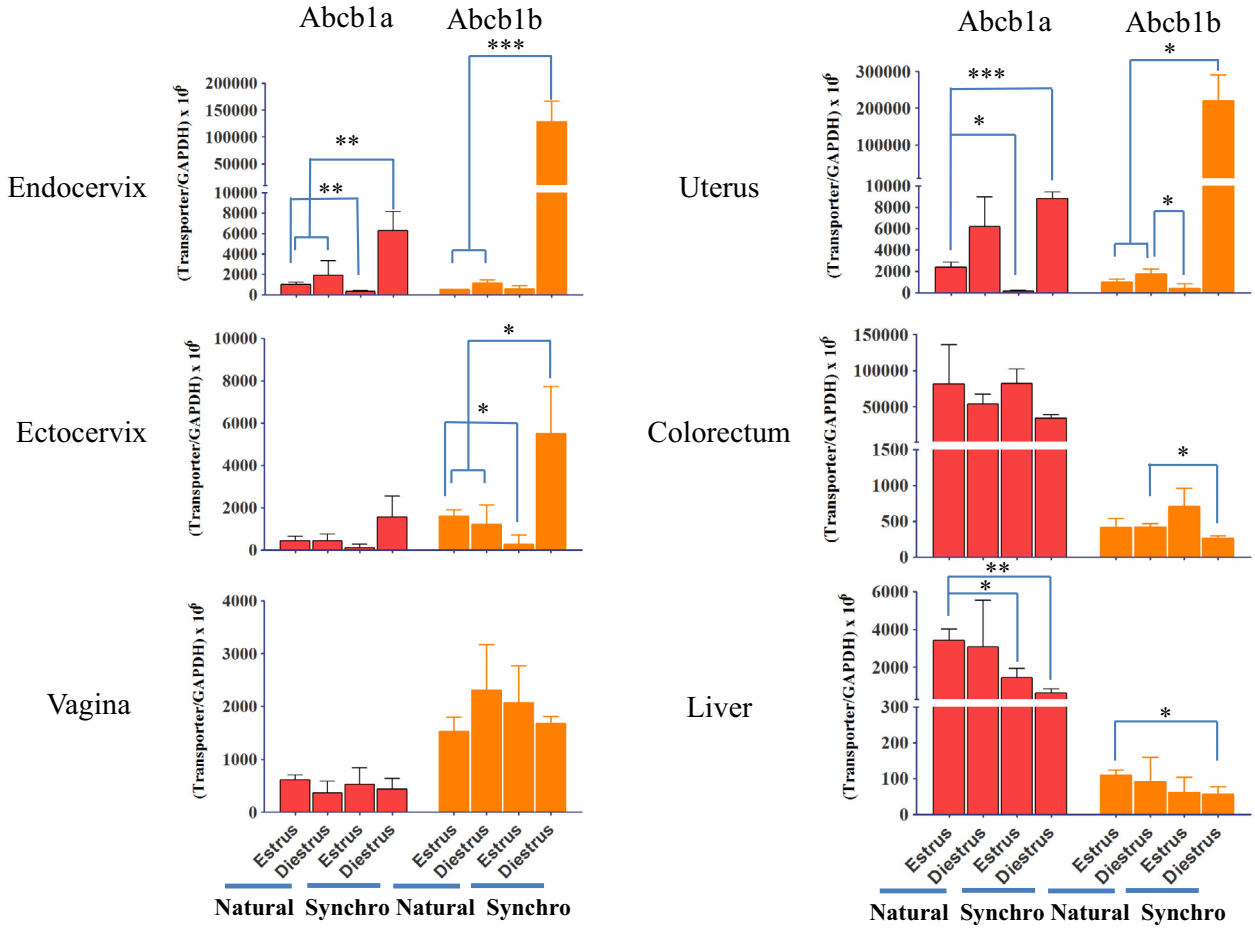


Fig. 1. Effect of estrus cycle and exogenous hormones on P-gp (Abcb1a/1b) mRNA expression in mouse tissues. The mouse counterpart to human P-gp consists of two isoforms, Abcb1a and Abcb1b. Therefore, the mRNA levels of both isoforms are shown. Mouse tissues were collected under 4 conditions: estrus stage and diestrus stage during natural estrous cycle (Natural), estrus and diestrus stages reached after PMSG and Depo-Provera synchronization (Synchro). The expression was examined in tissues from 3 to 5 mice. The Gapdh-normalized transporter levels (generated using the $2^{-\Delta Ct}$ method) were plotted in various tissues. To examine the effect of the natural estrous cycle, the mRNA level was compared between the estrus and diestrus stages of naturally cycling mice. To examine the effect of exogenous hormones, mRNA level at a synchronized status (synchronized to estrus by PMSG or synchronized to diestrus by Depo-Provera) was compared with the level at either stage of the natural estrous cycle. Student's *t*-test was used for comparisons. Red columns, Abcb1a. Yellow columns, Abcb1b. Results represent mean ± SD from all mouse tissues. $^{*}p < 0.05$; $^{**}p < 0.01$, $^{***}p < 0.001$. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

pharmacokinetics (PK) study, two kinds of gels were formulated; for the safety study, three kinds of gels were formulated. Components of these gels are shown in Table 4. The administered TFV gel contained a mixture of ^{3}H-TFV and non-radiolabeled TFV. The purpose of adding non-radiolabeled TFV into the gel was to ensure that the concentration of total TFV was not too low for the Mrp4 transporter to exert efflux effect.

2.6. Transporter function study in mice

All animal procedures were approved by the University of Pittsburgh IACUC. Female Swiss Webster mice (6 weeks old, around 23 g body weight) were synchronized with Depo-Provera on Day 1 and Day 5, and used for the PK or safety study on Day 8. To study the role of Mrp4 in the PK of vaginally administered TFV, mice were divided into three groups. Group 1 was dosed IP with 100 μL of saline, followed by 20 μL of TFV gel administered vaginally 30 min later; Group 2 was dosed IP with 100 μL of saline containing 7.5 mg/mL MK571 (Sigma-Aldrich), followed by 20 μL of TFV gel administered vaginally 30 min later; Group 3 was dosed IP with 100 μL of saline, followed by 20 μL of (TFV + MK571) gel applied vaginally 30 min later. Components of these gels are listed in Table 4.

TFV was administered at low dose. The purpose of selecting this dose was to simulate a situation in which the tissue drug concentration is dramatically decreased from its initial level, with increasing drug exposure expected to increase effectiveness. The selection of the MK571 dose was based on the dose range of MK571 in published animal studies [36–38]. Five minutes prior to vaginal gel dosing, the mice were injected IP with 100 μL of saline containing 10 mg/mL ketamine HCl (Henry Schein Animal Health Inc., Dublin, Ohio, USA) and 20 mg/mL xylazine (Sigma-Aldrich), to sedate the mice and facilitate vaginal gel administration. The dosed mice were euthanized with CO_2, followed by cervical dislocation, at 0.5 h and 1 h post gel administration, for all the three groups.

Cervicovaginal lavage (CVL) was collected immediately after euthanasia. CVL was obtained through eight sequential vaginal washes with 25 μL of saline (200 μL in total), using a pipette with a 200-μL tip. Around 0.25 cm of the sharp end of the tip was cut off to account for the high viscosity of the lavage. Blood was collected from the inferior vena cava, using 1-mL disposable syringe capped with heparinized 25G needles. The collected blood was centrifuged at 5000 rpm for 5 min (Eppendorf MiniSpin Plus), and 100 μL of the supernatant was collected as plasma samples. Mouse tissues—uterus, endocervix, ectocervix, vagina, colorectum, and kidney—were collected using surgical scissors. The harvested

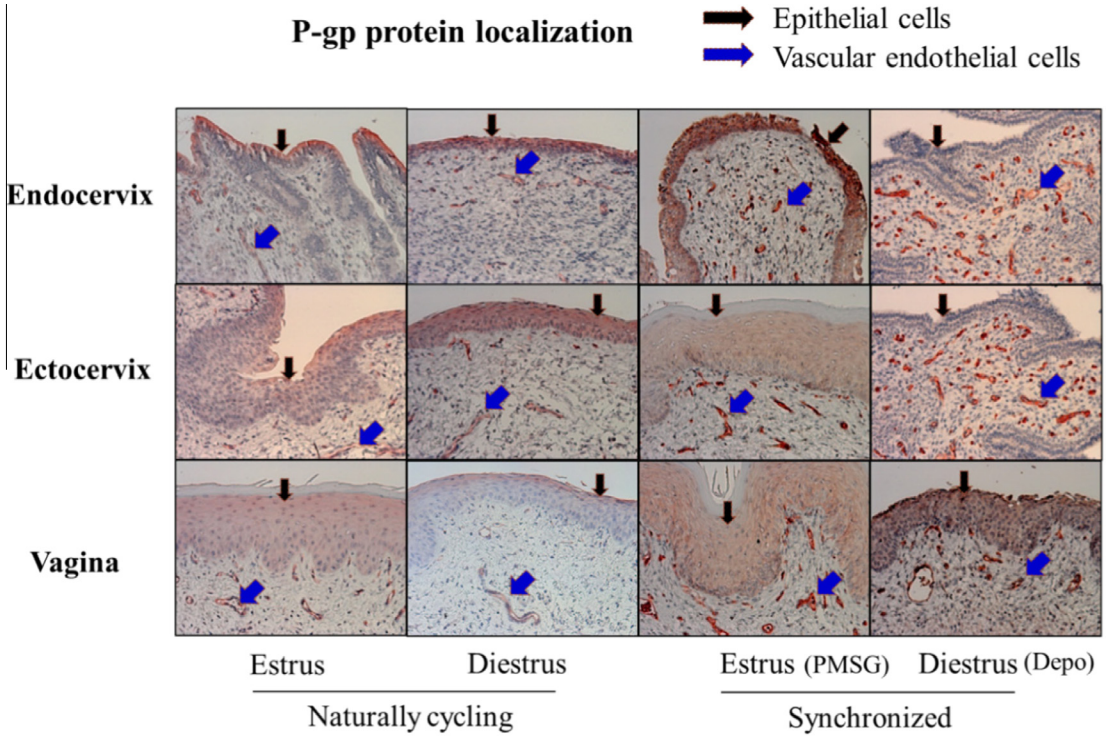


Fig. 2. Effect of estrus cycle and exogenous hormones on P-gp (Abcb1a/1b) protein expression in mouse tissues. Tissues were collected from mice euthanized under the 4 conditions described in Fig. 1. The majority of pictures shown are representative of the staining results from more than 3 mice. Color development was with AEC; red color indicates positive staining. Black arrows indicate the position of epithelial cells, and blue arrows point to positively stained vascular endothelial cells. Magnification is 20× for all pictures. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

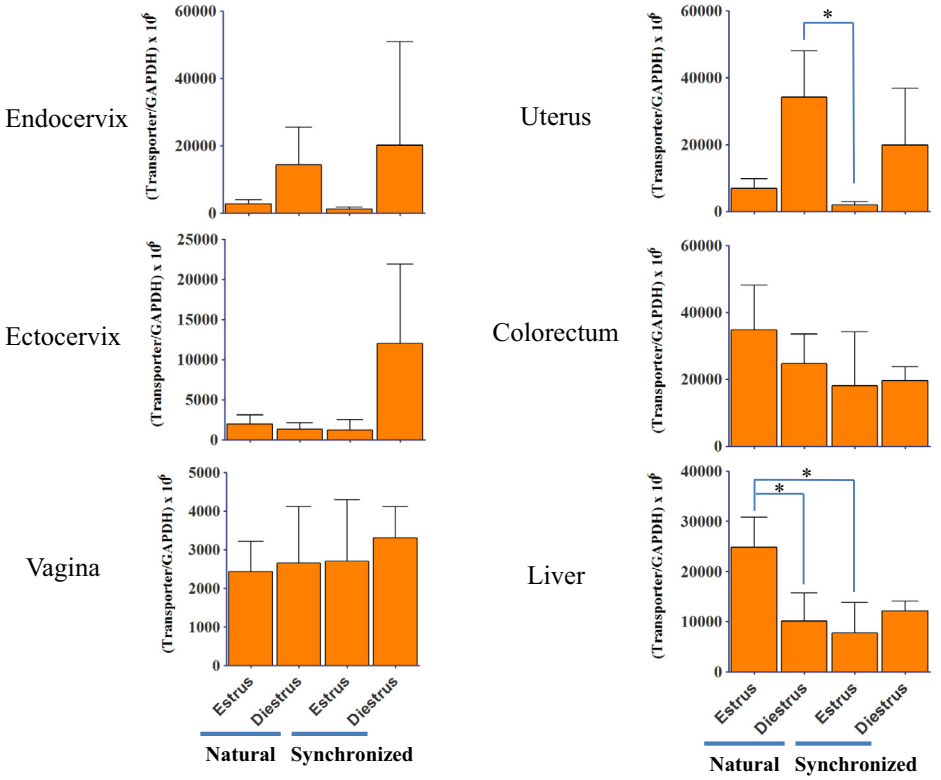


Fig. 3. Effect of estrus cycle and exogenous hormones on Bcrp mRNA expression in mouse tissues. Bcrp mRNA level was examined in mouse tissues collected under the 4 conditions described in Fig. 1. Tissue collection and data analysis were performed as described in Fig. 1. Results represent mean ± SD from all mouse tissues. $^{*}p < 0.05$.

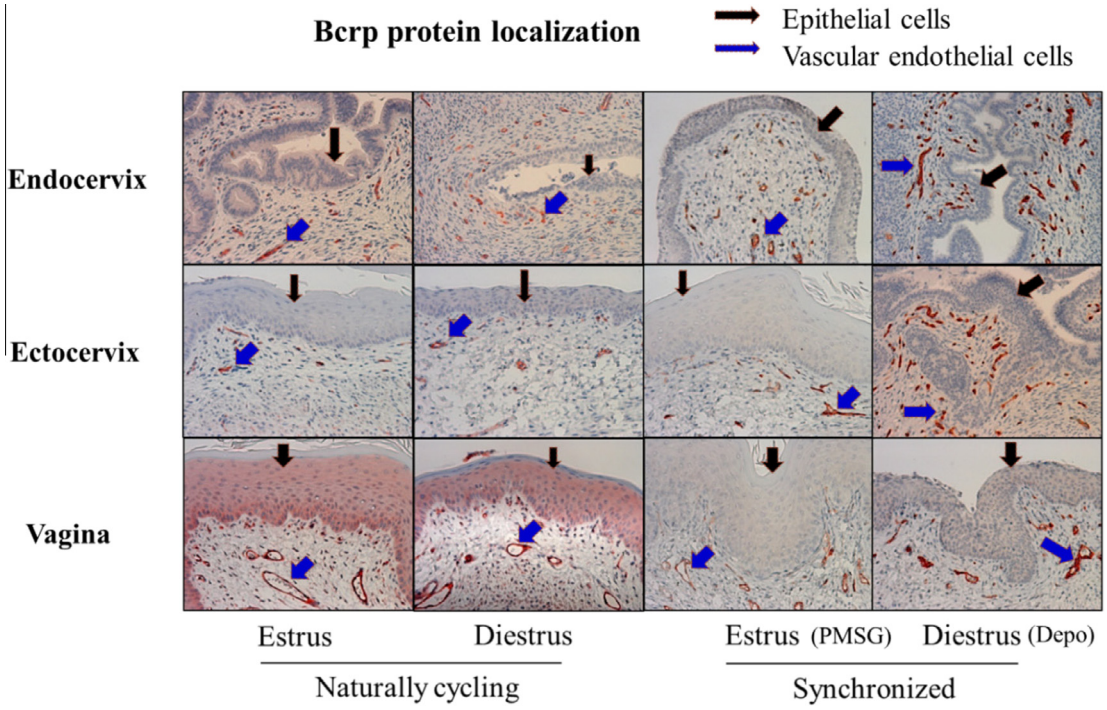


Fig. 4. Effect of estrus cycle and exogenous hormones on Bcrp protein expression in mouse tissues. Tissues were collected from mice euthanized under the 4 conditions described in Fig. 1. The majority of pictures shown are representative of the staining results from more than 3 mice. Color development was with AEC; red color indicates positive staining. Black arrows indicate the position of epithelial cells, and blue arrows point to positively stained vascular endothelial cells. Magnification is 20× for all pictures. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

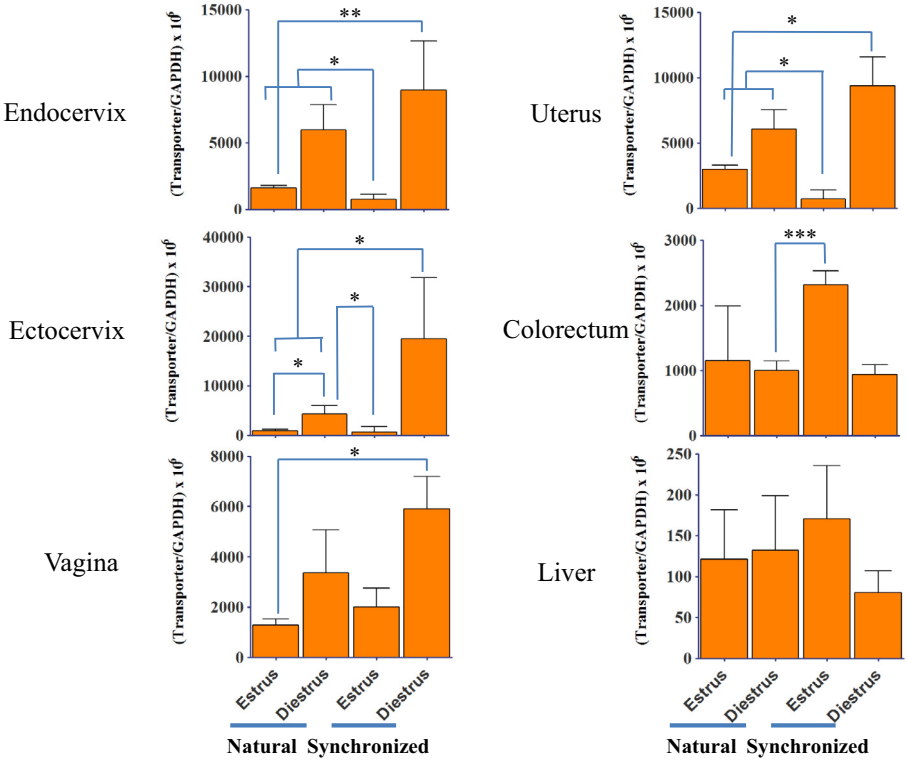


Fig. 5. Effect of estrus cycle and exogenous hormones on Mrp4 mRNA expression in mouse tissues. Mrp4 mRNA level was examined in mouse tissues collected under the 4 conditions described in Fig. 1. Tissue collection and data analysis were performed as described in Fig. 1. Results represent mean ± SD from all mouse tissues. $^{*}p < 0.05$; $^{**}p < 0.01$, $^{***}p < 0.001$.

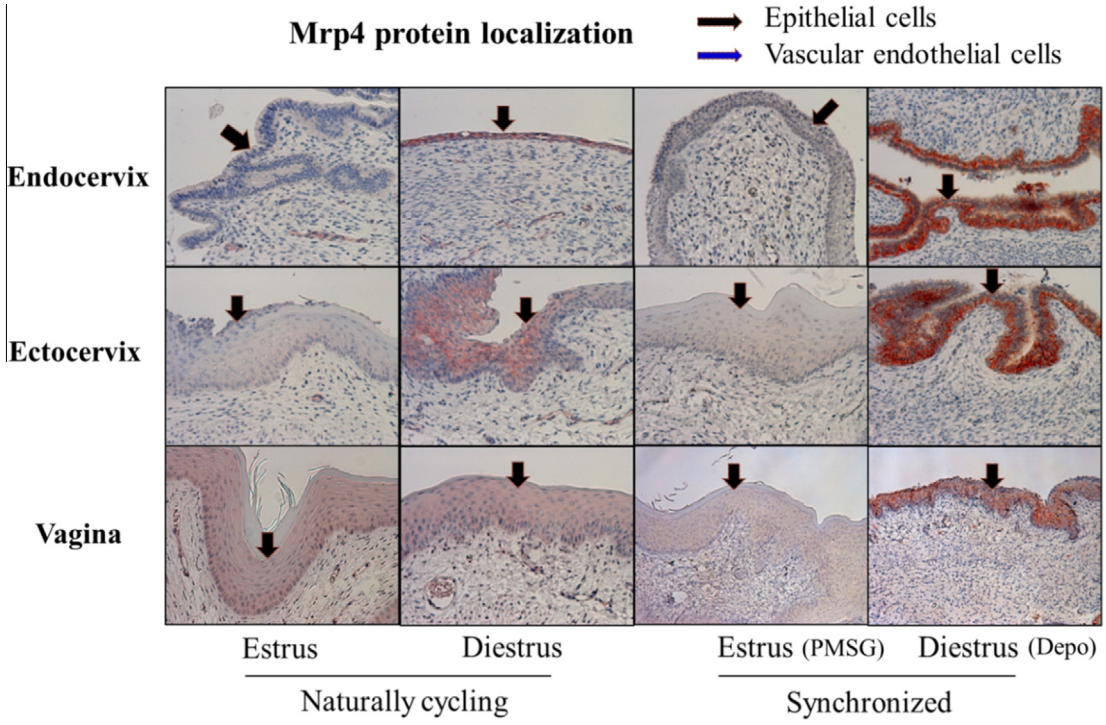


Fig. 6. Effect of estrus cycle and exogenous hormones on Mrp4 protein expression in mouse tissues. Tissues were collected from mice euthanized under the 4 conditions described in Fig. 1. The majority of pictures shown are representative of the staining results from more than 3 mice. Color development was with AEC; red color indicates positive staining. Black arrows indicate the position of epithelial cells, and blue arrows point to positively stained vascular endothelial cells. Magnification is 20× for all pictures. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

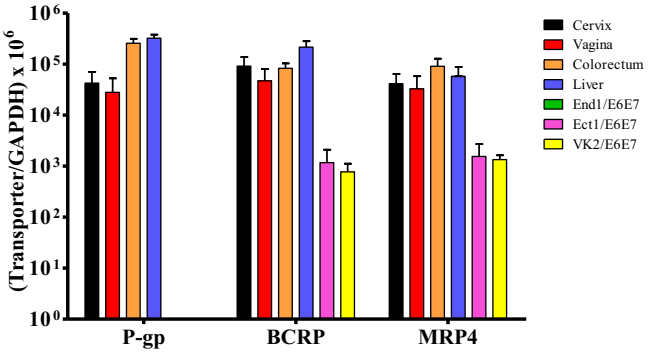
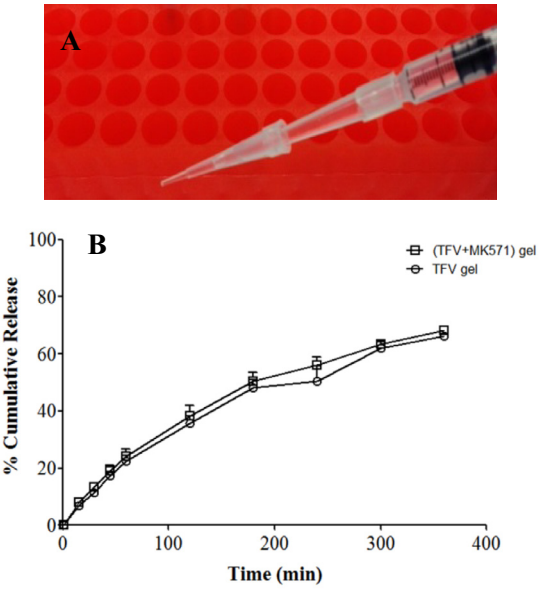


Fig. 7. Transporter mRNA expression in rabbit tissues and cervicovaginal cell lines. mRNA expression was examined in rabbit tissues (cervix, vagina, colorectum, liver) and human epithelial cell lines (End1/E6E7, Ect1/E6E7, VK2/E6E7). Uterus, cervix, and liver tissues were collected from 3 rabbits; colorectum was collected from 6 rabbits; vaginal tissue was collected from 7 rabbits. Rabbit endocervix and ectocervix were difficult to distinguish; therefore, the part of the rabbit genital tract connecting the vagina and uterine horns was obtained to represent the whole cervix. The GAPDH-normalized transporter levels (generated using the $2^{-\Delta Ct}$ method) were plotted in rabbit tissues and cell lines. Results represent mean ± SD from all rabbit tissues or from at least 3 culture flasks.

tissues were rinsed with saline, and the surface water was gently absorbed using gauzes. The tissues were then put into 1.5-mL tubes, and the weight of a tissue sample was obtained by subtracting the weight of the empty tube from the weight of the tube with the tissue.

To study the role of Mrp4 in the PK of IP-administered TFV, synchronized mice were divided into two groups. Group 1 was administered IP with 100 μL of saline, followed by IP injection of 100 μL of ^{3}H-TFV saline solution (0.94×10^{-5} mmol/mL, 125 μCi/mL) 15 min later. Group 2 was administered IP with MK571-containing saline (7.5 mg/mL), followed by the same



	% Release at 30 min	% Release at 60 min	P_{app} (cm/sec)
TFV gel	11.45 ± 0.93	22.38 ± 2.12	1.06 x 10^{-5} ± 5.67 x 10^{-7}
(TFV+MK571) gel	13.46 ± 1.21	24.05 ± 2.64	1.09 x 10^{-5} ± 6.87 x 10^{-7}

Fig. 8. Preparation and injection of gels containing ^{3}H-TFV and TFV. (A) Tip-capped syringe used for gel administration into mouse vagina. (B) Release kinetics of TFV from the gel. Gels, with or without MK571 incorporation, were applied to the donor chamber of a Franz cell apparatus, and the TFV concentration in the acceptor chamber medium (PBS) was measured to generate the kinetics of TFV release from the gels. P_{app}, permeability coefficient. Results represent mean ± SD from 3 gels in each group.

Table 3
Preparation of 2.7% universal placebo gel.

Ingredient	Amount added to make 100 g gel
Sorbic acid	0.1
Sodium chloride	0.85
Hydroxyethyl cellulose 250 HX	2.7
Sodium hydroxide 18%	As needed
MilliQ water	Supplement to 100
Total weight (g)	100

^{3}H-TFV solution as in Group 1. The TFV dosing level was again low for the reason given above. Mice were euthanized 1.5 h after TFV administration. CVL was collected using 60 μL of saline (20 μL saline per wash × 3 times). Blood and tissues were collected as described above for the gel-administered mice.

2.7. Measurement of radioactivity in mouse samples

The CVL (200 μL from the vaginal dosing group or 60 μL from the IP dosing group), plasma (100 μL), and tissue (5–50 mg) samples were transferred to scintillation vials. Five hundred μL of Solvable tissue lysis buffer (PerkinElmer Inc., Waltham, MA, USA) was added to the vials containing the CVL, plasma, or tissues. The vials were then incubated overnight in a 50 °C water bath to completely dissolve the tissues. Following this, the sample vials were taken out, and 100 μL of H_2O_2 (Thermo Fisher Scientific) was added to each vial. The vials were then incubated at 50 °C for 1 h. The purpose of adding H_2O_2 was to decolorize the samples in order to eliminate the influence of sample color on subsequent scintillation counting, and the 1 h of 50 °C incubation was to completely remove the remaining H_2O_2, which would also affect the activity of the scintillation cocktail. After H_2O_2 treatment, the vials were cooled to room temperature, and 2.5 mL of ScintiSafe Plus cocktail (Thermo Fisher Scientific) were added. Then the vials were vortexed and placed in a scintillation counter (PerkinElmer) for radioactivity measurement.

The count per minute (CPM) values generated by the scintillation counter were converted to decay per minute (DPM) using the following formula: $\mathrm{DPM} = \frac{CPM}{Counting\ efficiency}$. The radioactivity (μCi) of samples was calculated as $\mu\mathrm{Ci} = \mathrm{DPM}/(2.22 \times 10^6)$. The molar concentrations of ^{3}H-TFV in CVL, plasma, and tissue samples were calculated as

$$(\mathrm{fmol}/\mu\mathrm{L}\ \text{or}\ \mathrm{fmol}/\mathrm{mg}\ \text{tissue}) = [\mathrm{CPM}/\text{counting efficiency}/(2.22 \times 10^6)/\text{specific activity}] \times 10^6.$$

Total TFV concentration (^{3}H-TFV + TFV) in the sample was calculated based on the ratio of radiolabeled to non-radiolabeled TFV. Total TFV concentration for each sample was presented as mean ± standard deviation, and used for plotting and statistical analysis.

2.8. Examination of the safety of gels and solutions

To evaluate the safety of TFV and MK571 administration and to rule out the possibility that TFV concentration change might be caused by dosing and handling, mice were administered with non-radioactive TFV vaginal gels or IP solutions, with or without MK571 co-administration. The molar quantity of TFV of the gels and solutions used in these safety evaluations was the same as in the PK experiments, as detailed in Table 4. A 4% nonoxynol (N-9, Spectrum Chemicals Inc., New Brunswick, NJ) gel was used as a positive control because it is a well-known disruptor of cervicovaginal tissue epithelium [34]. The vehicle gel (universal placebo gel) was used as a negative control because it is known to be safe to human and mouse cervicovaginal tissues [34]. The mice were euthanized at 1 h for the vaginal dosing group, or at 1.5 h for the IP dosing group. The endocervix, ectocervix, and vagina were collected, fixed, and processed for H&E staining. The staining was performed as previously described [39]. Pictures were taken using a Zeiss Axioskop 40 microscope with AxioVision Software.

2.9. Statistical methods

Statistical analyses were conducted using the GraphPad Prism software. Student's *t*-test was used to compare the mRNA level of a transporter between different estrous stages. For vaginal substrate (TFV) administration, TFV concentration in each type of sample was compared among the three dosing groups, using a one-way analysis of variance (ANOVA). For IP substrate administration, Student's *t*-test was used to compare the two dosing groups. For all tests, $p < 0.05$, $p < 0.01$, and $p < 0.001$ were considered statistically significant, very significant, and extremely significant, respectively.

3. Results

3.1. Real-time RT-PCR examination of mouse transporter mRNA levels

The three transporters P-gp, Bcrp, and Mrp4 were positively expressed in the mouse tissues examined (Figs. 1, 3 and 5). The expression levels were moderate to high compared to mouse liver. For each transporter, the Gapdh-normalized transporter mRNA levels depended on the type of tissue, the stage of natural estrous cycle, or the type of exogenous hormone administered (Figs. 1, 3 and 5). The mouse counterparts of human P-gp are Abcb1a and Abcb1b. Abcb1a is mainly responsible for pumping out xenobiotics, while Abcb1b is mainly involved in the homeostasis of endogenous substances. During the natural estrous cycle, mouse P-gp and Bcrp levels were not significantly different between the two stages, although the large increase from estrus to diestrus could be observed for Abcb1a in the uterus and for Bcrp in uterus and

Table 4
Preparation of gels for mouse PK and safety evaluations.

Ingredients	Amount added (μL)				
	PK study		Safety study		
	TFV gel	(TFV + MK571) gel	TFV gel	(TFV + MK571) gel	4% N-9 gel
^{3}H-TFV stock (8.8 Ci/mmol, 1 mCi/mL)	25	25	0	0	0
0.5% TFV solution (non-radiolabeled)	7.8	7.8	9.4	9.4	0
7.5 mg/mL MK571 saline solution	0	18	0	18	0
Saline (0.9% NaCl)	18	0	0	0	0
Nonoxynol-9 (N-9)	0	0	0	0	20
2.7% universal placebo gel	449	449	490	472	480
Total volume (μL)	499.8	499.8	499.4	499.4	500

Twenty μL of gel was administered to each mouse.

endocervix (Figs. 1 and 3). The effect of the estrous cycle was more evident for Mrp4. A significant increase in Mrp4 mRNA was observed in ectocervix from estrus to diestrus, and large but not significant increases in Mrp4 could be seen in uterus, endocervix, and vagina (Fig. 5).

Exogenous hormones PMSG and Depo-Provera exerted obvious effects on the tested transporters (Figs. 1, 3 and 5). For the following transporters and tissues, transporter mRNA level was significantly altered after PMSG treatment compared to that at either stage of the natural cycle: Abcb1a in uterus and endocervix; Abcb1b in uterus and ectocervix; Bcrp in uterus; Mrp4 in uterus, endocervix, and colorectum. For the following transporters and tissues, transporter mRNA level was significantly altered after Depo-Provera treatment compared to that at either stage of the natural cycle: Abcb1a in uterus and endocervix; Abcb1b in uterus, endocervix, ectocervix, and colorectum; Mrp4 in uterus, endocervix, ectocervix, and vagina. The majority of PMSG-induced alterations were decreases, while the majority of Depo-Provera-induced alterations were increases (Figs. 1, 3 and 5).

Although the effects of the estrous cycle and exogenous hormones were tissue- and transporter-dependent, some general tendencies appeared, especially in reproductive tissues. The mRNA levels of transporters tended to be higher in the diestrus stage and lower in the estrus stage during the natural cycle. PMSG treatment tended to decrease transporter levels, while Depo-Provera tended to increase transporter mRNA levels, compared to their levels during the stages of the natural cycle.

3.2. Protein expression of three transporters in mouse cervicovaginal tissues

P-gp (Abcb1a/1b) protein was distributed in both epithelium and stroma in mouse endocervix, ectocervix, and vagina, during the natural estrous cycle (Fig. 2). There was no marked difference in P-gp protein abundance and localization pattern. Compared to the natural estrous cycle, PMSG treatment did not exert an obvious effect on protein abundance or localization. Depo-Provera treatment did not affect P-gp protein in the vagina. However, Depo-Provera increased P-gp protein density in stroma and reduced P-gp density in epithelia of both endocervix and ectocervix (Fig. 2).

Bcrp protein was primarily localized in the vessel wall of the stromal part of mouse endocervix and ectocervix, during the natural estrous cycle (Fig. 4). The epithelium of the mouse vagina was also positively stained. There was no obvious difference in Bcrp protein abundance and localization between the two stages of the natural estrous cycle in any of the three segments of the mouse lower genital tract. Compared to the natural cycle, PMSG synchronization did not seem to cause a significant change in endocervix and ectocervix, while Depo-Provera appeared to increase the protein density in these two tissues, especially in the stromal part. In addition, PMSG and Depo-Provera synchronization appeared to decrease Bcrp protein density in vaginal epithelium compared to the natural estrous stages.

Mrp4 protein in mouse endocervix and ectocervix was primarily found at the diestrus stage, while the staining at estrus stage was not readily observed, during the natural estrous cycle (Fig. 6). Weak staining of Mrp4 protein was found in epithelium and stroma in the mouse vagina at both estrus and diestrus stages. Compared to the natural estrous cycle, PMSG treatment decreased the epithelial and stromal abundance of Mrp4 protein in mouse endocervix, ectocervix, and vagina. Depo-Provera treatment markedly increased Mrp4 density in the epithelial layers, while it decreased Mrp4 density in the stromal part, in all three tissue segments, compared to the natural estrous cycle.

3.3. Real-time RT-PCR examination of rabbit and cell line transporter mRNA levels

P-gp, Bcrp, and Mrp4 were found to be positively expressed in the uterus, cervix, vagina, and colorectum of rabbits, and the levels were compared to those in rabbit liver (Fig. 7). Overall, the liver-normalized transporter mRNA levels in the rabbit genital tract and colorectum appear to be lower than those normalized levels in human and macaque. For P-gp, the mRNA levels in rabbit cervix and vagina were significantly lower than the liver P-gp level. For BCRP, the cervix, vagina and colorectum levels were significantly lower than the liver level.

The same panel of efflux transporters were also tested in cell lines derived from human endocervical epithelium (End1/E6E7), ectocervical epithelium (Ect1/E6E7), and vaginal epithelium (VK2/E6E7). P-gp was not detectable in any of the three cell lines. BCRP and MRP4 could be detected in all the cell lines, but their expression levels were lower than in the corresponding tissues in humans, rabbits, or mice (for human data, refer to Zhou et al. [24]). There was no significant difference in transporter levels among the different epithelial cell lines.

3.4. The effect of MK571 on the tissue distribution of vaginally administered TFV

First, we measured the release kinetics of two kinds of TFV gels used in the vaginal dosing experiments to determine whether the incorporation of MK571 in the TFV gel would change the release profile of TFV. As shown in Fig. 8, compared to the gel containing TFV alone, the incorporation of MK571 into the gel did not have any impact on the *in vitro* release profile of TFV. The percentages of the cumulative amount released by 30 min and 60 min, which were the two time points employed in the PK experiments described above, were very similar in the two kinds of TFV gel. The permeability coefficient P_{app}, as calculated from the time-dependent TFV concentration appearing in the acceptor chamber of the Franz cell apparatus was almost identical for both kinds of gels.

Next, the TFV gels were administered to three groups of synchronized mice. The control group was administered the gel containing TFV only. The effect of MK571 co-administration on TFV distribution was tested in the other two groups: MK571 was either injected IP as a solution 30 min prior to gel administration or incorporated into the TFV gel. When injected as an IP solution, MK571 significantly increased the TFV concentration in endocervix, ectocervix, and vagina by up to several fold on at least one of the two time points post-vaginal administration of TFV gel. However, the concentrations in plasma, uterus, and colorectum were only slightly increased or remained unchanged (Fig. 9).

When incorporated into the TFV gel, MK571 exerted time-dependent effects on TFV distribution compared to the control group (Fig. 9). At 0.5 h post gel administration, the TFV concentration in mouse vaginal tissue was significantly higher in the MK571 group. The TFV concentration was slightly, but not significantly, increased in plasma, uterus, ectocervix, and colorectum. The TFV concentration in CVL and endocervix remained unchanged with the addition of MK571 (Fig. 9). At 1.0 h, the TFV concentration in the colorectum was significantly increased, while its CVL concentration was significantly decreased. There was also a decrease in the TFV concentration in endocervix, ectocervix, and vagina, but the differences were not statistically significant. The TFV concentration in plasma and uterus remained unchanged with the incorporation of MK571 into the TFV gel (Fig. 9).

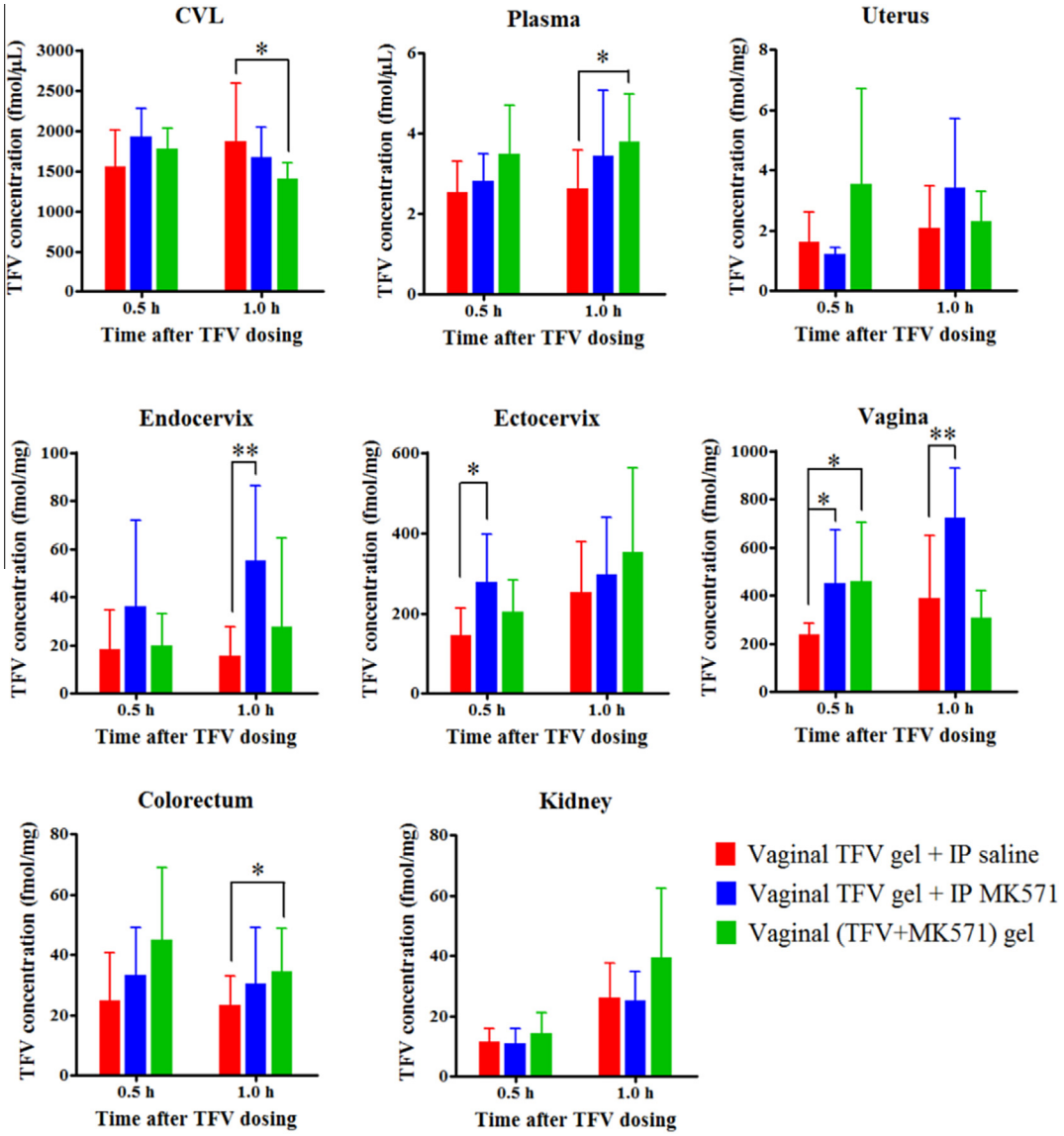


Fig. 9. Effect of MK571 on tissue distribution of vaginally administered TFV. The effect of MRP transporter inhibitor MK571 on TFV distribution was tested using vaginal gels containing ^{3}H-TFV, with or without co-administration of MK571. Mice were euthanized 0.5 h and 1.0 h post TFV gel administration; CVL, plasma, and tissue samples were collected; and radioactivity was measured in a scintillation counter. TFV molar concentrations in these samples were plotted for the 2 time points tested. Red columns, mice were administered IP with saline, followed by vaginal administration of TFV gel 30 min later; blue columns, mice were administered IP with MK571 solution, followed by vaginal administration of TFV gel 30 min later; green columns, mice were administered IP with saline, followed by vaginal administration of TFV + MK571 gel 30 min later. For each time point of each group, 7–13 mice were used. Data represent mean ± SD. $^{*}p < 0.05$, $^{**}p < 0.01$. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

3.5. The effect of MK571 on the tissue distribution of IP-administered TFV

To examine the functional role of the Mrp4 transporter in the cervicovaginal/colorectal tissue distribution of systemically administered TFV, mice were given TFV solution through the IP route, with or without MK571 co-administration via the same route 15 min prior to TFV dosing (Fig. 10). The TFV concentrations in CVL fluid, tissues, and plasma were measured 1.5 h post-TFV administration. As shown in Fig. 10, compared to its plasma concentration, TFV was preferentially distributed into cervicovaginal tissues and fluid after IP administration. MK571 increased TFV concentration in CVL and vagina ($p = 0.04$), while slightly decreasing TFV plasma concentration (−13%).

The vagina/plasma ($p = 0.03$) and colorectum/plasma ($p = 0.006$) ratios were significantly increased by MK571 co-administration (Fig. 10), suggesting that the observed increase in vagina and colorectum tissue concentrations found after MK571 application was not due to increased plasma concentration but to local tissue transporter inhibition, thus providing evidence for the functional role of Mrp4 transporter in limiting TFV distribution from blood to these tissues. MK571 significantly increased the kidney/plasma ratio by 33% ($p = 0.002$), consistent with the published report that the MRP4 transporter limits TFV accumulation in kidney tissue [20].

3.6. The effect of TFV and MK571 administration on tissue morphology

In evaluating the safety of MK571 co-administration, we performed H&E staining to examine the morphology of mouse endocervix, ectocervix, and vagina under the same conditions described above for the Mrp4 function study. In published reports and our preliminary tests, the vehicle gel had no effect on tissue morphology; therefore, we used it as a negative control. After

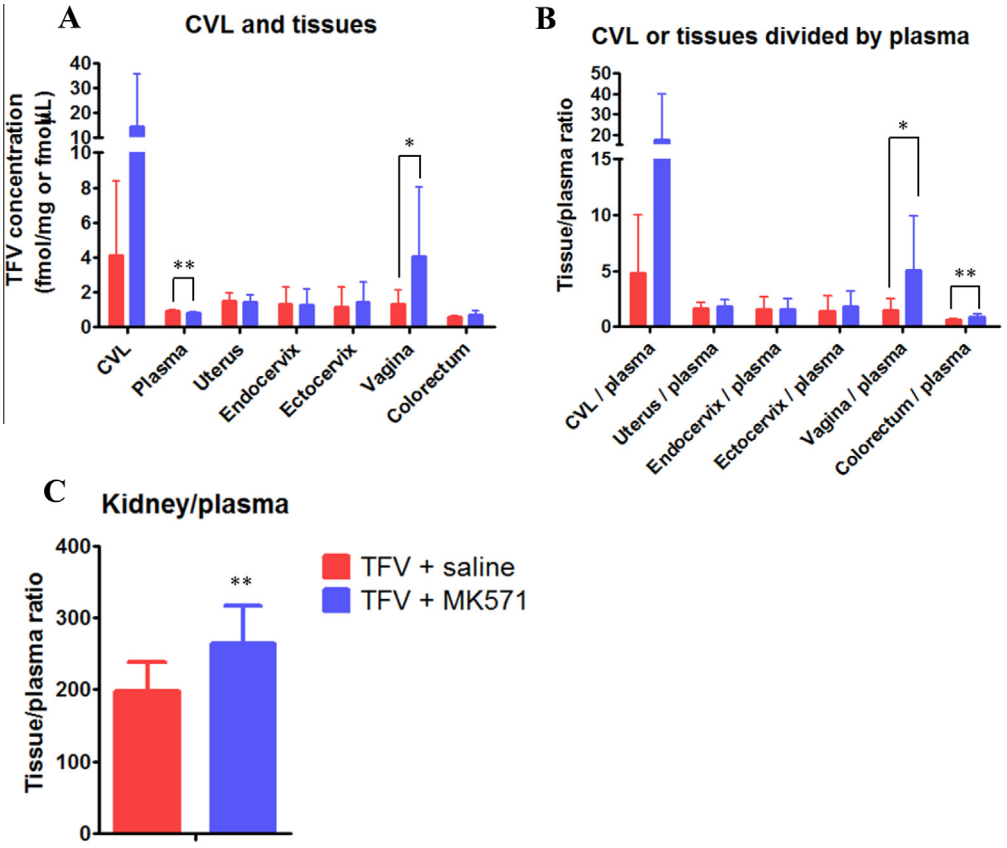


Fig. 10. Effect of MK571 on tissue distribution of systemically administered TFV. Mice were administered IP with saline (red columns) or MK571 solution (blue columns), followed by IP administration of ^{3}H-TFV saline solution 15 min later. Mice were euthanized 1.5 h post ^{3}H-TFV solution administration. Radioactivity in CVL, plasma, and tissue samples was measured in a scintillation counter. Absolute TFV molar concentrations in these samples (A) and plasma-normalized sample concentrations (B, C) were plotted. Data represent mean ± SD from 12 to 13 mice. $^{*}p < 0.05$, $^{**}p < 0.01$. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Depo-Provera synchronization, the ectocervix epithelia became single-layer columnar cells, but the intactness of this single layer was maintained. The vaginal epithelia still contained several layers after synchronization. The vehicle gel treatment did not result in any change in the morphology of mouse tissues, while the positive control 4% N-9 gel negatively affected the morphological intactness of the epithelia of all tissues. N-9-caused detachment of columnar and squamous epithelial cells from the basal lamina could be readily observed in a significant portion of the epithelium-lining regions of these tissues (Fig. 11). In the conditions mimicking the vaginal and IP administration of TFV and MK571, there was no difference, in the morphology of tissues, between the IP-administered mice and vehicle gel-treated mice.

These results demonstrated that the agents used in the transporter function study, including TFV and MK571, did not negatively affect the intact epithelia of the mouse cervicovaginal tissues, which are important natural barriers against sexually transmitted infectious pathogens [40]. In addition, the effect of vaginally and IP administered MK571 on TFV distribution was not likely to be due to its effect on vaginal epithelial intactness and passive permeability.

4. Discussion

In this study, we have examined the expression and regulation of P-gp (Abcb1a/1b), Bcrp, and Mrp4 in mouse cervicovaginal and colorectal tissues, under the influence of the mouse estrous cycle and of exogenous hormones. In addition, we have provided proof of concept that cervicovaginal and colorectal Mrp4 could affect TFV tissue absorption/disposition under certain circumstances, and that this effect may be reversed by co-administration of an MRP inhibitor MK571. To our knowledge, this is the first report revealing the effect of menstrual (estrous) cycle and exogenous hormones on the expression of the three transporters in cervicovaginal tissues. In addition, this is the first report of cervicovaginal tissue MRP4 function in an *in vivo* model.

Among the tested models, the synchronized mice appeared to be the most suitable model for the study of MRP4 function in cervicovaginal tissue. We examined transporter expression in rabbit tissues because it is another *in vivo* model used by microbicide researchers [41]. We also tested some cell lines because they have been used in microbicide safety screening [35]. We found that rabbit transporter expression is too deviant from human expression levels, especially for the TFV transporter MRP4. In addition, the rabbit cervicovaginal tract morphology is quite different from that of humans, which may affect the baseline permeability of the tissue and mask the transporter functionality. The cell lines tested cannot form multiple layers when cultured *in vitro*, and they lack tight junction expression observed in human cervicovaginal tissue and are therefore of little use in predicting what would happen *in vivo*. Compared to the rabbits and cell lines, the transporter expression profile in Depo-Provera-synchronized mouse tissues was found to be closer to human tissue levels. Mouse tissue morphology is also closer to that of humans because the mouse ectocervix and vagina have multiple epithelial layers and mouse endocervix has a single epithelial layer. The mRNA and protein expression of cervicovaginal-tissue Mrp4 was highest after Depo-Provera synchronization, compared to the mice undergoing natural cycling or synchronized by PMSG, suggesting that Mrp4 function

may be most prominent under this condition, and therefore, that this is the condition under which the effect of transporter inhibition is most likely to be observed. Although the Depo-Provera-synchronized mouse model is not perfect and may not be appropriate for data extrapolation, it appears to be a suitable model to use, among the available convenient preclinical models, for the proof-of-concept study of cervicovaginal tissue transporters. Therefore, this is the model we chose for the study of Mrp4 in cervicovaginal tissue.

In the mouse model, the estrous cycle, PMSG, and Depo-Provera demonstrated regulatory effects on the expression of several efflux transporters in mouse cervicovaginal tissues. The effects of these factors were found to be dependent on the type of transporter and type of tissue. The general tendency was that the mRNA expression of these transporters was higher at diestrus, compared to the level at estrus, during the natural estrous cycle. In addition, PMSG, the estrogen-stimulating hormone, tended to decrease transporter expression compared to transporter levels during the natural cycle. Depo-Provera synchronization, on the other hand, tended to increase transporter expression compared to the transporter levels during the natural cycle. The mechanism may involve the expression and activity of nuclear receptors (NRs) in cervicovaginal tissues. MPA has been demonstrated to bind and activate multiple NRs [42]. While MPA does not bind significantly to the NRs for estrogen and mineralocorticoids, it is an agonist of progesterone receptor (PR), androgen receptor (AR), and glucocorticoid receptor (GR) [42]. The approximate EC_{50} values are 0.01, 1, and 10 nM, for PR, AR, and GR, respectively [43]. We examined the mRNA expression of these NRs in mouse cervicovaginal tissues and found they were highly expressed compared to the levels in mouse liver (Ongoing research). These NRs have been shown to regulate the expression of P-gp, BCRP, and MRPs in human cell lines and rodent models. For example, AR activation has been reported to upregulate MRP4 gene expression in normal human prostate cells, a prostate cancer cell line, and tumor tissue collected from prostate cancer patients [44–47]. However, NR-mediated regulation of downstream genes is tissue dependent, and the NR-mediated regulation of transporters in cervicovaginal and colorectal tissues has not been experimentally validated. More studies will be needed to confirm the role of NRs in cervicovaginal tissues.

TFV is a substrate of two efflux transporters (MRP4, MRP7) and two uptake transporters (OAT1, OAT3). OAT1 and OAT3 were found to be undetectable in the cervicovaginal and colorectal tissues of humans and mice, and the GAPDH-normalized mRNA levels of MRP7 were at least 100-fold lower than the Mrp4 level in the endocervix, ectocervix, and vagina of the Depo-Provera synchronized mice (Ongoing research). The Mrp7 mRNA level in mouse colorectum was more than 10-fold lower than the Mrp4 level (Ongoing research). Based on the expression levels, it was most likely that Mrp4 was the major efflux transporter for TFV in mouse tissues, and the observed effects of MK571 on TFV distribution in this study were due to the inhibition of Mrp4 transporter in these tissues.

In both vaginal and IP administration, a large variability in TFV concentration in the female genital tract was observed among individual mice at all the time points post-administration. Notably, this variability in the mouse reproductive tract was much larger than the variability in mouse plasma or other internal organs. This is in line with the clinical observation that a high degree of intra- and inter-subject variability may be seen in human and non-human primate studies of TFV and other antiretroviral drugs, such as maraviroc [14,48–56]. The variability is probably due to the complexity of drug absorption and disposition in the female genital tract. Many factors, such as epithelial layer thickness, tight junction abundance, vaginal fluid level, transporters, and metabolizing enzymes might affect TFV distribution in cervicovaginal tissue and plasma [10,40,57]. These physiologic determinants are regulated by various factors such as hormone status, age, genital tract co-infections, which have a large variability among individuals. The observed large variability in mouse tissue concentration is therefore not surprising.

When TFV was given vaginally, the administration route of the MRP inhibitor MK571 mattered to its effect on TFV distribution in the mouse lower genital tract. When MK571 was administered IP, it increased TFV concentration up to 3-fold in endocervix, ectocervix, and vagina, at one time point at least (0.5 or 1.0 h) post-TFV administration. However, when MK571 was incorporated into the TFV gel and administered vaginally, it resulted in only an 80% increase in vaginal TFV concentration at 0.5 h. The release profile of TFV was not altered by MK571 incorporation as was determined using a Franz cell apparatus, which is the standard USP method of testing drug release from semisolid dosage forms. Therefore, the differential effects of MK571 were not caused by differences in TFV release in the two scenarios. Instead, it is possible that the different pharmacokinetic profile of MK571 when dosed via different routes resulted in its differential effects on TFV. If so, it may be that, when MK571 was given IP, it was distributed evenly among all the parts of the lower female genital tract due to the relatively high blood perfusion of reproductive tissues; however, when MK571 was given vaginally, the primary site of its absorption was the vagina, and MK571 exposure in the ecto- and endocervix was much lower, resulting in a more obvious effect of MK571 on TFV distribution in vaginal tissue but less in the ectocervix and endocervix. The effect of MK571 on TFV distribution in CVL, plasma, and internal organs (liver, kidney) was also different when MK571 was administered via different routes. It is possible that, when MK571 was given intravaginally, this facilitated TFV entry into, and concentration in, vaginal tissue and plasma at the earlier time point (0.5 h); since TFV penetrated more quickly, less TFV gel remained in the vaginal lumen at the later time point (1.0 h), resulting in a lesser amount of TFV in the CVL samples at the 1.0 h time point and reduced vaginal tissue concentration at 1.0 h.

Additional experiments will consolidate the findings and strengthen this study. For the detection of transporter expression under different conditions, Western blot may be utilized to directly visualize the protein expression of transporters. This technique can also be employed to visualize the expression of nuclear receptors that are likely involved in the hormonal regulation of cervicovaginal tissue transporters observed in this study. Several future studies will provide more evidence on the role of Mrp4 and other transporters in cervicovaginal and colorectal tissues. In this study, the MK571 concentration was not measured in the tissues due to the lack of a validated method of quantifying MK571 tissue concentration. Future studies will first need to address this shortcoming. Second, the dose and time dependency of TFV and MK571 will need to be further explored; i.e., more doses and more time points should be tested in future studies. Third, more types of transporter substrates and modulators should be tested in order to validate the role of transporters in drug pharmacokinetics. To overcome the non-specificity of chemical inhibitors, mouse models with transporter gene knockouts (e.g., Abcc4 [Mrp4]) may be used. In addition, the interspecies difference in transporter activity warrants further investigations in humans or animal models that are more clinically relevant. It should be noted that TFV must be phosphorylated by intracellular enzymes to TFV diphosphate (TFV-DP) to become an inhibitor of HIV reverse transcriptase and exert anti-HIV activity [2]. In the current study, the concentration of the parent drug TFV rather than TFV-DP was measured. It would be beneficial to monitor the tissue level of TFV-DP in future endeavors, and examine whether the altered TFV tissue level under Mrp inhibitor treatment could impact the intracellular kinetics of TFV-DP.

In conclusion, our studies have revealed the effect of the menstrual (estrous) cycle, PMSG, and Depo-Provera on the mRNA and protein expression of three efflux transporters, highly relevant to antiretroviral drugs, in the cervicovaginal and colorectal tissues of a mouse model utilized in PrEP/microbicide testing. In comparison with rabbit and three human cell lines, this mouse model appeared to be more appropriate for use in the study of cervicovaginal tissue transporters. Using this model, we have also provided preliminary evidence to demonstrate Mrp4 function in TFV distribution into cervicovaginal tissues. The data also suggest that co-administration of an MRP inhibitor can alter TFV distribution when the inhibitor is dosed at the proper level and via an appropriate route. However, it should be noted that, in the clinical use of PrEP products, adherence is the key to drug exposure and efficacy, especially for once-daily products. The functional role of MRP4 transporter and the effectiveness of MRP4 inhibitor could be observed only when adherence is kept at a high level. Overall, the information generated from this study will inform the development of novel strategies of drug delivery that aim to overcome transporter efflux, and will facilitate PrEP optimization in a number of aspects.

Conflict of interest

The authors declare no conflict of interest with the contents of this article.

Acknowledgements

We would like to acknowledge Dr. Eric Romanowski for his assistance with the acquisition of rabbit tissues. We would also like to thank Dr. Ian McGowan and members of his group for their help with instrument use. In addition, we would like to thank Ms. Galit Frenkel and Ms. Marilyn Cost for their help in the animal experiments, and Mr. Bruce Campbell and Ms. Taryn Serman for their assistance in language editing. Research reported in this publication was supported by the United States National Institute of Allergy and Infectious Diseases (NIAID) of the National Institutes of Health (NIH) under Award Number AI082639. The content is solely the responsibility of the authors and does not necessarily represent the official views of the NIH.

References

[1] UNAIDS, UNAIDS Report on the Global AIDS Epidemic, 2013.
[2] Q. Abdool Karim, S.S. Abdool Karim, J.A. Frohlich, A.C. Grobler, C. Baxter, L.E. Mansoor, A.B. Kharsany, S. Sibeko, K.P. Mlisana, Z. Omar, T.N. Gengiah, S. Maarschalk, N. Arulappan, M. Mlotshwa, L. Morris, D. Taylor, C.T. Group, Effectiveness and safety of tenofovir gel, an antiretroviral microbicide, for the prevention of HIV infection in women, Science 329 (5996) (2010) 1168–1174.
[3] MTN Statement on Decision to Discontinue Use of Tenofovir Gel in VOICE, a Major HIV Prevention Study in Women, MTN, Pittsburgh, PA, 2011. <www.mtnstopshiv.org/node/3909>.
[4] R.M. Grant, J.R. Lama, P.L. Anderson, V. McMahan, A.Y. Liu, L. Vargas, P. Goicochea, M. Casapia, J.V. Guanira-Carranza, M.E. Ramirez-Cardich, O. Montoya-Herrera, T. Fernandez, V.G. Veloso, S.P. Buchbinder, S. Chariyalertsak, M. Schechter, L.G. Bekker, K.H. Mayer, E.G. Kallas, K.R. Amico, K. Mulligan, L.R. Bushman, R.J. Hance, C. Ganoza, P. Defechereux, B. Postle, F. Wang, J.J. McConnell, J.H. Zheng, J. Lee, J.F. Rooney, H.S. Jaffe, A.I. Martinez, D.N. Burns, D.V. GliddenT. iPrEx Study, Preexposure chemoprophylaxis for HIV prevention in men who have sex with men, N. Engl. J. Med. 363 (27) (2010) 2587–2599.
[5] J.M. Baeten, D. Donnell, P. Ndase, N.R. Mugo, J.D. Campbell, J. Wangisi, J.W. Tappero, E.A. Bukusi, C.R. Cohen, E. Katabira, A. Ronald, E. Tumwesigye, E. Were, K.H. Fife, J. Kiarie, C. Farquhar, G. John-Stewart, A. Kakia, J. Odoyo, A. Mucunguzi, E. Nakku-Joloba, R. Twesigye, K. Ngure, C. Apaka, H. Tamooh, F. Gabona, A. Mujugira, D. Panteleeff, K.K. Thomas, L. Kidoguchi, M. Krows, J. Revall, S. Morrison, H. Haugen, M. Emmanuel-Ogier, L. Ondrejcek, R.W. Coombs, L. Frenkel, C. Hendrix, N.N. Bumpus, D. Bangsberg, J.E. Haberer, W.S. Stevens, J.R. Lingappa, C. Celum, E.P.S.T. Partners Pr, Antiretroviral prophylaxis for HIV prevention in heterosexual men and women, N. Engl. J. Med. 367 (5) (2012) 399–410.
[6] M.C. Thigpen, P.M. Kebaabetswe, L.A. Paxton, D.K. Smith, C.E. Rose, T.M. Segolodi, F.L. Henderson, S.R. Pathak, F.A. Soud, K.L. Chillag, R. Mutanhaurwa, L. I. Chirwa, M. Kasonde, D. Abebe, E. Buliva, R.J. Gvetadze, S. Johnson, T. Sukalac, V.T. Thomas, C. Hart, J.A. Johnson, C.K. Malotte, C.W. Hendrix, J.T. Brooks, T.D.F. S. Group, Antiretroviral preexposure prophylaxis for heterosexual HIV transmission in Botswana, N. Engl. J. Med. 367 (5) (2012) 423–434.
[7] L. Van Damme, A. Corneli, K. Ahmed, K. Agot, J. Lombaard, S. Kapiga, M. Malahleha, F. Owino, R. Manongi, J. Onyango, L. Temu, M.C. Monedi, P. Mak'Oketch, M. Makanda, I. Reblin, S.E. Makatu, L. Saylor, H. Kiernan, S. Kirkendale, C. Wong, R. Grant, A. Kashuba, K. Nanda, J. Mandala, K. Fransen, J. Deese, T. Crucitti, T.D. Mastro, D. Taylor, F.E.-P.S. Group, Preexposure prophylaxis for HIV infection among African women, N. Engl. J. Med. 367 (5) (2012) 411–422.
[8] J.M. Marrazzo, G. Ramjee, G. Nair, T. Palanee, B. Mkhize, C. Nakabiito, M. Taljaard, J. Piper, K. Gomez, M. Chirenje, Pre-exposure prophylaxis forHIV in women: daily oral tenofovir, oral tenofovir/emtricitabine, or vaginal tenofovir gel in the VOICE study (MTN 003), CROI 2013 abstract #26LB.
[9] J.L. Adams, A.D. Kashuba, Formulation, pharmacokinetics and pharmacodynamics of topical microbicides, best practice & research, Clin. Obstet. Gynaecol. 26 (4) (2012) 451–462.
[10] J. Romano, A. Kashuba, S. Becker, J. Cummins, J. Turpin, F. VeroneseOn Behalf of the Antiretroviral Pharmacology in HIV Prevention Think Tank Participants, Pharmacokinetics and pharmacodynamics in HIV prevention: current status and future directions: a summary of the DAIDS and BMGF sponsored think tank on pharmacokinetics (PK)/pharmacodynamics (PD) in HIV prevention, AIDS Res. Human Retroviruses (2013).
[11] M.L. Cottrell, A.D. Kashuba, Topical microbicides and HIV prevention in the female genital tract, J. Clin. Pharmacol. 54 (6) (2014) 603–615.
[12] F. Hladik, G.F. Doncel, Preventing mucosal HIV transmission with topical microbicides: challenges and opportunities, Antiviral Res. 88 (Suppl. 1) (2010) S3–S9.
[13] I. McGowan, C. Dezzutti, Rectal microbicide development, Curr. Top. Microbiol. Immunol. (2013).
[14] S.S. Karim, A.D. Kashuba, L. Werner, Q.A. Karim, Drug concentrations after topical and oral antiretroviral pre-exposure prophylaxis: implications for HIV prevention in women, Lancet 378 (9787) (2011) 279–281.
[15] N. Richardson-Harman, C. Mauck, I. McGowan, P. Anton, Dose-response relationship between tissue concentrations of UC781 and explant infectibility with HIV type 1 in the RMP-01 rectal safety study, AIDS Res. Hum. Retroviruses 28 (11) (2012) 1422–1433.
[16] R.H. Ho, R.B. Kim, Transporters and drug therapy: implications for drug disposition and disease, Clin. Pharmacol. Ther. 78 (3) (2005) 260–277.
[17] C. International Transporter, K.M. Giacomini, S.M. Huang, D.J. Tweedie, L.Z. Benet, K.L. Brouwer, X. Chu, A. Dahlin, R. Evers, V. Fischer, K.M. Hillgren, K.A. Hoffmaster, T. Ishikawa, D. Keppler, R.B. Kim, C.A. Lee, M. Niemi, J.W. Polli, Y. Sugiyama, P.W. Swaan, J.A. Ware, S.H. Wright, S.W. Yee, M.J. Zamek-Gliszczynski, L. Zhang, Membrane transporters in drug development, Nat. Rev. Drug Discovery 9 (3) (2010) 215–236.
[18] O. Kis, K. Robillard, G.N. Chan, R. Bendayan, The complexities of antiretroviral drug-drug interactions: role of ABC and SLC transporters, Trends Pharmacol. Sci. 31 (1) (2010) 22–35.
[19] S. Abel, D.J. Back, M. Vourvahis, Maraviroc: pharmacokinetics and drug interactions, Antivir. Ther. 14 (5) (2009) 607–618.
[20] T. Imaoka, H. Kusuhara, M. Adachi, J.D. Schuetz, K. Takeuchi, Y. Sugiyama, Functional involvement of multidrug resistance-associated protein 4 (MRP4/ABCC4) in the renal elimination of the antiviral drugs adefovir and tenofovir, Mol. Pharmacol. 71 (2) (2007) 619–627.
[21] S. Rodriguez-Novoa, P. Labarga, V. Soriano, D. Egan, M. Albalater, J. Morello, L. Cuenca, G. Gonzalez-Pardo, S. Khoo, D. Back, A. Owen, Predictors of kidney tubular dysfunction in HIV-infected patients treated with tenofovir: a pharmacogenetic study, Clin. Infect. Dis. 48 (11) (2009) e108–e116.
[22] J. Goole, D.J. Lindley, W. Roth, S.M. Carl, K. Amighi, J.M. Kauffmann, G.T. Knipp, The effects of excipients on transporter mediated absorption, Int. J. Pharm. 393 (1–2) (2010) 17–31.
[23] G. Minuesa, I. Huber-Ruano, M. Pastor-Anglada, H. Koepsell, B. Clotet, J. Martinez-Picado, Drug uptake transporters in antiretroviral therapy, Pharmacol. Ther. 132 (3) (2011) 268–279.
[24] T. Zhou, M. Hu, A. Pearlman, D. Patton, L. Rohan, Expression and localization of p-glycoprotein, multidrug resistance protein 4, and breast cancer resistance protein in the female lower genital tract of human and pigtailed macaque, AIDS Res. Hum. Retroviruses 30 (11) (2014) 1106–1116.
[25] C. Grammen, M. Baes, S. Haenen, J. Verguts, K. Augustyns, T. Zydowsky, P. La Colla, P. Augustijns, J. Brouwers, Vaginal expression of efflux transporters and the potential impact on the disposition of microbicides in vitro and in rabbits, Mol. Pharm. 11 (12) (2014) 4405–4414.
[26] K. Hijazi, A.M. Cuppone, K. Smith, M.A. Stincarelli, J. Ekeruche-Makinde, G. De Falco, G.L. Hold, R. Shattock, C.G. Kelly, G. Pozzi, F. Iannelli, Expression of genes for drug transporters in the human female genital tract and modulatory effect of antiretroviral drugs, PLoS One 10 (6) (2015) e0131405.
[27] M.R. Nicol, Y. Fedoriw, M. Mathews, H.M. Prince, K.B. Patterson, E. Geller, K. Mollan, S. Mathews, D.L. Kroetz, A.D. Kashuba, Expression of six drug transporters in vaginal, cervical, and colorectal tissues: implications for drug disposition in HIV prevention, J. Clin. Pharmacol. 54 (5) (2014) 574–583.
[28] T. Zhou, M. Hu, M. Cost, S. Poloyac, L. Rohan, Short communication: expression of transporters and metabolizing enzymes in the female lower genital tract: implications for microbicide research, AIDS Res. Hum. Retroviruses 29 (11) (2013) 1496–1503.

[29] I. Mukhopadhya, G.I. Murray, S. Berry, J. Thomson, B. Frank, G. Gwozdz, J. Ekeruche-Makinde, R. Shattock, C. Kelly, F. Iannelli, G. Pozzi, E.M. El-Omar, G.L. Hold, K. Hijazi, Drug transporter gene expression in human colorectal tissue and cell lines: modulation with antiretrovirals for microbicide optimization, J. Antimicrob. Chemother. (2015).
[30] M. Hu, S.K. Patel, T. Zhou, L.C. Rohan, Drug transporters in tissues and cells relevant to sexual transmission of HIV: implications for drug delivery, J. Controlled Release 219 (2015) 681–696.
[31] M.E. Morris, H.J. Lee, L.M. Predko, Gender differences in the membrane transport of endogenous and exogenous compounds, Pharmacol. Rev. 55 (2) (2003) 229–240.
[32] C.S. Caligioni, Assessing reproductive status/stages in mice, in: Jacqueline N. Crawley et al. (Eds.), Current Protocols in Neuroscience/Editorial Board, 2009. Appendix 4, Appendix 4I.
[33] MTN, Injectable Contraception and HIV Risk in VOICE. Secondary Analysis Results QA, 2014.
[34] B.J. Catalone, T.M. Kish-Catalone, L.R. Budgeon, E.B. Neely, M. Ferguson, F.C. Krebs, M.K. Howett, M. Labib, R. Rando, B. Wigdahl, Mouse model of cervicovaginal toxicity and inflammation for preclinical evaluation of topical vaginal microbicides, Antimicrob. Agents Chemother. 48 (5) (2004) 1837–1847.
[35] B.J. Catalone, T.M. Kish-Catalone, E.B. Neely, L.R. Budgeon, M.L. Ferguson, C. Stiller, S.R. Miller, D. Malamud, F.C. Krebs, M.K. Howett, B. Wigdahl, Comparative safety evaluation of the candidate vaginal microbicide C31G, Antimicrob. Agents Chemother. 49 (4) (2005) 1509–1520.
[36] Y. Hara, Y. Sassi, C. Guibert, N. Gambaryan, P. Dorfmuller, S. Eddahibi, A.M. Lompre, M. Humbert, J.S. Hulot, Inhibition of MRP4 prevents and reverses pulmonary hypertension in mice, J. Clin. Invest. 121 (7) (2011) 2888–2897.
[37] J.F. Blain, P. Sirois, Involvement of LTD(4)in allergic pulmonary inflammation in mice: modulation by cysLT(1)antagonist MK-571, Prostaglandins Leukot. Essent. Fatty Acids 62 (6) (2000) 361–368.
[38] A. Maekawa, Y. Kanaoka, W. Xing, K.F. Austen, Functional recognition of a distinct receptor preferential for leukotriene E4 in mice lacking the cysteinyl leukotriene 1 and 2 receptors, Proc. Natl. Acad. Sci. U.S.A. 105 (43) (2008) 16695–16700.
[39] L.C. Rohan, B.J. Moncla, R.P. Kunjara Na Ayudhya, M. Cost, Y. Huang, F. Gai, N. Billitto, J.D. Lynam, K. Pryke, P. Graebing, N. Hopkins, J.F. Rooney, D. Friend, C.S. Dezzutti, In vitro and ex vivo testing of tenofovir shows it is effective as an HIV-1 microbicide, PLoS One 5 (2) (2010) e9310.
[40] L.C. Rohan, A.B. Sassi, Vaginal drug delivery systems for HIV prevention, AAPS J. 11 (1) (2009) 78–87.
[41] R.S. Veazey, Animal models for microbicide safety and efficacy testing, Curr. Opin. HIV AIDS 8 (4) (2013) 295–303.
[42] A.E. Schindler, C. Campagnoli, R. Druckmann, J. Huber, J.R. Pasqualini, K.W. Schweppe, J.H. Thijssen, Classification and pharmacology of progestins, Maturitas 61 (1–2) (2008) 171–180.
[43] Joint FAO/WHO Expert Committee on Food Additives, Meeting (62nd: 2004: Rome Italy), Food and Agriculture Organization of the United Nations, World Health Organization, in: Residues of Some Veterinary Drugs in Animals and Foods: Monographs Prepared by the Sixty-Second Meeting of the Joint FAO/WHO Expert Committee on Food Additives, Rome, 4-12 February 2004, World Health Organization: Food and Agriculture Organization of the United Nations, Rome, 2004.
[44] T. Huynh, M.D. Norris, M. Haber, M.J. Henderson, ABCC4/MRP4: a MYCN-regulated transporter and potential therapeutic target in neuroblastoma, Front. Oncol. 2 (2012) 178.
[45] C.A. Heinlein, C. Chang, Androgen receptor in prostate cancer, Endocr. Rev. 25 (2) (2004) 276–308.
[46] C. Cai, J. Omwancha, C.L. Hsieh, L. Shemshedini, Androgen induces expression of the multidrug resistance protein gene MRP4 in prostate cancer cells, Prostate Cancer Prostatic Dis. 10 (1) (2007) 39–45.
[47] L.L. Ho, J.G. Kench, D.J. Handelsman, G.L. Scheffer, P.D. Stricker, J.G. Grygiel, R.L. Sutherland, S.M. Henshall, J.D. Allen, L.G. Horvath, Androgen regulation of multidrug resistance-associated protein 4 (MRP4/ABCC4) in prostate cancer, Prostate 68 (13) (2008) 1421–1429.
[48] M.R. Nicol, A.D. Kashuba, Pharmacologic opportunities for HIV prevention, Clin. Pharmacol. Ther. 88 (5) (2010) 598–609.
[49] C.G. Thompson, M.S. Cohen, A.D. Kashuba, Antiretroviral pharmacology in mucosal tissues, J. Acquir. Immune Defic. Syndr. 63 (Suppl. 2) (2013) S240–S247.
[50] K.C. Brown, K.B. Patterson, S.A. Malone, N.J. Shaheen, H.M. Prince, J.B. Dumond, M.B. Spacek, P.E. Heidt, M.S. Cohen, A.D. Kashuba, Single and multiple dose pharmacokinetics of maraviroc in saliva, semen, and rectal tissue of healthy HIV-negative men, J. Infect. Dis. 203 (10) (2011) 1484–1490.
[51] J.B. Dumond, K.B. Patterson, A.L. Pecha, R.E. Werner, E. Andrews, B. Damle, R. Tressler, J. Worsley, A.D. Kashuba, Maraviroc concentrates in the cervicovaginal fluid and vaginal tissue of HIV-negative women, J. Acquir. Immune Defic. Syndr. 51 (5) (2009) 546–553.
[52] J.B. Dumond, R.F. Yeh, K.B. Patterson, A.H. Corbett, B.H. Jung, N.L. Rezk, A.S. Bridges, P.W. Stewart, M.S. Cohen, A.D. Kashuba, Antiretroviral drug exposure in the female genital tract: implications for oral pre- and post-exposure prophylaxis, AIDS 21 (14) (2007) 1899–1907.
[53] D.T. Evans, G. Silvestri, Nonhuman primate models in AIDS research, Curr. Opin. HIV AIDS 8 (4) (2013) 255–261.
[54] C.M. Fennessey, B.F. Keele, Using nonhuman primates to model HIV transmission, Curr. Opin. HIV AIDS 8 (4) (2013) 280–287.
[55] K.B. Patterson, H.A. Prince, E. Kraft, A.J. Jenkins, N.J. Shaheen, J.F. Rooney, M.S. Cohen, A.D. Kashuba, Penetration of tenofovir and emtricitabine in mucosal tissues: implications for prevention of HIV-1 transmission, Sci. Transl. Med. 3 (112) (2011). 112re4.
[56] C.W. Hendrix, B.A. Chen, V. Guddera, C. Hoesley, J. Justman, C. Nakabiito, R. Salata, L. Soto-Torres, K. Patterson, A.M. Minnis, S. Gandham, K. Gomez, B.A. Richardson, N.N. Bumpus, MTN-001: randomized pharmacokinetic cross-over study comparing tenofovir vaginal gel and oral tablets in vaginal tissue and other compartments, PLoS One 8 (1) (2013) e55013.
[57] C.D. Blaskewicz, J. Pudney, D.J. Anderson, Structure and function of intercellular junctions in human cervical and vaginal mucosal epithelia, Biol. Reprod. 85 (1) (2011) 97–104.