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

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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

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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