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Amanda James, Emory University
Edward P Acosta, University of Alabama at Birmingham
Jennifer R King, University of Alabama at Birmingham
Ighoverha Ofotokun, Emory University
Anandi Sheth, Emory University

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Uptake of tenofovir and emtricitabine into non-monocytic female genital tract cells with and without hormonal contraceptives

Amanda Marie James¹
Jennifer R King¹
Ighovwerha Ofotokun²
Anandi N Sheth²
Edward P Acosta¹

¹Division of Clinical Pharmacology,
²Emory University School of Medicine, Department of Medicine, Division of Infectious Diseases, The University of Alabama at Birmingham, Birmingham, AL, USA

Background: Pre-exposure prophylaxis is becoming a strategic component used to control the human immunodeficiency virus (HIV-1) epidemic. The goal of this study was to characterize intracellular uptake of tenofovir and emtricitabine using five surrogate cell lines of the female genital tract and determine whether exogenous hormones influence their uptake.

Methods: Surrogate cell lines, ie, THP-1 (representing macrophages), BC-3 (CD8+), Ect1/E6E7 (squamous epithelial), HeLa (CD4+), and TF-1 (dendritic), were incubated for one hour with tenofovir and emtricitabine to assess uptake. In separate experiments, ethinyl estradiol (EE) and etonogestrel (ET) individually and together (EE/ET) were added prior to, simultaneously, and after incubation. Intracellular phosphorylated tenofovir and emtricitabine were quantified using validated tandem mass spectrometry methods.

Results: HeLa and Ect1/E6E7 cells showed significantly increased uptake relative to THP-1 controls for both antiretrovirals. Individually, ethinyl estradiol and etonogestrel significantly altered antiretroviral uptake across all cell lines, except Ect1/E6E7 for tenofovir and HeLa for emtricitabine. Cellular uptake of tenofovir and emtricitabine in BC-3 and TF-1 cells were significantly lower when dosed one hour prior to EE/ET administration compared with each antiretroviral administered in the absence of EE/ET (tenofovir, 80 versus 470 fmol/10⁶ for BC-3 and 77 versus 506 fmol/10⁶ cells for TF-1; emtricitabine, 36 versus 12 fmol/10⁶ for BC-3 and 75 versus 5 fmol/10⁶ cells for TF-1; P < 0.01 for each).

Conclusion: These data suggest that intracellular uptake of tenofovir and emtricitabine within the female genital tract varies by cell type and in the presence of hormonal contraceptives. The potential clinical implications of these findings should be further evaluated in vivo.

Keywords: pre-exposure prophylaxis, tenofovir, emtricitabine, in vitro, hormones

Introduction

The use of antiretrovirals by individuals negative for human immunodeficiency virus (HIV) to prevent HIV-1 infection, also known as pre-exposure prophylaxis (PrEP), has shown variable results in reducing the rate of HIV-1 transmission.¹,² The Center for the AIDS Program of Research in South Africa 004 trial, which examined topical 1% tenofovir gel in women, showed a 39% overall reduction in HIV-1 transmission.³ The PrEP Initiative study demonstrated a 42% decrease in HIV-1 transmission when daily oral tenofovir disoproxil fumarate (TDF) and emtricitabine were administered to men who have sex with men,⁴ while the TDF2 and Partners PrEP studies demonstrated over 60% efficacy against acquisition of HIV-1 in discordant heterosexual men and women when daily TDF was used with or without emtricitabine.⁵,⁶ In contrast, no significant difference was demonstrated between TDF and placebo.
in their ability to prevent HIV-1 infection in participants enrolled in the Vaginal and Oral Interventions to Control the Epidemic trial.7 Similarly, an oral combination of TDF and emtricitabine was not able to demonstrate effectiveness in preventing HIV-1 infection in HIV-negative women enrolled into the FEM-PrEP study who were at high risk of being exposed to HIV-1.8 As a result, these two PrEP trials were prematurely terminated.

Differences in outcomes between prevention trials could be due to several factors. Each study evaluated once-daily administration of TDF with or without emtricitabine, but the routes of administration differed. The data suggest that vaginal administration produces 100 times greater tenofovir concentrations in endocervical cells than oral dosing, thus possibly providing better protection against transmission of HIV-1.9 Adherence to therapy likely also plays an important role, because studies have reported >90% adherence by participants, whereas antiretroviral plasma concentrations suggest only 50% adherence.10 Finally, concomitant use of hormonal contraceptives was not an exclusion criterion. Although oral hormonal contraceptives do not alter the plasma pharmacokinetics of tenofovir or emtricitabine,11 tenofovir and emtricitabine require intracellular phosphorylation to the active diphosphate or triphosphate moieties, respectively.12,13 The possibility that hormones influence cellular uptake of nucleoside reverse transcriptase inhibitors has not been extensively evaluated.14

These factors support integrating intracellular pharmacology into clinical evaluations of PrEP studies. Several studies to date have quantified intracellular concentrations of tenofovir and emtricitabine in the female genital tract to determine the concentrations required to prevent HIV-1 transmission. Analytical methods to quantify intracellular concentrations in this compartment may differ, potentially leading to inconsistent results across studies. For example, most methods use macrophage-like peripheral blood mononuclear cells as controls and standards. However, cells of the female genital tract also include squamous epithelial, dendritic, CD4+, and CD8+ cells.15-17 The female genital tract can be divided into five major sections, ie, the vagina, cervix, uterus, fallopian tubes, and ovaries. The cervix and vagina are commonly sampled to quantify antiretrovirals because these sections are highly vulnerable to HIV-1 transmission and collection techniques, such as cytobrushing, direct aspirate, and TearFlo strips that target cells in these areas.18 Therefore, antiretroviral uptake in cell lines that match cell types in the endocervix, ectocervix, and vagina should be evaluated.19-21

Our hypotheses were that uptake of tenofovir and emtricitabine into these surrogate cell lines would not differ, and that administration of ethinyl estradiol and/or etonogestrel would not alter intracellular anabolite concentrations. The objectives of this study were to assess intracellular concentrations of tenofovir and emtricitabine in THP-1 cells,22 which represent macrophages, and four surrogate cell lines of the female genital tract, ie, BC-3 (representing CD8+ cells), Ect1/E6E7 (squamous epithelial cells), HeLa (CD4+ cells), and TF-1 (dendritic cells),23-26 and to compare intracellular tenofovir and emtricitabine concentrations across these surrogate cell lines when dosed prior to, simultaneously, and after ethinyl estradiol and/or etonogestrel.

Materials and methods
Materials
Tenofovir and emtricitabine were obtained for laboratory use only from the National Institutes of Health AIDS Research and Reagent Program. Ethinyl estradiol was purchased from Sigma-Aldrich (St Louis, MO, USA) and etonogestrel from Santa Cruz Biotechnology Inc (Santa Cruz, CA, USA). Isotopically-labeled tenofovir and emtricitabine were purchased from Moravek Inc (Brea, CA, USA) and used as the internal standard. All additional solvents were purchased from commercial suppliers and were of high performance liquid chromatography grade. The five different cell lines (THP-1, HeLa, BC-3, TF-1, and Ect1/E6E7) were obtained from the American Type Culture Collection (Rockville, MD, USA) and grown in medium prepared in accordance with American Type Culture Collection protocols in a freestanding Isotemp incubator (Fisher Scientific, Waltham, MA, USA) at 37°C with 5% CO2. The cells were passaged between five and 12 times at 95%-100% confluence. Confluent monolayers of adherent cell lines were subcultured by treatment with 0.35% trypsin containing EDTA, while confluent monolayers of suspension cell lines were subcultured with a one to three splitting technique.

Cell dosing studies
Uptake of tenofovir and emtricitabine in the absence of hormones
Each cell line was dosed (with six replicates) using either 0.3 µg/mL of tenofovir or 1.8 µg/mL of emtricitabine, representing the peak plasma concentration (Cmax) recorded in the literature for adults.27,28 After one hour, the cells were harvested by removing the medium and pelleted using centrifugation (5000 rpm for 10 minutes at 25°C). The pellet was resuspended in 70% methanol, vortexed, placed on ice for
15 minutes, and centrifuged. The supernatant, representing the cellular matrix, was collected and stored at −80°C until analysis. Cell viability was checked prior to and after dosing by cell count, visual inspection, and the Trypan Blue Cell Viability test. Interday variability was also assessed.

Uptake of tenofovir and emtricitabine in the presence of hormones
Each cell line (5–6 replicates) was dosed with 0.3 µg/mL of tenofovir or 1.8 µg/mL of emtricitabine. However, in this set of experiments, the cells were also dosed with 34.7 pg/mL of ethinyl estradiol and/or 1716 pg/mL of etonogestrel, representing the plasma C_{\text{max}} recorded in the literature for the NuvaRing® contraceptive (Merck, Whitehouse Station, NJ, USA). Dosing experiments with ethinyl estradiol and/or etonogestrel were conducted relative to the time of tenofovir and emtricitabine dosing. All cell lines were dosed with either tenofovir or emtricitabine one hour prior to, simultaneously, or one hour after dosing with the hormonal contraceptives. In each case, the hormones were allowed to incubate with the drug for one hour. The effect of EE/ET on antiretroviral uptake across a 25-fold range of tenofovir and emtricitabine doses was also examined. In addition to the initial doses of 0.3 µg/mL of tenofovir and 1.8 µg/mL of emtricitabine, the cells were also dosed with a concentration representing the plasma C_{\text{min}} of 0.06 µg/mL for tenofovir and 0.09 µg/mL for emtricitabine. A 25-fold increase from the C_{\text{min}} of 1.5 µg/mL for tenofovir and 2.25 µg/mL for emtricitabine. All samples were processed as described in the previous paragraph.

Sample preparation and mass spectrometry
Cellular lysate samples were subjected to anion exchange solid phase extraction with a QMA cartridge (Accell Plus QMA, 3 mL, Waters Corporation, Milford, MA, USA) using a potassium chloride salt gradient of 5–100 mM to isolate the phosphorylated forms of the drugs. The samples were then dephosphorylated from the active tenofovir diphosphate and emtricitabine triphosphate to measurable tenofovir and emtricitabine using sweet potato acid phosphatase XA (Sigma-Aldrich)/acetate buffer at 37°C for one hour. Cellular lysate containing only tenofovir and emtricitabine was then desalted by a solid phase extraction with a reverse phase Oasis HLB cartridge (3 mL, Waters Corporation). Desalted cellular lysate samples were then evaporated under nitrogen at 37°C and reconstituted in double-distilled H_{2}O to concentrate the samples. Quantification of tenofovir and emtricitabine was performed using a method developed on a Prominence Ultra-Fast liquid chromatography system (UFLC_{x,r} Shimadzu, Tokyo, Japan) coupled to an MDS Sciex API 5500 triple quadrupole mass spectrometer. Chromatographic separation was performed on an Atlantis dC18, 2.1 × 100 mm, 3 µm particle size, analytical column (Waters Corporation). The mobile phase consisted of 0.01% trifluoroacetic acid buffer and acetonitrile run isocratically at a ratio of 90:10 (by volume). Detection and quantification were achieved by multiple reaction monitoring. Tenofovir, tenofovir-internal standard, emtricitabine, and emtricitabine-internal standard were detected using the following transitions for protonated daughters [M + H]⁺: m/z tenofovir 288→176.1 and 288→159, m/z tenofovir-internal standard 293→181, m/z emtricitabine 248.1→130 and 248.1→113, m/z emtricitabine-internal standard 251→133. The analytical concentration range for intracellular tenofovir and emtricitabine was 5–2500 fmol/10⁶ cells. Standard curves, quality controls, and blanks for each cell line were prepared to ensure accurate quantitation throughout the analysis. Acceptance criteria for the assay were based on Good Clinical Laboratory Procedures standards, which consisted of standard curves and quality controls within ±15% of their nominal value along with the blank of each cell line demonstrating no measurable contamination response from preparation of samples.

Data analysis
THP-1 cells were used as the initial control cell line for statistical comparisons with the other cell lines using the unpaired nonparametric t-test (Figure 1). Uptake of tenofovir or emtricitabine from each cell line dosed with ethinyl estradiol and/or etonogestrel prior to, simultaneously, or after antiretroviral administration were matched by cell line and compared with tenofovir and emtricitabine only controls using the unpaired nonparametric t-test. The one-way analysis of variance test was used to assess interday variability. All statistical tests were two-sided, with significance set at 0.05. Data are reported as the mean ± standard deviation and associated P values. Statistical parameters were derived using GraphPad Prism version 5.04 (La Jolla, CA, USA).

Results
Determination of uptake of tenofovir and emtricitabine
Intracellular concentrations of tenofovir and emtricitabine in the five representative female genital tract cell lines are shown in Figure 1A and B. Differences in the uptake of tenofovir and emtricitabine across the cell lines versus controls were
observed. Mean intracellular tenofovir concentrations were highest in HeLa cells (13,022 ± 4503 fmol/10⁶ cells, \( P = 0.002 \)) and Ect1/E6E7 cells (10,778 ± 3713 fmol/10⁶ cells, \( P = 0.002 \)), representing a 32-fold and 27-fold increase, respectively, compared with tenofovir uptake by THP-1 macrophage control cells (403 ± 183 fmol/10⁶ cells). Intracellular tenofovir concentrations were comparable between THP-1, BC-3, and TF-1 cells. Emtricitabine uptake was also highest in HeLa (1152 ± 587 fmol/10⁶ cells, \( P = 0.002 \)) and Ect1/E6E7 cells (1143 ± 582 fmol/10⁶ cells, \( P = 0.002 \)) compared with THP-1 (18.1 ± 12.0 fmol/10⁶ cells), BC-3 (19.5 ± 12.3 fmol/10⁶ cells), and TF-1 cells (44.1 ± 36.7 fmol/10⁶ cells). Uptake in HeLa and Ect1/E6E7 cells was 63-fold higher compared with THP-1 cells. The average percentage uptake of tenofovir for the five cell lines was higher than the uptake of emtricitabine (4.85% of the tenofovir dose versus 0.07% of the emtricitabine dose). The median intracellular tenofovir and emtricitabine concentration across all five lines was 579 and 40 fmol/10⁶ cells, respectively.

**Uptake of tenofovir and emtricitabine prior to dosing with ethinyl estradiol or etonogestrel**

BC-3 and TF-1 cells, when treated with tenofovir for one hour prior to adding ethinyl estradiol, demonstrated decreased intracellular tenofovir concentrations of 87% and 78%, respectively, compared with tenofovir only controls. Similarly, when dosed with tenofovir prior to etonogestrel, intracellular tenofovir concentrations decreased by 87% in BC-3 cells and 79% in TF-1 cells. No uptake effect was seen for THP-1, HeLa, or Ect1/E6E7 cells when dosed prior to ethinyl estradiol or etonogestrel. BC-3 and TF-1 cells also showed significantly decreased emtricitabine uptake when dosed prior to ethinyl estradiol (78% and 85%, respectively) and etonogestrel (69% and 96%, respectively), with no differences in the remaining three cell lines.

**Uptake of tenofovir and emtricitabine with simultaneous dosing of ethinyl estradiol or etonogestrel**

Simultaneous dosing of tenofovir and exogenous hormones produced a mixture of decreased and increased tenofovir uptake. When ethinyl estradiol or etonogestrel was dosed concurrently with tenofovir in HeLa cells, a 57% and 65% decrease, respectively, in intracellular tenofovir concentrations was observed. TF-1 cellular uptake decreased by 35% and BC-3 cellular uptake decreased by 85% with simultaneous tenofovir/etonogestrel administration. For emtricitabine, the most pronounced effect was seen in BC-3 cells, where an 8.5-fold and 2.5-fold increase in intracellular emtricitabine

### Figure 1

**Uptake of TFV (A) and FTC (B) in five prominent cell types of the female genital tract.** Concentrations of TFV-DP (A) and FTC-TP (B) in five cell lines representing the female genital tract. Cell lines are THP-1 (macrophages, control), HeLa (CD4+), BC-3 (CD8+), TF-1 (dendritic), and Ect1/E6E7 (squamous epithelial cells) following a one hour incubation. Mean ± standard deviation values are reported * indicates significantly different from control. Each cell line represents 6 replicates except the BC-3 and TF-1 for TFV-DP which each had 5 replicates.

**Abbreviations:** TFV, tenofovir; TFV-DP, tenofovir diphosphate; FTC, emtricitabine; FTC-TP, emtricitabine triphosphate.
Table 1 Mean ± SD concentration of TFV and FTC in five cell types of the female genital tract when dosed prior to, simultaneously, and after ET or EE administration

<table>
<thead>
<tr>
<th>Cell line</th>
<th>TFV only EE</th>
<th>TFV dosed 1 hr prior EE</th>
<th>TFV dosed simultaneously EE</th>
<th>TFV dosed 1 hr after EE</th>
</tr>
</thead>
<tbody>
<tr>
<td>THP-1</td>
<td>393.6 ± 19.9</td>
<td>374.7 ± 7.3</td>
<td>377.1 ± 8.9</td>
<td>309.9 ± 7.9**</td>
</tr>
<tr>
<td>BC-3</td>
<td>469.7 ± 14.6</td>
<td>61.9 ± 1.5**</td>
<td>63.2 ± 1.7**</td>
<td>387.3 ± 12.6**</td>
</tr>
<tr>
<td>Ect1/E6E7</td>
<td>11,912 ± 720.4</td>
<td>12,551 ± 757.8</td>
<td>14,945 ± 3054</td>
<td>9457 ± 687.2</td>
</tr>
<tr>
<td>HeLa</td>
<td>18,456 ± 2294</td>
<td>71,680 ± 41,798</td>
<td>22,597 ± 652**</td>
<td>38,199 ± 850*</td>
</tr>
<tr>
<td>TF-1</td>
<td>505.7 ± 16.4</td>
<td><strong>111.8 ± 2.8</strong></td>
<td>108.4 ± 2.5**</td>
<td>315.6 ± 10.7**</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>FTC Only</th>
<th>FTC dosed 1 hr prior EE</th>
<th>FTC dosed simultaneously EE</th>
<th>FTC dosed 1 hr after EE</th>
</tr>
</thead>
<tbody>
<tr>
<td>THP-1</td>
<td>74.8 ± 2.6</td>
<td>78.8 ± 3.0</td>
<td>72.5 ± 3.5</td>
</tr>
<tr>
<td>BC-3</td>
<td>35.7 ± 2.5</td>
<td>7.7 ± 0.2**</td>
<td>11.1 ± 0.6**</td>
</tr>
<tr>
<td>Ect1/E6E7</td>
<td>1357 ± 211.9</td>
<td>1228 ± 54.6</td>
<td>1110 ± 38.1</td>
</tr>
<tr>
<td>HeLa</td>
<td>2001 ± 123.1</td>
<td>2067 ± 73.7</td>
<td>1877 ± 61.4</td>
</tr>
<tr>
<td>TF-1</td>
<td>75.1 ± 4.5</td>
<td>11.1 ± 0.6**</td>
<td>3.2 ± 0.3**</td>
</tr>
</tbody>
</table>

Influence of EE/ET on tenofovir and emtricitabine uptake over a 25-fold range of antiretroviral doses

The significant difference (seen in Figure 2) in cellular uptake of tenofovir and emtricitabine, specifically for both BC-3 and TF-1 cells, lead to their evaluation of cellular uptake with additional antiretroviral doses in conjunction with hormonal contraceptives. In BC-3 cells, mean intracellular tenofovir concentrations increased approximately 275% when the dose was increased from 0.06 μg/mL to 0.3 μg/mL and increased approximately 372% when the dose increased from 0.3 μg/mL to 1.5 μg/mL. Mean intracellular emtricitabine concentrations in BC-3 cells increased approximately 200% when the dose increased from 0.09 μg/mL to 1.8 and increased approximately 35% when the dose increased from 1.8 μg/mL to 2.25 μg/mL. Similar to BC-3 cells, tenofovir uptake by TF-1 cells increased approximately 330% and 324% when...
In contrast with BC-3 cells, mean intracellular emtricitabine concentrations in TF-1 cells increased only 24% when the dose was increased to 1.8 µg/mL and 228% when increased to 2.25 µg/mL. Intraday and interday variability (coefficients of variation) of the uptake of tenofovir and emtricitabine by the cell lines were 2.5% to 69% and 6.9% to 62%, respectively.

**Discussion**

This study evaluated the uptake of tenofovir and emtricitabine in vitro in representative cell types found in the female genital tract, and examined their uptake in the presence of hormonal contraceptives. Compared with the THP-1 control cell line, which represents macrophages, intracellular tenofovir and emtricitabine concentrations were increased in HeLa cells, which represent CD4+ T cells, and Ect1/E6E7 cells, a surrogate for squamous epithelial cells. Previous work has shown that these two cell lines are more rapidly invaded by HIV-1. In the presence of exogenous sex hormones, the most significant and consistent effect was observed for both tenofovir and emtricitabine when dosed one hour prior to EE/ET in BC-3 and TF-1 cells. These results indicate that differential uptake and phosphorylation of tenofovir and emtricitabine exists across major cellular subtypes in the female genital tract, and that sex hormones can influence cellular uptake and/or phosphorylation rates of these nucleoside analogs.

The five distinct surrogate female genital tract cells lines we evaluated in the absence of exogenous hormones achieved median intracellular tenofovir and emtricitabine concentrations of 579 and 40 fmol/10⁶ cells, respectively.
which for tenofovir is considerably higher than what has been reported to reduce viral transmission from male patients by 90% in plasma peripheral blood mononuclear cells (15.6 fmol/10^6 cells). However, our data, were derived from in vitro cellular uptake studies using surrogate female genital tract cell lines and not plasma peripheral blood mononuclear cells. An in vivo study that collected steady-state HIV-1 negative cervical cell samples reported a tenofovir diphosphate concentration of 337 fmol/10^6 cells and an emtricitabine triphosphate concentration of 4300 fmol/10^6 cells. Our median emtricitabine triphosphate concentration across the two cell lines with the highest uptake (HeLa and Ect1/E6E7) was 934 fmol/10^6 cells. Importantly, our goal was to examine uptake across these cell lines and assess the impact that exogenous hormones may have on uptake, so our in vitro results are not necessarily comparable with clinical data with regard to exposures required for prevention of transmission.

Penetration of nucleoside reverse transcriptase inhibitors into the female genital tract is dictated by facilitated diffusion and active transport. One potential explanation for differences in observed antiretroviral uptake is that various cell types in the female genital tract may express transporters differently and these transporters likely have varying degrees of functional activity. The data suggest that uptake of tenofovir occurs through organic anion

![Diagram of TFV and FTC concentrations](image-url)
transporter (OAT) 1 and 3 while uptake of emtricitabine occurs through organic cation transporter (OCT) 1.\textsuperscript{35,36} Expression and/or activity of these transporters relative to cells vulnerable to HIV-1 transmission in the female genital tract have not been extensively evaluated. However, these transporters can be manipulated by cyclic nucleotides and steroids.\textsuperscript{37–39} Progesterone upregulates OAT 1, which is used by tenofovir for entry into the cell, and downregulates OCT 1 and multidrug resistance protein (MRP) 1, 4, and 5, which is used by emtricitabine for entry into the cell. Through downregulation of MRP, the cellular efflux of tenofovir diphosphate and emtricitabine triphosphate is inhibited, thus potentially increasing the intracellular anabolites. Estrogen downregulates OAT 1 and 3 and OCT 1, which are both required by tenofovir and emtricitabine. Coadministration of ethinyl estradiol, etonogestrel, tenofovir, and emtricitabine may alter uptake of tenofovir and emtricitabine and potentially affect clinical outcomes. Our results suggest that intracellular tenofovir and emtricitabine concentrations, particularly in BC-3 and TF-1 cells, were altered in the presence of exogenous sex hormones. The percent uptake of tenofovir in the presence of EE/ET was similar as tenofovir doses increased. However, percent uptake of emtricitabine in the presence of EE/ET was variable as emtricitabine doses increased. Taken together, our data support the need for future in vivo studies designed to assess whether these effects occur in patients, and if so, their clinical significance.

The effect of synthetic sex hormones on the intracellular phosphorylation of nucleosides has not been directly studied. However, studies have been conducted with some of the intracellular enzymes responsible for phosphorylation and dephosphorylation of both tenofovir and emtricitabine and other nucleoside reverse transcriptase inhibitors in the presence of sex hormones. Human adenylate kinase, which phosphorylates tenofovir to its monophosphate anabolite, is not affected by estrogen,\textsuperscript{40} indicating that the difference in uptake and phosphorylation of tenofovir is more closely regulated by transporters for cell entry or the enzyme responsible for the second stage of phosphorylation, ie, nucleotide diphosphate kinase. The effect of the three enzymes responsible for the phosphorylation of emtricitabine has not been evaluated with ethinyl estradiol and etonogestrel in vitro. However, the enzyme (5′-nucleosidase) responsible for the dephosphorylation of emtricitabine monophosphate to emtricitabine shows altered enzymatic activity when dosed with estradiol and progesterone in homogenated rat brain and liver.\textsuperscript{41} The liver showed decreased enzymatic activity in the presence of both synthetic hormones, while estradiol in the brain increased the activity of the enzyme. A decrease in the efflux of emtricitabine would theoretically lead to an increase in the intracellular phosphorylated moiety. Another study has shown that high physiologically relevant concentrations of progesterone decrease (25%–68%) the phosphorylated forms of nucleoside reverse transcriptase inhibitors, while estrogen shows no significant change.\textsuperscript{42} These data suggest that intracellular tenofovir and emtricitabine may be altered by exogenous progesterone, which is consistent with our results, but the direction and magnitude of the change is variable.

There are some limitations to the studies we have conducted. Antiretroviral uptake was determined in only one female genital tract cell line for each cell type, and immortal cells were used. Our findings should be validated against additional cell lines representing these and other cell subtypes from the female genital tract and by primary female genital tract cells obtained in a clinical setting. Of note, our experiments were conducted using a one-hour incubation time. Earlier studies evaluating the mechanistic and kinetic activity of antiretrovirals or hormones suggest that one-hour incubations are sufficient for uptake of antiretrovirals and synthetic hormones.\textsuperscript{43–45} However, varying incubation times may be needed to account for any changes in cellular antigen, receptor, and/or transporter activity potentially caused by the addition of tenofovir, emtricitabine, ethinyl estradiol, or etonogestrel. Only one concentration of ethinyl estradiol and etonogestrel was included in the cell medium. Evaluating various doses of ethinyl estradiol and etonogestrel would determine if uptake of tenofovir and emtricitabine into the surrogate female genital tract cells is dependent on the functional activity of transporters induced and inhibited by ethinyl estradiol and etonogestrel. Importantly, the concentrations of ethinyl estradiol and etonogestrel used represent the plasma C\textsubscript{max} in humans. Intravaginal concentrations could be considerably different and vary from woman to woman, so differences in the magnitude of effect at the local site may still exist. Concentrations of ethinyl estradiol and etonogestrel have been quantified in the female genital tract following use of the NuvaRing contraceptive.\textsuperscript{46} The mean female genital tract concentrations (from the cervical region) of ethinyl estradiol after one cycle (17–21 days) was 68 pg/0.5 g tissue, which assuming 1 g is equivalent to 1 mL, is approximately 75% higher than the concentration used in this study (34.7 pg/mL). The mean etonogestrel concentration was 327 pg/0.5 g tissue, which is approximately 62% lower compared with our study (1716 pg/mL). Etonogestrel, which is uniquely found in the
hormonal contraceptive NuvaRing, was the only progesterone studied. Most hormonal contraceptives use a combination of ethinyl estradiol and different synthetic progesternes, such as norethindrone, norgestimate, or levonorgestrel. Given that the mechanism of action of etonogestrel is similar to that of other synthetic progestenes, a similar effect on cellular uptake of tenofovir and emtricitabine would be expected.

In conclusion, our data suggest that intracellular concentrations of tenofovir and emtricitabine differ across surrogate cell types of the female genital tract and these concentrations may be altered in the presence of exogenous sex hormones. Furthermore, in vitro studies using THP-1 cells only to make in vivo correlations between antiretroviral dosing and intracellular concentrations in the female genital tract may not be sufficient. The design of future PrEP studies should factor in patient use of hormonal contraceptives, because exogenous hormones can change the cellular makeup of the cervical mucus and endometrium. These changes shift the prevalence of certain cell types in the female genital tract and may alter the total amount of intracellular drug present. As tenofovir, emtricitabine, and additional PrEP agents are clinically evaluated in women, the pharmacologic consequences of exogenous hormone therapy should be considered.

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Disclosure
The authors report no conflicts of interest in this work.

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