The role of menopause in tenofovir diphosphate and emtricitabine triphosphate concentrations in cervical tissue

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Objective: Although postmenopausal (post-M) women have behavioral and biological risk factors for HIV infection, the activity of preexposure prophylaxis (PrEP) agents in older adults has not been well studied.

Design: We used an ex-vivo approach to compare the tissue concentrations of tenofovir (TFV) diphosphate (TFVdp) and emtricitabine (FTC) triphosphate (FTCtp) in cervical tissues from premenopausal (pre-M) and post-M women.

Method: Cervical explants from 16 pre-M and 11 post-M women were incubated in 10–300 μg/ml TFV or FTC for 24 h. Explants were then snap frozen in liquid nitrogen and stored until analysis. TFVdp and FTCtp were quantified using tandem liquid chromatography–mass spectrometry.

Results: Active metabolite concentrations of TFVdp were more than nine-fold lower in post-M explants ($P < 0.05$). The percentage of TFV converted to TFVdp in pre-M explants was 0.0038 [below the limit of quantification (BLQ)-0.5886] compared with 0.0004 (BLQ-0.0706) in post-M explants. The majority of FTCtp concentrations were BLQ. For both TFVdp and FTCtp, there was a trend for more unquantifiable concentrations in post-M vs. pre-M (TFV: 38 vs. 21%, $P = 0.2$; FTC: 71 vs. 52%, $P = 0.2$).

Conclusion: These findings could have implications in the use of nucleotide-based PrEP strategies targeted to older women. If validated *in vivo*, lower exposures of active nucleoside/tide metabolites could mean post-M women need higher doses of TFV-based PrEP to achieve protective efficacy.

AIDS 2018, **32**:11–15

Keywords: emtricitabine, explants, menopause, preexposure prophylaxis, tenofovir

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Received: 18 July 2017; revised: 5 October 2017; accepted: 16 October 2017.

DOI:10.1097/QAD.0000000000001678

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Introduction

Preexposure prophylaxis (PrEP) with antiretrovirals has proven that with consistent use, the risk of HIV acquisition in high-risk individuals can be reduced. Efficacy was first demonstrated in a 2010 study when daily use of Truvada (Gilead, Foster City, California, USA) [fixed dose oral combination of tenofovir (TFV) disoproxil fumarate (TDF) and emtricitabine (FTC)] reduced HIV infections by 44% in men or transgender women who have sex with men [1]. This finding was later validated in heterosexuals and injection drug users [2–4]. Regulatory agencies from several countries including the United States, Canada, Kenya, and South Africa [5] have approved the use of TDF/FTC for PrEP, and the WHO has endorsed and published guidelines for its use [6].

Not all PrEP initiatives, however, have met with success. Two studies in heterosexual women were stopped early for futility based on recommendation from the Data Safety Monitoring Boards [7,8]. Adherence in these studies when assessed by random blood concentrations suggested less than 30% of patients assigned to the active arms were taking daily doses. Further pharmacologic analyses have demonstrated that the level of adherence needed to achieve protective concentrations differs based on the route of HIV exposure, with stricter adherence to TDF/FTC-based PrEP required for protection from vaginal compared with colorectal acquisition [9,10].

To date, clinical trials that have evaluated PrEP in women have focused on younger women [7,8,11]. However, the Centers for Disease Control and Prevention reports 26% of new HIV diagnoses in the United States are in adults over the age of 45; of these, 29% are in women acquired through heterosexual contact [12]. High-risk behaviors such as unprotected sexual intercourse is common among older adults both in the United States [13,14] and in hyper endemic areas such as South Africa [15], possibly due to lack of concern about pregnancy protection after menopause and an under appreciation of risk for sexually transmitted infections. In addition, menopausal changes may alter the biological risk by increasing target cell expression, vaginal pH, epithelial function, and innate immunity [16–18]. Given that current dosing for oral PrEP achieves concentrations in the female genital tract (FGT) that may be close to the threshold for efficacy [10,19], we sought to evaluate the metabolite concentrations of TFV and FTC in cervical tissues from postmenopausal (post-M) women using a cervical explant model [19].

Methods

Tissue procurement

Cervical specimens were procured by the University of Minnesota Biological Materials Procurement Network (BioNet, Minneapolis, Minnesota, USA) from HIV-negative women undergoing gynecologic surgery. All women consented to tissue collection for research prior to their surgeries. Clinical information including age, race, comedications, and menopause status, defined as 12 months amenorrhea, were recorded from medical records. Specimens were collected fresh and transferred in culture medium prepared with: Iscove's Modified Dulbecco's Medium (Gibco, Carlsbad, California, USA), 10% fetal bovine serum (Gibco), 240 U/ml nystatin suspension (Sigma, St. Louis, Missouri, USA), 100-U/ml penicillin/streptomycin (Gibco), and MEM Vitamin Solution (Sigma). Tissues were typically brought to laboratory for processing within 30 min of surgery. Tissues were cleaned and dissected leaving only the epithelial layer and underlying submucosa for explant cultures. Endocervix and ectocervix were separated. Biopsy punches (Integra Miltex, Plainsboro, New Jersey, USA) were used to create 3-mm^2 explants. Hematoxylin and eosin staining was used on explants fixed in Safefix II (Fisher Scientific, Fair Lawn, New Jersey, USA) to confirm correct identification of ectocervical vs. endocervical portions.

Ex-vivo metabolism of nucleotide reverse transcriptase inhibitors

Drug stocks of 1-mg/ml TFV and FTC (NIH AIDS Reagent Program, Division of AIDS, NIAID, NIH, Germantown, Maryland, USA) were reconstituted in sterile water. Drug was then diluted in culture media in dilutions of 10, 30, 100, or 300 μg/ml. Explants were incubated in 500 μl in a 48-well tissue culture plate for 24 h. Tissues were weighed and then snap frozen in liquid nitrogen and stored at −80 °C. TFV diphosphate (TFVdp) and FTC triphosphate (FTCtp) were measured in tissue explant homogenates by tandom liquid chromatography-mass spectrometry over a concentration range of 0.02–20 ng/ml. To prevent degradation of intracellular metabolites, acetonitrile was added to frozen tissues before homogenization. Samples were extracted by protein precipitation with isotopically labeled $^{13}C_5$-TFVdp as the internal standard. Calibration standards and quality control (QC) samples were prepared in human vaginal tissue homogenate. Calibration standards and QC samples met acceptance criteria of ±15% of nominal concentrations for all analyses.

Statistical analysis

When applicable, values from replicate samples were averaged. Measured TFVdp and FTCtp concentrations were normalized for tissue weight. To normalize for incubation dose, dose-adjusted concentrations were calculated (TFVdp or FTCtp expressed as a molar percentage of TFV or FTC incubation concentrations.) Differences in metabolite concentrations were tested for significance using the Wilcoxon rank-sum test. Differences in the proportions of unquantifiable samples were tested using the Chi-square test. Statistics were performed

Table 1. Demographics of tissue donors.

		Premenopausal, $n=16$	Postmenopausal, $n=11$
Age	Median (range)	38.5 (21–56)	56 (52–68)
Race	White	13	9
	African-American	1	1
	Asian	2	0
	Unknown	0	1

using SAS 9.4 (Cary, North Carolina, USA). Data are reported as median (range) unless otherwise noted.

Results

Demographic information of tissue donors is shown in Table 1. No patients were on hormonal therapy at the time of surgery with the exception of one premenopausal (pre-M) patient who had been receiving testosterone injections prior to a sex reassignment procedure. A second pre-M patient was diagnosed with Turner Syndrome (45, X karyotype), a condition that commonly presents with ovarian insufficiency resulting in reduced estrogen production [20]. As metabolite concentrations in these patients were similar to median values, they were not excluded from analysis.

Tenofovir

Conversion of TFV to TFVdp in explants is shown in Fig. 1a and Supplemental Table 1, http://links.lww.com/QAD/B182. Measurements from 28% of explants were below the limit of assay quantification (BLQ) for TFVdp (21% of pre-M vs. 38% of post-M, $P=0.18$). BLQ samples were distributed across the doses and frequency of proportion unquantifiable did not decrease with increasing dose. TFVdp conversion in post-M explants was more than nine-fold lower than in pre-M explants in ectocervix ($P=0.007$). This trend was also observed in endocervix although it was not statistically significant due to fewer samples available ($P=0.7$) (Fig. 1a). An overall correlation was noted between age and TFVdp phosphorylation in ectocervical tissue ($r=-0.5$, $P=0.001$), but there was no correlation within pre-M ($r=-0.3$, $P=0.2$) or post-M ($r=-0.14$, $P=0.7$) groups. There was no correlation between age and TFV phosphorylation in endocervical tissue. Sensitivity analyses confirmed higher TFV phosphorylation in pre-M tissues even when BLQ samples were removed from analysis ($P=0.04$) and when the patients receiving hormonal treatment were excluded ($P=0.03$). Similar findings were also obtained when comparing TFVdp concentrations rather than dose-normalized values ($P=0.01$).

Emtricitabine

Conversion of FTC to FTCtp in explants is shown in Fig. 1b and Supplemental Table 2, http://links.lww.com/QAD/B182. Consistent across all doses, 56% of all FTCtp concentrations were BLQ. Although the difference was not statistically significant, unquantifiable FTCtp concentrations were more frequent in the post-M group compared with the pre-M group (71 vs. 52%, $P=0.2$). Unlike TFVdp, there was no correlation between age and FTCtp concentrations ($r=-0.1$, $P=0.5$).

Discussion

As the rollout of nucleoside/tide-based PrEP continues to expand, it becomes increasingly important to understand

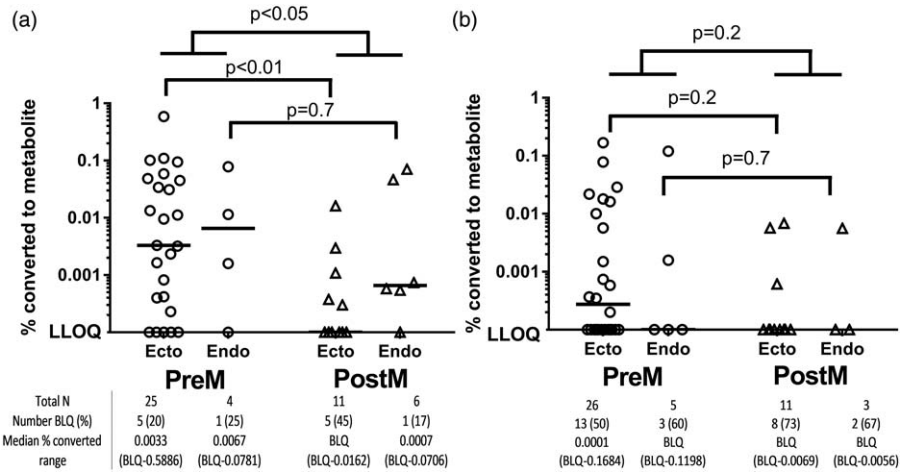

Fig. 1. Reduced nucleotide metabolite concentrations in postmenopausal explants. The measured tenofovir diphosphate (a) or emtricitabine triphosphate (b) following a 24-h incubation in tenofovir or emtricitabine 10–300 μg/ml is shown as a percentage of the parent incubation concentration. Circles (premenopausal) and triangles (postmenopausal) represent individual explant concentrations and the lines represent median vales. The uppermost *P* values represent comparisons between premenopausal and postmenopausal when ectocervix and endocervix were pooled together, whereas the middle and lower *P* values represent comparison between ectocervix and endocervix, respectively.

the factors modulating efficacy and identify populations at risk for drug-failure. In this study, we found reduced TFVdp concentrations in cervical tissues from post-M women. As the majority FTCtp concentrations were BLQ, we were not able to adequately assess differences of this metabolite between pre-M and post-M tissue, although the trend for a greater percentage of unquantifiable concentrations in the post-M group was consistent with the lower TFVdp exposures.

Our findings of decreased TFVdp in the estrogen-deficient state of menopause are consistent with Shen *et al.* [21] who found phosphorylation of TFVdp increased in epithelial cells isolated from the FGT when treated with estradiol. The authors also found differential phosphorylation in different FGT cell types with the greatest TFVdp/cell quantified in epithelial cells followed by fibroblasts, $CD14^+$ cells, and then $CD4^+$ cells. Thinning of the vaginal epithelium has been well documented in women past the age of menopause [17]. In our study, we were not able to assess the relative distribution of TFVdp in different cell types, and therefore, it is possible that, although we normalized for tissue weight, a smaller proportion of epithelial cells in our post-M explants contributed to the overall lower TFVdp exposure. The role epithelial cells play in providing a TFV reservoir for HIV target cells is not clear [21] and further studies are required.

Hormonal modulation of nucleotide-based PrEP could also have implications outside of the post-M population. Depo-Provera (medroxyprogesterone acetate, Pfizer, New York City, New York, USA) is a progestin-only injectable contraceptive that, similar to menopause, significantly reduces plasma estradiol exposures [22]. Whether Depo-Provera users on TDF have reduced cervical TFVdp is unknown. Following multiple oral doses of TDF, trough TFVdp concentrations in PBMCs are 240% lower in women taking oral or injectable hormonal contraception compared with a nonhormonal group following multiple oral doses of TDF [23]. This raises implications for both PrEP and treatment of HIV as Depo-Provera is widely used in sub-Saharan Africa, where the highest rates of HIV infection occur and young women of reproductive age are particularly vulnerable [24]. Notably, a post-hoc (not powered) analysis of the Partners PrEP study found Depo-Provera users still received protective benefit from oral TDF/FTC or TDF alone; however, there was an observed 10% increase in HIV incidence in Depo-Provera users assigned to active PrEP arms compared with female PrEP users with no hormonal contraception, suggesting more data is needed in this area [25].

We cannot rule out that the observed differences are due to other processes of aging rather than hormonal regulation. Although overall correlations with age were significant, we did not observe any correlation between age and concentration within either pre-M or post-M groups, making it difficult to separate menopausal effects from overall aging. A pilot study assessing antiretroviral pharmacokinetics in HIV+ adults over the age of 55 years reported higher TFVdp and lower FTCtp in PBMC compared with younger historic controls [26]. However, only one-half of the 12 patients in this pilot study were women, and sex differences were not reported. This differential effect of aging on TFVdp compared with FTCtp was hypothesized to be due to TFV phosphorylation being more influenced by cellular activation [27,28]. Decreased innate immunity in mucosal tissues of post-M women may be one explanation for the lack of similar findings in mucosal tissues [18]. Further analysis is needed to separate the effects of aging from the hormonal changes due to menopause and to determine whether effects are tissue specific.

There are limitations to this study. First, hormone concentrations were not directly measured. It is possible some of our pre-M patients were perimenopausal with declining estrogen levels. In addition, we could not control for hormonal variability across the menstrual cycle. Second, other factors not accounted for, such as microbial populations, cell populations, and inflammatory state, may also contribute to variability in phosphorylation or activity.

In conclusion, in this ex-vivo analysis, we found a decrease in TFVdp concentrations in post-M tissues. If validated *in vivo*, these findings could have implications on the use of TFV-based PrEP regimens in older women.

Acknowledgements

Author contributions: M.R.N. contributed to conception and design of the work; M.R.N., L.M.B., C.S. contributed to data collection; M.R.N., C.S., and A.D.M.K. contributed to data analysis and interpretation; M.R.N. drafted the article; M.R.N., L.M.B., C.S., and A.D.M.K. provided critical review of the article and final approval of submission.

The work was financially supported by the Department of Experimental and Clinical Pharmacology, College of Pharmacy, University of Minnesota and the Deborah E Powell Women's Center, University of Minnesota. Tissue specimens were collected under the University of Minnesota BioNet Tissue Procurement Facility. Tenofovir and emtricitabine was obtained through the NIH AIDS Reagent Program, Division of AIDS, NIAID, NIH.

Conflicts of interest

There are no conflicts of interest.

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