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Antiviral Activity of Genital Tract Secretions following Oral or Topical Tenofovir Pre-exposure Prophylaxis for HIV-1

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Abstract

Background—Surrogate markers of HIV-1 pre-exposure prophylaxis (PrEP) and microbicide efficacy are needed. One potential surrogate is the antiviral activity in cervicovaginal lavage (CVL) after exposure to candidate products. We measured CVL antiviral activity in women using oral or vaginal tenofovir-based PrEP and correlated activity with drug and immune mediator levels.

Methods—Inhibitory activity against HIV-1 and HSV-2 and concentrations of interleukin (IL)-1 β , IL-6, IL-8, interferon- γ , induced protein 10 (IP-10), macrophage inflammatory protein (MIP)-1 α , MIP-3 α , lactoferrin, secretory leukocyte protease inhibitor, and defensins were measured in CVL obtained from 60 women at baseline and after 6 weeks of a randomized sequence of oral and topical tenofovir. CVL tenofovir concentrations were measured by mass spectrometry.

Results—The number of women with CVL anti-HIV activity 90% increased significantly from 5.0% at baseline to 89.1% following daily use of 1% tenofovir gel (RR=17.85, $p<0.001$), but there was no increase following daily oral tenofovir. The CVL anti-HIV activity correlated with drug levels (Spearman correlation coefficient 0.64 following tenofovir gel; $p<0.001$), but not with the concentrations of mucosal immune mediators. No increase in CVL anti-HSV activity was observed following either drug regimen, an observation consistent with the higher concentrations of tenofovir needed to inhibit HSV-2 infection. The CVL anti-HSV activity correlated with lactoferrin, defensins, IP-10, IL-8 and detectable levels of MIP-1 α , but not with drug levels.

Conclusions—CVL may provide a surrogate for local but not systemic drug efficacy and a tool to better understand mucosal factors that modulate antiviral activity in genital tract secretions.

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Introduction

Oral and topical pre-exposure prophylaxis (PrEP) with tenofovir (TFV)-based drugs can prevent HIV acquisition. However, clinical trials outcomes have been variable. Oral Truvada™ (combination of TFV disoproxil fumarate (TDF) and emtricitabine) and TDF were protective in HIV serodiscordant couples in the Partners PrEP Study¹ and Truvada™ significantly decreased HIV acquisition in TDF-2, a study among high-risk African men and women^{1,2}. In contrast, the oral TDF, Truvada™ and vaginal TFV gel arms of the Vaginal and Oral Intervention to Control the Epidemic (VOICE) trial did not demonstrate efficacy³. Another oral PrEP trial, FemPrEP, conducted in a population of young women similar to those enrolled in VOICE was stopped early after a planned interim analysis determined that Truvada™ was unlikely to demonstrate protection; subsequent analyses of drug levels in these trials suggest that poor adherence contributed to the negative outcomes⁴. In contrast, partial efficacy was observed with pericoital intravaginal dosing of 1% TFV gel in CAPRISA 004, illustrating the potential to deliver safe and effective vaginal prevention products⁵. A 39% [95% CI 6–61%] and 54% [21–70%] reduction in HIV-1 (herein designated HIV) and HSV-2 seroconversion, respectively, were observed in women who were randomized to apply 1% TFV vaginal gel before and after sex⁵. While adherence clearly plays a major role in modifying efficacy, biological factors that modulate the relationship between pharmacokinetics (PK) (drug levels), pharmacodynamics (PD) (drug activity) and host susceptibility to HIV may also contribute.

Phase 2B proof-of-concept studies to assess microbicide and PrEP efficacy are complex and expensive, and better surrogates of product efficacy are needed to provide some indication of the potential protective effect prior to conducting effectiveness trials. The optimal surrogate may be to expose mucosal tissue obtained from participants taking active product *ex vivo* to HIV and compare susceptibility to tissue obtained at baseline or in those taking placebo⁶. However, limitations to this approach include the feasibility of collecting multiple biopsies and need to standardize the number and activation status of immune cell populations between samples, which could impact susceptibility of the tissue to HIV infection.

An alternative or complementary strategy is to measure antiviral activity of genital tract secretions collected by cervicovaginal lavage (CVL) or swab. The capacity of secretions to inhibit HIV reflects luminal drug and the anti-viral activity of antimicrobial peptides in the collected secretions^{7–10}. Measuring the antiviral activity of secretions is technically easy to perform and allows for assessment of antiviral activity in settings where collection of biopsy samples for *ex vivo* challenge is not feasible. However, while this may provide a biomarker of efficacy for drugs that act extracellularly or rapidly transit into and out of cells, it is less clear how informative measuring antiviral activity is for a drug such as TFV, which requires cellular uptake for phosphorylation to the active metabolite, tenofovir diphosphate (TFV-DP), which is retained intracellularly for a prolonged time¹¹. The antiviral activity of genital tract secretions attributable to oral or topically administered TFV likely reflects drug released from tissue or cells that has never been phosphorylated or has been dephosphorylated. For topical dosing, it also reflects residual unmetabolized drug in the vaginal lumen from previous gel application(s).

Thus, the objectives of this study were to measure the antiviral activity of CVL obtained from women using oral and topical TFV and to determine if activity correlated with CVL TFV drug levels or concentrations of immune mediators.

Methods

Sampling procedures

MTN 001 was a Phase 2 open-label crossover PK study in which all subjects were assigned to take a randomized sequence of daily TDF orally (300 mg), TFV vaginally (40 mg), or both over three 6-week periods; local IRB approval was obtained at each site¹². Thirty U.S. (3 sites) and 30 South African (2 sites) participants from the parent study were randomly selected for this substudy. CVL was obtained by washing the cervix and vaginal walls with 10 mL of sterile saline at enrollment (prior to product use) and after 6 weeks of prescribed daily vaginal gel and oral product use. Samples were collected at research clinic visits at one of the following times relative to observed final product dosing: pre-dose or 2, 4, or 6 hours post-dose. Primary study data indicated no difference across time in the CVL TFV concentrations¹². Women were asked to abstain from sexual intercourse and other vaginal products 48 h prior to sampling. CVL supernatants were processed as described⁸, divided into aliquots, stored at -80°C , and shipped on dry ice to central testing facilities.

Measurement of CVL immune mediators

Total protein was determined using a MicroBCA assay (Thermo Fisher Scientific, Rockford, IL). Concentrations of interleukin (IL)-1 β , IL-6, IL-8, interferon- γ (IFN- γ), induced protein 10 (IP-10), macrophage inflammatory protein (MIP)-1 α , and MIP-3a, were quantified using a multiplex proteome array with beads from Millipore using a Luminex 100 instrument (Luminex, Austin, TX) and analyzed using StarStation software (Applied Cytometry Systems, Sacramento, CA). ELISAs were used to quantify lactoferrin (EMD Chemicals, Gibbstown, NJ), secretory leukocyte protease inhibitor (SLPI; R&D Systems, Inc., Minneapolis, MN), human β defensins (H β D) 1, 2, and 3 (Alpha Diagnostics, San Antonio, TX), and human neutrophil peptides 1–3 (HNP1–3; HyCult Biotechnology, Uden, The Netherlands). Samples with values above the upper limit of detection (LOD) of the standard curve were diluted and retested. Values below the lower LOD (LLOD) were given a numerical value of half the LLOD. The soluble mediators were selected because in prior studies they have been associated with HIV risk and/or have antiviral activity in vitro^{13–15}.

Antiviral activity of CVL

TZM-bl cells were infected with HIV-1_{BaL} (approximately 10^3 TCID₅₀) mixed 1:1 with CVL or control buffer (normal saline containing 200 $\mu\text{g}/\text{ml}$ bovine serum albumin) and infection monitored by assaying for luciferase activity 48 h post-infection. Data are presented as mean percent reduction in relative luciferase units (RLU) compared to control. All samples were tested in triplicate in at least two independent experiments. To evaluate anti-HSV activity, Vero cells were infected with ~50–200 plaque forming units (pfu) of HSV-2(G) mixed 1:1 with each CVL or control buffer (see above) and plaques were counted after 48 hours. All samples were tested in duplicate in two independent experiments. To measure whether CVL interfered with drug activity, exogenous TFV was added to a subset of CVL or to control buffer and then tested in the infectivity assays.

Measurement of CVL TFV levels

Following the addition of isotopically-labelled internal standard, TFV was extracted from CVL using the Oasis^{AE} MCX solid phase extraction plate (Waters, Milford MA). Analytes were eluted and subjected to ultra-high-performance-liquid chromatographic-tandem mass spectrometric (UPLC-MS/MS). TFV quantification was performed using a previously described UPLC-MS/MS method using an AB-Sciex API4000 mass spectrometer interfaced with a Waters Acquity LC system¹². The analytical measuring range for the assay ranged

from 5 to 1280 ng/ml; values below 5 ng/ml were reported as below the limit of quantitation. Values above the highest calibrator were diluted and retested.

Statistical analyses

To reduce skewness, cytokines were $\log_{10}$ transformed except for IL-1 β , MIP-1 α and MIP-3 α , in which >18% of observations were below the LLOD such that skewness could not be reduced through a $\log_{10}$ transformation; the latter were dichotomized as detectable or not detectable. The percent inhibition of HIV and HSV infection also could not be analyzed as continuous variables since they could not be transformed to follow a normal distribution and were therefore dichotomized above and below the 67th percentile; e.g. for anti-HIV at 90% and for anti-HSV at 70%. Groups were compared using GEE models with a Gaussian link (for continuous outcomes) or a Poisson link (for binary outcomes), an exchangeable correlation structure and robust errors. For comparisons of anti-HIV and anti-HSV the coefficients for continuous mediators represent the change in risk of having high anti-HIV or anti-HSV for every 1 $\log_{10}$ change in the mediator; coefficients for binary mediators represent the risk of having high anti-HIV or anti-HSV for samples above the LLOD for that mediator compared to those below the LLOD. Interaction terms with TFV levels were estimated for all mediators, and, if statistically significant, analyses stratified by high and low TFV levels (dichotomized at the median TFV level).

In the analyses of the effects of TFV use on mucosal immune mediators, for models assessing continuous outcomes, the coefficient presented is the average difference from baseline for that outcome for the different modes (vaginal or oral) of administration. For dichotomous outcomes, the value presented is the risk of being above the LLOD for the different modes compared to baseline; data are presented as univariate analyses and after correction for multiple comparisons (Bonferroni). SPSS Version 20.0 (IBM Corp.) or Stata Version 12.0 (StatCorp LP) was used for all analyses.

Results

Characteristics of study participants

Study participants have been previously described¹². Briefly, mean age of the 30 U.S. participants was 31 years; 53% used hormonal contraception (HC) and 6% had bacterial vaginosis (BV) (defined using clinical criteria¹⁶) at baseline. Mean age of the 30 South African women was 31 years, with 87% using HC and 12% with BV at baseline. CVLs were available from all participants at baseline and in 27 U.S. and 28 South African participants following vaginal TFV gel, and 25 U.S. and 28 South African participants following oral TDF.

Vaginal TFV, but not oral TDF, is associated with increased CVL anti-HIV activity

Consistent with prior studies^{7,8,10,17}, baseline CVL activity against HIV was variable ranging from -174% (i.e. enhancement) to 100% (inhibition) (Fig. 1a) and did not differ among clinical sites. It is unlikely that the enhancement observed here and in other studies reflects HIV DNA or RNA in CVL from sexual exposure as all of the women remained HIV negative throughout the study and no increase in background luciferase activity was observed in pilot studies with enhancing CVL unless exogenous virus was added.

There was a large increase in the percent HIV inhibition in CVL obtained after vaginal (mean $\pm$ sd; 92.8 $\pm$ 25.1%), but not oral (29.5 $\pm$ 72.8%) product use, compared to baseline (32.2 $\pm$ 58.0%) (Fig. 1a). The proportion of participants with 90% HIV inhibitory activity increased from 5.0% (3/60) at baseline to 89.1% (49/55) after 6 weeks of vaginal gel, but there was no increase following 6 weeks of prescribed daily oral TDF (1.9%; 1/53). The

likelihood of detecting anti-HIV activity of 90% in CVL was 47.2 [95% CI: 6.80, 327.30] times higher in women using vaginal compared to oral product ($p < 0.001$).

Similarly, the concentrations of TFV were higher following vaginal compared to oral product usage, with median [25%, 75%] of 5.07 [4.48, 5.51] $\log_{10}$ ng/ml following vaginal gel compared to 1.34 [0.86, 1.63] following oral TDF (Fig. 1b). The percent inhibition of HIV correlated with the CVL TFV concentrations following vaginal gel (Spearman correlation coefficient (SCC)=0.64, $p < 0.001$), but not following oral PrEP (SCC= 0.17) (Fig. 1c).

Neither product increased CVL antiviral activity against HSV-2

There was no increase in antiviral activity of CVL against HSV-2 following vaginal or oral product use compared to baseline (Fig. 2). Rather, the median [25%, 75%] percent inhibition of HSV-2 plaque formation fell from 66% [17, 88] at baseline to 39% [23, 85] and 31% [17, 45] following vaginal and oral product use, respectively, although the decline was not statistically significant. The percentage of participants with 70% CVL HSV-2 inhibitory activity was 48% (29/60) at baseline and decreased to 21.8% (12/55) and 34.0% (18/53) following vaginal and oral product use, respectively. There was no correlation between anti-HSV activity of CVL and TFV drug levels (SCC -0.02, $p = 0.8$), which is consistent with the concentration of TFV recovered. The concentrations were less than the IC_{50} and IC_{90} of 5.73 $\log_{10}$ and 6.4 $\log_{10}$ ng/ml, respectively, for TFV in this HSV-2 plaque assay¹⁸. However, complete inhibition of HSV-2 plaque formation was observed if exogenous TFV was added to a subset of 8 randomly selected CVL to achieve a final concentration of TFV that exceeded the *in vitro* IC_{90} , indicating that CVL does not interfere with the anti-HSV activity of TFV (data not shown).

Anti-HSV, but not anti-HIV activity correlated with concentrations of soluble mucosal immune mediators

To further investigate what might contribute to the antiviral activity of CVL, concentrations of a subset of immune mediators were measured (Table 1). Using a GEE model, concentrations of lactoferrin, H β D1, 2 and 3, HNP1-3, IP-10, IL-8 and detectable levels of MIP-1 α were significantly associated with anti-HSV activity 70% following oral or vaginal application ($P = .03$). In contrast, none of the immune mediators were associated with anti-HIV activity either at baseline or following use of oral or vaginal product (Table 2A).

Interactions between mucosal immune mediators and antiviral activity

To assess whether any mediators tested modified the effect of TFV on antiviral activity, the mediators were evaluated as predictors of anti-HIV activity 90%, adding $\log_{10}$ TFV as a main effect and an interaction term with each mediator using the same GEE model. None of the mediators modified the effect of TFV on anti-HIV activity. In contrast, there was a significant effect of or a trend for inflammatory mediators to increase the likelihood of having high anti-HSV activity 70% at both low ($\log_{10}$ TFV < 3.0) and high ($\log_{10}$ TFV > 3.0) drug levels (Table 2B), with the effect of each mediator enhanced by higher drug levels. For example, among samples with low drug levels, for every 1 $\log_{10}$ increase in HNP1-3 there was a 2.99-fold greater likelihood of having high anti-HSV activity. However, among those samples with high drug levels, this increased to 6.16-fold for each 1 $\log_{10}$ increase in HNP1-3. These results suggest that while the inflammatory mediators are driving the overall anti-HSV activity, if drug levels are higher, the effect of these mediators on anti-HSV activity is increased.

Impact of oral or vaginal TFV on genital tract soluble mucosal immune mediators

There were no significant differences in concentrations of immune mediators or total protein recovered from CVL following oral product compared to baseline (Table 3). However, after correction for multiple comparisons, six weeks of vaginal TFV gel use was associated with a significant decline in concentrations of protein ($-0.21 \log_{10}\mu\text{g/ml}$), lactoferrin ($-0.38 \log_{10}\text{ng/ml}$), H β D-1 ($-0.18 \log_{10}\text{pg/ml}$), and IL-8 ($-0.35 \log_{10}\text{pg/ml}$), as well as a 24% decreased likelihood of having IL-1 β above the LLOD compared to baseline. The decline in lactoferrin levels remained significant after correcting for total protein recovered and for multiple comparisons, suggesting that the decline was not due to potential differences in dilutional effects of the lavage.

Discussion

Vaginal TFV, but not oral TDF, was associated with a significant increase in anti-HIV activity of CVL. These findings, coupled with results from PK studies showing that vaginal gel achieves ~100-fold higher active drug concentrations in vaginal tissue compared to oral dosing¹², suggest that daily TFV gel would provide more mucosal protection than oral TDF against HIV acquisition in adherent women. However, findings in this and other studies¹⁹ suggest that biological factors may also contribute to drug activity.

To protect against HIV, vaginally applied TFV must cross the epithelium and be transported into and metabolized by immune cells to TFV-diphosphate, which competes with intracellular pools of 2'-deoxyadenosine-triphosphate (dATP) for incorporation into the HIV-DNA chain¹¹. High CVL TFV levels correlate with high vaginal tissue levels²⁰ and TFV concentrations greater than 1000 ng/ml in genital tract secretions were associated with increased efficacy in CAPRISA 004⁵. However, no data bridge CVL drug levels and antiviral activity with protection at sites of infection (submucosal immune cells) and dissemination (lymphoid tissue). High CVL drug levels and antiviral activity (observed here following vaginal application) may not always translate to protection if insufficient intracellular TFV-diphosphate levels are achieved at sites of infection and dissemination. Conversely, low CVL drug levels and activity (observed here following oral dosing) may not translate into ineffectiveness as evidenced by high levels protection observed with oral PrEP in some studies^{1,2}. Orally administered drug may enter the vaginal tissue either directly from the circulation or following the recruitment into the genital tract of circulating TFV-diphosphate-containing immune cells. Oral PrEP may work by protecting immune cells that are recruited into the genital tract possibly following unprotected sexual intercourse²⁷⁻²⁹ or preventing HIV dissemination within lymphoid tissue.

Ideally measurements of drug levels and antiviral activity in both CVL and biopsies would provide complementary data of the potential for systemic and topical PrEP to be effective. Adherence in MTN-001 was heterogeneous, but not dissimilar to the adherence in several highly effective randomized controlled trials (Partners in Prevention and TDF2) making MTN-001 a reasonable comparison. Therefore, re-scaling of antiviral effect is needed when making comparisons of *ex vivo* challenge results and naturally occurring clinical HIV protection, whether with CVL challenge as in this report or with tissue explants.

Several lines of evidence support the hypothesis that the mucosal environment may modulate drug activity. First, while the majority of CVL with high TFV levels inhibited HIV by >90%, there were a few outliers that, despite having concentrations of TFV exceeding the IC₉₀, provided only modest antiviral activity (Fig. 1C). Occasional outliers have been observed in other studies⁸ and could reflect effects of CVL on TFV transport and metabolism or intracellular dATP levels, although we did not detect any modulatory effect of measured soluble immune mediators on the anti-HIV activity in the current study.

Second, in a nested case control sub-study of CAPRISA 004, higher levels of systemic inflammatory immune mediators were associated with increased risk of HIV acquisition, independent of TFV gel use¹⁹. Moreover, in another sub-study, TFV gel provided little or no protection against HIV in a subset of women who had higher levels of genital tract immune mediators²¹.

Whether these observations reflect interference with TFV activity and/or an increase in susceptibility to infection sufficient to overcome any protective effects of TFV requires further investigation. Inflammatory responses induce T cell activation, which, in addition to increasing the risk of HIV infection, may impact TFV PK/PD. TFV-diphosphate accumulated 2- to 3-fold lower and the intracellular half-life was 3-times shorter in activated compared to resting PBMCs^{22,23}. Thus, in the setting of inflammation, activated lymphocytes may be less protected and for a shorter time period than quiescent T cells. Activation also may increase intracellular dATP pools, leading to an unfavorable alteration of the TFV-diphosphate/dATP stoichiometry^{24,25}. Moreover, inflammation may modulate the expression of cellular transporters. The organic anion transporters involved in TFV uptake in the kidney have been characterized²⁶, but little is known about transporters and metabolizing enzymes involved in TFV trafficking in and out of genital tract epithelial and immune cells.

There was no increase in anti-HSV activity of CVL following oral or vaginal TFV dosing compared to baseline inhibitory activity. These results are consistent with the concentrations of drug recovered in CVL, which were less than the IC₅₀ needed to inhibit HSV-2 *in vitro*¹⁸. These findings are also consistent with the observation that oral TDF has no impact on HSV shedding³⁰, but do not explain the protection against HSV-2 observed CAPRISA 004. Possibly, the local concentrations of TFV achieved with BAT24 dosing in CAPRISA 004 exceeded the concentrations achieved with daily dosing in the current study. Moreover, CVL levels may not reflect the concentrations of intracellular TFV-diphosphate in genital tract epithelium, a major site of HSV acquisition. Greater drug uptake and or metabolism of TFV to TFV diphosphate by epithelial compared to immune cells could explain why there was more protection against HSV-2 than HIV in CAPRISA 004 (54% compared to 39%), despite the 100-fold greater *in vitro* drug levels needed to inhibit HSV-2 compared to HIV. Additional studies are needed to better define the potential role of TFV-based PrEP for HSV-2 prevention.

While the anti-HSV activity of CVL did not correlate with drug levels, it did correlate with the concentration of pro-inflammatory immune mediators including lactoferrin and HNP1-3, which is consistent with results obtained in other studies and suggests that anti-HSV activity may provide a biomarker of mucosal inflammation³¹⁻³³. In contrast to the results obtained for HIV, there was a significant positive modulatory effect of inflammatory mediators on the anti-HSV activity of TFV. Further studies are needed to elucidate the underlying mechanisms but the findings suggest that inflammatory mediators may augment TFV uptake or metabolism within epithelial cells. A more intensive bioinformatics study of the mucosal proteome is also needed to better define the association between anti-HSV activity and specific mucosal proteins.

A significant decrease in lactoferrin levels in CVL was observed after controlling for differences in total protein recovered (to correct for dilutional effects of gel and CVL) and for multiple comparisons following six weeks of TFV gel. There was also a trend towards less lactoferrin being recovered from CVL after 14 days of TFV compared to placebo gel ($p=0.09$) in a prior study⁸. The decline may have contributed to the non-significant decrease in CVL anti-HSV activity (Fig. 2). The specific proteins measured represent only a fraction of total protein, and repeated gel applications may affect expression of other mucosal

proteins¹². Whether the decline in lactoferrin levels represents an as yet unrecognized toxicity of the formulation or drug itself or other environmental factors such as shifts in the microbiome requires further study. Concerns have been raised about the hyperosmolarity of TFV gel and alternative formulations are being explored^{34,35}. The primary clinical toxicity associated with oral TDF is renal proximal tubule damage, which likely reflects mitochondrial toxicity from accumulation of drug in renal cells where the expression of TFV transporters is high^{36,37}. TFV-diphosphate inhibits DNA polymerases (primarily mitochondrial DNA polymerase- γ) by the same mechanisms that inhibit HIV reverse transcriptase and could impact RNA and protein expression. The decreased recovery of protein in CVL may reflect local exposure to high concentrations of drug.

Biorepositories of swabs, CVL, and vaginal or cervical biopsies collected in ongoing clinical trials of both systemic and topical PrEP will provide the opportunity to validate the utility of measuring antiviral activity as a surrogate of drug efficacy, compare matrices, and identify the factors that limit product efficacy among adherent women. If mucosal mediators modulate drug efficacy, efforts must be directed at developing combination products that overcome this limitation. Different classes of antiretroviral drugs may be differentially impacted by the mucosa depending on their site (intracellular or luminal) and mechanism of action, requirement for active transport and/or metabolism.

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Conflicts of Interest and Source of Funding

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Figure 1. Anti-HIV activity and TFV concentrations in CVL obtained following six-weeks of oral TDF or topical 1% TFV vaginal gel

(a) The percent inhibition of HIV infection by CVL samples from participants at baseline (enrollment, circles) and after six weeks of oral TDF (squares) or six weeks of daily vaginal TFV gel (triangles). The lines indicate the median and interquartile range for the group; values below zero indicate enhancement. (b) The concentration of TFV detected in CVL. The line indicates median and interquartile range. (c). Correlation of anti-HIV activity with TFV concentrations. The Spearman correlation coefficient following vaginal TFV gel is 0.64, $p < 0.001$.

Figure 2. Anti-HSV activity of CVL following six-weeks of oral TDF or six weeks of TFV 1% vaginal gel

The percent inhibition of HSV-2 plaque formation using CVL obtained HIV from participants at baseline (circles) or following oral (squares) or vaginal (triangles) product use. The lines indicated median and interquartile range for the group; values below zero indicate enhancement.

Table 1

Concentrations of total protein and immune mediators in CVL at baseline and following six weeks of TDF (oral) or six weeks of tenofovir 1% gel (vaginal) presented as the median (interquartile range) or % (n) > lower LOD as indicated.

Mediator	Baseline n=60	Six weeks oral n=53	Six weeks vaginal n=55
Log ₁₀ Total protein (µg/ml)	2.5 (2.3, 2.7)	2.4 (2.2, 2.6)	2.2 (1.9, 2.5)
Log ₁₀ SLPI (pg/ml)	5.1 (4.7, 5.3)	4.9 (4.5, 5.3)	4.8 (4.5, 5.1)
Log ₁₀ Lactoferrin (ng/ml)	2.7 (2.2, 3.0)	2.6 (1.9, 3.1)	2.2 (1.7, 2.8)
Log ₁₀ HBD-1 (pg/ml)	3.2 (2.9, 3.6)	3.1 (2.8, 3.5)	2.9 (2.7, 3.3)
Log ₁₀ HBD-2 (pg/ml)	3.4 (3.0, 3.7)	3.3 (3.0, 3.8)	3.1 (2.9, 3.5)
Log ₁₀ HBD-3 (pg/ml)	3.0 (2.7, 3.3)	3.0 (2.6, 3.3)	2.9 (2.3, 3.2)
Log ₁₀ HNP1-3 (pg/ml)	4.8 (3.9, 5.0)	4.7 (4.2, 5.0)	4.4 (4.0, 5.0)
Log ₁₀ IL-6 (pg/ml)	0.6 (0.4, 1.0)	0.6 (0.3, 1.2)	0.5 (0.3, 1.2)
Log ₁₀ IP-10 (pg/ml)	1.9 (1.3, 2.4)	1.9 (1.5, 2.3)	1.7 (1.4, 2.1)
Log ₁₀ IL-8 (pg/ml)	2.7 (2.3, 2.9)	2.6 (2.2, 3.1)	2.3 (1.9, 2.6)
IL-1β > lower limit	88% (53)	89% (47)	67% (37)
MIP-1α > lower limit	47% (28)	53% (28)	40% (22)
MIP-3α > lower limit	77% (46)	76% (40)	82% (45)
Anti-HIV 90%	5% (3)	2% (1)	89% (49)
Anti-HSV 70%	48% (29)	34% (18)	22% (12)

Table 2

Associations between percent inhibition of HSV-2 and HIV-1 and concentrations of immune mediators in CVL (GEE model) (A) and between mediators and HSV inhibition 70% after stratification by TFV levels (B).

A.						
Mediator	Risk Ratio	95% CI	p-value	Risk Ratio	95% CI	p-value
	HSV-2			HIV-1		
Log ₁₀ (TFV)	0.92	0.79, 1.08	0.3	2.11	1.89, 2.15	<0.001
Log ₁₀ SLPI	1.49	0.84, 2.65	0.2	0.97	0.81, 1.16	0.7
Log ₁₀ Lactoferrin	2.51	1.55, 4.06	<0.001	1.00	0.82, 1.21	1.0
Log ₁₀ HBD-1	1.89	1.06, 3.37	0.03	0.88	0.76, 1.02	0.08
Log ₁₀ HBD-2	3.21	0.83, 5.61	<0.001	1.17	0.91, 1.52	0.2
Log ₁₀ HBD-3	2.80	1.71, 4.59	<0.001	0.99	0.82, 1.18	0.9
Log ₁₀ HNP1-3	4.01	2.78, 5.80	<0.001	0.92	0.77, 1.09	0.3
IL-1β > lower limit	3.43	0.90, 13.11	0.07	0.81	0.59, 1.11	0.2
Log ₁₀ IL-6	1.24	0.83, 1.85	0.3	0.90	0.78, 1.05	0.2
Log ₁₀ IP-10	2.44	1.76, 3.40	<0.001	1.01	0.88, 1.15	0.9
MIP-1α > lower limit	3.23	1.45, 7.20	0.004	0.89	0.71, 1.11	0.3
Log ₁₀ IL-8	2.96	1.79, 4.90	<0.001	0.93	0.75, 1.13	0.5
MIP-3α > lower limit	3.71	0.96, 14.26	0.06	1.03	0.76, 1.39	0.8
B.						
Mediator	Risk Ratio	95% CI	p-value	Risk Ratio	95% CI	p-value
	Log ₁₀ TFV < 3.0			Log ₁₀ TFV > 3.0		
Log ₁₀ SLPI	1.07	0.51, 2.26	0.9	1.89	0.90, 3.98	0.09
Log ₁₀ Lactoferrin	1.40	0.79, 2.47	0.3	5.45	2.35, 12.64	<0.001
Log ₁₀ HBD-2	2.20	1.04, 4.63	0.04	6.41	3.20, 12.84	<0.001
Log ₁₀ HBD-3	1.84	0.94, 3.61	0.08	6.54	3.30, 12.96	<0.001
Log ₁₀ HNP1-3	2.99	1.75, 5.11	<0.001	6.16	2.74, 13.85	<0.001
Log ₁₀ IL-6	0.93	0.53, 1.64	0.8	1.54	0.88, 2.70	0.1

B.						
Mediator	Risk Ratio	95% CI	p-value	Risk Ratio	95% CI	p-value
	Log ₁₀ TFV < 3.0			Log ₁₀ TFV > 3.0		
Log ₁₀ IP-10	1.76	1.22, 2.54	0.003	5.29	2.51, 11.17	<0.001
Log ₁₀ IL-8	3.04	1.52, 6.05	0.002	4.88	2.75, 8.66	<0.001

Table 3

Changes in immune mediators following six weeks of oral or vaginal product use. For the continuous outcomes, the coefficient presented is the average difference from baseline and for the dichotomous outcomes the value presented is the risk of being above the lower limit compared to baseline.

Continuous Outcomes	Oral Coefficient (95% CI) P-value	Vaginal Coefficient (95% CI) P-value
Log ₁₀ Protein (ug/ml)	-0.02 (-0.13, 0.09)	-0.21 (-0.33, -0.09) *
Log ₁₀ SLPI (pg/ml)	-0.03 (-0.18, 0.11)	-0.17 (-0.31, -0.02)
Log ₁₀ Lactoferrin (ng/ml)	-0.05 (-0.17, 0.07)	-0.38 (-0.51, -0.25) **
Log ₁₀ HBD-1 (pg/ml)	-0.06 (-0.20, 0.08)	-0.18 (-0.29, -0.06) *
Log ₁₀ HBD-2 (pg/ml)	0.03 (-0.15, 0.21)	-0.27 (-0.45, -0.09)
Log ₁₀ HBD-3 (pg/ml)	0.08 (-0.09, 0.25)	-0.13 (-0.26, 0.01)
Log ₁₀ HNP1-3 (pg/ml)	0.06 (-0.10, 0.22)	-0.16 (-0.34, 0.02)
Log ₁₀ IL-6 (pg/ml)	-0.01 (-0.18, 0.16)	-0.04 (-0.24, 0.16)
Log ₁₀ IP-10 (pg/ml)	0.12 (-0.05, 0.30)	0.03 (-0.18, 0.23)
Log ₁₀ IL-8 (pg/ml)	0.04 (-0.08, 0.17)	-0.35 (-0.51, -0.18) *
IL-1 β > lower limit	1.01 (0.93, 1.11)	0.76 (0.64, 0.91) *
MIP-1 α > lower limit	1.09 (0.86, 1.38)	0.85 (0.64, 1.13)
MIP-3 α > lower limit	0.99 (0.84, 1.17)	1.07 (0.90, 1.28)
Anti-HIV 90%	0.43 (0.12, 1.56)	18.17 (6.17, 53.53) *
Anti-HSV 70%	0.71 (0.48, 1.05)	0.46 (0.26, 0.81)

* significance retained after Bonferroni correction

** significance retained after also controlling for protein recovered