

Topical Tenofovir, a Microbicide Effective against HIV, Inhibits Herpes Simplex Virus-2 Replication

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SUMMARY

The HIV reverse-transcriptase inhibitor, tenofovir, was recently formulated into a vaginal gel for use as a microbicide. In human trials, a 1% tenofovir gel inhibited HIV sexual transmission by 39% and, surprisingly, herpes simplex virus-2 (HSV-2) transmission by 51%. We demonstrate that the concentration achieved intravaginally with a 1% tenofovir topical gel has direct antiherpetic activity. Tenofovir inhibits the replication of HSV clinical isolates in human embryonic fibroblasts, keratinocytes, and organotypic epithelial 3D rafts, decreases HSV replication in human lymphoid and cervicovaginal tissues *ex vivo*, and delays HSV-induced lesions and death in topically treated HSV-infected mice. The active tenofovir metabolite inhibits HSV DNA-polymerase and HIV reverse-transcriptase. To exert dual antiviral effects, tenofovir requires topical administration to achieve a drug concentration higher than systemic levels achieved by oral treatment. These findings indicate that a single topical treatment, like tenofovir, can inhibit the transmission of HIV and its copathogens.

INTRODUCTION

Unprotected heterosexual intercourse remains a major transmission mode of HIV-1. In the absence of a protective vaccine, an efficient topical vaginal microbicide would be critical to the prevention of male-to-female HIV-1 transmission and to curbing the worldwide AIDS epidemic (Balzarini and Van Damme, 2007; Klasse et al., 2008). HIV-1 infection is commonly associated with other sexual infections, such as HSV, that facilitate the risk of HIV acquisition and worsen the clinical course of HIV disease (Blower and Ma, 2004; Corey, 2007; Buvé, 2010). Therefore, it would be beneficial if a future microbicide was efficient not only against HIV-1, but also against other sexually transmitted infections.

Unfortunately, until recently, all microbicide formulations that successfully passed preclinical testing failed to demonstrate efficiency in the early stages of clinical development (Van Damme et al., 2002, 2008; Abdool Karim, 2010), and some of them apparently promoted HIV-1 infection (Van Damme et al., 2002, 2008).

Instead of testing new compounds as potential microbicides, tenofovir, an efficient nucleotide HIV reverse-transcriptase (RT) inhibitor (Balzarini et al., 1993) widely used in HIV therapy, was recently formulated as a 1% gel and tested in a double-blind placebo-controlled study in 889 women (CAPRISA 004 [Abdool Karim et al., 2010]). Tenofovir convincingly diminished HIV-1 transmission (by 39%). Surprisingly, in the CAPRISA 004 trial, a significant 51% reduction of the risk of acquiring HSV-2, a common HIV-1 copathogen that facilitates HIV transmission, was also observed (Cates, 2010). This effect of tenofovir gel on HSV was rather unanticipated because this highly potent antiretroviral and antihepadnaviral drug has been previously shown to exhibit minimal, if any, anti-HSV activity (Balzarini et al., 1993). Therefore, one might have envisioned a complicated and indirect mechanism to explain this phenomenon. It turned out not to be the case.

In the present study, we report on the resolution of this apparent contradiction. We provide compelling evidence that, at the concentrations achieved intravaginally by the topical administration of a 1% gel, tenofovir exhibits direct antiherpetic activity. These data and the therapeutic principles emerging from our study are important for the development of new drug formulations and administration protocols to design and/or optimize future microbicide trials.

RESULTS

Tenofovir Inhibits HSV-1 and HSV-2 Replication in Various Cell Cultures

Tenofovir inhibited cytopathicity of the laboratory strains of HSV-1 and HSV-2 in human embryonic lung (HEL) fibroblasts and in primary human keratinocytes (PHKs) with EC₅₀ values of 103–193 μg/ml (Table 1). In HEL fibroblasts, tenofovir consistently inhibited the cytopathic effects of a variety of

Table 1. Antiviral Activity against Wild-Type and Acyclovir-Resistant Laboratory Strains and Clinical Isolates of HSV-1 and HSV-2 Using the CPE Reduction Assay

Cell Type	Viral Strain	EC ₅₀ ^a (μg/ml)						
		Acyclovir	Tenofovir	Adefovir	Cidofovir			
HSV-1 Laboratory Strain								
HEL	Kos	0.053 ± 0.046	132 ± 20	6.0 ± 3.5	0.63 ± 0.36			
PHKs	Kos	0.42 ± 0.15	141 ± 53	9.2 ± 0.1	1.4 ± 0.03			
HSV-2 Laboratory Strain								
HEL	G	0.10 ± 0.10	103 ± 31	3.6 ± 0.1	0.70 ± 0.37			
PHKs	G	0.45 ± 0.07	193 ± 17	13.0 ± 2.1	4.4 ± 2.8			
HSV-1 Wild-Type Clinical Isolates								
HEL	RV-132	0.031 ± 0.005	114 ± 8	6.7 ± 2.4	0.90 ± 0.15			
	RV-134	0.063 ± 0.040	160 ± 57	10.2 ± 2.5	1.19 ± 0.57			
	RV-6	0.075 ± 0.040	131 ± 29	4.3 ± 0	0.36 ± 0.01			
	C559142	0.018 ± 0.012	101 ± 14	3.6 ± 0.8	0.22 ± 0.17			
	RV-174	0.039 ± 0.002	110 ± 0	5.8 ± 1.1	0.43 ± 0.11			
	RV-175	0.018 ± 0.020	109 ± 2	5.0 ± 0	0.82 ± 0.30			
HSV-1 TK ⁻ Clinical Isolates ^b								
HEL	RV-117 (stop codon at position R281)	22.6 ± 4	≥ 159 ± 48	6.4 ± 3.0	0.36 ± 0.18			
	328058 (Nts 548-553, C deletion in a string of 6Cs)	>20 ± 0	117 ± 25	3.1 ± 0.9	0.13 ± 0.01			
	RV-36 (substitution T245M)	≥ 20 ± 0	179 ± 29	6.0 ± 5.7	0.42 ± 0.30			
	RV-179 (Nts 430-436, G insertion in a string of 7Gs)	10 ± 5	100 ± 0	3.2 ± 0.7	0.14 ± 0.01			
HSV-2 Wild-Type Clinical Isolates								
HEL	RV-124	0.086 ± 0.021	163 ± 52	7.3 ± 0.9	0.90 ± 0.15			
	RV-24	0.12 ± 0.02	125 ± 22	7.5 ± 1.7	1.07 ± 1.11			
	NA	0.045 ± 0.032	≥ 134 ± 76	3.4 ± 1.2	0.32 ± 0.26			
	PB	0.11 ± 0.03	176 ± 34	5.2 ± 3.2	0.60 ± 0.39			
	NS	0.05 ± 0	147 ± 7	3.0 ± 1.4	0.53 ± 0.18			
	HSV-47	0.037 ± 0.004	176 ± 34	5.4 ± 4.2	0.62 ± 0.54			
HSV-2 TK ⁻ Clinical Isolates ^b								
HEL	RV-129 (Nts 433-439, G insertion in a string of 7Gs)	≥ 20 ± 0	163 ± 52	7.5 ± 3.5	0.49 ± 0.24			
	BA 19026589 (Nts 792-796, G deletion in a string of 5 Gs)	7.5 ± 3.5	179 ± 29	10.0 ± 0	1.43 ± 0			
	LU C557672 (Nts 808-811, C insertion in a string of 4Cs)	>20 ± 0	131 ± 6	7.5 ± 3.5	0.50 ± 0			
	RV-101 (Nts 551-556, C insertion in a string of 6Cs)	>20 ± 0	144 ± 80	5.1 ± 2.6	0.33 ± 0.16			
	HSV-44 (Nts 433-439, G deletion in a string of 7Gs)	>20 ± 0	142 ± 82	6.0 ± 5.7	0.35 ± 0.40			
	RV-184 (Nts 551-556, C deletion in a string of 6Cs)	12 ± 3	87 ± 18	3.3 ± 0.6	0.35 ± 0.04			
	RV-185 (Nts 433-439, G insertion in a string of 7Gs)	21 ± 9	85 ± 8	4.1 ± 1.3	0.32 ± 0.07			
Cell Toxicity (μg/ml)								
Cell Type	Acyclovir		Tenofovir		Adefovir		Cidofovir	
	CC ₅₀ ^c	MCC ^d	CC ₅₀	MCC	CC ₅₀	MCC	CC ₅₀	MCC
HEL	166 ± 17	>100	547 ± 39	>400	100 ± 57	>100	82 ± 57	>100
PHKs	132	>50	>50	>200	17 ± 6	>50	34 ± 20	>50

Confluent HEL and PHK cell cultures were exposed to 100 TCID₅₀ of virus in the presence of different drug concentrations and incubated for 3 days at 37°C. Then, the cytopathicity was determined microscopically and the EC₅₀ values determined. Cell toxicity (CC₅₀ and MCC) was measured in parallel in noninfected cell cultures.

^aEC₅₀, 50% effective concentration or compound concentration required to reduce virus-induced cytopathicity (CPE) by 50%. Data shown are the mean values (±SD) of at least two independent experiments.

^bNucleotide position refers to the coding region of the TK gene.

^cCC₅₀, 50% cytostatic concentration or compound concentration required to reduce cell growth by 50%.

^dMCC, minimum cytotoxic concentration that causes a microscopically detectable alteration of cell morphology.

wild-type HSV-1, thymidine kinase-deficient (TK⁻) HSV-1, wild-type HSV-2, and HSV-2 TK⁻ clinical isolates with mean EC₅₀ values of 123 μg/ml (range of 101–160 μg/ml), ≥ 139 μg/ml (100 to ≥ 159 μg/ml), 154 μg/ml (125–176 μg/ml), and 133 μg/ml (85–179 μg/ml), respectively. The antiherpesvirus activity of tenofovir was revealed at a concentration range

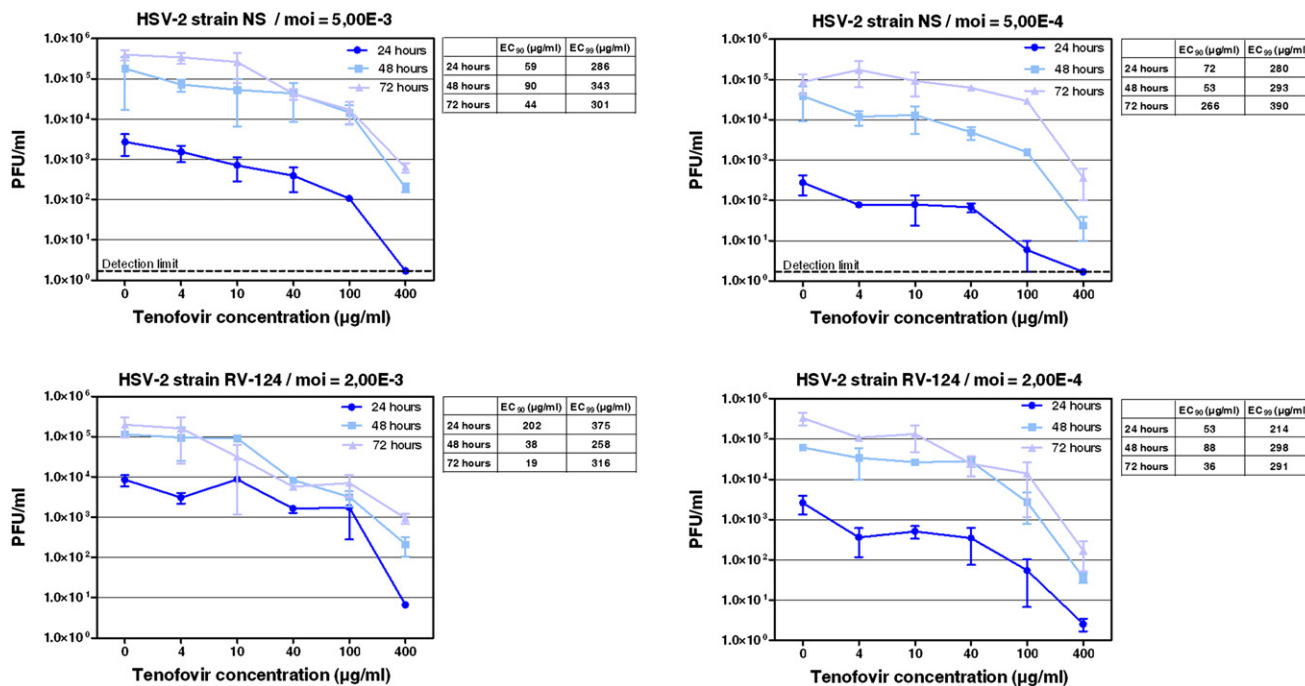


Figure 1. Inhibitory Activity of Tenofovir against HSV-2 Replication Cytopathicity in HEL Cell Cultures Using Different Multiplicities of Infection Virus yield was determined at 24, 48, and 72 hr postinfection, and EC₅₀ and EC₉₉ values were calculated from the graphical plots. Error bars represent \pm SD.

that is markedly higher than those established for antiherpetic drugs, such as acyclovir and its closely related acyclic nucleoside phosphonate congeners, adefovir and cidofovir (Table 1).

There was no marked difference in the suppression of HSV-1 and HSV-2 replication by tenofovir and the reference antiherpesvirus agents. Importantly, there were no signs of tenofovir cytotoxicity, as assessed from HEL cell morphology, even at the highest drug concentrations used (500 μ g/ml). The effects of tenofovir on HSV-2 yield in HEL cells were also determined at 24, 48, and 72 hr postinfection to confirm the data obtained using the cytopathic effect (CPE) reduction assay. The dose-response curves of tenofovir for two clinical HSV-2 isolates (RV-124 and NS) at two different multiplicities of infection (moi) were determined using the plaque-forming unit (PFU) reduction assay and are depicted in Figure 1. Tenofovir inhibited the replication of both viruses with EC₉₉ values (i.e., the drug concentration that decreases the virus titers by two orders of magnitude) in the range of 214–390 μ g/ml, whereas no changes in cell number were noted at any drug concentration.

The effect of tenofovir was also evaluated against HSV-2 in primary monocyte/macrophage (M/M) cell cultures using two different methods, CPE reduction and PFU reduction assays. Tenofovir dosage of 500 or 100 μ g/ml completely suppressed HSV-2 replication in M/M. HSV-2 production dropped from 3.7×10^5 TCID₅₀/ml (\sim 95% cytopathicity) in the culture supernatants of untreated cells by more than one log₁₀ in 20 μ g/ml and an approximate half log₁₀ in 5 μ g/ml tenofovir-treated cultures. At 1 μ g/ml or 0.2 μ g/ml drug concentrations, no significant protective effect of tenofovir was observed (2.4 – 3.0×10^5

TCID₅₀/ml; 66%–81% cytopathicity) (Table 2 and Figure S1 available online). As expected, the structurally related reference compound adefovir, which was included as a control in the assay, proved to be more potent in its anti-HSV-2 activity in the M/M cell cultures (full CPE suppression at 5 μ g/ml) than tenofovir (Table 2).

Tenofovir Suppresses Viral Replication in Organotypic Epithelial Raft Cultures

Because differentiated keratinocytes are the main target cells for productive infection of HSV in vivo, we evaluated the antiviral activity of tenofovir in organotypic raft cultures of keratinocytes. The organotypic epithelial raft cultures were infected after 10 days of differentiation and treated with tenofovir. After 5 days of drug treatment, histological examination of the noninfected raft cultures revealed completely differentiated epithelium with characteristic layers, whereas HSV-infected rafts revealed a pronounced viral infection that had spread all along the epithelium (Figure 2). At a concentration of 200 μ g/ml, tenofovir caused a 2.6 (HSV-1) and a 5.4 (HSV-2) log₁₀ reduction in virus production and complete protection of virus-induced cytopathicity (Figure 3). A 0.81 (HSV-1) and a 1.75 (HSV-2) log₁₀ reduction was recorded at a concentration of 50 μ g/ml of tenofovir. At 20 and 5 μ g/ml, tenofovir was partially protective, with areas of a normal epithelium and areas of destructed rafts. At a concentration of 2 μ g/ml, tenofovir was inactive against HSV-2 (Figure 3). The higher activity of tenofovir against HSV-2, but not HSV-1, can be due to the different levels of replication between these two viruses: in the untreated control cultures, the HSV-2 titers were lower ($\sim 1.5 \times 10^6$ PFU/raft) than the HSV-1 titers ($\sim 9 \times 10^6$ PFU/raft).

Table 2. Inhibitory Activity of Tenofovir and Adefovir against HSV-2 in Primary Monocyte/Macrophage Cultures Using the CPE Reduction Assay and the PFU Reduction Assay

Treatment	Virus Production (TCID ₅₀ /ml)	Inhibition of Virus Production (% of Control) CPE Reduction Assay	Virus Production (PFU/ml)	Inhibition of Virus Production (% of Control) PFU Reduction Assay	Cytopathicity (CPE) (%)
None	3.7 × 10 ⁵		9 × 10 ⁴		95
Tenofovir (μg/ml)					
500	No virus	100	No virus	100	None
100	No virus	100	No virus	100	None
20	1.3 × 10 ⁴	96	2.2 × 10 ³	98	5
5	7.2 × 10 ⁴	80	1.9 × 10 ⁴	79	60
1	2.4 × 10 ⁵	34	6.6 × 10 ⁴	27	70
0.2	3 × 10 ⁵	19	7.3 × 10 ⁴	19	80
0.04	3.2 × 10 ⁵	13	8.5 × 10 ⁴	6	85
Adefovir (μg/ml)					
500	No virus	100	No virus	100	None
100	No virus	100	No virus	100	None
20	No virus	100	No virus	100	None
5	No virus	100	No virus	100	None
1	8 × 10 ²	99	2.5 × 10 ²	99	None
0.2	6 × 10 ⁴	84	9.9 × 10 ³	89	5
0.04	1 × 10 ⁵	73	1.8 × 10 ⁴	80	40

Virus production was evaluated on day 6 postinfection. HSV-2 was quantified in the supernatants of drug-treated virus-infected cell cultures by titration in Vero cell cultures using the CPE reduction assay (calculation of TCID₅₀/ml) and the PFU reduction assay (calculation of PFU/ml). See also Figure S1.

Tenofovir Suppresses HSV-2 in Singly Infected and in HIV-1-Coinfected Human Lymphoid and Cervicovaginal Tissue Ex Vivo

The effect of tenofovir on the replication of HSV-1_F, HSV-2_G, and HSV-2_{MS} was investigated in infected human tonsillar tissues ex vivo (Figure 4). Upon inoculation in human tonsillar tissues ex vivo, HSV-1_F, HSV-2_G, or HSV-2_{MS} replicated efficiently, as shown by accumulation of viral DNA in culture medium. The median accumulation throughout the 9 days of culture was 7.8 log₁₀ DNA copies/ml (interquartile range [IQR] 7.1–8.1, n = 5); 7.25 log₁₀ DNA copies/ml (IQR 7.2–7.9, n = 6); and 6.4 log₁₀ DNA copies/ml (IQR 6.1–7.5, n = 3) for HSV-1_F, HSV-2_G, and HSV-2_{MS}, respectively.

Tenofovir suppressed replication of HSV-1_F, HSV-2_G, and HSV-2_{MS} in a dose-dependent manner (Figure 4). On the basis of the real-time PCR data presented above, we calculated the EC₅₀ for HSV suppression of tenofovir as 7 μg/ml (95% confidence interval [CI] 10–44) for HSV-1_F; 14 μg/ml (CI 10–163) for HSV-2_G (Figure 4A); and 19 μg/ml (CI 27–127) for HSV-2_{MS}. The EC₅₀ calculated by this technique is in agreement with that obtained with the PFU reduction assay (Thi et al., 2006). Accordingly, tenofovir at a concentration of 66 μg/ml reduced HSV-1_F, HSV-2_G, and HSV-2_{MS} replication by 99% ± 0.1%, 87% ± 12%, and 91.7% ± 3.2%, respectively, compared to infected donor-matched untreated tissue (p < 0.01). At the 66 μg/ml tenofovir concentration, the suppression of HSV-1_F, HSV-2_G, and HSV-2_{MS} replication was not associated with measurable tonsillar depletion of either total T cells (CD3⁺), total B cells (CD19⁺), or subsets of naive and memory T cells compared to donor-matched untreated tissues (n = 3, p > 0.4), in which the loss of these cells between days 1 and 12 in culture was negligible (Grivel et al.,

2000). In tonsillar tissues coinfecting with HSV-2_G and HIV-1_{LAI}, 66 μg/ml tenofovir (maintained throughout the entire culture period) inhibited replication of both viruses: in the untreated control tissues, HSV-2_G DNA release into culture medium was 7.3 log₁₀ DNA copies/ml (IQR 6.8–7.4), whereas in donor-matched tissues treated with 66 μg/ml tenofovir HSV-2_G replication was suppressed by 96% ± 1% (n = 6; p < 0.01). In these tissues, HIV-1_{LAI} was inhibited completely (Figure 4B). The antitherpetic effect of tenofovir is not a general property of the nucleoside reverse-transcriptase inhibitors (NRTIs) as we found no effect of lamivudine (3TC) on HSV-2_G replication (data not shown). Finally, we found no effect of tenofovir (at 66 μg/ml) on the release of 15 cytokines (IL-1α, IL-6, IL-8, IL-15, IL-16, CCL3/MIP-1α, CCL4/MIP-1β, CCL20/MIP-3α, CCL5/RANTES, CXCL12/SDF-1β, CCL2/MCP-1, CCL11/Eotaxin, CXCL9/MIG, CXCL10/IP-10, and GM-CSF) into the culture medium (p > 0.15). Tenofovir at a concentration of 150 μg/ml also inhibited HSV-2_G replication in cervicovaginal tissue ex vivo from 6.6 log₁₀ DNA copies/ml (IQR 5.3–8.2) to 5.5 log₁₀ DNA copies/ml (IQR 4.8–5.7, n = 5) (Figures 4C and 4D), reflecting 78% ± 9% reduction when the reductions of viral replication in each experiment were averaged (p < 0.01).

Activity of Tenofovir in HSV-Infected Mice

The anti-HSV-1- and HSV-2 activities of tenofovir were evaluated in virus-infected athymic nude mice (lumbosacral scarification model). The drug was administered to HSV-1- and HSV-2-infected mice at a concentration of 1% in DMSO for 5 days, starting at the day of infection. Mice treated with placebo (DMSO) developed lesions in the lumbosacral area, leading to paralysis of the hind legs and, finally, death. Treatment with tenofovir (1%) resulted in a statistically significant delay of morbidity and

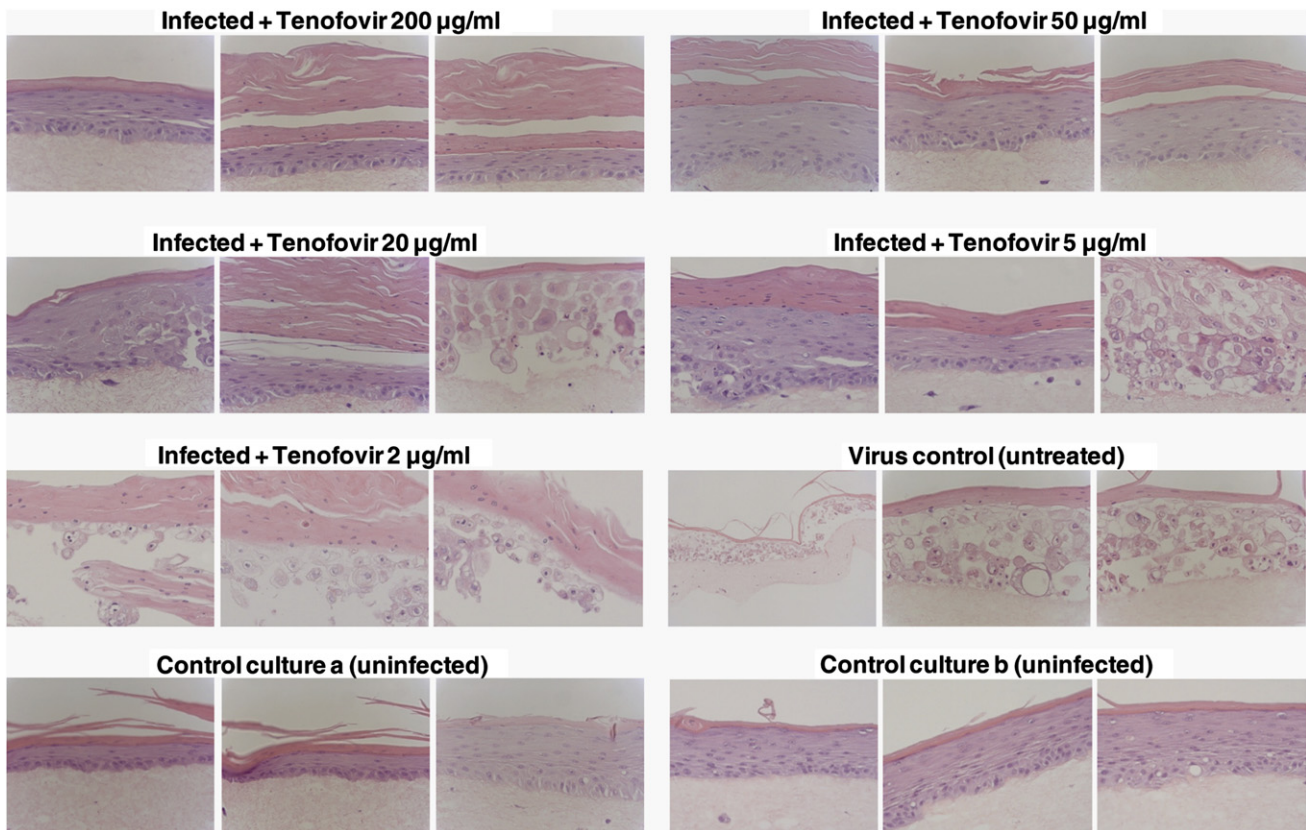


Figure 2. Effects of Tenofovir on Organotypic Epithelial Rafts Cultures Infected with HSV-2_G at 10 Days after Lifting

Compounds were added to the culture media on the day of infection and remained in contact with the cells until the rafts were fixed (i.e., at 15 days after lifting). Magnification, 40x.

prolonged the survival of the mice infected with HSV (Figure 5A). Tenofovir was somewhat more active against HSV-1 than HSV-2, for unknown reasons. Also, when formulated in a 1% gel (as used in the CAPRISA 004 microbicide trial), tenofovir significantly ($p < 0.01$) delayed the appearance of herpesvirus-related

lesions and subsequent death of the animals compared to placebo (same gel formulation without drug) (Figure 5B). Adefovir performed markedly better against HSV-1 than HSV-2 in the 1% gel-treated mice, but was virtually not superior to tenofovir against HSV-2 (Figure 5B).

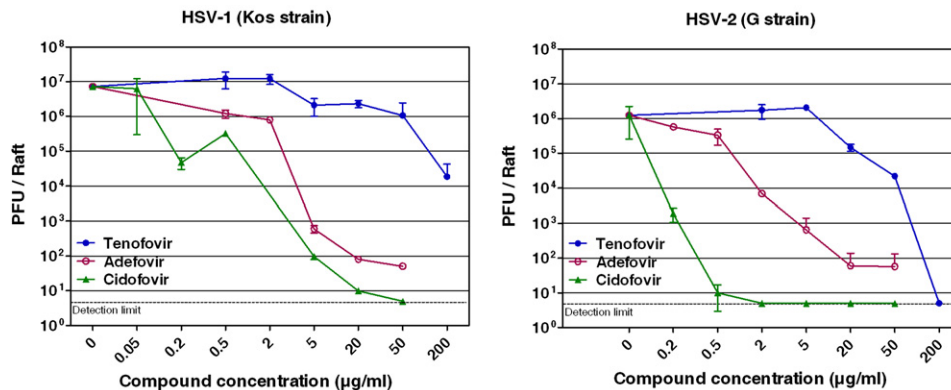


Figure 3. Quantification of Virus Yield in Organotypic Epithelial Raft Cultures Infected with HSV-1 or HSV-2 at Day 10 after Initiation of Cell Cultures

Compounds were added to the culture media on the day of infection (i.e., 10 days after initiating differentiation) and remained in contact with the cells for 5 days until the rafts were frozen for determination of virus production by a plaque assay in HEL cell cultures. Two independent rafts were used for the quantification of virus production to take into account the variation of epithelial thickness among the rafts. Error bars represent \pm SD.

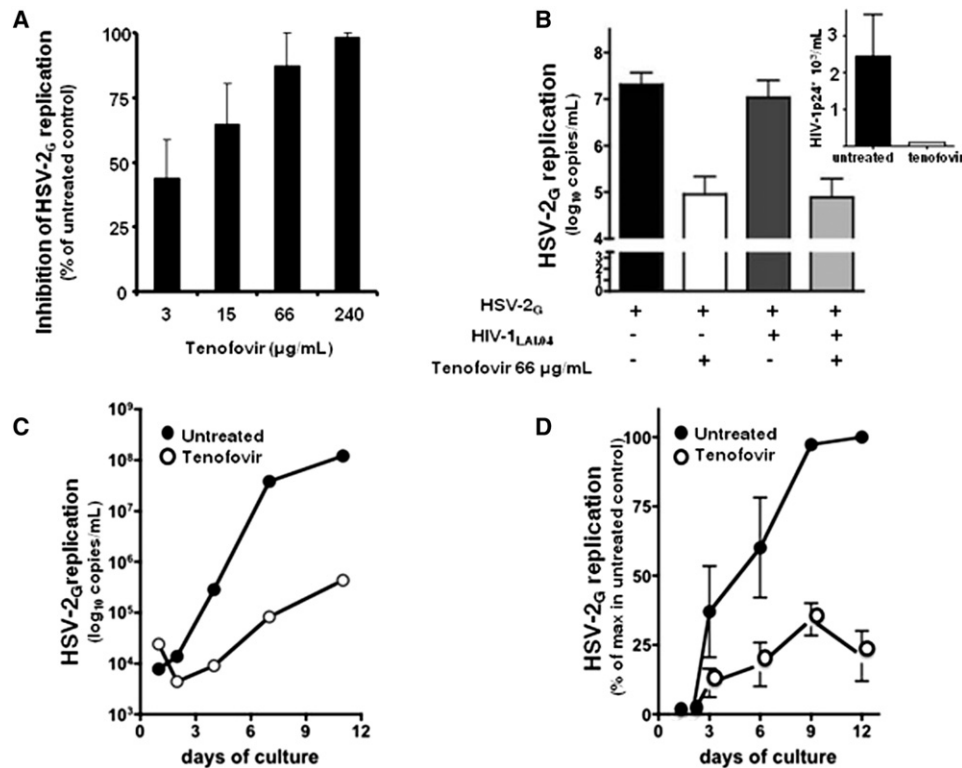


Figure 4. Suppression of HSV-2 in Singly Infected and HIV-1-Coinfected Human Ex Vivo Lymphoid Tissue by Tenofovir

(A) Blocks of human tonsillar tissue were inoculated ex vivo with HSV-2_G and treated or not treated with tenofovir (3, 15, 66, or 240 µg/ml). HSV-2_G replication was monitored by measuring viral DNA in culture media (day 9 postinfection). Presented are means ± SEM of the results with tissues from 14 donors. For each donor, each data point represents pooled viral release from 27 tissue blocks.

(B) Blocks of human tonsillar tissue were coinoculated ex vivo with HSV-2_G and HIV-1_{LAI} and treated or not treated with tenofovir (66 µg/ml). HIV-1 replication was monitored by measuring p24_{gag} accumulated in culture media over 3 day periods. Presented are means ± SEM of the results with tissues from six donors. For each donor, each data point represents pooled viral release from 27 tissue blocks.

(C) Blocks of human cervicovaginal tissues were inoculated ex vivo with HSV-2_G and treated or not treated with tenofovir (150 µg/ml). HSV-2_G replication was monitored by measuring viral DNA accumulated in culture media at different time points throughout the culture period. Presented is a representative experiment (out of five) with a tissue from an individual donor. Each data point represents pooled viral release from 16 tissue blocks.

(D) Blocks of human cervicovaginal tissues were inoculated ex vivo with HSV-2_G and treated or not treated with tenofovir (150 µg/ml). HSV-2_G replication was monitored by measuring viral DNA accumulated in culture media at different time points throughout the culture period. Presented are means ± SEM of the results with tissues from five donors. For each donor, each data point represents pooled viral release from 16 tissue blocks.

Tenofovir Is Converted to Its Antivirally Active Metabolite in Relevant Cell Cultures

In lymphocytic CEM, epithelial TZM-BI, and fibroblast HEL cultures, [2,8-³H]tenofovir (0.60 µg/ml, applied for 24 hr starting from 72 hr postinitiation of the cultures) was converted into tenofovir-diphosphate. Concentrations of this metabolite reached 5.4 ± 0.6, 5.2 ± 2.2, and 19 ± 17 ng/10⁹ cells in lymphocytic cells, epithelial cells, and fibroblasts, respectively. We also demonstrated that, at increasing tenofovir concentrations (i.e., 0.6, 6, 60, or 600 µg/ml), concomitantly higher tenofovir diphosphate concentrations were formed (5.18 ± 2.2, 103 ± 15.2, 1,026 ± 103, and 10,400 ± 800 ng/10⁹ TZM-BI cells, respectively). Thus, epithelial cells exposed to 600 µg/ml tenofovir concentrations produced as much as 10.4 ± 0.8 ng of tenofovir diphosphate/10⁶ cells (this corresponds to an ~3 µg/ml if we consider a cell volume to be 4 pl (Alberts et al., 1994)) without any sign of toxicity, as measured upon microscopic inspection. Also, MTT dye viability testing of epithelial cell cultures that were

exposed to up to 1,000 µg/ml tenofovir did not show measurable toxicity (data not shown). Such a linear relationship between external tenofovir concentrations and intracellular tenofovir diphosphate concentrations has been observed earlier (Balzarini et al., 1991). This characteristic of tenofovir allows sufficiently high HSV-2-suppressive levels of tenofovir diphosphate metabolite upon application of 1% tenofovir (10 mg/ml) gel. Thus, tenofovir is efficiently converted to its antivirally active metabolite in multiple different cell types that represent relevant target cells for either HIV or HSV infection in vivo.

The Active Metabolite of Tenofovir Efficiently Inhibits Both HSV DNA Polymerase and HIV Reverse-Transcriptase

The active tenofovir metabolite, tenofovir diphosphate, has been evaluated for its inhibitory activity against the HIV-1 reverse-transcriptase and HSV DNA polymerase using activated calf thymus DNA as the primer/template and [2,8-³H]

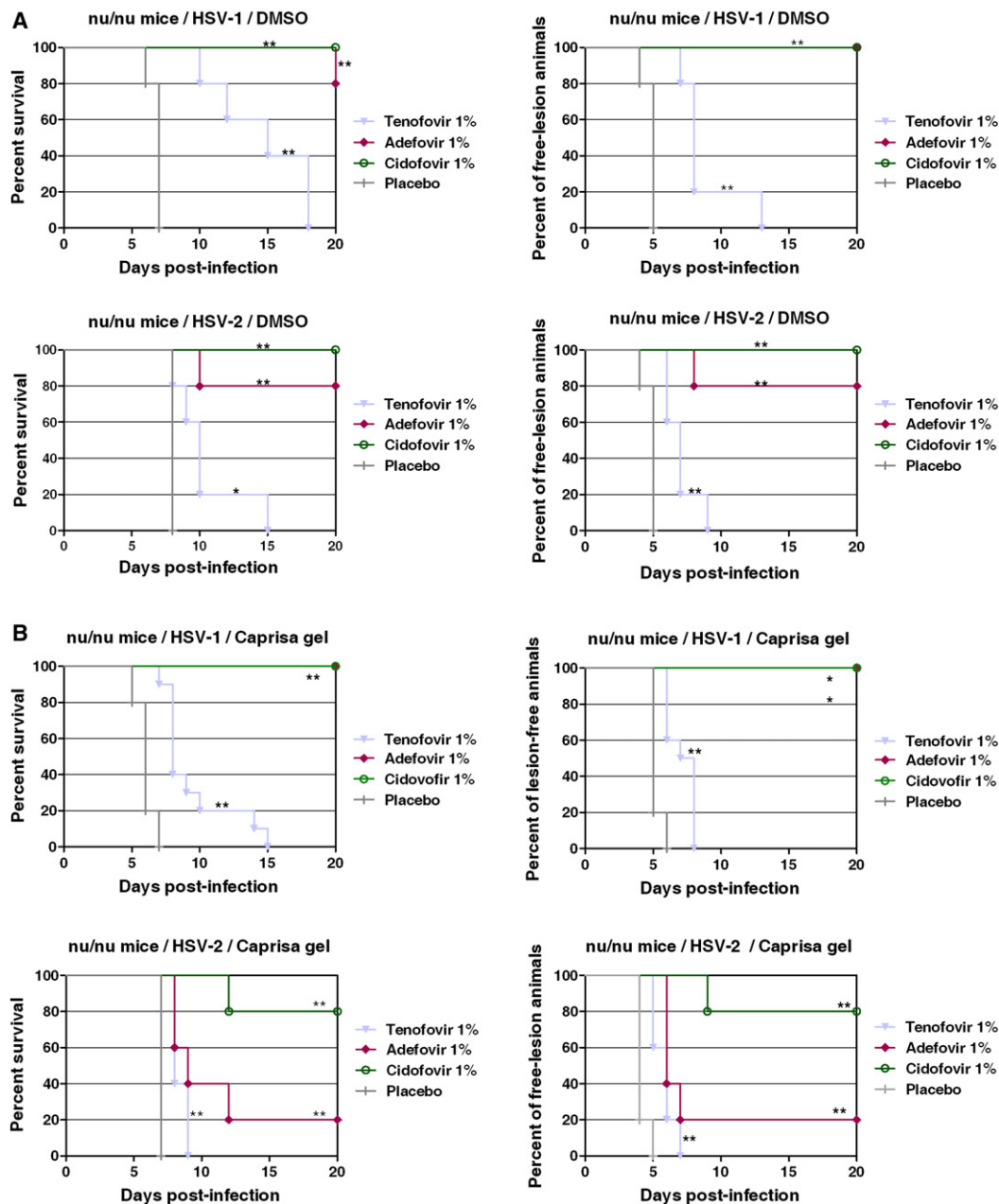


Figure 5. Effect of Tenofovir, Adefovir, and Cidofovir Treatment in DMSO Formulation or in Gel Identical to that Used in the CAPRISA 004 Trial on Development of Lesions and Mortality in Mice Inoculated with HSV-1 or HSV-2

(A and B) Groups of five nu/nu mice were inoculated with HSV-1 or HSV-2 on the lumbosacral area. Each cohort was then subjected to topical treatment twice daily for 5 consecutive days, starting the day of viral infection. The placebo groups received a similar treatment with the test formulation without drug. Development of skin lesions, paralysis, and mortality were recorded over a period of 20 days. Animals were euthanized when more than 30% loss in body weight or development of paralysis occurred. Survival rates were estimated according to the Kaplan-Meier method and were compared using the log-rank test (Mantel-Cox using GraphPad Prism). * $p < 0.05$; ** $p < 0.01$.

2'-deoxyadenosine triphosphate (dATP) as the competing substrate. Tenofovir diphosphate efficiently inhibited HIV-1 RT with an IC_{50} of 1.3 $\mu\text{g/ml}$ in the presence of dATP (3.2 μM) as the competing deoxynucleotide triphosphate (dNTP). Tenofovir diphosphate also inhibited herpesvirus DNA polymerase-catalyzed polymerization at IC_{50} s of 0.38 ± 0.03 $\mu\text{g/ml}$, 7.1 ± 5 $\mu\text{g/ml}$, 8.5 ± 3.8 $\mu\text{g/ml}$, and 25 ± 1.8 $\mu\text{g/ml}$ in the presence of

competing dATP (3.2 μM), dGTP (2.8 μM), dTTP (1 μM), or dCTP (2.5 μM), respectively.

Thus, the antitherpetic activity of tenofovir in cell cultures, organotypic epithelial raft cultures, human lymphoid and cervicovaginal ex vivo tissues, and virus-infected mice can be fully explained by the inhibition of the viral DNA polymerase by its active metabolite, tenofovir diphosphate.

DISCUSSION

Tenofovir is a common anti-HIV drug that, in the currently approved dose (300 mg tablet), suppresses HIV-1 replication *in vivo* and *in vitro* but is not reported to markedly affect herpes viruses (Balzarini et al., 1993). Recently, however, it was reported that, in the microbicide CAPRISA 004 trial, topical vaginal administration of tenofovir significantly diminished the acquisition not only of HIV-1, but also of HSV-2. We hypothesized that the discrepancy between the earlier reported lack of significant antitherpetic activity and the CAPRISA 004 data is explained by the striking differences in drug concentrations between systemic and topical applications of tenofovir.

We demonstrated that the antiretroviral drug tenofovir is indeed endowed with a direct antitherpetic activity in a variety of experimental models at drug concentrations that are lower than the median concentration achieved in cervicovaginal fluid following the administration of 1% tenofovir gel and that were nontoxic for the exposed cells (Rohan et al., 2010 and data of this study). Indeed, tenofovir levels in the cervicovaginal fluid were reported as 18.6 mg/ml.hr, measured over a 24 hr time period (AUC_{24hr}) after topical administration, and levels were still at ~ 100 μ g/ml at 24 hr after drug exposure (Schwartz et al., Abstract LBPEC03, 5th IAS, Cape Town, South Africa, 19-22 July 2009). Rather, it was shown by Dumond et al. (2007) that the steady-state genital concentration of tenofovir was ~ 100 ng/ml (24 hr), and peak concentrations of tenofovir reached ~ 500 ng/ml (6 hr) after administration of tenofovir by oral route.

Several recent publications report lower concentrations of tenofovir in the female genital tract upon *in vivo* application, with some concentrations equal to or even slightly higher than the EC_{50} that we determined *in vitro* and *ex vivo*. In this respect, our findings might explain why HSV-2 transmission prevention by tenofovir was not absolute but, rather, reached 51% (Abdool Karim, 2010), although the extrapolation of active drug concentrations *ex vivo* to the situation *in vivo* has its limitation.

We observed tenofovir activity against both laboratory and clinical HSV-1 and HSV-2 isolates (wild-type and drug-resistant thymidine kinase-deficient virus strains) in: (1) HEL cell fibroblasts, (2) primary macrophages and keratinocytes, (3) organotypic epithelial raft cultures, (4) human lymphoid and cervicovaginal tissues *ex vivo*, and (5) HSV-1- and HSV-2-infected mice. The most pronounced antitherpetic activity of tenofovir was observed in macrophages. Although HSV targets in tissues are still poorly understood and it is not known whether macrophages are important HSV targets *in vivo*, it seems that both HIV-1 and HSV-2 can infect macrophages. HIV-1 has been recovered frequently from genital herpes lesions in coinfecting individuals (Schacker et al., 1998). Cells of the M/M lineage reside in genital mucosal tissues and are thought to be reservoirs of HIV-1 in the genital tract (Lehner et al., 1991; Spira et al., 1996). Moreover, there is evidence that HSV infection can also stimulate macrophages *in vitro* and induce HIV-1 replication in these cells (Moriuchi et al., 2000). The observed marked inhibitory activity of tenofovir against HSV-2 in M/M cultures is likely due to the low endogenous dNTP pools (Perno et al., 1996) and/or to low HSV-2 replication in this cell type. Low endogenous dNTP pools give tenofovir a competitive advantage to interact with the herpetic DNA polymerase activity. Furthermore, we deciphered

that the molecular mechanism of the tenofovir antitherpetic activity: tenofovir diphosphate, to which tenofovir converted in various human cell types, efficiently inhibits HSV DNA polymerase. Concentrations of this compound in the cells were in the range of concentrations that inhibited HSV DNA polymerase in a cell-free system.

Thus, our findings provide a direct explanation for the dual anti-HIV/HSV activity reported by the CAPRISA 004 microbicide trial (Abdool Karim et al., 2010; Cates, 2010). Indeed, in our experiments, tenofovir suppressed HSV activity at concentrations of approximately ~ 10 – 200 μ g/ml, which are in the range of the drug concentrations reached in cervicovaginal fluid upon application of 1% tenofovir gel (Schwartz et al., Abstract LBPEC03, 5th IAS, Cape Town, South Africa, 19-22 July 2009). Such tenofovir concentrations were not found to be toxic in our cell models, in agreement with previous findings that a 1% tenofovir gel does not affect either PBMCs or epithelial cells (Rohan et al., 2010). Neither was any toxicity observed in the CAPRISA 004 trial.

Shortly after the publication of the CAPRISA 004 results, oral Truvada, a combination of tenofovir disoproxil fumarate (TDF) and emtricitabine, was reported to provide a 44% reduction in the incidence of HIV in case of pre-exposure chemoprophylaxis in men who have sex with men (Grant et al., 2010). In contrast to topical application, steady-state tenofovir concentrations in the genital tract following oral administration (300 mg/day) have been shown to be ~ 100 ng/ml (Dumond et al., 2007). Although tenofovir concentrations generated during oral drug administration may be sufficient for an effective systemic inhibition of HIV infection, they are substantially lower than those necessary to inhibit the replication of herpesviruses. Accordingly, prevention of HSV-2 acquisition was not reported in this trial. Also, no epidemiological evidence has emerged of concomitantly decreased incidence of HSV-2 infection in HIV-infected individuals treated with systemic (oral) tenofovir DF. In fact, it was very recently reported that oral tenofovir administered as part of combination antiretroviral therapy had no suppressive effect on HSV shedding in HIV/HSV-coinfecting asymptomatic adults (Tan et al., 2011). Also, another recent report showed that daily oral tenofovir DF (in combination with FTC) did not reduce HSV-2 acquisition among high-risk men who have sex with men, likely because TDF concentrations in the rectal or penile tissues insufficiently decrease acquisition of HSV-2 infection (Lama et al., Abstracts of the 18th Conference on Retroviruses and Opportunistic Infections, Boston, Massachusetts, USA, 27 February-2 March 2011, # 1002).

Surprisingly, in contrast to the partial prevention of HIV acquisition upon topical intravaginal administration of a 1% tenofovir gel (Abdool Karim et al., 2010) and upon oral drug administration in men who have sex with men (Grant et al., 2010), the FEM-PrEP HIV prevention study with oral Truvada has been stopped due to inability to determine effectiveness (<http://sciencespeaksblog.org/2011/04/18/fem-prep-hiv-prevention-study-halted-due-to-futility/>). No significant differences on the number of new HIV infections were observed between placebo- and drug-treated individuals. Although the reasons for this apparent discrepancy have not been revealed yet, it should be clear that the oral administration of tenofovir does not affect the rate of HSV-2 sexual transmission (Tan et al., 2011), in contrast with the topical drug administration. This conclusion will be further tested in the ongoing VOICE clinical trial, which has both topical and systemic application arms.

Although we have consistently demonstrated the antitherpetic activity of tenofovir in different systems, its potency is predictably lower than that of specific antitherpetic drugs like acyclovir. Indeed, tenofovir was designed to suppress HIV-1, not herpesviruses. However, when the drug was tested upon oral administration, the high concentrations achieved with topical application were not considered. With the CAPRISA 004 trial, it became clear that these concentrations have now become clinically relevant and have been shown to suppress not only HIV-1, but also HSV. Here, we showed that tenofovir affects HSV directly, rather than through a complex, indirect mechanism. Together, these findings argue that “marginal” antiviral activities of a variety of existing drugs should be revisited in light of possibly missed antiviral activities in topical applications as new microbicides with dual or multiple antiviral properties. In this respect, it would be important to consider acyclovir prodrugs as potential microbicidal candidates. Indeed, acyclovir, which traditionally has been regarded only as a potent antiherpesvirus inhibitor, has recently been shown to have dual antiviral properties: in lymphoid and cervicovaginal tissues coinfecting with herpesviruses, it efficiently inhibits HIV-1 as well (Lisco et al., 2008).

Thus, in this respect, like tenofovir, acyclovir prodrugs can become potential dual-targeted microbicides. Importantly, it has been shown that prodrugs of phosphorylated acyclovir that bypass the requirement of the presence of herpesvirus for drug activation (phosphorylation) release the activated form of acyclovir intracellularly and are endowed with both antitherpetic and anti-HIV activity (Derudas et al., 2009; Vanpouille et al., 2010). However, findings on the antiviral activities of various compounds in *ex vivo* models (and even in *ex vivo* human tissues that closely reflect human tissues *in vivo*) have their limitations and should be verified in clinical trials.

In conclusion, our data provide a plausible explanation for the unexpected antitherpetic activity of 1% tenofovir gel observed among treated African women participating in the CAPRISA 004 trial. Furthermore, our results provide specific considerations for designing new microbicides with a dual antiviral effect and indicate that topical creams, rather than oral administration of anti-HIV compounds, particularly tenofovir and its derivatives, may be efficient in preventing transmission of HIV-1 and its copathogens.

EXPERIMENTAL PROCEDURES

Cells

Human embryonic lung HEL-299 fibroblasts were obtained from the American Type Culture Collection (ATCC). Primary human keratinocytes (PHKs) were isolated from neonatal foreskins and cultured as previously described (Andrei et al., 2005). TZM-BI cells (Montefiori, 2009) were kindly provided by Dr. G. Van Ham, Institute for Tropical Medicine (ITG), Antwerp, Belgium.

Viruses

The HSV-1 strains KOS and F and the HSV-2 strains G and MS were used as reference herpesviruses. Several HSV-1 wild-type (RV-6, RV-132, RV-134, C559143, RV-174, RV-175), HSV-1 thymidine kinase-deficient (TK⁻) (RV-36, RV-117, 328058, RV-179), HSV-2 wild-type (RV-24, RV-124, NA, PB, NS, HSV-47) and HSV-2 TK⁻ (RV-101, RV-129, BA 19026589, LU C557672, HSV-44, RV-184, RV-185) clinical strains isolated from virus-infected individuals in Belgium were used. They were obtained as part of a translational research program (<http://www.regavir.org>) granted by the Belgian Ministry of Health as part of the National Cancer Plan for the diagnosis of drug resistance

in herpesviruses. All viruses were obtained and used as approved by the Belgian equivalent of IRB (Departement Leefmilieu, Natuur en Energie, protocol SBB 219 2011/0011, and the Biosafety Committee at K.U. Leuven). The genotype of the TK⁻ virus strains is included in Table 1. HIV-1 strains III_B and Ba-L were provided by Drs. R.C. Gallo and M. Popovic (at that time at the National Institutes of Health, Bethesda, MD).

Compounds

The sources of compounds were acyclovir (ACV, 9-2-hydroxyethoxymethyl, GlaxoSmithKline, Stevenage, UK; (S)-HPMPC (cidofovir, CDV, (S)-1-(3-hydroxy-2-phosphonylmethoxypropyl)cytosine) PMEAPMEA, (adefovir, ADV, 9-[2-(phosphonylmethoxyethyl)adenine] and (R)-PMPA (tenofovir, TFV, (R)-9-[2-(phosphonylmethoxypropyl)adenine]) Gilead Sciences, Foster City, CA. Tenofovir diphosphate (TFV-DP) and acyclovir triphosphate (ACV-TP) were obtained from Moravex Biochemicals, Brea, CA.

Radiochemicals

[³H]tenofovir (radiospecificity: 15 Ci/mmol) and [2,8-³H]dATP (radiospecificity: 153 Ci/mmol) were obtained from Moravex Biochemicals, Brea, CA.

HSV Cytopathic Effect (CPE) Measurements

The HSV-induced cytopathic effect (CPE) was evaluated in HSV-infected HEL and PHK cultures as described (Andrei et al., 2000, 2005). Briefly, cells were infected with each viral strain at 100 TCID₅₀ (1 TCID₅₀ being the 50% tissue culture infective dose, or virus dose, required to infect 50% of the number of virus-exposed cell cultures) and cultured in 96-well microtiter plates for 3 days in the presence of several concentrations of the test compounds. After the incubation period at 37°C in a CO₂-controlled (5%) humidified atmosphere, CPE was visually assessed, and the 50% effective concentration (EC₅₀, compound concentration required to reduce viral CPE by 50%) was determined.

Cytotoxicity Assays

Cytostatic activity measurements were based on the inhibition of cell growth. HEL cells and PHKs were seeded at 5 × 10³ cells/well into 96-well microtiter plates and allowed to proliferate for 24 hr. Then, medium containing different concentrations of the test compounds was added. After 3 days of incubation at 37°C, the cell number was determined with a Coulter counter. The cytostatic concentration was calculated as the CC₅₀, or the compound concentration required reducing cell proliferation by 50% relative to the number of cells in the untreated controls. CC₅₀ values were estimated from graphic plots of the number of cells (percentage of control) as a function of the concentration of the test compounds. Alternatively, cytotoxicity of the test compounds was measured on day 3 after exposure of the test compounds and expressed as the minimum cytotoxic concentration (MCC), or the compound concentration that caused a microscopically detectable alteration of cell morphology.

Cytotoxicity of tenofovir in epithelial TZM-BI cell cultures has also been measured by the MTT method. Confluent cell cultures in 96-well microtiter plates were exposed to different concentrations of tenofovir for 3–4 days, after which the cell cultures were exposed to the MTT tetrazolium dye. Viable cells convert the compound to a blue formazan derivative, which can be quantified by optical density measurements (Pannecouque et al., 2008).

Virus Yield Reduction Assays

These assays were carried out in HEL cell monolayers at different times post-infection. Cells were grown in 24-well microtiter plates and infected with two HSV-2 clinical isolates (NS and RV-124) at the indicated multiplicity of infection (moi). After 2 hr at 37°C, the cells were washed and the medium containing different concentrations of tenofovir (in duplicate) was added. Following 24, 48, and 72 hr of incubation, the viruses were released by freeze thawing and then titrated by plaque assay in HEL cells. The EC₉₀ and EC₉₉ are defined as the drug concentrations causing a 90% or 99% reduction, respectively, in virus production as measured following viral titration by plaque assay.

Herpesvirus Infection of Primary Monocyte/Macrophage Cell Cultures

Human peripheral blood mononuclear cells (PBMCs) obtained from the blood of healthy seronegative donors were seeded into 48-well plates

(1.8×10^6 cells/well). Monocytes/macrophages were separated by adherence onto plastic and estimated to be 10^5 /well. They were cultured for an additional 3 days and infected with HSV-2 (100 TCID₅₀) in the absence or presence of tenofovir. The drug was maintained throughout the experiment. CPE for macrophages was evaluated at day 6 postinfection. The amount of infectious virus in the supernatants was determined by a classical cytopathogenicity (CPE) reduction assay and by a plaque-forming units (PFU) reduction assay on Vero cell cultures (Brand et al., 2001). Details on the monocyte/macrophage preparation and antiviral activity measurements are provided in the [Supplemental Information](#).

Organotypic Epithelial Raft Cultures

Primary human keratinocyte (PHK) cells were seeded on the top of collagen gels in 24-well microtiter plates and maintained in a submerged state for 24–48 hr. The collagen rafts were then raised and placed into stainless steel grids at the interface between air and liquid culture medium. The epithelial cells were allowed to stratify as described (Andrei et al., 2005). Rafts were infected with 5,000 PFU of HSV-1_{KOS} or HSV-2_G at 10 days after lifting and treated with tenofovir. Five days later, one series of rafts was fixed in 10% buffered formalin, embedded in paraffin, and stained with hematoxylin and eosin for histological evaluation. Another series of rafts was used to quantify virus production. For that purpose, each raft was frozen in 3 ml phosphate buffered saline (PBS) and thawed to release the virus from the infected epithelium. Supernatants were clarified by centrifugation at 1,800 rpm and titrated by plaque assay in HEL cells as follows: 10-fold serially diluted samples were added to confluent monolayers of HEL cells in 96-well plates (100 μ l/well and 6 wells per dilution). The cultures were incubated at 37°C for 2 days, and the numbers of plaques were counted. Titers were calculated as PFU per milliliter of virus suspension. Virus production per raft was then calculated. Two rafts per drug concentration were used to determine the effects of the compounds on virus yield.

Human Ex Vivo Tissues

Human tonsillar tissues were obtained from patients undergoing routine tonsillectomy at the Children's National Medical Center (Washington, DC) under Institutional Review Board (IRB)-approved protocol. Cervical tissues were obtained through the National Disease Research Interchange (NDRI, Philadelphia, PA). Tissues were dissected into about $2 \times 2 \times 2$ mm blocks and placed onto collagen sponge gels in culture medium at the air-liquid interface as described earlier (Grivel and Margolis, 2009). For tonsillar tissue for each experimental condition, 27 tissue blocks (9 blocks/well filled with 3 ml of medium) were inoculated with HSV-1 (strain F) or HSV-2 (strains G and MS). In coinfection experiments, tissue blocks were sequentially infected with HSV-2_G and HIV-1_{LAI} (obtained from the Rush University Virology Quality Assurance Laboratory, Chicago, IL). For cervicovaginal tissue, 16 blocks were infected by immersion in HSV-2_G-containing medium and maintained on the gelfoam rafts. Tenofovir was added to the culture medium 12 hr prior to viral infection and replenished at each culture medium change. HSV replication was evaluated by the release of viral DNA into the culture medium as measured by quantitative real-time PCR (Lisco et al., 2008). HIV-1 replication was evaluated by the release of p24 capsid antigen using a bead-based assay (Biancotto et al., 2009).

In Vivo Antiviral Activity of HSV-1- and HSV-2-Infected Mice

Female adult NMRI athymic nude mice or hairless mice (weighing ~20 g and ~4 weeks of age) were sacrificed on the lumbosacral area over a surface of about 1 cm² with 5×10^3 PFU of HSV-1_{KOS} or 5×10^2 of HSV-2_G.

Topical formulations of tenofovir, adefovir, and cidofovir (1% in 100% DMSO to ascertain prolonged local exposure to the drugs or in a gel identical to that used in the CAPRISA 004 trial) were applied topically twice a day for 5 days, starting 1–2 hr postinfection. In each experiment, a group of animals was treated with a placebo formulation that contained exactly the same vehicle but without drug. All animal procedures were approved by the K.U. Leuven Animal Care Committee. Development of lesions and mortality were recorded over a 1 month period. Survival rates were estimated according to the Kaplan-Meier method and compared using the log-rank test (Mantel-Cox) using GraphPad Prism.

Metabolism of Tenofovir in Lymphocyte, Fibroblast, and Epithelial Cell Cultures

Metabolism of radiolabeled tenofovir was monitored as follows: CEM, HEL, or TZM-BI cells were seeded at 4×10^5 , 5.1×10^5 and 17×10^5 cells/ml, respectively, in 5 ml culture flasks (25 cm²) and incubated with 0.6 μ g/ml [^{2,8-³H}]tenofovir (10 μ Ci/flask) for 24 hr. Cells were collected and washed by centrifugation, and [³H]tenofovir and its metabolites in the culture supernatants were quantified by HPLC analysis (Balzarini et al., 1991). In a second set of experiments, tenofovir-diphosphate levels were measured in TZM-BI cell cultures that were exposed to 0.6, 6, 60, and 600 μ g/ml tenofovir for 24 hr.

HSV-1 DNA Polymerase and HIV-1 Reverse-Transcriptase Assay

The reaction mixture (40 μ l) for the HSV-1 DNA polymerase and HIV-1 RT assays contained 4 μ l Premix (200 mM Tris.HCl [pH 7.5]; 2 mM DTT; 30 mM MgCl₂), 4 μ l BSA (5 mg/ml), 1.6 μ l activated calf thymus DNA (1.25 mg/ml), 0.8 μ l dCTP (5 mM), 0.8 μ l dTTP (5 mM), 0.8 μ l dGTP (5 mM), 2 μ l radiolabeled [³H]dATP (1 mCi/ml) (3.2 μ M), 18 μ l H₂O, and 4 μ l tenofovir-DP at different concentrations (i.e., 200, 20, 2, or 0.2 μ M). In the HSV DNA polymerase assays, the inhibitory effect of tenofovir-diphosphate on herpesvirus DNA polymerase-catalyzed polymerization was also determined in the presence of radiolabeled [³H]dGTP (1 mCi/ml) (2.8 μ M), [³H]dTTP (1 mCi/ml) (1 μ M), or [³H]dCTP (1 mCi/ml) (2.5 μ M) as competing dNTP in the presence of 0.8 μ l (5 mM) of the other dNTPs in the reaction mixture as described above. The reaction was started by the addition of 4 μ l recombinant HSV-1 DNA polymerase (kindly provided by M.W. Wathen [Pfizer, Kalamazoo, MI]) or recombinant HIV-1 RT (in 20 mM Tris.HCl [pH 8.0]; 1 mM DTT; 0.1 mM EDTA; 0.2 M NaCl; or 40% glycerol), and the reaction mixture was incubated for 60 min (HSV-1 DNA polymerase) or 30 min (HIV-1 RT) at 37°C. Then, 1 ml ice-cold 5% TCA in 0.02 M Na₄P₂O₇·10 H₂O was added to terminate the polymerisation reaction, after which the acid-insoluble precipitate (radiolabeled DNA) was captured onto Whatman glass fiber filters, type GF/C (GE Healthcare UK Limited, Buckinghamshire, UK) and further washed with 5% TCA and ethanol to remove free radiolabeled dATP. Radioactivity was determined in a Perkin Elmer Tri-Carb 2810 TR liquid scintillation counter.

ACCESSION NUMBERS

The GenBank accession number for the viral *TK* sequences reported in this paper for HSV-1 mutants is JN15116–JN415119.

The GenBank accession number for the viral *TK* sequences reported in this paper for HSV-2 mutants is JN415120–JN415126.

SUPPLEMENTAL INFORMATION

Supplemental Information includes [Supplemental Experimental Procedures](#) and one figure and can be found with this article online at doi:10.1016/j.chom.2011.08.015.

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