

## Herpes Simplex Virus Type 2 Suppressive Therapy with Acyclovir or Valacyclovir Does Not Select for Specific HIV-1 Resistance in HIV-1/HSV-2 Dually Infected Persons

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**Recent in vitro studies suggest that acyclovir may directly inhibit HIV-1 replication and can select for a specific HIV-1 reverse transcriptase mutation (V75I) with concomitant loss of an anti-HIV-1 effect. We tested for HIV-1 genotypic resistance at reverse transcriptase codon 75 in plasma from 168 HIV-1-infected persons from Botswana, Kenya, Peru, and the United States taking daily acyclovir or valacyclovir for between 8 weeks and 24 months. No V75I cases were detected (95% confidence interval, 0%–2.2%). These prospective in vivo studies suggest that standard-dose acyclovir or valacyclovir does not select for HIV-1 resistance.**

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Herpes simplex virus type 2 (HSV-2) infection is common among persons with HIV-1 [1]. Acyclovir and valacyclovir are routinely used as episodic and daily suppressive treatment against genital ulcer disease caused by HSV-2. Symptomatic and asymptomatic HSV-2 reactivation are associated with increased HIV-1 levels in plasma and genital secretions, suggesting HSV-2 may heighten HIV-1 infectiousness and accelerate HIV-1 disease [2]. Five short-term studies among HIV-1/HSV-2 dually infected persons not taking antiretroviral therapy found standard doses of daily HSV-2 suppressive therapy (400–800 mg of acyclovir or 500 mg of valacyclovir twice daily) reduced plasma HIV-1 levels by .25–.5 log<sub>10</sub> copies/mL [3–7]. Another recent trial found that 400 mg of acyclovir twice daily reduced plasma HIV-1 levels by .25 log<sub>10</sub> copies/mL and the risk of HIV-1 disease progression by 16% but failed to reduce HIV-1 transmission to sexual partners [1, 8].

Acyclovir and its prodrug valacyclovir are guanidine analogues that are highly herpesvirus-specific chain terminators; they are phosphorylated intracellularly by an HSV thymidine kinase that converts them to an active form that is preferentially incorporated by HSV DNA polymerase. The effect of HSV-2 suppressive therapy in reducing HIV-1 concentrations has been thought to be mediated through decreasing HSV-2 replication and related immune activation [3–7]. However, in vitro studies have suggested that acyclovir may directly inhibit HIV-1 replication, with acyclovir exposure selecting for a single base pair mutation in HIV-1 reverse transcriptase (RT) that results in an amino acid substitution at codon 75 (V75I) that confers resistance to the anti-HIV-1 effect of acyclovir [9, 10]. Two other mutations (T69N and M184I) were also detected in a minority of sequences in these in vitro experiments. Both in vitro studies used high acyclovir concentrations; as such, whether HSV-2 suppression selects similar HIV-1 RT resistance in vivo is unknown. Using a highly sensitive oligonucleotide ligation assay (OLA) [11], we screened for the V75I mutation in plasma samples from 168 HIV-1 infected persons from Botswana, Kenya, Peru, and the United States exposed to daily acyclovir or valacyclovir for periods of 8 weeks to 24 months.

### METHODS

Subjects were from 3 cohorts of HIV-1/HSV-2 dually infected persons participating in clinical trials of daily HSV-2 suppressive therapy:

#### US Cohort

An ongoing, randomized, open-label, crossover trial of HSV-2 suppressive therapy dose on plasma HIV-1 levels is being

conducted among HIV-1/HSV-2 dually infected persons (ClinicalTrials.gov identifier NCT00527618). Participants were randomized to 1000 mg of valacyclovir or 400 mg of acyclovir twice daily for 12 weeks, and after a 2-week washout, the alternative arm for 12 weeks. Participants were  $\geq 18$  years of age, not pregnant, and not taking antiretroviral or anti-HSV medications. For this analysis, plasma from the final follow-up visit in the study was tested from 14 participants who had completed the study.

### Peru Cohort

Two randomized, double-blind, placebo-controlled, crossover trials of HSV-2 suppressive therapy were conducted among 39 HIV-1/HSV-2 dually infected men and women between 2003 and 2005 (ClinicalTrials.gov identifiers NCT00378976 and NCT00465205) [3, 5, 12]. Participants were randomized to 500 mg of valacyclovir or placebo twice daily for 8 weeks, followed by a 2-week washout, and then the alternative arm for 8 weeks. Participants were  $\geq 18$  years of age, had CD4 cell counts of  $>200$  cells/ $\mu\text{L}$ , and were not taking antiretroviral or anti-HSV medications. Valacyclovir decreased HIV-1 concentrations in plasma (by .33 [men] and .26 [women]  $\log_{10}$  copies/mL) as well as in seminal, cervical, and rectal samples. A plasma sample from the last follow-up visit during the valacyclovir period was selected.

### Botswana and Kenya Cohort

Between 2004 and 2008, 3408 African HIV-1- and HSV-2-infected persons and their HIV-1 seronegative heterosexual partners were enrolled and followed up in the Partners in Prevention HSV/HIV Transmission Study, a randomized, double-blind, placebo-controlled trial of acyclovir HSV-2 suppressive therapy to prevent HIV-1 transmission (ClinicalTrials.gov identifier NCT00194519) [1]. HIV-1-infected partners were  $\geq 18$  years of age, had a CD4 cell count of  $\geq 250$  cells/ $\mu\text{L}$ , and were not taking antiretroviral therapy at study entry. HIV-1 infected partners were randomized to 400 mg of acyclovir or placebo twice daily and seen monthly for up to 24 months. The trial demonstrated no significant effect of acyclovir in preventing HIV-1 transmission. For this analysis, plasma samples from the final study visit were tested for 138 randomly selected HIV-1 infected partners from the acyclovir arm; to increase the likelihood of identifying HIV-1 resistance mutations as a result of acyclovir exposure, samples were chosen from participants with ongoing replication of  $>25,000$  copies/mL of HIV-1 RNA by polymerase chain reaction (PCR). Participants were from 1 country in southern Africa (Botswana,  $n = 46$ ) and 1 country in East Africa (Kenya,  $n = 92$ ) to capture different HIV-1 subtypes, specifically subtype C in Botswana and subtypes A and D in Kenya. For these 138 participants, adherence to the study product was high (96.9% of dispensed doses taken), and, compared to placebo arm participants, the incidence of genital ulcer disease due to HSV-2 was reduced by 74% (95% CI,

43%–88%;  $P < .001$ ; log-linear regression), an effect similar to the 73% reduction for the overall acyclovir arm [1]. Additional samples from the placebo arm were tested to preserve blinding of laboratory technicians (data not shown).

For all cohorts, participants were receiving acyclovir or valacyclovir suppressive therapy on the day the plasma sample was obtained. Study protocols were approved by institutional review boards at the University of Washington and collaborating sites. Participants provided written informed consent.

**Laboratory analyses.** HIV-1 and HSV-2 serostatus was confirmed by Western blot analysis. CD4 quantification was performed using standard flow cytometry, and plasma HIV-1 RNA was quantified using real-time PCR assays [1, 3, 5].

HIV-1 viral RNA was obtained from plasma via silica extraction, and cDNA from the *pol* gene was generated (GeneAmp RNA PCR Core kit, Applied Biosystems). A 1010-bp DNA fragment extending from nucleotides 2256 through 3265 of the HXB-2 numbering system (amino acid 2 in protease to amino acid 239 in RT) was amplified, as described elsewhere [13].

For all 3 cohorts, detection of the V75I mutation was performed using a highly sensitive OLA (<http://depts.washington.edu/idimmweb/faculty/frenkel/OLAmanual1305april04.pdf>) [11] for HIV-1 subtypes A, B, C, and D, developed using 2 differentially modified genotype-specific probes (5'-digoxigenin-AYAGCACTAARTGGAGRAAATTAG-3' for wild-type, and 5'-fluorescein-AYAGCACTAARTGGAGRAAATTAA-3' for mutant) and a biotin-labeled common probe (5'-phosphate-TAGATTTYAGRGARCTCAATAAAAGA-biotin-3'). In brief, the genotype-specific oligonucleotide probes were annealed to their complementary sequence in the subject's PCR product and covalently joined by a thermostable Ampligase DNA Ligase (Epicentre Technologies) to the biotinylated common probe. The ligated products were then captured on a streptavidin-coated microtiter plate, where enzyme-linked immunosorbent assay, using reporter-specific antibodies labeled with alkaline phosphatase or horseradish peroxidase, allowed sequential colorimetric detection of both genotypes in a single well. PCR-amplified plasma HIV-1 RNA from clinical specimens with the mutant or wild-type sequence were cloned into the pCR4-TOPO vector (TOPO-TA Cloning Kit, Invitrogen) and used as controls in the OLA. Amplicons were tested in duplicate in 96-well plates containing standards with mixtures of the mutant and wild-type plasmids at concentrations of 0%, 2%, 5%, and 100% mutant. Samples with optical density values of  $\geq 2\%$  mutant control were considered positive for a G to A mutation encoding V75I, whereas samples with negative reactions for both the mutant and wild-type genotypes were defined as indeterminate, which implied genetic polymorphisms within 2–3 bases of the ligation site. All specimens from the US and Peru cohorts, as well as samples with indeterminate OLA results from the Botswana and Kenya cohort, were also analyzed by consensus sequencing (sensitivity

**Table 1. Characteristics of 168 HIV-1/HSV-2 Dually Infected Persons Exposed to 8 Weeks to 24 Months of Daily HSV-2 Suppressive Therapy with Acyclovir or Valacyclovir**

	US (n = 14)	Peru (n = 31)	Botswana and Kenya (n = 123)
Age [y], median (IQR)	47 (39–53)	29 (25–33)	31 (27–36)
Male, n (%)	12 (86%)	16 (52%)	44 (36%)
CD4 [cells/ $\mu$ L], median (IQR)	463 (349–732)	377 (316–492)	361 (264–479)
Plasma HIV-1 RNA, at the time of HIV-1 resistance testing [ $\log_{10}$ copies/mL], median (IQR)	4.2 (3.6–4.7)	4.5 (4.1–5.0)	4.9 (4.6–5.2)
Acyclovir or valacyclovir dosage and duration of exposure	Acyclovir 400 mg twice daily $\times$ 12 weeks and Valacyclovir 1000 mg twice daily $\times$ 12 weeks	Valacyclovir 500 mg twice daily $\times$ 8 weeks	Acyclovir 400 mg twice daily $\times$ up to 24 months (median 22 months, IQR 18–24 months)

**NOTE.** IQR, interquartile range.

estimated to be ~20% of viruses in the sample) with fluorescence-labeled dideoxy-chain terminators (ABI PRISM BigDye Terminator Cycle Sequencing Ready Reaction Kit, Applied Biosystems) [13].

## RESULTS

OLA testing for the HIV-1 RT V75I mutation was performed on plasma samples from 168 HIV-1/HSV-2 dually infected persons exposed to daily acyclovir or valacyclovir for between 8 weeks and 24 months, including 14 persons from the United States, 31 from Peru, and 123 from Botswana/Kenya (Table 1). An additional 8 Peru and 15 Botswana/Kenya samples did not amplify by PCR and could not be evaluated. No cases of the V75I substitution were detected (95% CI, 0%–2.2%). Seven samples had indeterminate results by OLA (ie, negative reactions for both the mutant and wild-type strains). Sequence analysis of these 7 samples demonstrated no cases of the GTA  $\rightarrow$  ATA substitution encoding V75I (Table 2). Three of these 7 had a synonymous polymorphism at position 75 (GTA  $\rightarrow$  GTG) encoding the wild-type amino acid valine.

By consensus genotyping, no US or Peru samples had the V75I substitution. Only 2 US samples had mutations conferring resistance to nucleoside or nonnucleoside reverse transcriptase

inhibitors (NRTIs or NNRTIs)—1 with M184V and 1 with K103N; both of these mutations were present in enrollment visit samples, prior to acyclovir or valacyclovir exposure. No cases of T69N or M184I were observed.

## DISCUSSION

In this prospective evaluation of HIV-1/HSV-2 dually infected men and women from Botswana, Kenya, Peru, and the United States exposed to daily HSV-2 suppressive therapy for between 8 weeks and 24 months, we found no evidence of selection of HIV-1 genotypic resistance, specifically the V75I substitution. Our study is the among the first assessments of whether standard doses of acyclovir and valacyclovir suppressive therapy select for specific HIV-1 resistance mutations in vivo, and our results thus do not confirm in vitro studies that found that high-dose acyclovir had direct anti-HIV-1 activity and selected V75I mutants resistant to acyclovir.

The 2 recent in vitro studies that found that acyclovir may directly inhibit HIV-1 replication hypothesized that either inefficient cellular enzymes or viral kinases from other ubiquitous herpes-group viruses (eg, human herpesvirus 6) phosphorylate acyclovir [9, 10]. In one of these reports, HIV-1 culture in the presence of acyclovir resulted in selection of a specific HIV-1 RT

**Table 2. Consensus Genotyping Results for 7 Samples with Indeterminate OLA Results at Codon 75**

Wild-type	AYAGCACTAARTGGAGRAAATTA <b>GT</b> AGATTTYAGRGARCTCAATAAAAGA
Mutant	----- <b>A</b> -----
Botswana (subtype C)	-C--T---G-----A-----C--T--G--G--T-----
Kenya1 (subtype CRF01A_E)	-T---A-----G----- <b>G</b> ---C--A--G--T-----
Kenya2 (subtype A)	-T--Y---A-----A-----C--A--G-----
Kenya3 (subtype A)	-C--TG---A-----A----- <b>G</b> ---C--A--G-----G
Kenya4 (subtype A)	-T---R--A-----G---G---Y--C--A--G-----
Kenya5 (subtype CRF01A_E)	-T-----A-----A--GC--- <b>G</b> ---C--A--G-----
US (subtype B)	-C--T---A-----A---G-----C--A--A--T---G---

**NOTE.** Codon 75 sequences are given in boldface font.

mutation (V75I) that became the predominant genotype by 94 days [9]. In a single-cell HIV-1 infectivity assay, virus with the V75I mutation demonstrated phenotypic resistance to acyclovir. These results suggested that therapy with acyclovir in the absence of antiretroviral therapy could lead to HIV-1 that is resistant to the effect of acyclovir on decreasing HIV-1 replication. One explanation for the difference between those in vitro findings and our in vivo results may be that the in vitro studies used high concentrations of acyclovir, concentrations not achieved with standard doses used for HSV-2 treatment [14].

The mutations selected in the in vitro experiments confer cross-resistance to NRTIs: V75I contributes resistance to multiple NRTIs in the presence of a concurrent Q151M mutation, T69N and other variants at position 69 are selected by didanosine [15] and other NRTIs but their susceptibility profiles are not well described (<http://hivdb.stanford.edu/>), and the variants M184I/V confer high-level resistance to lamivudine and emtricitabine. Thus, the authors of the in vitro studies suggested caution in the use of acyclovir for HIV-1 infected persons not on effective antiretroviral therapy. While more studies are warranted, potentially including subcloning and sequencing to definitively document that no HIV-1-resistant variants evolve under acyclovir pressure, our results reassuringly suggest that HIV-1 resistance does not commonly arise in HIV-1 infected persons taking standard doses of acyclovir or valacyclovir who are not taking antiretroviral therapy.

Our study is unique in the geographic diversity of study subjects, reflecting HIV-1 subtypes A, B, C, and D. The duration of acyclovir or valacyclovir exposure was long (up to 24 months of daily therapy, including a median 22 months for the 123 African participants), and the cohorts had high adherence to daily HSV-2 suppression [1, 3, 5]. Of note, in our multisite African study [1], including the participants from Botswana and Kenya who were included in the present analysis, acyclovir reduced plasma HIV-1 levels by an average of .25 log<sub>10</sub> copies/mL, and this effect persisted throughout 24 months of follow-up. This finding suggests that standard HSV-2 suppression does not result in loss of an acyclovir effect on plasma HIV-1 levels over up to 24 months of daily dosing, arguing against the development of HIV-1 resistance as a result of acyclovir exposure.

In conclusion, daily HSV-2 suppressive therapy with standard-dosage acyclovir or valacyclovir for up to 2 years did not result in HIV-1 genotypic resistance when provided to HIV-1/HSV-2 dually infected persons from the United States, Peru, and Africa. As such, clinicians can be reassured that suppressive therapy for HSV-2 infection does not compromise the effectiveness of antiretroviral therapy in persons with HIV-1.

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## Authorship

All authors contributed to the design and execution of the study. Data and specimens were collected by J.M.B., J.L., C.C., A.W., K.F., E.W., N.M., J.S., M.E., J.M., J.K., C.F., and L.C.; I.B., L.F., and G.P. developed the assay and performed the laboratory testing. J.M.B. completed the first draft of the manuscript. All authors contributed to the analysis and interpretation of the data and to the final draft of the article.

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