Valacyclovir Decreases Plasma HIV-1 RNA in HSV-2 Seronegative Individuals: A Randomized Placebo-Controlled Crossover Trial

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Background. Acyclovir (ACV), a highly specific anti-herpetic drug, acts as a DNA chain terminator for several human herpesviruses (HHVs), including HHV-2 (HSV-2), a common human immunodeficiency virus (HIV)-1 copathogen. Several trials demonstrated that HSV-2 suppressive therapy using ACV or its prodrug valacyclovir (valACV) reduced plasma HIV-1 viral load (VL) in HIV-1/HSV-2 coinfected persons, and this was proposed to be due to a decrease in generalized immune activation. Recently, however, we found that ACV directly suppresses HIV-1 ex vivo in tissues free of HSV-2 but endogenously coinfected with other HHVs. Here, we asked whether valACV suppresses VL in HIV-1 infected HSV-2-seronegative persons.

Methods. Eighteen HIV-1 infected HSV-2-seronegative individuals were randomly assigned in a double blind placebo-controlled, crossover trial. Eligible participants had CD4 cell counts of ≥500 cells/µL and were not taking antiretroviral therapy. Subjects in group A received 12 weeks of valACV 500 mg given twice daily by mouth followed by 2 weeks of no treatment washout and then 12 weeks of placebo; subjects in group B received 12 weeks of placebo followed by 2 weeks of no treatment washout and then 12 weeks of valACV 500 mg twice daily.

Results. HIV-1 VL in plasma of patients treated with valACV 500 mg twice daily for 12 weeks was reduced on average by 0.37 log10 copies/mL.

Conclusions. These data indicate that the effects of valACV on HIV-1 replication are not related to the suppression of HSV-2-mediated inflammation and are consistent with a direct effect of ACV on HIV-1 replication.

Keywords. HIV-1; HSV-2; acyclovir; herpesvirus; reverse transcriptase inhibitor.

Acyclovir (ACV) was the first safe, potent, and specific antiviral nucleoside analogue to be approved for clinical use [1]. It is a guanosine analog that is efficiently phosphorylated by herpesvirus kinases and acts as specific chain terminator for several human herpesviruses (HHV), including HHV-1, -2 and -3 (respectively, herpes simplex viruses [HSV]-1 and 2 and Varicella zoster virus [VZV]). In the early years of the AIDS epidemic, ACV was tested in vitro for activity against human immunodeficiency virus type 1 (HIV-1) and found to be ineffective [2–4].

HHVs, in particular HSV-2, are among the most common co-pathogens in HIV-1 infected persons, and ACV is commonly used in HIV-1/HSV-2 coinfected patients to treat and prevent symptomatic HSV-2 infection. Several retrospective studies found that ACV treatment of HIV-1 infected individuals was associated with increased survival [5, 6]. These early observations were then corroborated by numerous more recent randomized trials, which demonstrated that HSV-2 suppressive therapy using ACV or its prodrug valacyclovir (valACV) reduced plasma HIV-1 viral load (VL) by 0.25 to 1.23 log10 RNA...
copies/mL in HIV-1/HSV-2 coinfected persons and delayed HIV-1 disease progression [7–20].

Because ACV has been thought to be inactive against HIV-1, these results were attributed to a decrease of general inflammation due to the suppression of HSV-2 replication (reviewed in [21]).

Surprisingly, we found that in human tissues studied ex vivo, ACV suppresses HIV-1 by directly inhibiting HIV-1 reverse transcriptase (RT) provided that ACV is phosphorylated [22]. These results were confirmed in another ex vivo system [23]. HHV thymidine kinases (TK) are particularly efficient in phosphorlyating ACV, and coinfecting herpesviruses present in tissues can supply T cells with sufficient phosphorylated ACV to inhibit HIV [22]. Coinfecting herpesviruses may not be necessary to activate ACV as McMahon et al (2011) recently showed that triphosphorylated ACV could also be detected within cells even in the absence of HHVs [24].

In the present study, we hypothesized that if a similar mechanism occurred in vivo, the suppressive effect of ACV on HIV-1 replication should not be limited to HSV-2 coinfect individuals but should also be demonstrable in HSV-2-seronegative persons. Here, we tested this hypothesis. We undertook a randomized, placebo-controlled, crossover trial to evaluate the impact of valACV on HIV-1 viremia in HSV-2-seronegative persons not receiving antiretroviral therapy (ART).

**METHODS**

**Participants**

Twenty-one HIV-1–infected HSV-2 seronegative subjects 18 years and older were enrolled at Asociación Civil Impacta Salud y Educación, in Lima (Peru) and at University Hospitals Case Medical Center in Cleveland Ohio between June 2009 and July 2012. All participants provided written informed consent. The IRBs of the participating institutions approved the study. Inclusion criteria were confirmed HIV-1 infection, absence of serum antibodies for HSV-2, plasma HIV-1 RNA ≥1000 copies/mL, and CD4 cell count ≥500 cells/mm³. We excluded women who were breast-feeding and those of reproductive potential who were not using a reliable contraceptive method, persons with AIDS-defining illnesses, and individuals who had received or were receiving ART, and those who had previous adverse reactions to ACV or valACV.

**Study Design**

This was a randomized, double-blind, placebo-controlled clinical trial with a crossover design to evaluate the effect of 12 weeks of valACV administration on VL in chronically HIV-1 infected subjects not receiving ART. Twenty-one HIV-infected male and female subjects were divided in 2 groups: Group A received 12 weeks of valACV 500 mg given twice daily by mouth followed by 2 weeks of no treatment washout and then 12 weeks of placebo; Group B received 12 weeks of placebo followed by 2 weeks of no treatment washout and then 12 weeks of valACV 500 mg given twice daily by mouth. ValACV and matched placebo were provided by Glaxo SmithKline Inc. and were stored and distributed by the site pharmacies.

**Screening and Follow-up**

Baseline laboratory evaluations were obtained and blood was also collected in EDTA containing tubes at weeks 11, 12, 14, 25, and 26. All samples were frozen on site and at the end of the trial were sent for laboratory evaluation. For each patient, a 1 mL sample of coded plasma was shipped to Case Western Reserve University, Cleveland, Ohio for HHV serological tests and a second 1 mL sample was shipped to the Laboratory of Biochemical Pharmacology, Department of Pediatrics at Emory University, Atlanta, Georgia for HIV-1 ultra-deep sequence analyses of RT. Peripheral blood monocular cells (PBMCs) were cryopreserved on site and shipped to the National Institutes of Health (NIH) for HHV DNA VL quantification. Adherence was assessed by participant’s self-report.

**Laboratory Analyses**

*Nucleic Acid Extraction and Real-Time Quantitative Polymerase Chain Reaction (PCR)*

HHV DNA extraction and VL measurement were performed at NIH as described elsewhere [22] (see Supplementary Data).

**HIV-1 Viral Load Measurement**

Levels of HIV-1 RNA in plasma were measured in the 2 clinical laboratories, in Lima (Peru) and Cleveland (Ohio) using Food and Drug Administration (FDA)-approved commercial assays (Roche, Abbott).

**Ultra-deep Sequencing and Data Analysis**

These assays were performed to search for antiretroviral drug resistance mutations (DRMs). Plasma samples from 15 HIV-1 infected subjects were stored in aliquots at −80°C until further processing (see Supplementary Data).

**Statistical Analysis**

We used medians and interquartile ranges (IQRs) to describe the data. The outcome of interest was the change in plasma HIV-1 RNA from baseline (at study entry) to week 12 and from the end of the washout period (at week 14) to week 26. To assess the effects of the treatment and period, we used graphical exploration as previously described [25]. To analyze the effect of treatment without correction, we used the approach of Senn [26] to compare the change in plasma HIV-1 RNA during treatment with valACV to the change during treatment with placebo. We then fitted an appropriate regression model to account for the possible effect of the treatment period. We tested for the presence of carryover and other treatment-by-period
interaction and found no effects on the significance of the viral load difference in the two groups of patients. All tests were 2-sided without correction for multiple comparisons, and P-values <.05 were reported as statistically significant.

RESULTS

Study Participants

The characteristics of the 21 subjects who enrolled in the study are presented in Table 1. The groups were well balanced with regard to demographic characteristics, CD4+ T-cell count, and plasma HIV-1 RNA level at baseline. There were no significant imbalances by study site other than ethnic background.

Two subjects discontinued the study after randomization and before week 12 and were excluded from the analyses. Another subject was lost to follow-up after randomization and returned to the site only at week 25, when a premature discontinuation visit was completed. There were no study-related toxicities.

Valacyclovir Decreases Plasma HIV-1 RNA Levels

The HIV-1 levels pre- and post-valACV treatment for the above-described 18 individuals are shown in Table 2. The median VL pre- and post-valACV treatment were respectively 4.09 (IQR, 3.42–4.62) and 3.77 (IQR, 3.15–4.33). As shown in Figure 1, administration of valACV was associated with a modest but statistically significant decline in HIV RNA (estimated HIV RNA change during active treatment −0.37 log10 copies/mL, 95% confidence interval, −0.62, −0.11; P = .009) (Figure 1). We then assessed the model for the presence of a period effect, following the Hills–Armitage approach [27] and found that there was no evidence for a significant period effect (P = .958). Similarly, we fitted a model including an estimate of carryover effect and did not find statistically significant evidence for it (P = .06). Of note, one subject became seropositive for HSV-2 during follow-up. Because the seroconversion was discovered when HSV-2 serologies were repeated at week 26, we could not determine when in the course of trial the seroconversion occurred. Plasma HIV-1 RNA in this patient increased during ACV treatment. When in the course of trial the seroconversion occurred. Plasma HIV-1 RNA in this patient increased during ACV treatment. If we had done so, the overall plasma HIV-1 RNA decrease would have been −0.40 log10.

Table 1. Subject Characteristics

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Arm A</th>
<th>Arm B</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>ValACV Then Placebo, n = 11</td>
<td>Placebo Then ValACV, n = 10</td>
</tr>
<tr>
<td>Age, yearsa</td>
<td>30.4 (22.5–44.9)</td>
<td>30.9 (24.5–38.5)</td>
</tr>
<tr>
<td>Male sex, n (%)b</td>
<td>10 (90.9)</td>
<td>7 (70)</td>
</tr>
<tr>
<td>Race, n (%)b</td>
<td></td>
<td></td>
</tr>
<tr>
<td>White</td>
<td>0 (0)</td>
<td>2 (20)</td>
</tr>
<tr>
<td>Black</td>
<td>2 (22.2)</td>
<td>1 (10)</td>
</tr>
<tr>
<td>Mixed</td>
<td>7 (77.8)</td>
<td>7 (70)</td>
</tr>
<tr>
<td>Hispanic ethnicity, n (%)b</td>
<td>7 (77.8)</td>
<td>7 (70)</td>
</tr>
<tr>
<td>Body mass index, kg/m²b</td>
<td>26.2 (23–28.8)</td>
<td>26.5 (24.5–27.9)</td>
</tr>
<tr>
<td>Baseline CD4+ T-cell count, cells/mm³b</td>
<td>538 (534–650)</td>
<td>635 (594–792)</td>
</tr>
<tr>
<td>Baseline plasma HIV-1 RNA, log₁₀ copies/mLb</td>
<td>4.39 (3.62–4.87)</td>
<td>4.36 (3.76–4.61)</td>
</tr>
</tbody>
</table>

Abbreviations: HIV-1, human immunodeficiency virus type 1; ValACV, valacyclovir.

a Presented are medians and IQR.

Table 2. Changes in Plasma HIV-1 RNA Levels

<table>
<thead>
<tr>
<th>Patients</th>
<th>HIV-1 Viral Load at Baseline</th>
<th>HIV-1 Viral Load After ValACV Treatment</th>
<th>HIV-1 VL Changes (log₁₀)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>3.34</td>
<td>3.78</td>
<td>0.44</td>
</tr>
<tr>
<td>2</td>
<td>3.45</td>
<td>3.73</td>
<td>0.28</td>
</tr>
<tr>
<td>3</td>
<td>4.09</td>
<td>4.36</td>
<td>0.27</td>
</tr>
<tr>
<td>4</td>
<td>4.86</td>
<td>5.04</td>
<td>0.18</td>
</tr>
<tr>
<td>5</td>
<td>3.94</td>
<td>3.99</td>
<td>0.05</td>
</tr>
<tr>
<td>6</td>
<td>4.93</td>
<td>4.67</td>
<td>−0.26</td>
</tr>
<tr>
<td>7</td>
<td>3.28</td>
<td>3.01</td>
<td>−0.27</td>
</tr>
<tr>
<td>8</td>
<td>4.65</td>
<td>4.38</td>
<td>−0.27</td>
</tr>
<tr>
<td>9</td>
<td>4.61</td>
<td>4.32</td>
<td>−0.29</td>
</tr>
<tr>
<td>10</td>
<td>2.92</td>
<td>2.50</td>
<td>−0.42</td>
</tr>
<tr>
<td>11</td>
<td>4.10</td>
<td>3.65</td>
<td>−0.45</td>
</tr>
<tr>
<td>12</td>
<td>3.82</td>
<td>3.32</td>
<td>−0.50</td>
</tr>
<tr>
<td>13</td>
<td>4.76</td>
<td>4.21</td>
<td>−0.55</td>
</tr>
<tr>
<td>14</td>
<td>4.46</td>
<td>3.79</td>
<td>−0.67</td>
</tr>
<tr>
<td>15</td>
<td>4.44</td>
<td>3.76</td>
<td>−0.68</td>
</tr>
<tr>
<td>16</td>
<td>3.53</td>
<td>2.69</td>
<td>−0.84</td>
</tr>
<tr>
<td>17</td>
<td>3.34</td>
<td>2.43</td>
<td>−0.91</td>
</tr>
<tr>
<td>18</td>
<td>4.38</td>
<td>3.20</td>
<td>−1.18</td>
</tr>
</tbody>
</table>

Abbreviations: HIV-1, human immunodeficiency virus type 1; valACV, valacyclovir; VL, viral load.

HHV DNA in PBMC of the Enrolled Subjects

HHV DNAs were measured in PBMCs at enrollment. The limits of detection of the PCR assays for HHV DNAs were between 5 and 10 copies depending on the virus [22]. HSV-2 positive individuals were excluded from the trial; as expected, we did not find HSV-2 DNA in PBMC of any enrolled individuals. Also, HSV-1, VZV, or cytomegalovirus (CMV) DNA was not found. Other HHV DNAs were identified however. Epstein–Barr virus (EBV) DNA was present in PBMC of all 18 subjects. HHV-7 and HHV-6 DNAs were found in PBMCs samples of 15 and 6 individuals, respectively. HHV-8 DNA was found in one
patient sample. Levels of viral DNA varied significantly for each HHV. EBV DNA content ranged from 127 to 8692 copies per $10^6$ erv-3 cellular genes; HHV-7 ranged from 447 to 7067 copies per $10^6$ erv-3 cellular genes; HHV-6 ranged from 44 to 146 copies per $10^6$ erv-3 cellular genes. No correlations were observed between the HHV DNA copy number and the change in HIV-1 VL during the treatment.

Seroprevalence for HHVs was determined at enrollment and at completion of the study. We found a prevalence of anti-EBV, anti-CMV, anti-HHV-6, and anti-HSV-1 antibodies, respectively, of 94% (17/18), 89% (16/18), 83% (15/18), and 78% (14/18) at enrollment. One patient seroconverted for HSV-2 during the study.

The concordance between the HHV DNA in PBMC and seropositivity varied for different HHVs: EBV DNA and serology were concordant in all tested patients except one in which serology was negative while PCR was positive for EBV DNA. A similar pattern was observed for HHV-6 in which a positive serology was found in 5 out of the 6 patients who had a viral DNA PCR.

PCR and serology were discordant for CMV and HSV-1 as no viral DNA was detected by PCR in any of the tested samples, whereas serology was positive in 89% and 78% of the study subjects.

**Emergence of Drug Resistance Mutations in HIV-1 RT**

Prolonged exposure to high dose ACV in vitro has been associated with the development of resistance mutations in HIV-1 RT [28, 29]. Here, we sequenced the HIV-1 RT in plasma samples from enrolled subjects to investigate whether ACV selective pressure is associated with the emergence of resistance mutations in vivo. Conventional sequencing determined that all subjects were infected with clade B HIV-1 (data not shown). Ultra deep pyrosequencing was successfully performed in a total of 34 samples. Nine samples (from 6 different subjects) of the 34 samples analyzed (26%) exhibited DRM with a score of $\geq 1\%$ based on Stanford v6.3.

DRM were found in 9 samples from 6 subjects. In group A, one subject had V118I (2.5%) at week-26 post-placebo; a second subject had D67N (19.9%) and K70R (99.7%) at week-0 pre-valACV treatment, and a third subject had 5 DRM (T69D (1.1%), T69N (98.4%), K101E (13.7%), V108I (5.1%), V118I (15.4%)) at week-0 pre-valACV and 3 DRM at week-12 post-valACV (T69D (29.2%), T69N (70.2%), V118I (13.7%)), and the same 3 DRM at week-26 post-placebo (T69D (14.8%), T69N (84.8%), V118I (36.7%)).

In group B, in 3 subjects with DRM, there was V75I (19.9%) at week-0 pre-placebo, and K70R (98.3%) at week-26 post-valACV in 1 subject. The second subject also had V75I (5.9%) at week-26 post-valACV. The third subject had DRM K70R (5.4%) at week-26 post-valACV.

**DISCUSSION**

Accumulating evidence suggests that a number of copathogens including viruses can affect HIV transmission and pathogenesis. In particular, HSV-2 infection increases the efficiency of both acquisition and transmission of HIV-1 and is associated with...
increased plasma and genital HIV-1 levels (reviewed in [21]). The effect on acquisition and transmission was attributed to the local disruption of epithelial genital barriers and to the enrichment of submucosal layers with activated immune cells, whereas the effect on HIV-1 replication was ascribed to recruitment of target cells and increased systemic inflammation [21]. Therefore, suppression of HSV-2 with ACV seemed to be a meaningful strategy for both reducing HIV-1 transmission and delaying HIV-1 disease progression by reducing HIV-1 levels.

Over the past 7 years, at least 14 clinical trials have demonstrated that ACV treatment, though ineffective in preventing HIV-1 transmission and acquisition [13, 14, 30], resulted in reduced genital and plasma HIV-1 levels [7–20] and in some cases, delayed HIV-1 disease progression in coinfected individuals [5, 6, 14, 19, 31].

Until recently, the decrease of HIV-1 VL by ACV has been attributed to the suppression of HSV-2-triggered systemic inflammation as no direct effect of ACV on HIV-1 replication could be documented in in vitro studies.

We recently reported in studies utilizing human tissues ex vivo that ACV suppresses HIV-1 replication directly and acts as an HIV-1 RT inhibitor [22]. In those ex vivo systems, HIV-1 suppression did not require HSV-2 but was conditioned to the presence of other HHVs.

Here we evaluated the effect of valACV, a prodrug of ACV, on plasma HIV-1 RNA levels in HIV-1-infected individuals not co-infected with HSV-2. ValACV was used because of its enhanced bioavailability relative to ACV.

On average, valACV reduced HIV-1 VL in plasma by 0.37 log10 copies/mL. Plasma HIV-1 concentrations rebounded to pre-enrollment levels within 2 weeks of termination of valACV treatment, consistent with short drug half-life and lack of intracellular accumulation. Although the effect of valACV on plasma HIV-1 RNA is modest, it is comparable to monotherapy with other antiretrovirals. For example, an approximately 0.5 log10 decrease in HIV-1 viremia was reported for either zidovudine or other antiretrovirals. For example, an approximately 0.5 log10 decrease in HIV-1 viremia was reported for either zidovudine or other antiretrovirals.

ValACV had no impact on the fraction of activated CD8+ T cells, hsCRP, interleukin 6 (IL-6), or sICAM-1, or on other immune and inflammatory markers [36]; (ii) Although treatment with valACV of HIV-1 coinfected pregnant women was associated with lower plasma HIV-1 RNA levels, the frequencies of both CD4+ and CD8+ CD38+ HLA-DR+ T cells in these subjects was not different from those in placebo recipients [37]; (iii) Although both ACV and valACV completely suppressed genital HSV-2 shedding, valACV treatment was associated with a greater reduction in HIV-1 VL than ACV [20].

Our trial supports the evidence that ACV directly inhibits HIV-1 in vivo and that HSV-2 is not required for this effect. As expected, however, we found that almost every subject enrolled in this study carried one or several other HHVs, as evaluated by the presence of HHV DNAs. Moreover, serology showed the presence of anti-HHV antibodies, an evidence of HHV infection history. Although we previously demonstrated that HHVs mediate phosphorylation of ACV and are sufficient and necessary to confer anti-HIV-1 activity to ACV ex vivo, we did not find any correlation between the pattern of infection with different HHVs and the extent of HIV-1 VL reduction in our trial. It remains unknown whether the TK of these HHVs is sufficient to mediate ACV phosphorylation, or if an unknown cellular kinase [24] is critical for ACV phosphorylation and the subsequent anti-HIV-1 effect observed in vivo.

Another important question is whether ACV administration may lead to the selection of resistance mutations. In in vitro studies, a V75I mutation in RT renders HIV-1 less sensitive to ACV; this mutation was selected under the pressure of supra-therapeutic dose of ACV or its produg derivative in both single cell cultures of CD4+ lymphoblasts and in the MT-4 cell line [28, 29]. In contrast, in vivo HSV-2 suppressive therapy using therapeutic concentrations of ACV or valACV did not select for specific HIV-1 resistance in HIV-1/HSV-2 coinfected persons [38–40]. To assess whether or not HIV-1 DRM were selected by valACV therapy in this study, we performed ultradeep sequencing of the HIV-1 RT. Although our findings of decreased HIV-1 levels in plasma imply that there was some selection pressure on HIV-1 and presumably on its RT, our findings were consistent with the 3 previously published studies, which showed that valACV did not select for specific HIV-1 resistance mutations [38–40]. Indeed, in the present in vivo study, the V75I mutation was only found in 2 individuals and at low frequency (<20%); one appeared only at baseline of week-0 pre-placebo, and the other only at week-26 post-ACV. Despite these findings, we cannot discount the possibility that a more pronounced evolution of specific HIV-1 ACV resistance mutations might have been observed following a longer period (>12 weeks) of valACV monotherapy. The discrepancy between in vitro and in vivo results may be explained by the weaker selective pressure exerted by low-dose ACV and a significant fitness cost.
that may outweigh the advantage of ACV resistance. Indeed, a comparison of the fitness of wild-type vs V75I virus in the presence of ACV in the single round infectivity assay showed that replication of wild-type virus is favored over the mutant [29].

Currently, the primary indication for use of ACV or valACV in HIV-1 infected individuals remains treatment of HSV-2 clinical infection. Our data indicate that valACV may decrease HIV-1 VL in HIV-1-infected HSV-2-negative patients, and thus the drug’s benefits for HIV-infected patients may go beyond the suppression of HSV-2. Larger randomized trials and cost effectiveness analyses could be warranted to further explore the potential of this antiviral agent in the context of HIV-1 infection, in particular in combination with other antivirals.

Supplementary Data

Supplementary materials are available at Clinical Infectious Diseases online (http://cid.oxfordjournals.org). Supplementary materials consist of data provided by the author that are published to benefit the reader. The posted materials are not copyedited. The contents of all supplementary data are the sole responsibility of the authors. Questions or messages regarding errors should be addressed to the author.

Notes

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References