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A COMMON ANTI-CMV DRUG, GANCICLOVIR, INHIBITS HIV-1 REPLICATION IN HUMAN TISSUES EX VIVO

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Abstract

Cytomegalovirus (CMV) is a common HIV-1 copathogen. Since CMV infection is an important contributor to immune activation, the driving force of HIV disease, an anti-CMV strategy might be beneficial to HIV-infected patients. Shin et al. reported that anti-CMV therapy with valganciclovir in coinfecting individuals results in a decrease of HIV viral load that is not accompanied by a decrease of immune activation. This suggests an alternative mechanism for HIV inhibition other than suppression of CMV-mediated inflammation. Here, we show that ganciclovir, the active form of valganciclovir, has a direct suppressive activity on HIV replication in human tissues *ex vivo*, including HIV-1 of laboratory strains, drug-resistant and primate isolates. We deciphered the mechanism of this inhibition and showed that GCV-TP is incorporated in the nascent DNA chain and acts as a delayed chain terminator.

Keywords

CMV; ganciclovir; HIV-1; reverse transcriptase; delayed-chain termination

INTRODUCTION

Human cytomegalovirus (CMV) is a beta human herpesvirus (HHV) with high prevalence in both the general population (~65%) and in HIV-infected individuals (~90%) [1, 2]. Like

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AUTHOR CONTRIBUTIONS STATEMENT

CV, AL, AA, ES, and LM designed *ex vivo* experiments performed by CV, AL, AA, and ES. JB and MG designed and performed the experiments on GCV-TP incorporation by HIV RT. CV and JB wrote the paper and LM edited it. All authors reviewed the manuscript.

ADDITIONAL INFORMATION

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HSV-2 and other sexually transmitted copathogens such as *neisseria gonorrhea*, *chlamydia trachomatis*, *treponema pallidum* and *trichomonas vaginalis*, CMV has been considered a cofactor in HIV transmission [3–8]. In particular, the compartmentalized reactivation of CMV in genital mucosa has been associated with recruitment of immune cells [9–12], local changes in chemokine spectra [13, 14], and mucosal inflammation that is associated with increased HIV-1 viral loads in cervicovaginal secretion and semen [13, 15–19]. More recent data have shown that CMV infection may also contribute to systemic immunoactivation in HIV-infected individuals whether they are treated or not. This immunoactivation contributes to the prevalence of cardiovascular disease, cancer, fragility, and general aging [20, 21]. These findings generated interest in evaluating the possible clinical benefits of anti-CMV strategies for prevention of both HIV infection and HIV comorbidities.

Towards this goal, Shin et al. (2014) assessed the effect of CMV therapy on HIV genital levels by evaluating the impact of valganciclovir (a valine ester prodrug of gancyclovir, GCV) on HIV-infected, ART-naïve men in an open label observational study [22]. Valganciclovir treatment resulted in the reduction not only of CMV but also of HIV-1 viral load (VL) in blood and in semen. The decrease in the semen HIV VL was not associated with immune parameters (activated T cells and inflammatory cytokines), suggesting that the reduced HIV VL in semen could not be solely explained by the suppression of immune activation but rather by alternative mechanisms, as hypothesized by the authors [22]. Here, we describe such a mechanism: we showed that GCV has a direct suppressive effect on HIV-1 replication in human lymphoid and cervical tissues *ex vivo*. The molecular mechanism of this suppression involves GCV-triphosphate (GCV-TP), the active form of GCV, being incorporated into the nascent DNA chain by HIV-1 reverse transcriptase (RT) and then acting as a delayed chain-terminator.

MATERIALS AND METHODS

Tissue culture and viral infection

Tonsillar tissues were obtained from the Children's National Medical Center (Washington, DC) and infected with HIV_{LAI.04}. Cervico-vaginal tissues were obtained through the National Disease Research Interchange (Philadelphia, PA) and infected with HIV_{BaL}.

Primary isolates (HIV-1_{97USNG30}, HIV-1_{96USNN20}, and HIV-1_{ME1}), AZT-resistant virus, nevirapine-resistant virus (HIV-1_{N119}), fusion inhibitor-resistant virus (HIV-1_{pNL4-3 gp41(36G)/V38A/N42D}), protease inhibitor-resistant virus (HIV-1_{L10R/M46I/L63P/V82T/I84V} and HIV-1_{M46I/L63P/V82T/I84V}), and multi-nucleoside reverse transcriptase inhibitor (NRTI)-resistant viruses (HIV-1₇₃₂₄₋₄, HIV-1₇₃₀₃₋₃) were obtained through the NIH AIDS Research and Reference Reagent Program [23–27].

All tissues were obtained according to IRB-approved protocols. Tissues were dissected into 2-mm³ blocks and cultured as described earlier [28]. Tissue inoculations with HIV-1 and CMV were performed as described earlier [29, 30]. GCV (Bedford Laboratories, Bedford, OH) was present during the entire period of culture and was replenished with each medium change (every 3 days).

Cytokines/chemokines quantification

Concentrations of 32 cytokines/chemokines were measured in supernatants of tissue cultures using a multiplex bead-array-assay [31] (See supplementary data for details).

Viability assay in human lymphoid tissue

Cell viability in human tonsillar tissues treated with GCV (5 or 50 μ M) was assessed as [32] (See supplementary data for details).

Exogenous template RT assay

HIV-1 reverse transcriptase was expressed and purified as described previously [33]. T50A (CCAATATTCACCATCAAGGCTTGACGTGACTTCACTCCACTATACCACTC), and T50A6 (CCAATATTCACCATCAAGGCTTGATGAACTTCACTCCACTATACCACTC) were used as templates for DNA synthesis. P1 (GAGTGGTATAGTGGAGTGAA), P1a (GAGTGGTATAGTGGAGTGAAGT), and P1f (GAGTGGTATAGTGGAGTGAAGTTT) were used as primers. We performed synthesis of oligonucleotides containing internal GCV-monophosphate (MP) residues enzymatically using HIV-1 RT in a standard primer extension reaction [34]. Samples were subsequently resolved via 12% denaturing polyacrylamide gel electrophoresis (PAGE), and the final DNA product was excised and eluted from the gel matrix in 0.5 M ammonium acetate and 0.1% SDS. Full-length DNA synthesis by HIV-1 RT and steady-state kinetics of single nucleotide incorporation by RT were performed as described in the supplementary file.

RESULTS

GCV suppresses CCR5- and CXCR4-tropic HIV-1 infection in human tissues *ex vivo*

To test the effect of GCV on HIV replication, we used tonsillar and cervico-vaginal tissues, cultured as described earlier [28]. Briefly, 27 human tissue blocks for each experimental condition from each of three donors were infected with a prototypic CXCR4- or CCR5-tropic variant of HIV, respectively X4_{LAI.04} or R5_{BaL}, and incubated with GCV for 12 days. Culture medium was changed and GCV was replenished every 3 days. Donor-matched infected tissues not treated with GCV were used as controls. We found that GCV significantly ($p < 0.01$) suppressed HIV-1 replication, evaluated as p24_{gag} accumulation in culture medium. On average, GCV treatment of *ex vivo* human tonsils reduced production of p24_{gag} in culture medium from $10,600 \pm 4,400$ pg/ml to $1,430 \pm 409$ pg/ml and in *ex vivo* human cervico-vaginal tissues from $2,057 \pm 445$ pg/ml to 174 ± 39 pg/ml ($n = 3$) (Fig.1). To account for donor-to-donor variability, we normalized these data by calculating the relative suppression of HIV-replication in each donor tissue. On average, in tonsils infected with X4_{LAI.04}, GCV suppressed replication by $83.9 \pm 5.8\%$, and in cervicovaginal tissues infected with R5_{BaL} by $90.0 \pm 4.03\%$ ($n = 3$).

Although both cellular viral kinases encoded by endogenous HHVs, which are reactivated during the culture of human tissues *ex vivo*, are able to phosphorylate nucleotide analogs [30], here we decided to infect tissues with CMV, as GCV is specifically activated by UL97-encoded kinase expressed by CMV. To investigate whether exogenous CMV would confer better anti-HIV activity of GCV, we compared the HIV-inhibitory effect of GCV in tonsillar

tissues that we infected with CMV and HIV and in donor-matched controls that were infected with HIV only. We found that the suppression of HIV-1 replication was dose-dependent and not statistically different whether we coinfect HIV-infected tissues with CMV or not. The 50% effective concentrations (EC_{50}) of GCV in tonsillar tissue blocks were respectively 4.7 μ M (95% confidence interval (CI): 0.9–24.9) in HIV-1-singly infected tissues and 5.4 μ M (95% CI: 1.5–19.0) in CMV-coinfecting tissues (Figure 1C).

GCV suppresses infection of HIV-1 of clades A, B and C in human tissues *ex vivo*

We evaluated the susceptibility of three primary isolates to GCV: HIV-1_{97USNG30} of clade C, HIV-1_{96USNN20} of clade A, and HIV-1_{ME1} of clade B. Primary isolate HIV-1_{96USNN20} has a broad coreceptor utilization, including CCR2B, CCR3, CCR4, CCR5, CXCR4, Bob, and Bonzo while HIV-1_{97USNG30} and HIV-1_{ME1} are exclusively CCR5 tropic. Human lymphoid tissues (27 tissue blocks per experimental condition) were inoculated with each of these three isolates and then treated for the entire culture period with 5 μ M or 50 μ M GCV (which is respectively the EC_{50} and a concentration that completely suppresses X4_{LAI.04} replication in this system). We found that GCV suppressed replication of all the three tested primary isolates and this suppression was not significantly different from that of laboratory isolate X4_{LAI.04} at both 5 and 50 μ M GCV ($P > 0.05$) (Figure 1D).

GCV suppresses replication of drug resistant HIV-1 in human tissues *ex vivo*

First, we investigated the sensitivity of NRTI-resistant virus to GCV (5 and 50 μ M) in human tissue *ex vivo*. In particular, we evaluated the sensitivity to GCV of a AZT-resistant HIV [35] and two NRTI-multidrug resistant molecular clones (clones 10076-4 and 7303-3). The molecular clones belong to a panel of six prototypical infectious multi-NRTI resistant HIV-1 molecular clones that carry several prototypical mutations conferring resistance to at least two of the following NRTIs: abacavir, zidovudine, stavudine, zalcitabine, didanosine, tenofovir, and lamivudine [36]. The replication of AZT-resistant virus, and of 10076-4 and 7303-3 molecular clones was respectively suppressed by 48.6 $\pm$ 21%, 80.6 $\pm$ 12.2%, 46.4 $\pm$ 14% at GCV concentration of 5 μ M and by 68.5 $\pm$ 3.9%, 92.4 $\pm$ 4.3%, and 73.5 $\pm$ 1.9% at GCV concentration of 50 μ M. No statistical difference between the inhibition of AZT-resistant HIV-1 or multi NRTI-resistant molecular clones and the inhibition of the laboratory strain X4_{LAI.04} was observed ($P > 0.05$) (Fig. 1D).

In a second set of experiments, we tested the sensitivity to GCV of the nNRTI resistant HIV-1_{N119} (resistant to nevirapine), of a fusion inhibitor resistant HIV (HIV-1_{pNL4-3 gp41(36G)/V38A/N42D}) (resistant to enfuvirtide (T-20)), and of two protease inhibitor resistant HIV-1 ((HIV-1_{L10R/M46I/L63P/V82T/I84V} and HIV-1_{M46I/L63P/V82T/I84V}), which are resistant to the structurally diverse protease inhibitors MK-639, XM323, A-80897, Ro31-8959, VX-478 and SC-52151 [23]. The replication of HIV-1_{N119}, HIV-1_{pNL4-3 gp41(36G)/V38A/N42D}, HIV-1_{L10R/M46I/L63P/V82T/I84V} and HIV-1_{M46I/L63P/V82T/I84V} was respectively suppressed by 47.9 $\pm$ 4.9%, 52.5 $\pm$ 23.3%, 38.2 $\pm$ 16.6%, and 36.2 $\pm$ 18.1% at GCV concentration of 5 μ M and by 88.3 $\pm$ 3.2%, 96.1 $\pm$ 2.1%, 86.1 $\pm$ 1.7%, and 80.6 $\pm$ 4.5% at GCV concentration of 50 μ M (Fig. 1D).

Incorporation of GCV-TP by HIV-1 RT results in downstream DNA synthesis inhibition

We hypothesized that GCV-TP interferes with the activity of HIV-1 RT and directly suppresses HIV-1 replication. To test this hypothesis, we evaluated the ability of GCV-TP to suppress HIV-1 RT by measuring the polymerizing activity of RT from purified recombinant enzyme HIV-1 using an exogenously added primer-template hybrid (Fig. 2A). In particular, we assessed the extension of a GCV-MP-terminated primer using a multiple nucleotide incorporation assay. Pausing in DNA synthesis was observed at positions $n+1$ and $n+3$ for HIV-1 RT when GCV-MP was located at position n . These pausing sites were overcome with increasing concentrations of nucleotides, suggesting that either compromised nucleotide binding following these pausing sites or hybrid dissociation was caused by GCV-MP that was located in the primer strand.

Kinetic comparison of GCV-TP and dGTP incorporation for HIV-1 RT

To probe the structural determinants governing the efficiency of GCV-TP incorporation, we employed a steady-state kinetic approach to compare GCV-TP incorporation with that of the natural dGTP substrate (Table 1). For RT, the rate of GCV-TP incorporation (denoted by k_{cat}) was similar to that for the natural dGTP substrate; however, HIV-1 RT displayed marked increases in K_m values for GCV-TP compared with dGTP (HIV-1 RT: 0.058 μ M for dGTP and 4.4 μ M for GCV-TP).

Difference in efficiency of nucleotide incorporation downstream of GCV-MP in the primer strand

In an effort to analyze the pattern of inhibition by GCV-TP, a steady-state kinetic analysis of single nucleotide incorporation by HIV RT was conducted; the positions for nucleotide incorporation selected were those immediately following the pausing sites seen in the full-length DNA synthesis experiment for HIV-1 RT (Figure 2B), namely positions $n+2$ and $n+4$. The kinetic analysis for these two primer systems with internal GCV-MP residues was compared with the control primer system having the natural dGMP located at position n . The results (Table 2) point to a distinct mechanism of inhibition for HIV-1 RT. While nucleotide incorporation is 27-fold less efficient at position $n+2$ when GCV-MP is located at position n than the control with dGMP at position n , the efficiency of nucleotide incorporation is over 1,000-fold less efficient at position $n+4$ when GCV-MP is found at position n , as compared with the control.

GCV does not decrease inflammation/activation in human tissues *ex vivo*

Although the data above indicates that GCV acts directly on HIV, we investigated alternative potential mechanisms of such an inhibition: (i) the decrease of general inflammation of the tissue and (ii) the decrease of the activation status of tonsillar cells, in particular of CD4+ T cells. We addressed these two possibilities in two different sets of experiments.

First, we measured the levels of 32 cytokines/chemokines in the supernatants of human tissues treated or not with GCV. Tissues were treated with 5 μ M GCV (EC_{50} of X4_{LAI.04}) or 50 μ M GCV (10 fold higher than EC_{50}). Among the 22 cytokines/chemokines detectable in tonsillar tissue cultures, none were significantly different in tissues treated with GCV

(whether tissues were treated with 5 μ M or 50 μ M GCV) compared to their counterparts in untreated tissue cultures (Table 3).

In the second set of experiments, we specifically investigated whether GCV affected the viability of tissue cells, in particular the viability of HIV target cells (CD4⁺ T cells expressing CXCR4 or CCR5) including activated T cells (CD25⁺ or CD38⁺) (Fig. 1D). After 12 days in culture, we isolated cells from tissue blocks treated with 5 or 50 μ M GCV and from donor-matched untreated tissue and stained them for CD3, CD4, CD8, CXCR4, CCR5, CD25, and CD38. We evaluated the cytopathicity of GCV by comparing the number of cells in GCV-treated tissues with those in matched untreated tissues using a Novocyte flow cytometer, which allowed us to acquire a similar volume for each experimental condition. To account for size differences in tissue blocks, we normalized the data by the weight of the tissues. Figure 1E showed that GCV, even at the concentration of 50 μ M, did not deplete any cell subset, including activated CD4⁺ T cells that are primary targets for HIV.

DISCUSSION

It is commonly accepted that persistent immune activation is a driving force of HIV disease. In HIV-1-infected individuals, CMV prevalence is around 90%, and a growing body of evidence suggests that asymptomatic replication of CMV, a member of the HHV family, may be an important contributor to this persistent immune activation (reviewed in [8, 21]) accelerating the development of HIV-triggered immunological abnormalities [8, 37–40]. In particular, CMV shedding in the genital tract has been associated with increased T-cell immune activation and proliferation in peripheral blood [9–11, 39], higher HIV-1 shedding in semen [13, 15–17, 19], and higher risk of HIV-1 transmission [3, 6, 7]. Both CMV and HIV are associated with inflammation-related morbidities [21]. Several studies have suggested that both direct and indirect interactions between CMV and HIV could influence their replication and the resulting disease pathogenesis. Freeman et al. [21] reported on several of such interactions making the contribution of CMV to HIV pathogenesis and vice versa difficult to tease apart.

Antiretroviral therapy (ART) has affected the course of HIV infection in a revolutionary way, dramatically decreasing the mortality of HIV-1-infected individuals and reducing complications associated with opportunistic infections. However, even with sustained suppression of HIV replication, abnormal immune activation persists in most of these individuals. This immune activation is associated with subsequent morbidity and earlier mortality [41]. It has been suggested that CMV contributes to immune activation even when ART has suppressed HIV replication [10, 19, 42–45].

These observations suggested that treating asymptomatic CMV coinfection may be beneficial by suppressing general T cell activation, and ultimately decreasing HIV-1 VL, as well as delaying HIV disease progression [10, 22]. In this context, Shin et al. [22] investigated the effect of anti-CMV therapy using valgancyclovir on immune activation and HIV-1 VL in semen. They found that GCV did indeed reduce HIV-1 VL. However, this

reduction was surprisingly not associated with decreases in T-cell activation and in production of inflammatory cytokines.

On the basis of GCV-mediated reduction of HIV-1 genital VL in the absence of significant genital changes in immunoactivation, we hypothesized along with Shin et al. [22] that GCV has a direct effect on HIV replication provided it is phosphorylated by cellular or HHV TK.

Here, we tested this hypothesis in human tissues *ex vivo*. These are adequate models for pre-clinical drug testing, as they support productive HIV infection without exogenous activation and stimulation. Moreover, *ex vivo* tissues retain cytoarchitecture as well as the pattern of expression of key cell-surface molecules relevant to HIV infection [28]. First, we tested the sensitivity of two laboratory isolates of HIV-1, of CXCR4-tropic X4_{LAI.04} and of CCR5-tropic R5_{BaL}. We demonstrated that GCV inhibited HIV-1 in both lymphoid and cervico-vaginal tissues *ex vivo* and that GCV inhibited both X4 and R5 HIV-1. HIV-1 inhibition was not limited to laboratory isolates as three HIV-1 primary isolates of different subtypes (A, B, and C) were also suppressed by GCV. Also, GCV sensitivity was not limited to the wild type HIV-1 as NRTI-, nNRTI-, fusion inhibitor- and protease inhibitor-resistant viruses were all sensitive to GCV.

To investigate whether HIV inhibition by GCV was the result of a direct inhibition on the virus itself rather than the result of a GCV-mediated decrease of inflammation of the tissue, we measured the levels of 32 cytokines/chemokines (including but not limited to inflammatory cytokines) in the supernatants of tonsillar tissues treated or not with GCV. No significant difference in any levels of cytokines/chemokines was found between treated and untreated tissues. Neither did we find that GCV affected the viability or the number of tonsillar cells, in particular of CD4⁺ T cells, which are the primary target cells for HIV infection. Thus, it seems that GCV inhibited HIV-1 replication by directly affecting the virus and not by decreasing inflammation or activation of the tissue.

GCV is a synthetic purine nucleoside analogue of guanine and also has to undergo triphosphorylation to become active, with the initial monophosphorylation catalyzed more efficiently by HHV-encoded kinase than by cellular kinases. HHVs seem to be necessary to inhibit HIV-1, as GCV did not inhibit HIV-1 in MT-4 cell cultures (data not shown), which are free of endogenous HHVs [30]. Moreover, we showed here that the EC₅₀ of GCV for HIV-1 was approximately 5 μ M, whether human tissues were exogenously coinfecting with CMV or not. Thus, it seems that kinases expressed by endogenous HHVs activate GCV by adding the first phosphate. HHV-6 U69-encoded kinase is one such enzyme that has been shown to phosphorylate ganciclovir [46].

Both the multisite incorporation assay and the steady-state kinetic analysis suggest that GCV-MP inhibits HIV-1 RT by acting as a delayed chain terminator. It is possible that the inhibitor's interaction with RT is more strongly compromised by the lack of the sugar ring stacking with the enzyme that is seen with the natural dGTP substrate.

Using a steady-state kinetic approach, we found that the major mechanism of inhibition of HIV-1 RT by GCV was through a block in nucleotide incorporation at position $n+4$, as the efficiency of nucleotide incorporation was over 1,000-fold less efficient at position $n+4$

when GCV-MP is found at position n . In comparison, the efficiency of nucleotide incorporation was over 27-fold less efficient at position $n+2$ when GCV-MP was found at position n .

GCV is not the first delayed chain terminator to be described, as entecavir has been shown to block DNA synthesis through inhibition at positions $n+1$ (minor) and $n+4$ (major) in HIV-1 RT [47]. However, both the chemical structures and the mechanisms of action of GCV and entecavir are different, indicating that diverse mechanisms of delayed chain termination can be obtained from chemical modification of the inhibitor.

The effect of GCV on HIV VL in CMV-coinfected tissues resembles that of acyclovir (ACV) in HSV-2-coinfected individuals. As with the inhibition of HIV by GCV, the inhibition of HIV by ACV was thought to proceed exclusively via an indirect mechanism. Indeed, the effect of ACV on HIV VL was initially attributed to the suppression of HSV-2 replication followed by the suppression of HSV-2-triggered inflammation [48, 49]. However, we and others [30, 50, 51] have found that ACV also has a direct effect on HIV-1 after the drug is phosphorylated by HHV. The direct inhibitory effect of ACV on HIV VL observed *ex vivo* was recently confirmed *in vivo* in a double-blind crossover clinical trial [52]. In this trial, we showed that valacyclovir (a prodrug of ACV) suppresses HIV-1 VL in HIV-1-infected HSV-2 seronegative persons, proving that the effects of valacyclovir on HIV-1 are consistent with a direct effect of ACV on HIV-1 replication [52].

As with what we previously described for ACV/valACV, here, we found that GCV inhibited HIV in human tissues *ex vivo* but not in cell lines. Indeed, no anti-HIV effect of GCV was observed here in MT-4 cell lines or in H9 cells as reported by Medina et al. [53]. The anti HIV activity of GCV may be directly related to endogenous HHVs present in tissues [30] but not in cell lines [30]. Indeed, HHVs are ubiquitous in the tonsillar tissues. Earlier we found that 96% of tonsillar tissues (received from the same hospital as in the current study) were positive for one or several HHVs. In particular, real-time PCR analysis revealed CMV in 15% of tissues; EBV in 52%; HHV-7 in 89%; and HHV-6 in 96% of tissues.

The anti-HIV activity of the GCV occurs at clinically relevant concentrations. Indeed, although GCV penetration efficiency and drug clearance were unknown for *ex vivo* tissues, the calculated EC₅₀ of 4.7 μ M is in the range of what has been reported *in vivo* [54]. For a dose of 900 mg of oral valganciclovir per day, the maximum mean plasma concentration is 26.3 μ M with a mean AUC_{0–24} of 51.2 μ gh/mL, and plasmatic concentrations remain higher than 4.9 μ M for the first 15 h after drug administration [54].

In conclusion, our results suggest that an anti-CMV strategy using valganciclovir in HIV-1-infected individuals may reduce HIV-1 VL directly by inhibiting HIV-1 RT. Given the potential impact of CMV in immune activation and the potential role of CMV in various human pathologies, in particular in cardiovascular disease, T-cell senescence, and aging, strategies to reduce CMV replication in HIV-infected individuals may turn out to be beneficial.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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**Cervico-vaginal
tissue** **Tonsillar tissue** **A**

C

D

E

Figure 1. GCV suppresses HIV infection in human tissues *ex vivo* but does not deplete HIV-1 CD4 T cells

A–B. Blocks of human tonsillar and cervico-vaginal tissues were respectively inoculated with X4_{LAI.04} and R5_{BAL} and treated or not with GCV. The medium was changed, and GCV was replenished every 3 days. HIV-1 replication was evaluated from measurements of p24_{gag} accumulated in culture media over 3-day periods. For each donor, each datum represents pooled p24_{gag} release from 27 tonsillar tissue blocks or 18 cervico-vaginal blocks. We evaluated the percentage of HIV-1 inhibition by comparing viral replication in drug-tested tissues with that in untreated donor-matched control tissues **A**. Means $\pm$ SEM of HIV inhibition; **B** data of individual with tissues from three donors.

C. GCV suppresses HIV infection in human tissues *ex vivo* in a dose response manner. HIV-1 replication was measured in tonsillar tissue blocks singly infected with X4_{LAI.04} or coinfecting with CMV. GCV was added at concentrations of 0.3, 1, 3, 10, and 30 μ M, and its anti-HIV activity was evaluated from the suppression of viral replication compared with control donor-matched HIV-infected tissues. The medium was changed, and GCV was replenished every 3 days. HIV-1 replication was evaluated by measuring p24_{gag} accumulated in culture media over 3-day periods. For each donor, each datum represents pooled viral release from 27 tonsillar tissue blocks. We estimated the 50% effective concentration (EC₅₀)

and 95% confidence interval by fitting the data to four-parameter logistic regression. EC₅₀s were estimated from independent experiments performed in samples from five different donors.

D. Inhibition of replication of primary isolates and drug-resistant HIV-1 by GCV in human lymphoid tissues. Donor-matched sets of tissue (27 blocks for each experimental condition from each of 2 to 4 donors) were incubated with GCV (5 or 50 μ M) for 12 days or used as untreated controls. Some of these sets were infected with primary isolates (HIV-1_{97USNG30}, HIV-1_{96USNN20}, and HIV-1_{ME1}), AZT-resistant virus, nevirapine-resistant virus (HIV-1_{N119}), fusion inhibitor-resistant virus (HIV-1_{pNL4-3 gp41(36G)/V38A/N42D}), and protease inhibitor-resistant viruses (HIV-1_{L10R/M46I/L63P/V82T/I84V} and HIV-1_{M46I/L63P/V82T/I84V}), and multi-nucleoside reverse transcriptase inhibitor (NRTI)-resistant viruses (HIV-1₁₀₀₇₆₋₄, HIV-1₇₃₀₃₋₃). Replication of HIV-1 was evaluated by p24_{gag} core antigen release in pooled medium bathing 27 tissue blocks using a bead-based assay. Presented are means $\pm$ SEM of HIV-1 replication relative to matched untreated tissues (n=4 for HIV-1_{97USNG30}, HIV-1_{96USNN20}, HIV-1_{ME1}, AZT-resistant HIV-1; n=3 for HIV-1₁₀₀₇₆₋₄, HIV-1_{L10R/M46I/L63P/V82T/I84V} and HIV-1_{M46I/L63P/V82T/I84V}; n=2 for HIV-1₇₃₀₃₋₃, HIV-1_{N119}, HIV-1_{pNL4-3 gp41(36G)/V38A/N42D}).

E. Cell toxicity of heterodimer-inoculated *ex vivo*-cultured human lymphoid tissues. Tissue blocks (27 from each individual human tonsil donor) were treated with 5 and 50 μ M GCV. At day 12, cells were stained with anti-CD3-QD605, anti-CD4-QD655, anti-CD8-QD800, anti-CXCR4-FITC, anti-CCR5-PE-Cy5, anti-CD25-APC, and anti-CD38-PE antibodies. The cytopathicity of GCV for different cell subsets was evaluated by comparing the numbers of cells in treated tissue blocks with those in matched untreated control tissue blocks. To account for size differences in tissue blocks, the data were normalized by the weight of the tissues. Presented are means $\pm$ SEM with tissues from five different donors.

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A

B

Figure 2. HIV-1 RT incorporates GCV-TP

A. GCV-TP inhibits HIV-1 RT in exogenous template reverse transcriptase assays. Multisite DNA synthesis was performed by HIV-1 RT using GCV-MP-chain-terminated primers hybridized to a DNA template (shown in figure). Pausing sites for DNA synthesis for RT are marked with arrows; increasing concentrations of nucleotide are able to rescue DNA synthesis, suggesting non-obligate chain termination by GCV. The full-length product is marked as “fl”.

Presented is one representative gel of 3 independent experiments.

B. Experimental design for steady-state kinetics of single nucleotide incorporation.

(a) The primer/template system used for comparing the incorporation of dGTP and GCV-TP used to generate the kinetic constants shown in Table 1 is shown. (b) The primer/template sequences used to generate the kinetic constants found in Table 2 are displayed. Sequence systems for positions $n+2$ and $n+4$ incorporation are listed. The number sign is used to indicate a position in the sequence where GCV-MP is located at position n . (c) A representative single-nucleotide incorporation experiment (out of three independent experiments) showing the primer and $n+1$ product of DNA synthesis resolved by denaturing

polyacrylamide electrophoresis is presented. Increasing nucleotide concentrations are added to the reactions at a fixed time point. A negative control lane is shown, in which divalent metal ions were omitted. (d) Graphical representation of the data from panel *C* fitted to the Michaelis-Menten equation.

Table 1
Steady-state kinetic determinants for dGTP and GCV-TP incorporation for HIV-1 RT.

Enzyme	Position of Incorporation	Substrate	k_{cat} (min^{-1}) ^{a,b}	K_m (μM) ^{a,c}	k_{cat}/K_m ($\text{min}^{-1}/\mu\text{M}$) ^d	Selectivity ^e
HIV-1 RT	N	dGTP	0.44 ± 0.029 ^f	0.058 ± 0.014 ^f	7.6	Ref
HIV-1 RT	N	GCVTP	0.27 ± 0.021	4.4 ± 1.1	0.061	125

^aValues displayed in the table are the result of at least three independent experiments, $\pm$ SEM

^b k_{cat} is the enzyme turnover constant, obtained by dividing V_{max} for the reaction by the enzyme concentration.

^c K_m is the substrate concentration required to reach half the maximum rate of the reaction

^dCatalytic efficiency is defined as k_{cat}/K_m .

^eThe selectivity of nucleotide incorporation is obtained by dividing the efficiency of nucleotide incorporation for dGTP by the efficiency of GCV-TP incorporation.

^fPreviously reported values for k_{cat} and K_m : 0.44 min^{-1} and $0.10 \mu\text{M}$, respectively (Marchand B. et al., JBC 2007).

Presented are values from an experiment performed in triplicate.

Table 2

Steady-state kinetic determinants for dCTP incorporation downstream of GCV-MP in the primer strand for four viral polymerases.

Enzyme	Position of Incorporation	Substrate	k_{cat} (min^{-1}) ^a	K_m (μM) ^a	k_{cat}/K_m ($\text{min}^{-1}/\mu\text{M}$)	Selectivity ^b
HIV-1 RT	<i>n</i> +2	dCTP	0.16 ± 0.011	0.11 ± 0.025	1.5	Ref
HIV-1 RT	<i>n</i> +2, GCVMP at <i>n</i>	dCTP	0.33 ± 0.021	6.0 ± 1.4	0.055	27
HIV-1 RT	<i>n</i> +4	dCTP	0.46 ± 0.011	0.029 ± 0.003	15.9	Ref
HIV-1 RT	<i>n</i> +4, GCVMP at <i>n</i>	dCTP	0.048 ± 0.00018	3.4 ± 0.74	0.014	1136

^a Values displayed in the table are the result of at least three independent experiments, $\pm$ S.M.E.

^b The selectivity of nucleotide incorporation for a given enzyme and position was determined by dividing the efficiency of nucleotide incorporation for dCTP incorporation in the absence of GCV-MP in the primer by the efficiency of nucleotide incorporation for dCTP incorporation in the presence of GCV-MP in the primer.

Presented are values from an experiment performed in triplicate.

Table 3Cytokine/chemokine expression in human tissue *ex vivo* treated or not with GCV.

	GCV 5 μ M		GCV 50 μ M	
	Fraction of control	Wilcoxon rank sum test	Fraction of control	Wilcoxon rank sum test
IL-1 α	0.94 $\pm$ 0.15	0.44	1.07 $\pm$ 0.12	0.99
IL-1 β	0.94 $\pm$ 0.04	0.19	1.04 $\pm$ 0.09	0.62
IL-2	1.02 $\pm$ 0.02	0.99	1.34 $\pm$ 0.38	0.99
IL-4	1.07 $\pm$ 0.47	0.99	1.03 $\pm$ 0.22	0.62
IL-6	1.06 $\pm$ 0.08	0.62	0.98 $\pm$ 0.17	0.81
IL-7	1.05 $\pm$ 0.05	0.99	0.97 $\pm$ 0.03	0.99
IL-8	1.10 $\pm$ 0.23	0.37	1.43 $\pm$ 0.49	0.99
IL-10	1.03 $\pm$ 0.03	0.62	1.01 $\pm$ 0.08	0.81
IL-17	1.27 $\pm$ 0.27	0.99	0.78 $\pm$ 0.22	0.99
IL-18	1.03 $\pm$ 0.05	0.99	1.08 $\pm$ 0.06	0.44
IL-21	0.95 $\pm$ 0.12	0.81	0.96 $\pm$ 0.11	0.31
GM-CSF	0.98 $\pm$ 0.09	0.81	1.05 $\pm$ 0.12	0.81
GRO α	1.01 $\pm$ 0.16	0.99	0.94 $\pm$ 0.04	0.50
IFN γ	0.91 $\pm$ 0.22	0.99	0.99 $\pm$ 0.38	0.75
IP-10	0.77 $\pm$ 0.09	0.06	0.81 $\pm$ 0.20	0.62
ITAC	1.13 $\pm$ 0.23	0.81	1.04 $\pm$ 0.19	0.99
MCP-1	0.94 $\pm$ 0.11	0.44	1.13 $\pm$ 0.14	0.99
MIG	1.03 $\pm$ 0.12	0.99	0.84 $\pm$ 0.15	0.44
MIP-1 α	1.16 $\pm$ 0.17	0.62	1.04 $\pm$ 0.15	0.87
MIP-1 β	1.10 $\pm$ 0.14	0.62	0.96 $\pm$ 0.15	0.37
MIP-3 α	0.96 $\pm$ 0.16	0.87	1.15 $\pm$ 0.06	0.25
RANTES	0.96 $\pm$ 0.07	0.31	1.15 $\pm$ 0.06	0.81
TNF- α	1.51 $\pm$ 0.28	0.37	2.31 $\pm$ 0.80	0.25

Levels of 32 cytokines/chemokines present in the supernatant of tonsillar tissue cultures untreated or treated with 5 or 50 μ M GCV were measured using a multiplex bead-assay as described in [31]. 10 cytokines/chemokines were not detected while 22 were detected but not statistically different in tissues treated with GCV versus untreated tissues control. For each cytokine and each experimental condition (untreated control, GCV 5 μ M and GCV 50 μ M), cumulative production over a 12-day period of culture was evaluated and results were expressed as fractions of control. Statistical significance was assessed by Wilcoxon signed-rank test. $P < 0.05$ was considered to be significant. Presented are means $\pm$ SEM from experiments with 5 different tissue donors.