A New Class of Dual-Targeted Antivirals: Monophosphorylated Acyclovir Prodrug Derivatives Suppress Both Human Immunodeficiency Virus Type 1 and Herpes Simplex Virus Type 2

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Background. Human immunodeficiency virus type 1 (HIV-1) and herpes simplex virus type 2 (HSV-2) are responsible for 2 intersecting epidemics in which the disease caused by 1 virus facilitates the transmission of and pathogenesis by the other. Therefore, suppression of one virus infection will affect the other. Acyclovir, a common antiherpetic drug, was shown to directly suppress both viruses in coinfected tissues. However, both antiviral activities of acyclovir are dependent on phosphorylation by the nucleoside kinase activity of coinfecting human herpesviruses.

Methods. We developed acyclovir ProTides, monophosphorylated acyclovir with the phosphate group masked by lipophilic groups to allow efficient cellular uptake, and investigated their antiviral potential in cell lines and in human tissues ex vivo.

Results. Acyclovir ProTides suppressed both HIV-1 and HSV-2 at median effective concentrations in the submicromolar range in ex vivo lymphoid and cervicovaginal human tissues and at 3–12 μmol/L in CD4^{+} T cells. Acyclovir ProTides retained activity against acyclovir-resistant HSV-2.

Conclusions. Acyclovir ProTides represent a new class of antivirals that suppress both HIV-1 and HSV-2 by directly and independently blocking the key replicative enzymes of both viruses. Further optimization of such compounds may lead to double-targeted antivirals that can prevent viral transmission and treat the 2 synergistic diseases caused by HIV-1 and HSV-2. To our knowledge, the acyclovir ProTides described here represent the first example of acyclic nucleoside monophosphate prodrugs being active against HIV-1.

Human immunodeficiency virus type 1 (HIV-1) infection is commonly associated with infection with other copathogens that greatly affect human immunodeficiency virus (HIV) transmission and disease progression [1–4]. Herpes simplex virus type 2 (HSV-2), one of the most common HIV-1 copathogens, establishes with HIV-1 a vicious circle in which each virus facilitates the replication, shedding, and acquisition of the other [2, 5, 6]. Epidemiological studies have demonstrated that HIV-1 and HSV-2 are responsible for 2 epidemics that, by overlapping in the populations at risk, reinforce the spread of both HIV-1 disease and genital herpes [1]. In particular, HSV-2 may have contributed substantially to the spread of HIV-1 in Africa, where genital herpes has been estimated to account for ~30% of HIV infections [7, 8].

We recently discovered that the antiherpetic drug acyclovir, a well-known inhibitor of herpesviral DNA polymerase [9], is also an HIV-1 reverse-transcriptase inhibitor after it has been intracellularly converted to

its 5′-triphosphate derivative. We demonstrated that acyclovir inhibits HIV in various human tissues coinfected with human herpesviruses (HHVs) [10]. However, the anti-HIV activity of acyclovir is limited by its dependence on acyclovir phosphorylation by coinfecting HHV-encoded kinases.

Here, we report on our study of acyclovir monophosphate–based prodrugs, acyclovir ProTides, with anti-HIV activities that are independent on HHV coinfection. We demonstrate that acyclovir ProTides inhibit replication of HSV-2 and HIV-1 in T cell lines. In addition, acyclovir ProTides suppress these viruses in various human tissues ex vivo that are infected with either of these viruses alone or in combination. Moreover, acyclovir ProTides suppressed HSV-2 variants that are resistant to acyclovir because of their lack of thymidine kinase activity, which demonstrates the effective intracellular delivery of the free acyclovir monophosphate.

Thus, the acyclovir ProTides described here constitute a new class of antivirals that uniquely combine 2 distinct anti-HIV strategies: direct suppression of HIV reverse transcriptase and an indirect effect through inhibition of HSV-2, a virus known to facilitate HIV infection. Importantly, they represent the first acyclic nucleosides that we know to inhibit HIV reverse transcriptase, and thus they probe new structural space in this key viral enzyme and drug target.

METHODS

Cell and human tissue culture. Human T lymphocyte MT-4 and CEM cell lines (American Type Culture Collection) were cultured in Roswell Park Memorial Institute 1640 medium (RPMI 1640) with 10% fetal bovine serum. Peripheral blood mononuclear cells (PBMCs) from healthy donors were stimulated with phytohemagglutinin at 2 μg/mL (Sigma) for 3 days at 37°C and then seeded at 0.5×10^6 cells per well into a 48-well plate in cell culture medium (RPMI 1640) containing 10% fetal calf serum and interleukin 2 (25 U/mL; R&D Systems Europe). Tonsillar tissues from routine surgery were obtained from the Children's National Medical Center (Washington, DC). Cervicovaginal tissues from routine hysterectomy were obtained through the National Disease Research Interchange (Philadelphia, PA) in accordance with protocols approved by the institutional review board. Tissues were dissected into 2-mm³ blocks and placed onto collagen sponge gels at the air-liquid interface and cultured as described elsewhere [11].

ProTides synthesis. ProTides were synthesized as described elsewhere [10, 12].

Viral infection and antiviral assays. Tissue blocks were inoculated with $X4_{LAI.04}$ or $R5_{BaL}$ HIV-1 (Rush University Virology Quality Assurance Laboratory, Chicago, IL) as described elsewhere [11, 13]. MT-4 cells (10^5 cells) were inoculated with 10 μL of viral stock of $X4_{LAI.04}$ containing 0.7 ng/mL of $p24_{gag}$ antigen and cultured for 3 days. HIV replication was evaluated

by $p24_{gag}$ antigen release in MT-4 cell culture medium or in pooled medium bathing 27 tissue blocks using a bead-based assay [10]. CEM cells (4.5×10^5 cells/mL) were suspended in fresh culture medium and infected with HIV-1 at 100 times the median cell culture infective dose ($CCID_{50}$)/mL of cell suspension, and viral replication was evaluated by giant cell formation 4–5 days later. PBMCs were infected with HIV-1 isolates of different clades (provided by Dr L. Lathey, BBI Biotech Research Laboratories, Gaithersburg, MD) at a final dose of 250 pg/mL (p24 or p27). For human immunodeficiency virus type 2 (HIV-2) p27 Ag detection, the Innotest from Innogenetics was used.

Twenty-seven tissue blocks were inoculated with 5 μL of viral stock of HSV-2 strain G (ATCC) or a clinical acyclovir-resistant HSV-2 isolate [14] containing 1.4 times the median tissue culture infective dose ($TCID_{50}$). Replication was evaluated by real-time polymerase chain reaction [10].

The antiviral activities of acyclovir ProTides were investigated in cell lines and in human tissues ex vivo. MT-4 cells were preincubated with acyclovir (Bedford Laboratories), acyclovir-monophosphate, or acyclovir-triphosphate (Moravek Biochemicals and Radiochemicals) at 100 μmol/L overnight, suspended in fresh medium containing various concentrations of compounds and infected with HIV-1. To test the anti-HIV activity of acyclovir ProTides, HIV-1–infected MT-4 cells, CEM cells, PBMCs, or human tissue blocks were incubated with test compounds, and viral replication in treated and untreated cells was evaluated. The results are given as the mean ± standard error of the mean of the concentration required to suppress viral replication by 50% (median effective concentration [EC_{50}]). The value of EC_{50} was calculated by fitting the data points to a sigmoidal dose-response curve, with Prism software, (version 4.0; GraphPad).

Viability assays. Viability assays were performed in the MT-4 cell cultures with the Nucleocounter automated cell counting system (ChemoMetec). The numbers of total and dead cells in untreated cultures and in cultures treated with acyclovir ProTides was determined using a propidium iodide–based assay according to the manufacturer's protocol. Data were collected and analyzed using Nucleoview software (version 1.0; ChemoMetec).

Flow cytometry. To assess the cytotoxicity of acyclovir ProTides in human tonsillar tissues after 12 days of culture, cells isolated from untreated tissue blocks and from those treated with acyclovir ProTides were stained for surface markers. Cells were stained with combinations of the following fluorescence-labeled monoclonal antibodies: anti–CD3-QD655, anti–CD4-QD605, anti–CD8-PacificBlue, anti–CD45RA-PE-Cy7, anti–CCR7-APC-Cy7, and anti–Ki67-PE antibodies (Caltag Laboratories). Detection and enumeration of HIV-1–infected cells were performed with intracellular staining by means
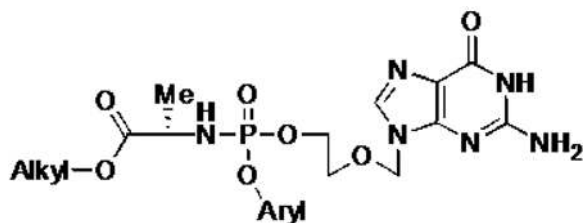
Figure 1. Generic structure of acyclovir ProTides, which consist of monophosphorylated acyclovir and of aryloxy and aminoacyl ester groups linked to the phosphate moiety.

of anti–p24-PE (Beckman Coulter). Data were acquired and analyzed as described elsewhere [10]. Cell depletion was quantified using Trucount beads (Becton Dickinson) for volumetric control, and cell numbers were normalized by tissue-block weights.

RESULTS

Treatment of HIV-1–infected cell lines with phosphorylated acyclovir. As an initial attempt to overcome acyclovir dependence on HHV-mediated phosphorylation, we evaluated the ability of already phosphorylated acyclovir (monophosphorylated acyclovir or triphosphorylated acyclovir) to inhibit HIV-1 replication. Treatment of HIV-1$_{LAI.04}$–infected MT-4 cell cultures with acyclovir-monophosphate or acyclovir-triphosphate suppressed HIV replication, albeit with low potency: values of EC$_{50}$ for acyclovir-monophosphate and acyclovir-triphosphate were 100 ± 11 and 58 ± 2.6 μmol/L, respectively, whereas the EC$_{50}$ value for acyclovir itself was >250μmol/L. Likewise, acyclovir did not efficiently suppress viral replication in HIV-1$_{IIIB}$– or HIV-2$_{ROD}$–infected CEM cells (data not shown). To improve the potency of phosphorylated forms of acyclovir in inhibiting HIV-1 replication, we designed phosphorylated prodrugs of acyclovir in which the phosphate moiety is protected by lipophilic groups (acyclovir ProTides).

Efficient inhibition by acyclovir ProTides of HIV-1 in CD4$^+$ T cell lines. We designed a variety of ProTides that were based on the structure of acyclovir-monophosphate and investigated their anti–HIV-1 activities. In these compounds, the phosphate group is attached to the acyclovir core and is protected from cleavage caused by extracellular enzymes by lipophilic aryl and aminoacyl ester groups [15] (Figure 1). For the current study, we chose 4 acyclovir ProTides, designated as Cf2648, Cf2649, Cf2676, and Cf2681 [10, 12] (Table 1). All acyclovir ProTides efficiently and dose-dependently inhibited HIV-1 replication in MT-4 and CEM cell cultures. Their EC$_{50}$ values varied from 1.7 to 12 μmol/L (Table 2).

The concentrations of ProTides that killed 50% of the MT-4 cells (CC$_{50}$) were >150 μmol/L (Table 2). Nonetheless, ProTides Cf2676, Cf2649, Cf2648, and Cf2681 reduced cell

proliferation by 50% (median inhibitory concentration [IC$_{50}$]) at the concentrations of 12, 19, 34, and 73 μmol/L, respectively. To study the reversibility of the cytostatic effect, we treated cell cultures with ProTide Cf2648 at 50 or 150 μmol/L for 3 days and then washed the drug away. Three days after Cf2648 removal, the number of cells in these cultures had increased 3-fold, an increase similar to that in untreated cultures (2.5-fold). Thus, acyclovir ProTides suppressed HIV-1 replication at concentrations that do not significantly decrease cell viability, whereas at higher concentrations they reversibly slow cell growth.

Inhibition by acyclovir ProTides of HIV-1 replication in PBMCs and in human tissues. As shown in Table 3, acyclovir ProTides Cf2648 and Cf2649 efficiently inhibited HIV-1 variants of subtypes A, B, C, D, and A/E, including R5 and X4 variants, in PBMCs. We also investigated the anti-HIV activity of ProTides in an in vivo–like system of human lymphoid and cervicovaginal tissue explants. This system retains tissue cytoarchitecture and supports HIV replication without exogenous activation [11, 13, 16, 17]. Blocks of human tonsillar tissue were treated with ProTides overnight and infected with a prototypical X4 variant of HIV-1 (HIV-1$_{LAI.04}$). Acyclovir ProTides were present during the entire culture period. The absolute HIV replication level in tissues from different donors varied from 3.1 to 15.8 ng/mL. The EC$_{50}$ value for acyclovir ProTides Cf2648 and Cf2649 was 1 ± 0.4 μmol/L ($n = 4$) and 0.14 ± 0.03 μmol/L ($n = 5$), respectively (Figure 2A). Next, we compared the numbers of HIV-infected cells in tissues treated with ProTide Cf2649 and in untreated tissues. On day 12 after infection, using flow cytometry we evaluated the fraction of p24$^+$CD8$^-$ T cells in ProTide-treated and control tissues (Figure 2B). This fraction includes CD4$^+$ T cells that down-regulated CD4 because of HIV infection [18]. A mean of 3.16% ± 1.63% CD8$^-$ T cells in ProTide-treated cultures were infected with HIV, whereas in control donor-matched cultures these cells

Table 1. Acyclovir ProTides Variants

Compound	Aryl	Ester	Amino acid	ClogP
Cf2648	Ph	Bn	L-Ala	0.89
Cf2649	1-Nap	Me	L-Ala	0.35
Cf2676	1-Nap	Et	L-Ala	0.88
Cf2681	1-Nap	iPr	L-Ala	1.19

NOTE. The aryl groups represent a 1-naphthyl or phenyl group, whereas the aminoacyl ester group could be a benzyl or alkyl (Me, Et, or iPr) group. The amino acid in the 4 ProTides used in this study was L-alanine. Compounds were synthesized as described in supplemental data. The value of ClogP is a measure of hydrophilicity, calculated using ChemDraw (version 11.0; CambridgeSoft) as ClogP = log($c_{octanol}$/c_{water}), where $c_{octanol}$ and c_{water} are concentrations of octanol and water, respectively, portioned in the 2-phase system.

Table 2. Inhibitory Activity of Acyclovir ProTides against Human Immunodeficiency Virus Type 1 (HIV-1) in CD4+ T Cell Cultures

Compound	EC$_{50}$, μmol/L		CC$_{50}$, μmol/L		IC$_{50}$, μmol/L		SI[a]
	MT-4	CEM	MT-4	CEM	MT-4	CEM	
Acyclovir	>250	>250	>150	>150	>150	>250	NA
Cf2648	5.7 ± 1.6	6.4 ± 5.8	>150	>150	34 ± 11	33 ± 7.1	>26
Cf2649	4.7 ± 2.1	10 ± 7.9	>150	>150	19 ± 3.2	57 ± 45	>32
Cf2676	1.7 ± 0.8	12 ± 9.8	>150	>150	12 ± 5.3	32 ± 7.8	>88
Cf2681	5.4	6.2 ± 5.4	>150	>150	73	36 ± 15	>28

NOTE. CC$_{50}$, concentration that reduced the viability of the MT-4 or CEM cells by 50%; EC$_{50}$, effective concentration that inhibited HIV-1 replication by 50%; IC$_{50}$, concentration that inhibited MT-4 or CEM cell growth by 50%; NA, not applicable; SI, selective index (ratio of CC$_{50}$[MT-4]/EC$_{50}$).

[a] Values were calculated for acyclovir, Cf2648, Cf2649, Cf2676, and Cf2681 from 3, 5, 3, 3, and 2 independent experiments, respectively.

constituted 11.71% ± 3.87% of the total CD8$^-$ T cells ($n = 4$; $P<.05$).

In addition, we used flow cytometry to evaluate the effect of acyclovir ProTides on tissue cell viability. For this purpose, we isolated cells from tissue blocks treated with 1 μmol/L Cf2649 (a concentration that completely suppresses HIV replication in this system) and from donor-matched untreated tissue and stained them for CD3, CD4, CD8, CD45RA, and CCR7. Because HIV-1 preferentially replicates in memory T cells, we evaluated cellular depletion in all 3 main subsets of memory CD4$^+$ T cells: central (T$_{CM}$; CD45RA$^-$CCR7$^+$), effector (T$_{EM}$; CD45RA$^-$CCR7$^-$), and terminally differentiated effector (T$_{EMRA}$; CD45RA$^+$CCR7$^-$), as well as in naive (CD45RA$^+$CCR7$^+$) CD4$^+$ T cells. On day 12 after infection, the amounts of total, naive, T$_{CM}$, T$_{EM}$, and T$_{EMRA}$ CD4$^+$ T cells in Cf2649-treated tissues were similar to those in untreated tissues (107% ± 5%, 98% ± 2%, 107% ± 3%, 111% ± 9%, and 97% ± 6% of untreated matched tissue controls, respectively; $n = 2$). Similarly, Cf2681 did not affect the numbers of these cells in treated tissues. Likewise, the fractions of CD8$^+$ T cells and their subsets (naive, T$_{CM}$, T$_{EM}$, and T$_{EMRA}$) in tissues treated with acyclovir ProTide Cf2649 or Cf2681 were similar to those in untreated control tissues (data not shown).

Next, we investigated the effect of acyclovir ProTides on HIV replication in cervicovaginal tissue. Although the absolute HIV replication level in tissues from different donors varied from 1 to 5 ng/mL, a mean of 1 μmol/L acyclovir ProTide Cf2649 consistently suppressed replication of HIV-1$_{BaL}$ by 75.8% ± 10.4% ($n = 4$; $P<.01$) relative to donor-matched tissues infected with HIV-1$_{BaL}$ alone (Figure 3A).

We also investigated whether acyclovir ProTides affected HIV replication after the compounds had been removed. Tonsillar tissue blocks were pretreated with Cf2649 at 1 μmol/L for 1 day; then the drug was removed, and HIV-1$_{LAI.04}$ was inoculated. On day 12 after infection, HIV-1 replication was still inhibited by 69.3% ± 2.3% ($n = 3$; $P<.05$). In other experimental conditions, blocks of cervicovaginal tissues were treated overnight with Cf2648 (1 μmol/L) and infected with HIV-1$_{BaL}$. For 1 set of tissue blocks, Cf2648 was replenished at each medium change, whereas in another set, it was removed on day 3 after infection and not replenished. In the first case, HIV replication was inhibited by 100% ($n = 2$), whereas in the second case, HIV-1 replication was suppressed by 57.1% ± 16.0% ($n = 4$; $P<.05$). If the concentration of Cf2648 was increased to 5 μmol/L, 3-day incubation followed by drug removal resulted in 90.3% ± 9.2% ($n = 3$; $P<.05$) inhibition of HIV replication on day 12 after infection.

Finally, in a complementary set of experiments ($n = 3$), ton-

Table 3. Inhibitory Activity of Acyclovir ProTides against Human Immunodeficiency Virus Type 1 (HIV-1) Strains and Clinical Isolates in Peripheral Blood Mononuclear Cell Cultures

ProTide	EC$_{50}$, μmol/L							CC$_{50}$, μmol/L	SI[a]
	NL4.3 X4	BaL R5	UG 275 R5 (clade A)	US 3 R5 (clade B)	ETH 2220 R5 (clade C)	UG 270 X4 (clade D)	ID12 R5 (clade A/E)		
Cf2648	13 ± 3.8	6.6 ± 1.4	25 ± 10	28 ± 12	19 ± 14	55 ± 40	16 ± 15	319	14
Cf2649	11 ± 3.1	5.2 ± 2.4	16 ± 11	4.9 ± 6.1	6.8 ± 3.9	10 ± 12	53 ± 4.4	352	23

NOTE. CC$_{50}$, 50% cytostatic concentration as measured by the 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) viability staining method; EC$_{50}$, 50% effective concentration required to inhibit p24 production in virus-infected peripheral blood mononuclear cells; SI, selectivity index (ratio of CC$_{50}$/EC$_{50}$).

[a] For EC$_{50}$, the mean EC$_{50}$ values for all virus strains were taken together (ie, 23.2 μmol/L for Cf2648 and 15.3 μmol/L for Cf2649).
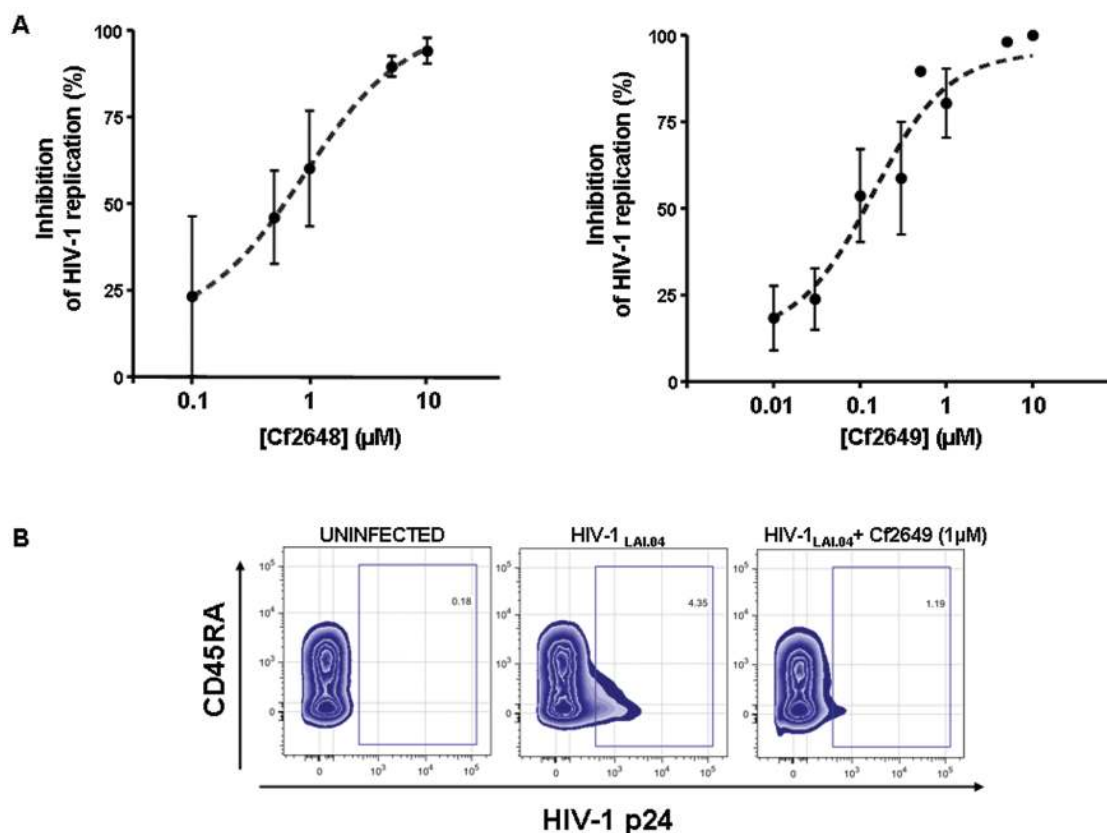
Figure 2. Inhibition by acyclovir ProTides of human immunodeficiency virus type 1 (HIV-1) replication in human lymphoid tissues. Human tonsillar tissues (27 blocks of tissue from each of *n* donors for each experimental condition) were infected with HIV-1$_{LAI.04}$ and treated with acyclovir ProTide Cf2648 or Cf2649 at various concentrations. The anti–HIV-1 activities of the ProTides were evaluated by comparing viral replication in drug-treated tissues with that in untreated donor-matched control tissues. *A*, HIV-1 inhibition at different concentrations of ProTides, as defined by the following formula: inhibition = $(1 - R_{ProTide}/R_{contr}) \times 100$, where $R_{ProTide}$ and R_{contr} are the amounts of p24 accumulated in the medium over the 12-day culture period in ProTide-treated cultures and in donor-matched untreated cultures, respectively. The median effective concentration (EC$_{50}$) for each acyclovir ProTide was estimated. The values of EC$_{50}$ for Cf2648 and Cf2649 were 1 ± 0.4 and 0.14 ± 0.03 μmol/L, respectively. Data are presented as the mean of the results, with tissues from 4 or 5 donors; error bars show the standard error of the mean. *B*, Flow cytometry density plots from a representative experiment of HIV-1$_{LAI.04}$–infected CD8$^-$ T cells on day 12 after HIV-1 infection. Isolated cells from donor-matched uninfected, HIV-1–infected, and HIV-1–infected acyclovir ProTide Cf2649–treated tissues were stained for CD3, CD4, CD8, and CD45RA surface markers as well as for intracellular p24. μM, micromoles per liter.

sillar tissue blocks were first infected with HIV-1$_{LAI.04}$ and then treated with acyclovir ProTide Cf2649 (1 μmol/L) at different time points between day 0 and day 6 after infection. HIV-1 replication was inhibited by 96.9% ± 3% if the treatment started simultaneously with infection, 94.4% ± 5.5% if the treatment started 2 days after infection, 90.2% ± 9.8% if the treatment started 4 days after infection, and 54.1% ± 13.5% if the treatment started 6 days after infection. Only a 6-day delay of treatment with acyclovir ProTides resulted in lower HIV-1 inhibition, although it was statistically not significant ($P =$ .055).

Inhibition by acyclovir ProTides of HSV-2 and HIV-1 replication in coinfected human tissues. We investigated the ability of acyclovir ProTides to suppress HSV-2 in singly infected human lymphoid tissues and in tissues coinfected with

HIV-1. We infected blocks of human tonsillar tissues with HSV-2 (strain G) and treated them with 1 μmol/L acyclovir ProTide Cf2648, which was maintained throughout the entire culture period. On day 12 after infection, HSV-2 replication was suppressed by 99.6% ± 0.3% ($n = 4$). Acyclovir ProTide Cf2648 was also active in suppressing an HSV-2 variant that does not encode for a functional thymidine kinase and thus is resistant to acyclovir [14]: replication of this HSV-2 variant was inhibited by 94.9% ± 5.1% ($n = 2$), whereas acyclovir showed no inhibition at 10 μmol/L.

In HIV-1– and HSV-2–coinfected human lymphoid tissue, the acyclovir ProTides retain their inhibitory activity against both viruses: 1 μmol/L Cf2648 inhibited the replication of HIV by 81.1% ± 5.9% ($n = 5$; $P < .01$) (Figure 3*B*) and that of HSV-2 by 99.6% ± 0.2% ($n = 5$; $P < .01$) (Figure 3*C*). Treatment
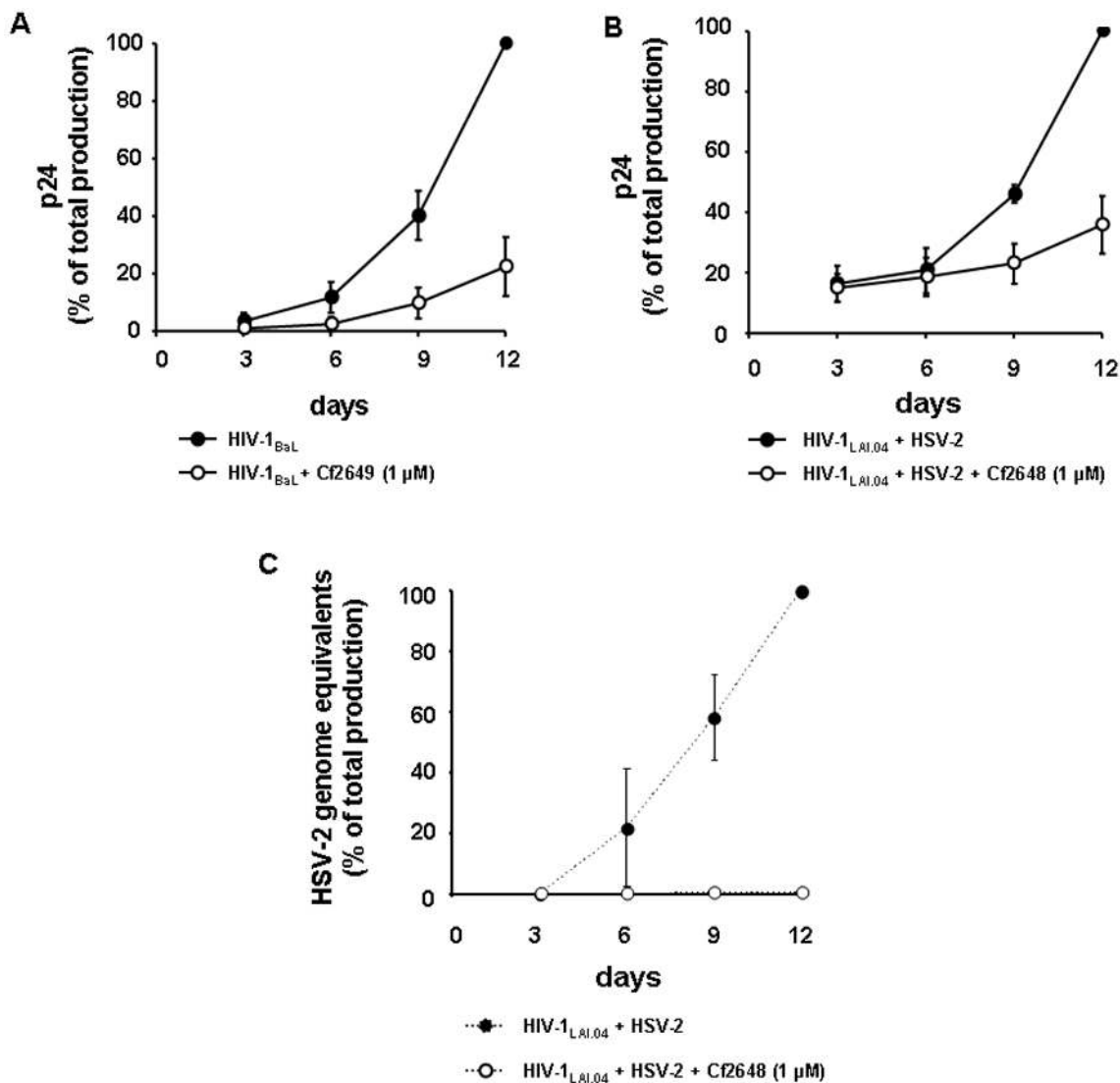
Figure 3. Suppression by acyclovir ProTides of human immunodeficiency virus type 1 (HIV-1) replication in human cervicovaginal tissues and in herpes simplex virus type 2 (HSV-2)–coinfected tonsillar tissues. *A*, Suppression by acyclovir ProTides of HIV-1 replication in human cervicovaginal tissues. Blocks of human cervicovaginal tissue were infected with HIV-1$_{BaL}$ and either treated with the acyclovir ProTide Cf2649 for 12 days at a concentration of 1 μmol/L or left untreated (control). Each measurement represents the amount of HIV-1 accumulated in culture medium over a 3-day period and is presented as the percentage of the maximal p24 concentration in untreated control tissues, to account for the variation in absolute replication levels in tissues from different donors. Data are presented as the mean of the results, with tissues from 4 donors; error bars show the standard error of the mean. *B*, Blocks of human tonsillar tissue were coinfected with HIV-1$_{LAI.04}$ and HSV-2 (strain G). Tissues were treated with the acyclovir ProTide Cf2648 at 1 μmol/L or left untreated (control). Replication of HIV-1$_{LAI}$ was evaluated by p24$_{gag}$ core antigen release in pooled medium bathing 27 tissue blocks using a bead-based assay. Data are presented as the mean of the percentage of maximal cumulative p24 production; error bars show the standard error of the mean. *C*, Blocks of human tonsillar tissue were coinfected with HIV-1$_{LAI.04}$ and HSV-2 (strain G). Tissues were treated with the acyclovir ProTide Cf2648 at 1 μmol/L or left untreated (control). Replication of HSV-2 was monitored with real-time polymerase chain reaction for viral DNA accumulated in the culture medium. Data are presented as the mean of the maximal of cumulative production of genome equivalent concentration; error bars show the standard error of the mean. μM, micromoles per liter.

with acyclovir ProTides reduced the mean production of p24$_{gag}$ in culture medium from 13.2 $\pm$ 4.6 to 3.6 $\pm$ 1.6 ng/mL in HIV-1– and HSV-2–coinfected tissues. Thus, acyclovir ProTides efficiently suppress replication of HIV-1 and its common co-pathogen HSV-2 in coinfected human tissues.

DISCUSSION

Drugs that directly suppress HIV play a major role in containing AIDS epidemics. However, it is becoming clear that other viruses that infect the human body before or after HIV infection

play a significant role in HIV transmission, pathogenesis, and disease. Therefore, the development of drugs that not only target HIV directly but also affect HIV by suppressing coinfecting viruses may be a valid strategy for therapy for HIV infection. Here, we describe such a drug that suppresses both HIV-1 and HSV-2, one of the viruses that is commonly associated with HIV-1 infection and that facilitates HIV-1 transmission and pathogenesis.

Acyclovir was designed to suppress HHVs, in particular HSV-2 [9]. Its potency and specificity against HSV-2 and some other HHVs are based primarily on the unique ability of HHV nucleoside kinases to phosphorylate acyclovir and thus enable its eventual conversion into the 5′-triphosphate form, which is an HHV DNA chain terminator. Nonherpetic viruses, including HIV, lack such nucleoside kinase activity, and therefore acyclovir is inactive against them. However, recently we found that phosphorylated acyclovir is capable of terminating not only the elongation of HHV DNA but also that of HIV-1 DNA [10]. This occurs in tissues coinfected with HSV-2 (or with other HHVs capable of phosphorylating acyclovir) and HIV-1, but not in HHV-free systems [10]. However, acyclovir would be inactive against HIV-1 in tissues infected with HSV variants that have nonfunctional thymidine kinase (acyclovir-resistant HSV) and in tissues in which HHVs are absent, are not replicating, or phosphorylate acyclovir inefficiently. Thus, the anti-HIV activity of acyclovir heavily depends on HHV nucleoside kinases.

Here, we describe masked phosphorylated acyclovir derivatives (acyclovir ProTides) that suppress HIV-1 replication independently of HHV nucleoside kinases. The ProTide strategy has been successfully used in designing an abacavir-based drug against hepatitis B virus [19], 2′,3′-didehydro-2′,3′-dideoxythymidine (d4T)– and L-carbocyclic-2′,3′-didehydro-2′,3′-dideoxyadenosine (L-C-d4A)–based agents against HIV-1 [20–22], and a 4′-azidonucleoside–based drug against hepatitis C virus [23], as well as in several cancer studies [24, 25].

In this study, we used the ProTide strategy to deliver intracellularly phosphorylated acyclovir. Inside the cells the ProTides undergo ester hydrolysis and spontaneous aryl loss, followed by P-N cleavage, resulting in a release of free acyclovir-monophosphate that is further converted into acyclovir-triphosphate, which terminates HIV-1 DNA elongation. In contrast to other nucleoside reverse-transcriptase inhibitors (NRTIs), such as tenofovir and adefovir, which are "stable" acyclic purine nucleoside phosphonate analogues, phosphorylated acyclovir is to our knowledge the first known nonphosphonate acyclic nucleoside analogue that inhibits HIV-1 reverse transcriptase.

We evaluated acyclovir ProTide activity in 2 experimental models of HIV infection: single-cell cultures (T cell lines or PBMCs) and ex vivo human lymphoid and cervicovaginal tissues in which the gross cytoarchitecture and local microenvironment are preserved and in which anti-HIV drugs have already been evaluated [17, 26]. In these tissues 1 μmol/L acyclovir ProTides was sufficient to inhibit HIV-1 replication by 75%–100%, and the EC_{50} values were as low as 0.14–1 μmol/L. In cell lines (MT-4 and CEM), acyclovir ProTides suppressed HIV-1 replication with an EC_{50} value between 1.7 and 12 μmol/L, whereas in PBMCs, the EC_{50} values ranged between 4.9 and 55 μmol/L. The lower potency of acyclovir ProTides in PBMCs and cell lines compared with that in tissues may be related to the level of cell activation and proliferation. Immortalized cell lines and phytohemagglutinin-stimulated PBMCs have high concentrations of intracellular pyrimidine and purine nucleotides, in particular 2′-deoxyguanosine-5′-triphosphate (dGTP) [27], which competes with acyclovir-triphosphate for DNA chain incorporation by HIV-1 reverse transcriptase. However, because the critical events of HIV pathogenesis in vivo occur in tissues, the system of human tissues ex vivo that supports HIV production without exogenous activation or stimulation appears to be more adequate.

To suppress HIV-1 replication in tissues, acyclovir ProTides do not need to be present permanently: despite removal of the drugs several days after infection, inhibition of HIV-1 replication was sustained. It is conceivable that the block of the initial infection or the maintenance of effective intracellular drug concentrations even after the drug is removed from the culture medium could be responsible for sustained HIV inhibition.

HIV replication was significantly inhibited either by brief exposure to an acyclovir ProTide followed by its removal or by delay of treatment. Thus, on the timescale of our experiments, acyclovir ProTides can be applied before or after HIV infection, or removed during the course of infection, and still significantly suppress HIV-1 replication in human tissues ex vivo. Moreover, acyclovir ProTides appeared to inhibit a variety of HIV-1 isolates, irrespective of viral subtype and coreceptor tropism.

Importantly, none of the tested acyclovir ProTides killed cells in tissues or cell lines. Flow cytometry of tissue cells treated with acyclovir ProTides, even at a concentration 10 times higher than the EC_{50} value, did not reveal depletion of $CD4^+$ and $CD8^+$ T cells or of their subsets, naive, T_{CM}, T_{EM}, and T_{EMRA} cells. Neither did we observe cell death in cell line cultures with the acyclovir ProTides at a concentration as high as 150 μmol/L. However, it cannot be excluded that prolonged application of these compounds in vivo may reveal mitochondrial poisoning, which often takes several months to materialize in the case of the most toxic NRTIs [28]. The tested compounds, although nontoxic, were cytostatic in vitro, but at concentrations clearly higher than their EC_{50} values. Furthermore, the cytostatic effect was reversible because after removal of the compound, cells proliferated at the same level as that in untreated controls. The cytostatic effect of the acyclovir ProTides was not observed in

ex vivo tissues, because presumably cell proliferation was negligible in this system [29].

Although acyclovir ProTides inhibit HIV, they also retain their antiherpetic activity. In coinfected lymphoid and cervicovaginal human tissues, acyclovir ProTides efficiently inhibited both viruses. Furthermore, they suppressed an acyclovir-resistant strain of HSV-2 both in cell lines [12] and in ex vivo human tissues. These findings clearly demonstrate that the activity of acyclovir ProTides is entirely independent on the virus-induced thymidine kinase.

The dual inhibitory activity of ProTides is important, because such drugs may interrupt a vicious circle of mutual facilitation of HSV-2 and HIV coinfections. In particular, HSV-2 infection results in an increased risk of HIV-1 acquisition and transmission because of HSV-induced disruption of genital mucosa, recruitment of activated cells, and increase of HIV-1 genital and plasma loads [30–33]. In turn, HIV-1 infection increases the frequency of HSV-2 reactivations and its mucosal shedding [34].

Strategies to control HSV-2 in HIV-1 prevention and care are now being tested in several clinical trials. As of this writing, 2 targeted clinical trials have failed to demonstrate a protective effect of HSV-2 suppression on HIV-1 acquisition [35, 36]. However, these trials were marked by low drug dosage, as well as by low adherence and behavioral disinhibition [37–40]. On the other hand, in HSV-2–positive individuals who were already infected with HIV-1, suppression of HSV-2 with acyclovir or its prodrug valacyclovir resulted in HIV-1 load decrease in plasma and genital, rectal, and seminal compartments [41–46]. Thus, the pronounced antiherpetic activity of acyclovir ProTides [12], in combination with their anti-HIV activity, seems to be an advantage of these compounds that can be used in the framework of these new strategies.

In conclusion, the anti-HIV activity of acyclovir ProTides, in conjunction with their potent antiherpetic activity, makes them prototypes of new dual-targeted antivirals. Such drugs allow the suppression of both HIV and HSV-2, a copathogen that significantly enhances HIV-1 sexual transmission and acquisition. These antivirals, structurally based on acyclovir and intracellularly delivered by means of the ProTide strategy, may become valuable components of future anti-HIV drug cocktails.

Acknowledgments

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