Sensitivity of Kaposi's Sarcoma–associated Herpesvirus Replication to Antiviral Drugs

Implications for Potential Therapy

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Abstract

Using a cell line (termed BCBL-1) derived from a peripheral effusion (body cavity–based) lymphoma latently infected with Kaposi's sarcoma–associated herpesvirus (KSHV), we recently reported the successful induction of KSHV replication in culture (Renne, R., W. Zhong, B. Herndier, M. McGrath, N. Abbey, D. Kedes, and D. Ganem. 1996. *Nat. Med.* 2:342–346). Here we report the first use of this system for establishing the susceptibility of KSHV to available antiviral drugs. Latently infected BCBL-1 cells were induced to lytic replication with phorbol esters; such cells secrete large numbers of KSHV virions into the culture medium. We assayed the ability of the antivirals to block KSHV production, as measured by the release of encapsidated viral DNA. The results show that KSHV replication is insensitive to acyclovir (9-[(2-hydroxyethoxy)-methyl]guanine) (50% inhibitory concentration [IC_{50}] = 60–80 μM), but sensitive to ganciclovir (9-[1,3-dihydroxy-2-propoxymethyl]guanine) (IC_{50} = 2.7–4 μM), foscarnet (trisodium phosphonoformate hexahydrate) (IC_{50} = 80–100 μM), and cidofovir (1-[(*S*)-3-hydroxy-2-(phosphonomethoxy)propyl]cytosine) (IC_{50} = 0.5–1 μM). (*J. Clin. Invest.* 1997. 99:2082–2086.) Key words: KSHV • HHV-8 • IC_{50} • BCBL-1

Introduction

Before the world-wide spread of the human immunodeficiency virus (HIV), Kaposi's sarcoma (KS)[1] was a rare tumor usually afflicting elderly Mediterranean and African men. However, since the onset of the HIV epidemic, it has become the single most frequent neoplasm of patients with the acquired immunodeficiency syndrome (AIDS). Although HIV infection by itself increases the risk of developing KS by 20,000-fold compared with that of the general population (1), this risk is not evenly distributed among AIDS subpopulations. Rather, it varies significantly by mode of HIV acquisition (1–3). Most dramatically affected are HIV-infected homosexual and bisexual men, 15–25% of whom will develop KS in the course of their HIV infection. In contrast, the rate of KS in those who contract HIV by percutaneous inoculation or vertical transmission is 1–3% or less (1). Women with AIDS also have a similarly low risk of KS (1, 4–8). Taken together, these findings suggest that a sexually transmitted cofactor other than HIV may be required for KS development. Consistent with this, KS also occurs in selected HIV-negative groups, including immunosuppressed transplant recipients (2, 9) and some African and Mediterranean populations (2, 3).

Over 2 yr ago, DNA sequences of a novel human herpesvirus, termed Kaposi's sarcoma–associated virus (KSHV) or human herpesvirus-8 (HHV-8), were first identified in the KS tumor of a patient with AIDS (10). Since then, KSHV DNA sequences have been regularly identified in all forms of KS (11) and in the rarer AIDS-associated body cavity–based B cell lymphomas (also known as peripheral effusion lymphomas) (12). Furthermore, viral infection precedes the onset of KS (13, 14), is predictive of an increased risk of tumor development (13–16), and is targeted to the spindle cells thought to be the central cell in KS pathogenesis (17, 18). Thus, the case for a major etiologic role for KSHV in KS is becoming increasingly strong. Accordingly, there is interest in the possibility that antiviral therapy of infected individuals might reduce the incidence of KS or delay its onset. However, to date little information is available on the susceptibility of KSHV replication to antiherpesvirus therapies (19).

To address this issue we used a culture system recently developed in our laboratory (20) in which the phorbol ester 12-*O*-tetradecanoylphorbol-13-acetate (TPA) efficiently induces lytic growth of KSHV in BCBL-1 cells, a latently infected lymphocyte line derived from a body cavity–based lymphoma. In this system, induced cells release large numbers of intact virions into the media (20). As a result, we were able to measure the effects of different antiherpesvirus drugs on KSHV release, focusing on four clinically available agents: acyclovir, ganciclovir, cidofovir, and foscarnet. The first two are nucleoside analogs, the third is a nucleotide analog, and the fourth is a pyrophosphate analog; all four act by inhibition of the viral DNA polymerase during lytic viral replication (21–24).

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1. *Abbreviations used in this paper:* CMV, cytomegalovirus; KS, Kaposi's sarcoma; KSHV, KS-associated herpesvirus; TPA, 12-*O*-tetradecanoylphorbol-13-acetate.

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Methods

Cell culture. BCBL-1 is a B cell line derived from a body cavity–based B lymphoma that is latently infected with HHV-8/KSHV; no EBV DNA is present in this line (20). The cells were maintained as described previously (20). Cell viability and growth were assessed by counting trypan blue–excluding cells on a hemocytometer. All cell counts were performed in triplicate.

KSHV induction and antiviral drug addition. On day 1, 10 ml of BCBL-1 cells at 2–3 $\times$ 10^5/ml were pelleted at 500 *g* for 5 min, washed once with 37°C PBS, and then resuspended to the original volume with fresh prewarmed media with or without TPA (Calbiochem-Novabiochem Corp., La Jolla, CA) (final concentration = 20 ng/ml) and the indicated antiviral drugs: acyclovir (Burroughs Wellcome Co., Research Triangle Park, NC), ganciclovir (Syntex Laboratories Inc., Palo Alto, CA), foscarnet (Astra USA, Inc., Westborough, MA), cidofovir (Gilead Sciences Inc., Foster City, CA), or A77003 (Abbott Laboratories, Abbott Park, IL). On day 2 (16–24 h after TPA addition), the cells were repelleted as above and resuspended in 10 ml fresh media with the same antiviral drug but without TPA. On day 4, 10 ml of additional fresh media with the appropriate antiviral drug was added to the flasks, thereby doubling the final volume. On day 7, the virus was harvested from the media (see below). Aliquots of each sample on days 1, 4, and 7 were removed to assess cell viability and growth (see above).

Viral isolation. KSHV was isolated essentially as we have described previously (20). To establish the amount of virus present in the media at baseline, media on day 2 were collected immediately after their addition to the washed cell pellet (see above). Free virus was isolated from these media as described (20). The remaining samples were incubated for a total of six additional days (see above), at which time released intact virus was similarly isolated. DNA was isolated from the viral pellets also as described previously (20).

Viral quantification. An aliquot of viral DNA from each sample (equivalent to 5 ml of culture supernatant; in the untreated control sample, this represented $\sim$ 5 $\times$ 10^6 viral genome-equivalents as judged by hybridization with cloned KSHV DNA standards) was denatured for 10 min at room temperature by adding an equal volume of 0.2 N NaOH and then diluting with nine additional volumes of 20$\times$ SSC (3 M sodium chloride; 0.3 M sodium citrate; Sigma Chemical Co., St. Louis, MO) before transferring immediately to a nylon filter (Hybond-N, International plc, Buckinghamshire, United Kingdom) using a dot blot apparatus. The bound DNA on the filter was then hybridized to a ^{32}P-labeled KSHV specific probe. The filter was then placed on a phosphor screen for 24–48 h and the signals were quantitated using a PhosphorImager (Molecular Dynamics, Milpitas, CA). The background signal from supernatants collected immediately after the washing step on day 2 (see above) was $<$ 1% of the value detected from the equivalent amount of supernatant from the day 7, no drug control. Values were expressed as a percentage of the no drug control.

Results

Assay for KSHV production. Since the replication of other herpesviruses is sensitive to the aforementioned antiviral drugs, we reasoned that KSHV might be similarly inhibited. Although no plaque assay or efficient de novo infection system exists for KSHV, we were able to assess effects on viral production by measuring directly the amount of viral DNA released into the media after TPA induction. Although the vast majority of BCBL-1 cells are latently infected, 1–3% of the untreated cells spontaneously enter the lytic cycle and release virus. To minimize the contribution from this basal level of virus release, we washed the cells and placed them into fresh media at the start of each experiment (see Methods). At various times thereafter, virus was pelleted from the medium and virion DNA extracted and quantitated. All viral preparations were first digested with micrococcal nuclease to eliminate free (unencapsidated) KSHV DNA released from dying cells; thus, we measured only encapsidated (virion) DNA in the assay (see Methods and reference 20).

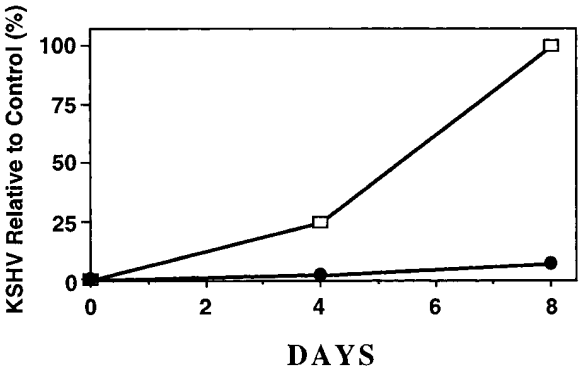

Figure 1. Time course of KSHV release from TPA-induced BCBL-1 cells in the absence (*open rectangles*) or presence (*closed circles*) of foscarnet (250 μM).

Time course of KSHV production. We began the analysis by determining the time course of KSHV release from TPA-induced BCBL-1 cells. Fig. 1 shows the results of one such experiment. Enhanced virion production is detected as early as 2 d after induction, and virions continue to accumulate in the medium for up to 8 d after induction. For convenience, all subsequent assays were scored at 6 d after TPA addition. Fig. 1 also shows the results of the addition of 250 μM foscarnet to a parallel set of cultures; this reduced the total amount of KSHV released into the media by $>$ 90% compared with the control.

Specificity and potency of antiherpesvirus drugs. To compare the relative potencies of the antiherpesvirus drugs on KSHV production, we first tested each one at three different concentrations (chosen with reference to the known sensitivities of herpes simplex virus and human cytomegalovirus [CMV]) (21, 24, 25). The effects of acyclovir, ganciclovir, cidofovir, and foscarnet on KSHV production are compared in Fig. 2 *A*. The addition of TPA alone led to a 25-fold induction of released virus compared with the low levels arising from spontaneous lytic replication. At sufficiently high concentrations, each of the four antiherpesvirus drugs inhibited this TPA-induced replication. 50% or greater inhibition resulted from acyclovir at only the highest concentration tested (160 μM), ganciclovir and foscarnet at the intermediate concentrations tested (4 and 100 μM, respectively), and cidofovir at the lowest concentration tested (1 μM). (Although in this experiment the level of KSHV inhibition with 25 μM cidofovir was slightly less than with 5 μM cidofovir, this finding was not reproduced in other experiments [data not shown].) In contrast to the four antiherpesvirus drugs, the HIV protease inhibitor A77003 (a ritonavir analog) in a separate experiment (Fig. 2 *B*) had no effect on KSHV production even at 1.5 μM, a concentration 10–20-fold higher than its IC_{50} in comparable HIV antigen release assays (26). To control for potential nonspecific cytotoxicity induced by the drugs, in each experiment we examined (at several time points) the viability of cells in treated and untreated cultures, using trypan blue exclusion. In all experiments, there was a 20–

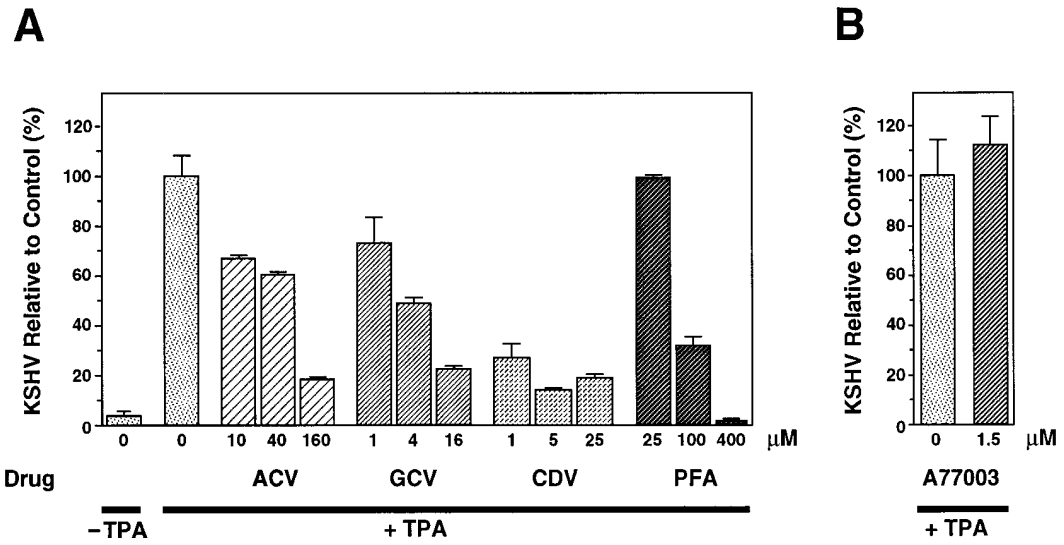

Figure 2. Effect of antiviral drugs on KSHV production in culture. (*A*) Dose-dependent inhibition of KSHV production by acyclovir (*ACV*), ganciclovir (*GCV*), cidofovir (*CDV*), and foscarnet (*PFA*) relative to no drug controls. (*B*) Effect of HIV-protease inhibitor, A77003, on KSHV release relative to the no drug control. Omission (*−TPA*) or inclusion (*+TPA*) of TPA is indicated below the graphs. All values are the mean, and error bars indicate the range of triplicate determinations.

30% decline in percentage of viable cells over the course of the experiment, but there was no difference between drug-treated and untreated cultures; presumably this reflects the toxic effects of TPA and possibly the inhibitory effects of early viral gene expression, which in other herpesviruses is known to shut off host gene expression. The growth rates of the viable cells in the cultures of treated and untreated cells were likewise identical (data not shown). Thus, no evidence for nonspecific drug-induced cytotoxicity was found.

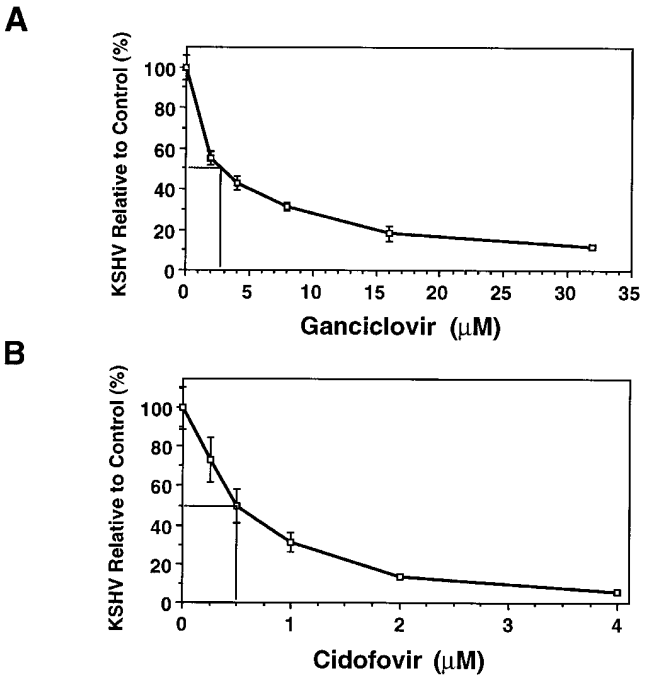

Figure 3. IC_{50} determinations for ganciclovir (*A*) and cidofovir (*B*) inhibition of KSHV production. IC_{50} values are indicated by the vertical lines intercepting the curves. Values are the mean, and error bars indicate the range of triplicate determinations.

IC_{50} determinations. Knowing the approximate range of concentration required for each drug's inhibition of KSHV production, we then determined more accurately the IC_{50} values, using similar assays but with five concentrations at twofold increments. The results of such experiments for ganciclovir and cidofovir are shown in Fig. 3; the IC_{50} for the two drugs were $\sim$ 2.7 and 0.5 μM, respectively. Although the absolute value of the IC_{50} measured for each drug varied up to twofold among repeated experiments, the shape of a single drug's dose–response curve and the relative potencies of one drug versus another were constant. Table I lists the IC_{50} determinations for all four drugs, with the ranges reflecting the interexperimental variation. Table II allows comparison of these IC_{50} values with the typical serum concentrations of these drugs after intravenous administration of standard (full) doses (22, 27). This reveals that even though the absolute values of the IC_{50} for acyclovir and foscarnet are similar, such concentrations are therapeutically achievable only for foscarnet.

Discussion

To date no efficient de novo infection system exists for KSHV (19, 28, and Renne, R., and D. Ganem, manuscript in preparation), preventing the development of a simple plaque assay for viral infectivity. Instead, to test the susceptibility of KSHV to antiherpesvirus drugs, we have developed an in vitro system that measures viral release into the media after TPA induction of latently infected BCBL-1 cells. In contradistinction to plaque-reduction assays, in which even subtle effects on viral growth can become greatly amplified through multiple rounds of replication and infection, measurements in our assay system reflect the properties of a single cycle of viral growth. As a result, the IC_{50} values for the four drugs we examined are not directly comparable with those determined by plaque-reduction assay for the same drugs against other herpesviruses (21, 24, 25) (they are likely to be higher than those that would arise from a plaque-reduction assay, were one available). Nonetheless, broadly speaking the sensitivity profile of KSHV resembles most closely that found for the β-herpesvirus CMV, which is similarly insensitive to acyclovir, but sensitive to the other

Table I. Comparison of IC_{50} Values (μM) for Acyclovir (ACV), Ganciclovir (GCV), Cidofovir (CDV) and Foscarnet (PFA) for the Inhibition of KSHV Production

Drug	KSHV (released virus)
ACV	60–80
GCV	2.7–4
CDV	0.5–1
PFA	80–100

three drugs we tested. To the extent that the potential therapeutic efficacy of each drug depends on the ratio of its peak achievable serum level ($C_s$max) to its IC_{50} ($C_s$max/IC_{50}), the predicted drug potency against KSHV would be: cidofovir ($C_s$max/IC_{50} = 140–30) > foscarnet ($C_s$max/IC_{50} = 74–10) > ganciclovir ($C_s$max/IC_{50} = 18.5–12.5) > acyclovir ($C_s$max/IC_{50} = 1.67–1.25). We emphasize that these conclusions are based upon the single isolate present in the BCBL-1 cell line; it is not yet possible to test the susceptibility of viruses found in primary clinical specimens and there are few if any other cell lines in culture that support replication sufficiently well to allow antiviral testing (19, 29).

Recently it has been observed that some AIDS-KS patients treated with HIV protease inhibitors (usually in combination with HIV reverse transcriptase inhibitors) experience arrest or even regression of their KS lesions while on this therapy (Lampiris, H., and T. Berger, personal communications). In contrast to ganciclovir, foscarnet, and cidofovir, the HIV protease inhibitor we tested (A77003, a ritonavir analog) had no effect on KSHV replication. This result is not unexpected, given the known specificity of ritonavir and the wide divergence between retroviral and herpesviral proteases. Our data suggest that the anti-KS benefits of HIV protease inhibitors are likely mediated by indirect effects, most probably reflecting the partial restoration of immune system function that accompanies effective inhibition of HIV replication.

What are the implications of these findings for patients with (or at risk for) KS? Since all antiherpesviral drugs are targeted to lytic rather than latent viral replication, this question reduces to the following: what is the role of lytic viral replication in KS tumorigenesis? Our earlier studies of KS tumors indicate that most spindle cells are latently infected (17); such cells would not be expected to be affected by antiviral therapy. However, a small subpopulation of cells in the tumor does support lytic viral growth (17, 30). If, for some reason, latently infected tumor cells are not capable of indefinite proliferation (for example, if they received only a transitory growth stimulus or if they underwent enhanced programmed cell death), then the propagation of a KS tumor could depend upon continuous de novo infection by virus released from lytically infected cells. Were this so, then inhibition of KSHV replication could have a delayed impact on the biology of an established KS tumor.

Table II. Average Peak Serum Levels of Each Drug after Intravenous Administration

Drug (mg/kg)	Serum peaks (μM)
ACV (10)	100
GCV (5)	50
CDV (5)	30–70
PFA (90)	990–5900

However, a more likely role for anti-KSHV therapy is in the prevention or retardation of KS development. Earlier studies (13, 16, 31) document that KSHV infection typically precedes the development of clinical KS by several years. Presumably, during this interval, virus produced by lytic replication spreads to spindle cells, where a latent infection is set up leading ultimately to growth deregulation. It is reasonable to assume that the risk of KS tumorigenesis will be proportional to the number of such latently infected cells produced; this, in turn, would be related to the intensity and duration of lytic viral growth. Accordingly, inhibition of viral growth during this period could diminish the number of infected spindle cell targets and thus retard or reduce the progression to overt KS. In this connection it is interesting to note that some retrospective clinical studies have examined whether antiherpesviral therapy in AIDS (usually for concomitant CMV infection) alters the natural history of KS development. Acyclovir routinely has had no beneficial effect (14, 32, 33), while ganciclovir has had in some cases no effect (14, 33) but in others a modest protective benefit (32). Foscarnet has shown the greatest potential, with both case reports of KS remissions after treatment (34) and larger observational studies describing associations between treatment and the inhibition of KS development (14, 32). (However, the potential benefit of foscarnet remains controversial [33].) In general, these findings are in accord with expectations based on our in vitro results. For cidofovir, the drug we found to have the greatest in vitro activity against KSHV, only anecdotal data exist on its effects on KS, but a clinical study addressing its use in KS treatment is currently underway (Cherrington, J., personal communication).

In light of the above considerations, it seems reasonable to consider conducting prospective clinical trials to determine if treatment with effective anti-KSHV drugs reduces the risk of KS development. The ideal population for such trials would be HIV-infected subjects who are seropositive for KSHV but who do not yet have overt KS. Unfortunately, given the long interval between KSHV seroconversion and the development of clinical KS, such trials would require long-term drug administration, mandating that the tested drugs be both nontoxic and orally bioavailable. None of the three drugs we have found to have anti-KSHV activity fulfills these criteria: most require intravenous administration to achieve potentially effective serum levels (though an oral preparation of ganciclovir has been developed recently) and each can engender significant systemic toxicity. However, several new, orally bioavailable antivirals are now being evaluated for other herpesviral infections; it will be of interest to examine the activity of these drugs against KSHV. Finally, we note that our in vitro system provides a generally applicable strategy for identifying novel compounds with anti-KSHV activity that might prove useful for such trials.

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