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Efficacy of antiviral compounds in human herpesvirus-6–infected glial cells

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The β-herpesvirus human herpesvirus-6 (HHV-6) is becoming increasingly recognized as an important pathogen in immunocompromised patients, particularly in post bone marrow transplant (BMT). Reactivation of latent HHV-6 resulting in encephalitis has been reported in BMT and stem cell transplant (SCT) patients. The development of HHV-6 encephalitis can be a fatal complication, the frequency of which is increasing likely due to improved diagnosis with quantitative polymerase chain reaction (PCR) of cerebrospinal fluid. There are currently no antiviral compounds approved for HHV-6, nor have any controlled clinical trials been conducted. The frequency and severity of HHV-6 encephalitis in both immunocompetent and immunocompromised patients necessitates studies on the usefulness of currently available anti-viral compounds. The authors compared the antiviral efficacy of four drugs currently used for cytomegalovirus (CMV) infection, a β-herpesvirus sharing homology with HHV-6. In HHV-6A– and HHV-6B–infected T cells, acyclovir, ganciclovir, foscarnet, and cidofovir exhibited antiviral activity consistent with that published in other studies. In HHV-6–infected human astrocytes (U251), however, only foscarnet and cidofovir exhibited antiviral activity and this effect was restricted to infection with HHV-6 variant A. In pathological brain sections from patients with neurological disorders such as multiple sclerosis and epilepsy, HHV-6 has been localized to glial cells. Determination of antiviral activity in human glial fibrillary acidic protein (GFAP)-positive astrocytes of currently used antiviral compounds is essential for potential treatment of HHV-6 and neurological disorders. Our data highlight the necessity for further study of antiviral compound in HHV-6–infected glial cells as well as the development of more selective compounds for HHV-6. *Journal of NeuroVirology* (2006) **12,** 284–293.

Keywords: anti-viral; CNS; glial cells; HHV-6; quantitative PCR

Introduction

Human herpesvirus-6 (HHV-6) is a β-herpesvirus originally isolated in 1986 from patients with lymphoproliferative disorders (Ablashi *et al*, 1987). HHV-6 shares homology with the other β-herpesviruses cytomegalovirus (CMV) and HHV-7, and exists as two distinct variants: 6A and 6B. HHV-6A and -6B share 90% sequence homology (Isegawa *et al*, 1999) but differ in tropism and etiology (De Bolle *et al*, 2005a). HHV-6B is the causative agent of the childhood disease exanthem subitum (Yamanishi *et al*, 1988); however, the more neurotropic strain, HHV-6A, has not been definitively linked to a disease. Exposure to HHV-6 occurs most often during the first 2 years of life, and the virus has been hypothesized to establish latency at various sites following primary infection. Possible sites of HHV-6 latency have been suggested to include peripheral blood mononuclear

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cells (PBMCs), salivary glands, and the central nervous system (CNS) (Campadelli-Fiume *et al*, 1999; Caserta *et al*, 1994). In the CNS, HHV-6 has been localized to glial cells, suggesting a susceptibility of glia to HHV-6 infection and the potential for glial cells to harbour latent virus (Challoner *et al*, 1995). Previous studies have suggested that reactivation of HHV-6 in the CNS may be associated with neurologic diseases (Kimberlin and Whitley, 1998), including epilepsy (Donati *et al*, 2003), multiple sclerosis (MS) (Akhyani *et al*, 2000; Challoner *et al*, 1995; Opsahl and Kennedy, 2005; Soldan *et al*, 1997), encephalitis/meningitis (Birnbaum *et al*, 2005; Isaacson *et al*, 2005), and post–bone marrow transplant encephalitis (Maeda *et al*, 2000; Yoshida *et al*, 2002). HHV-6 is becoming recognized as an important CNS pathogen and identification of clinically useful antiviral therapies against this virus may provide valuable information for management of HHV-6–associated complications.

The antiviral drugs currently used to treat herpesvirus infection are primarily active against herpes simplex virus 1, 2 (HSV-1, HSV-2), varicella-zoster virus (VZV), and CMV. Acyclovir and ganciclovir are guanosine analogs that require intracellular phosphorylation by a viral kinase (U69) for their activation. The triphosphate metabolites of acyclovir and ganciclovir compete with GTP to inhibit viral DNA polymerase. Acyclovir and ganciclovir both have higher affinity for viral than for cellular DNA polymerase, giving them selectivity for HHV-6 (De Bolle *et al*, 2005a; Matthews and Boehme, 1988). Ganciclovir is used primarily to treat CMV infection in neonates and immunosuppressed patients and has been used with some success to treat both CMV and HHV-6 infection post transplant. Acyclovir has less *in vitro* activity against HHV-6 than ganciclovir, but its low toxicity makes it useful in some cases (Burns and Sandford, 1990).

Foscarnet and cidofovir are both nonguanosine derivatives. Foscarnet reversibly binds to the pyrophosphate binding site of viral DNA polymerase and has higher affinity for viral than for cellular DNA polymerase, increasing its selectivity for viral polymerases. Foscarnet has been used to treat acyclovir-resistant HSV and VZV and both CMV and HHV-6 infection in immunocompromised patients. Previous studies have shown that foscarnet exhibits *in vitro* activity against HHV-6 in a number of T-cell lines with low toxicity and does not require activation by a viral kinase (Burns and Sandford, 1990; De Clercq, 2005; Manichanh *et al*, 2000; Reymen *et al*, 1995). Cidofovir, an acyclic nucleoside phosphonate analog of (deoxy) CMP, does not require metabolism for activation. Mechanistically, cidofovir competitively inhibits dCTP and has demonstrated broad-spectrum antiviral activity against most DNA viruses including herpes, polyoma, papilloma, adeno, and poxviruses. It is currently used to treat CMV retinitis in acquired immunodeficiencey syndrome (AIDS) patients (De Bolle *et al*, 2005a). Similar to the other antiviral compounds, cidofovir has higher affinity for viral than for cellular DNA polymerase. Efficacy of cidofovir against HHV-6 has been demonstrated using infected HSB T-lymphoblastoid cells (Reymen *et al*, 1995).

To date, *in vitro* studies investigating the antiviral efficacy of acyclovir, ganciclovir, foscarnet, and cidofovir against HHV-6 have primarily used virus infected T cells. Although originally isolated from lymphocytes, HHV-6 is a pleiotropic virus that infects a variety of cell types, including astrocytes (Donati *et al*, 2005; He *et al*, 1996), oligodendrocytes (Ahlqvist *et al*, 2005), monocytes (Janelle and Flamand, 2006), and dendritic cells (Niiya *et al*, 2004). As HHV-6 is a commensal virus in the CNS, the recent demonstration of increased HHV-6 DNA and viral transcripts in lesions of MS plaques in a subset of MS patients suggests a role for HHV-6 in disease pathogenesis (Cermelli *et al*, 2003; Opsahl and Kennedy, 2005). Additionally, a number of studies have shown HHV-6 has tropism for human primary astrocytes (Yao *et al*, 2006), progenitor cells–derived astrocytes (Donati *et al*, 2005; He *et al*, 1996), and other astrocytic cell lines (Yoshikawa *et al*, 2002), suggesting that CNS astrocytes may be an *in vivo* reservoir for HHV-6. Therefore, identification of clinically useful antiviral therapies against HHV-6 in cells of neural origins becomes increasingly important. In this study, we established an *in vitro* system of an HHV-6–infected astrocytic cell line to address the efficacy of four commercially available antiviral drugs. We compared two guanosine analogues (acyclovir and ganciclovir) and two nonguanosine derivatives (foscarnet and cidofovir) in inhibiting HHV-6 replication in neural glial cells.

Results

Infection of HHV-6 variants A and B in Astrocytes and T cells

HHV-6 was first described as a lymphotropic virus (Salahuddin *et al*, 1986); therefore, *in vitro* studies that have examined the anti-HHV-6 activity of guanosine analogues and nonguanosine derivatives targeting viral replication were primarily performed in T cells. The broad spectrum of neurological diseases associated with HHV-6 has necessitated the need for the development of an *in vitro* system. Accordingly, we evaluated the efficacy of antiviral drugs in controlling HHV-6 replication in cells of CNS origin. Based on previous studies demonstrating that HHV-6 can infect astrocytes (He *et al*, 1996), we used a glial fibrillary acidic protein (GFAP)-positive astroglioma cell line U251 that constitutively expresses the viral receptor CD46 (Figure 1) for infection with HHV-6.

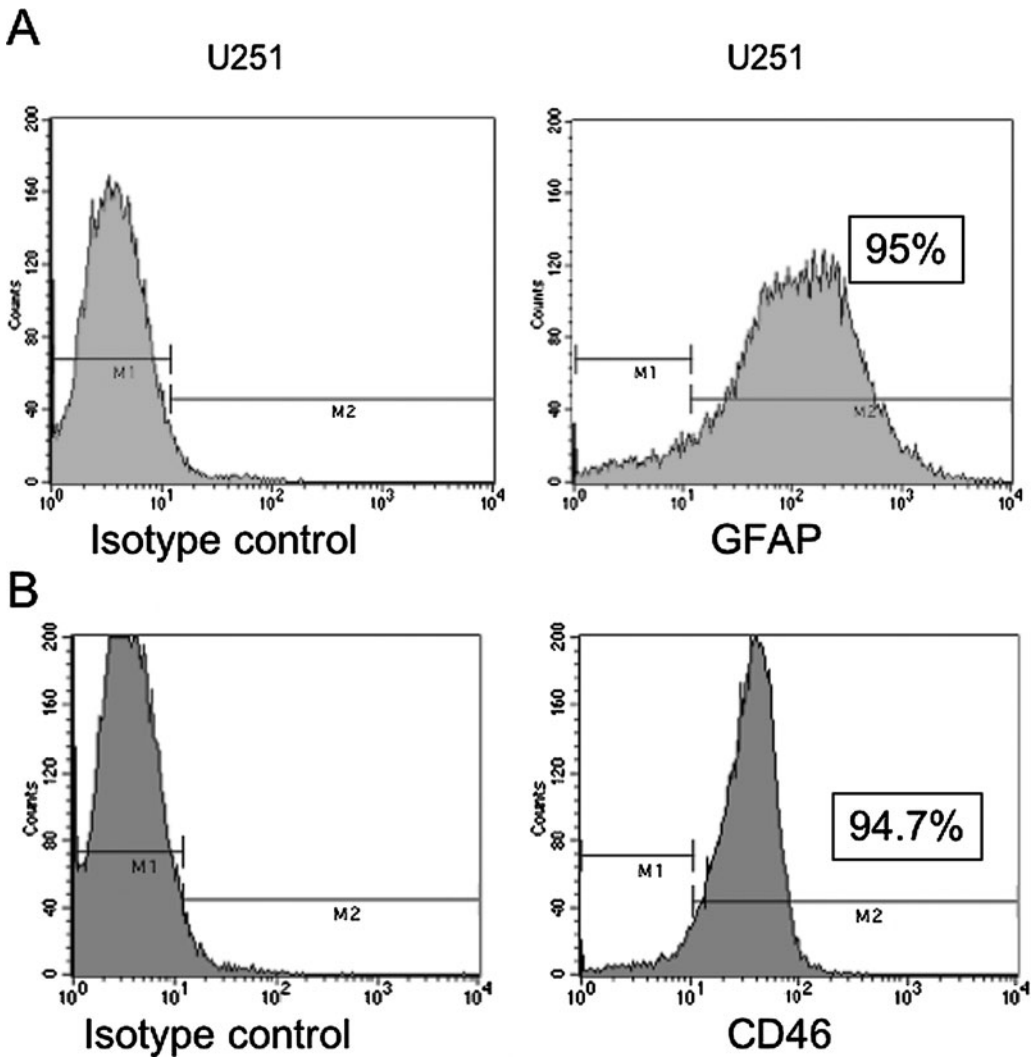

Figure 1 Expression of GFAP and CD46 on U251 astroglioma cell line. (**A**) FACS analysis demonstrated 95% of U251 cells expressed GFAP, a marker of astrocytic lineage, compared to staining with mouse IgG1 isotype control. (**B**) The expression of the HHV-6 receptor CD46 is on 94.7% of the U251 astroglioma cell line.

Both strains of HHV-6 replicated efficiently in a susceptible CD4+ T-cell line (SupT-1), as demonstrated by the consistent increase in viral loads over time (Figure 2A). Infectivity of HHV-6 variants A and B in U251 cells were monitored by measuring HHV-6 viral loads using a highly sensitive, strain specific, quantitative TaqMan polymerase chain reaction (PCR) analysis (Nitsche *et al*, 2001). As shown in Figure 2B, HHV-6 variant A infection of U251 cells was productive as demonstrated by over 2 logs increase in viral load from days 3 to 7 post infection. By contrast, HHV-6 variant B viral load decreased over time, suggesting a nonproductive infection. This observation is consistent with other published studies demonstrating variant-specific tropism of HHV-6 infection in glial cells (Donati *et al*, 2005). To confirm the infectibility of astroglioma cells by HHV-6, we performed reverse transcriptase (RT)-PCR analysis for *de novo* synthesis of viral messenger RNA. Expression of the HHV-6 U69 gene was detected only in HHV-6A–infected astroglioma cells but not in HHV-6B–infected cells (Figure 2C). This result is consistent with the quantitative TaqMan PCR analysis of viral DNA (Figure 2B), suggesting a productive infection of HHV-6A in astroglioma cells whereas HHV-6B infection was abortive. Moreover, we performed RT-PCR analysis of HHV-6 U12 transcript over time on HHV-6A–infected U251 cells. As shown in Figure 2D, expression of U12 mRNA increased exponentially from 4 to 56 h post infection. Collectively, these results demonstrate a differential tropism of HHV-6A and HHV-6B for astroglioma *in vitro*.

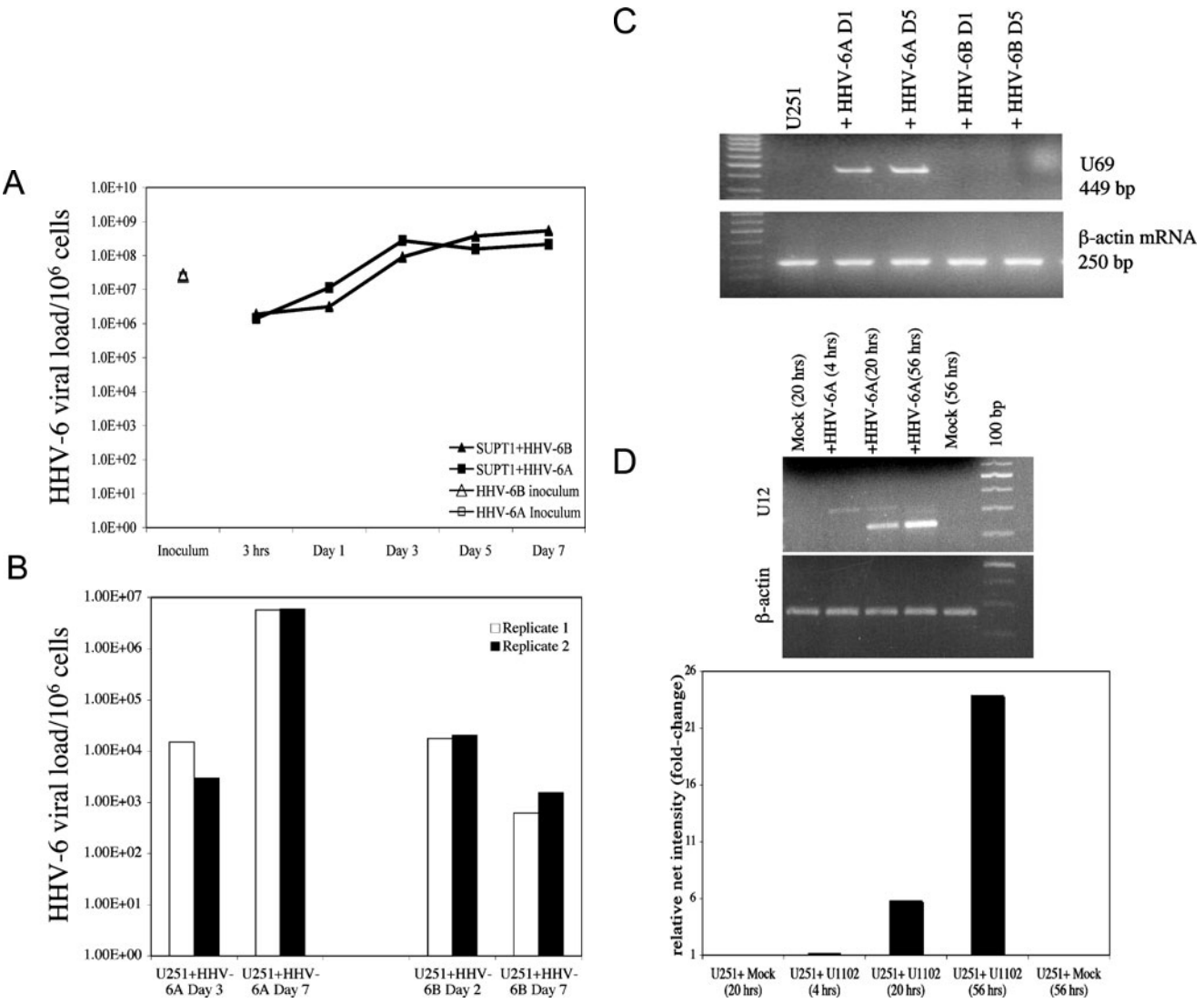

Figure 2 Quantitative real-time PCR detection of HHV-6A and HHV-6B viral loads in HHV-6 infected cells. (**A**) Viral loads analysis of HHV-6 variants A and B infected SupT-1 at 3 h, 1, 3, 5, and 7 days post infection. Replication of HHV-6 in SupT1 was demonstrated by the increase in viral load over time. (**B**) Viral loads analysis of HHV-6 variants A and B infected U251 astroglioma cell line. Productive infection of HHV-6A in U251 was demonstrated by increase in viral load from days 3 to 7 post infection. Decrease in HHV-6 variant B viral load indicated a non productive infection. (**C**) Analysis of the expression of immediate early viral transcript (U69) by RT-PCR in U251 cells infected with HHV-6 variant A or B. Top panel illustrates the RT-PCR analysis for U69 at days 1 and 5 post-infeciton. β-Actin was used as an internal control for RNA quality (*bottom panel*). (**D**) Quantitation of HHV-6A mRNA expression over time. Amount of U12 transcript was normalized to the β-actin housekeeping gene.

Evaluation of antiviral drug efficacy against HHV-6 infection in T cells and U251 astroglioma

A few studies have recently begun to examine the efficacy of antiviral compounds, including acyclovir, ganciclovir, foscarnet, and cidofovir, in controlling HHV-6 replication in T-cell lines (De Bolle *et al*, 2004). However, whether these drugs are effective at controlling HHV-6–infected glial cells is unknown. In this study, we addressed this question by comparing the efficacy of these antiviral compounds using a GFAP-positive astroglioma cell line (U251) and CD4+ T-cell line (SupT-1) infected with HHV-6. Astroglioma and CD4+ T-cell lines infected with HHV-6 variants A and B were treated with nontoxic doses of the various antiviral compounds experimentally determined (See Materials and Methods) to be acyclovir (70 μg/ml), ganciclovir (5 μg/ml), foscarnet (70 μg/ml), and cidofovir (5 μg/ml). Antiviral activity was assessed by monitoring the HHV-6A and HHV-6B viral loads over time. In the HHV-6A–infected CD4+ T-cell line, foscarnet and ganciclovir demonstrated the highest efficiency in inhibiting viral replication with 1.5- and 3-log decreases in viral loads, respectively, compared with infected cells not treated with drugs (Figure 3A). In contrast to HHV-6A–infected CD4+ T cells, foscarnet and cidofovir were found to

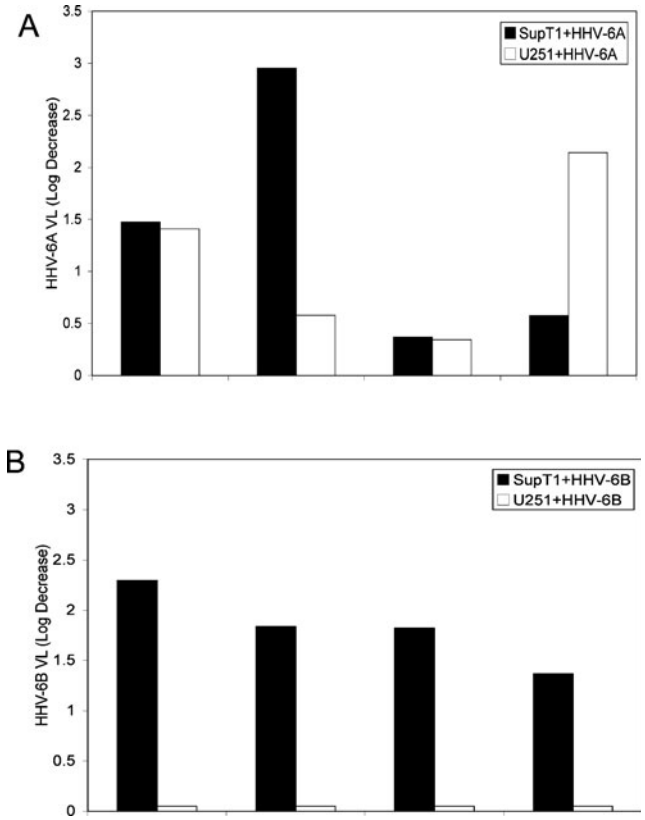

Figure 3 Evaluation of antiviral drug activity against HHV-6A and HHV-6B viral replication in human T cells and astrocytes. (**A**) SupT-1 T cells and U251 astroglioma cells were infected with HHV-6A (U1102) for 7 days in the presence or absence of nontoxic concentrations of ACV (70 μg/ml), PFA (70 μg/ml), GCV (5 μg/ml), or CDV (5 μg/ml). HHV-6 viral load in cells was measured by real-time TaqMan PCR and represented as log decrease compared with the HHV-6 viral load in non-treated cells. (**B**) Antiviral effect of nontoxic doses of PFA, GCV, ACV, and CDV in HHV-6B variant infected SupT-1 and U251.

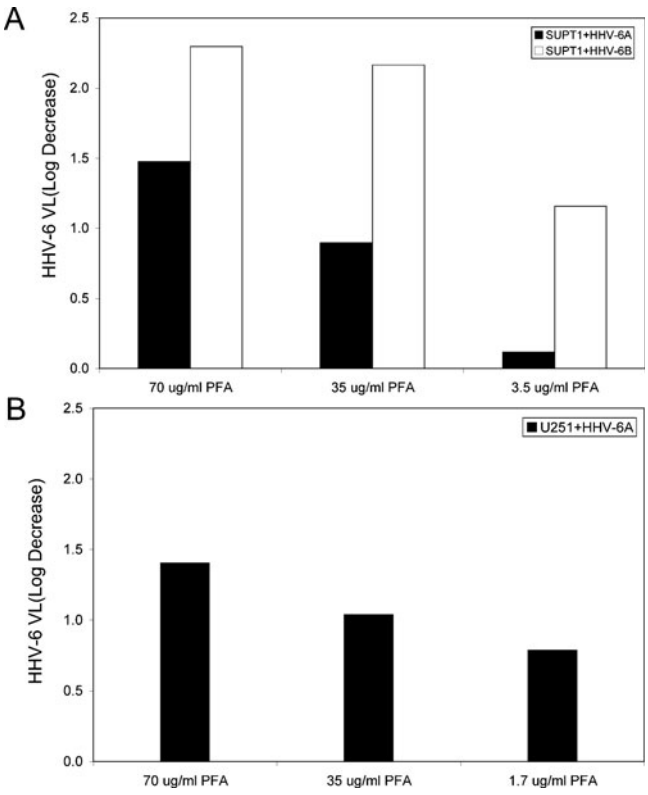

Figure 4 Antiviral activity of foscarnet (PFA) is dose-dependent in human T cell and U251 astroglioma. (**A**) SupT-1 cells infected with HHV-6A and B variants were treated with various concentrations of PFA as indicated for 7 days. Viral loads in cells were measured by HHV-6 strain specific real-time TaqMan PCR. (**B**) HHV-6 variant A infected U251 astroglioma cells were treated with 70, 35, and 1.7 μg/ml of PFA for 7 days and viral load was determined by real-time TaqMan PCR.

be most effective in inhibiting HHV-6 replication in U251 astroglioma cells. For HHV-6 B variant, all four antiviral compounds demonstrated significant anti-HHV-6 activity in CD4+ T-cell line (Figure 3B) (Supplementary material is available for this article. Go to the publisher's online edition of the **Journal of NeuroVirology** for an additional Table). However, because HHV-6B infection in astroglioma was abortive, antiviral activity against HHV-6 variant B infection in astrocytes could not be assessed. Together, these results demonstrate that antiviral compounds tested in this study may have differential efficacy in controlling HHV-6 infection in cells of lymphoid or glial origin.

Dose dependency of foscarnet

Among the four antiviral compounds tested, foscarnet was the only compound demonstrating the ability to control both HHV-6A– and HHV-6B–infected T cells and HHV-6A infection in glial cells. Therefore, the anti-HHV-6 property of foscarnet was further characterized by examining the dose dependency of this compound. As shown in Figure 4A, in CD4+ T cells, foscarnet demonstrated significant dose-dependent inhibition of HHV-6B viral replication. By contrast, significant inhibition of HHV-6A–infected T cells was observed only at higher concentrations (70 μg/ml and 35 μg/ml). A similar degree of inhibition could be seen in HHV-6A-infected astroglioma at high drug concentrations. However, at low concentrations (3.5 μg/ml in CD4+ T cells) and (1.7 μg/ml in astroglioma), foscarnet was more effective at inhibiting HHV-6A replication in astroglioma than in T cells. These data suggest that higher concentrations (70 μg/ml and 35 μg/ml) of foscarnet are effective at targeting both HHV-6A and HHV-6B in T cells, but the drug maybe more efficient in glial cells when used at low concentrations.

Mechanism of foscarnet antiviral activity in astroglioma cell line

The mechanism of antiviral activity of foscarnet has been described in lymphocytes (Reymen *et al*, 1995); however, no study to date has examined its mode of action in neural glial cells. It has been described that foscarnet inhibits viral replication by blocking the pyrophosphate binding site of viral DNA polymerase,

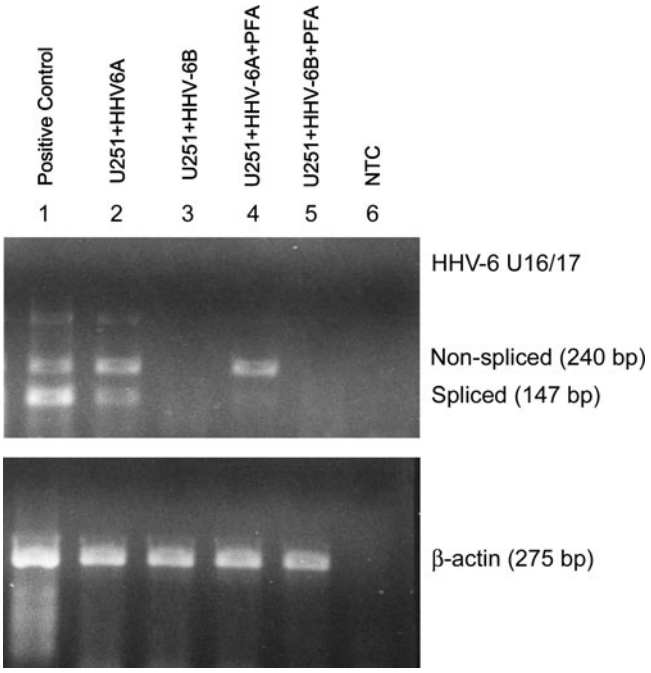

Figure 5 Detection of viral transcript by RT-PCR in U251 cells infected with HHV-6A and HHV-6B. Lane 1: positive control (HHV-6A infected T cells) expressed both immediate early (*upper band*) and late (*lower band*) transcripts of HHV-6 U16/17. Lanes 2 and 3: U251 cells infected with HHV-6A or B, respectively. Expressions of immediate early (*upper band*) and late (*lower band*) transcripts were detected in HHV-6A–infected U251 cells. U16/17 transcripts were undetectable HHV-6B–infected U251 cells. Lanes 4 and 5: HHV-6A– or HHV-6B–infected U251 cells treated with PFA. In HHV-6A–infected U251 cells treated with PFA, the late transcript (U16) was not detected despite the expression of immediate early transcript (U16/17). Lower panel shows β-actin as an internal control.

thereby preventing the cleavage of pyrophosphate from deoxynucleoside triphosphate and elongation of the viral DNA chain (De Bolle *et al*, 2005a). Similar to other β-herpesvirses, HHV-6 follows a strict temporal transcription process with initial expression of immediate early, followed by early, and then late genes (Mirandola *et al*, 1998; Oster and Hollsberg, 2002). To examine whether foscarnet interferes with HHV-6 viral DNA polymerase, we utilized primers specific for the HHV-6 U16/17 transcript. U16/17 has been described to be an immediate transcript that does not require *de novo* protein synthesis for its expression. The spliced product, U16, is expressed as a late gene and is dependent on protein expression (Flebbe-Rehwaldt *et al*, 2000). Shown in Figure 5 is RT-PCR analysis of U16/17 expression in HHV-6A–infected astroglioma with and without treatment with foscarnet for 5 days at 70 μg/ml. In HHV-6A–infected astroglioma cells, expressions of immediately early (U16/17) and late (U16) transcripts were detected. However, only the immediate early transcript was detected in HHV-6A–infected astrocytoma cells treated with foscarnet, suggesting that this drug acts on a process that is downstream of early viral proteins translation. As expected, neither transcript was detected in HHV-6B infected astrocytes. This observation is consistent with results shown in Figure 2C, where expression of U69 was nondetectable in HHV-6B–infected astroglioma cells. Collectively, this result demonstrates the efficacy of foscarnet in terminating HHV-6 replication in neural glial cells.

Discussion

HHV-6 is becoming increasingly recognized as a neurotropic virus that may play a role in a number of neurologic diseases including seizures, encephalitis, and multiple sclerosis (Dewhurst, 2004). Reactivation of virus associated with neurologic disease has been demonstrated by increased frequency of detection of HHV-6 DNA and gene transcripts in MS lesional tissue form post-mortem brains of patients with MS (Cermelli *et al*, 2003; Opsahl and Kennedy, 2005). This appeared specific for HHV-6 because no evidence of two other related herpesviruses, HHV-7 or HVV-8, were found in MS lesions (Opsahl and Kennedy, 2006). In addition, HHV-6 DNA and glycoproteins (gp116/54/64) have also been demonstrated in formalin-fixed paraffin-embedded mesial temporal lobe epilepsy surgical brain resections. In particular, HHV-6 gp116/54/64 antigen was colocalized with GFAP-positive cells, demonstrating infection of HHV-6 in astrocytes *in vivo* (Donati *et al*, 2003). Moreover, HHV-6 reactivation associated with encephalitis have also been reported in a number of immunocomprimised post–bone marrow transplant recipients (Mookerjee and Vogelsang, 1997; Rapaport *et al*, 2002) and immunocompetent adults (Isaacson *et al*, 2005).

The increasing number of reports suggesting a role for HHV-6 in a variety of neurologic diseases emphasizes the need for effective antiviral treatments that target HHV-6 in the CNS. Currently, there are no antiviral compounds approved for treatment of HHV-6 infections. Drugs that are used to treat HHV-6–associated clinical manifestations are the same as those used to treat the closely related β-herpesvirus human CMV (De Bolle *et al*, 2005a). The rationale being that compounds effective against one β-herpesvirus may be effective against all. Retrospective studies suggest that antiviral compounds with *in vitro* activity against HHV-6–infected T lymphocytes may decrease detectable viral DNA in post–bone marrow transplant patients and in some cases lead to resolution of neurological symptoms (Bethge *et al*, 1999; Birnbaum *et al*, 2005; Denes *et al*, 2004). However, drugs most effective against CMV may not be the most effective against HHV-6 given the differences these viruses exhibit with respect to genomic diversity, tissue tropism, and clinical outcomes. Most importantly, all antiviral studies to date have been tested in β-herpesvirus–infected lymphocytes. There is no information regarding the efficacy of these compounds in virus-infected glial cells. Because HHV-6 is also a neuropathogenic agent

demonstrated to infect glial cells *in vivo* and *in vitro*, studies directly examining the antiviral efficacy of clinically used compounds against HHV-6 infection in neural cells will provide valuable information for management of HHV-6 associated neurological complications.

Our data support those from previous studies demonstrating the antiviral efficacy of foscarnet and ganciclovir in HHV-6A–infected T cells and all compounds tested were effective at inhibiting HHV-6 B replication in lymphocytes (De Bolle *et al*, 2005a). In astrocytes, only the nonguanosine derivatives, cidofovir and foscarnet, exhibited the ability to decrease HHV-6A replication. It is possible that this difference in efficacy between the compounds tested reflects differential expression of HHV-6 genes in human astrocytes and T cells (De Bolle *et al*, 2005b; Yao *et al*, 2006). Using a multivirus array platform containing every open reading frame (ORF) of HHV-6 and -7, other viruses that allowed rapid simultaneous analysis of viral genes (Ghedin *et al*, 2004; Yao *et al*, 2006), we had previously reported that the viral transcripts of HHV-6A was differentially expressed in human primary astrocytes and CD4+ T cells despite comparable viral loads. This variance in viral gene expressions may reflect cellular differences as astrocytes and CD4+ T cells are derived from separate cell lineages. Alternatively, down-regulation of certain HHV-6 viral genes in astrocytes may favor establishment of viral latency in the CNS as has been demonstrated for other viruses (Schneider *et al*, 2005). Importantly, previous studies demonstrated the viral kinase U69 was differentially expressed in HHV-6A–infected T cells and astrocytes. U69 transcript expression was highly expressed in HHV-6–infected T cells but not in astrocytes (De Bolle *et al*, 2005b; Yao *et al*, 2006). As both ganciclovir and acyclovir require activation by the viral kinase U69, the guanosine derivative antivirals may be effective at inhibiting actively replicating virus in HHV-6–infected T cells while exhibiting minimal efficacy in HHV-6A–infected glial cells.

Although human astrocytes have previously been shown to be susceptible to HHV-6 infection (De Bolle *et al*, 2005b; Donati *et al*, 2005; He *et al*, 1996; Yao *et al*, 2006), viral particle production has been demonstrated to be 100-fold lower than in susceptible T-cell lines (He *et al*, 1996). This is consistent with our results demonstrating viral RNA in HHV-6A–infected U251 astrocytes (Figure 2C and D) associated with an increase in HHV-6A viral load (Figure 2B). Based on these observations, it is reasonable to suggest that *in vitro* infection of human astrocytes with HHV-6A may not produce adequate levels of U69 for activation of acyclovir or ganciclovir. As demonstrated in this study, neither acyclovir nor ganciclovir exhibited anti-HHV-6 activity in infected U251 astrocytes. By contrast, both foscarnet and cidofovir demonstrated significant efficacy at inhibiting HHV-6A replication in U251 cells. Because cidofovir is highly toxic to both SupT-1 and U251 astrocytes *in vitro*, rendering the nontoxic doses to a limited range (10 to 2.5 μg/ml) (De Bolle *et al*, 2005a), we focused our study on characterizing the mechanism of action of foscarnet to interfere with HHV-6 replication in U251 astrocytes. We found that high doses of foscarnet were equally effective at inhibiting HHV-6 replication in both T cells and astrocytes. However, at lower concentrations, foscarnet had more potent antiviral effect in HHV-6A–infected U251 astrocytes than HHV-6A–infected T cells. Foscarnet has been shown to penetrate the blood brain barrier demonstrated by measurable amount of foscarnet in the cerebrospinal fluid (CSF) (Hengge *et al*, 1993), and therefore may be more suitable for treating HHV-6–associated neurological complications than acyclovir, ganciclovir, and cidofovir. Moreover, although cidofovir also demonstrated significant inhibition of HHV-6A–infected U251 in this study, it is highly nephrotoxic and has been used only in treating acyclovir, ganciclovir, or foscarnet resistant strains of β-herpevirus infections (Dewhurst, 2004; Maschke *et al*, 2002).

Our observation that HHV-6A selectively replicate in U251 is consistent with previous studies demonstrating variant-specific tropism of HHV-6 in human astrocytes (Donati *et al*, 2005). The ability of HHV-6A to replicate in cells of astrocytic lineages is consistent with more neurotropic properties of HHV-6A suggested by frequent association of HHV-6A with CNS infections (Cuomo *et al*, 2001; Knox *et al*, 1995).

The mechanism of how foscarnet inhibits viral replication has been elucidated in T cells (De Bolle *et al*, 2005a). However, because T cells and astrocytes stem from different cell lineages, it is possible that cellular differences might alter the mode of action in astrocytes. Using HHV-6–specific primer set (U16/17) that differentiates between immediate early and late transcripts, we characterized the mechanism of action of foscarnet in inhibiting viral replication in human U251 astrocytic cell line. Our results demonstrated that detection of both the immediate early transcript (expression independent of protein synthesis) and the late transcripts (expression depends on protein synthesis) in HHV-6A–infected U251. However, the absence of the late viral transcript in foscarnet-treated cells suggests a disruption of the viral replication pathway that requires *de novo* protein synthesis. This is consistent with previous reports demonstrating that foscarnet reversibly binds to the viral DNA polymerase and prevents DNA chain elongation in T cells (thus blocking expression of late genes) (De Bolle *et al*, 2005a).

The association of HHV-6 with an increasing number of neurological complications highlights the importance to identify effective treatment for HHV-6. This study is the first to demonstrate efficacy of currently used antiviral compounds in inhibiting active HHV-6A replication in glial cells *in vitro*. Further

studies of *in vitro* activity in HHV-6–infected primary glial cells, and evaluation of both safety and efficacy of these antiviral drugs using suitable animal models, are essential steps towards identification of effective treatment(s). However, prospective studies of antiviral therapy efficacy are still required to elucidate fully any potential clinical benefit of these compounds in patients with HHV-6–related neurological diseases.

Materials and methods

HHV-6 infection and cell cultures
Cell-free HHV-6 virus stocks were prepared by growing HHV-6A (U1102 strain, kind gift from C. Cermelli, Modena University, Italy) and HHV-6B (Z29 strain, kind gift from P. Secchiero, Institute of Human Virology, Baltimore, MD) in T-cell lines. When cytopathic effect (CPE) was greater than 80%, the cell suspensions were centrifuged for 10 min at $200 \times g$. The supernatants were collected and centrifuged again for 10 min at $1800 \times g$. The cell-free virus stocks were aliquoted and stored at -70°C and the viral DNA was quantified by TaqMan PCR. The same protocol was used for virus-free supernatant by using uninfected T-cell line to prepare inoculum for mock infection.

Determination of cytotoxicity
CD4+ T-cell line (SupT-1) and astroglioma (U251) were plated on 6-well plates at a density of 2×10^4 cells/ml and were incubated with different doses of each antiviral compound for 7 days. Cell cultures were observed daily for morphological changes and induction of cell death. At day 3, cell cultures were subcultivated by twofold dilution with fresh medium containing fresh drug. After 7 days of incubation, CD4+ T cells were stained with Trypan blue to differentiate viable and nonviable cells. 7-Aminoactinomycin D, (7-ADD) a DNA intercalating dye that only enters membrane-compromised cells, was also used to measure cell death by flow cytometry–activated cell sorting (FACS) analysis. Similar results were observed with Trypan blue exclusion and 7-AAD analyses in CD4+ T cells. Drug induced cytotoxicity in U251 astroglioma cells was determined with Trypan blue exclusion. We determined the cytotoxic doses (CC50) as previous described (Manichanh *et al*, 2000). The nontoxic doses of each drug used in this study were acyclovir (ACV), 70 μg/ml; ganciclovir (GCV), 5 μg/ml; foscarnet (PFA), 70 μg/ml; cidofovir (CDV), 5 μg/ml. Efficacy of antiviral compounds at indicated concentrations was expressed as log decrease compared to non–drug-treated infected cells. Duplicate measurements of HHV-6 viral load decrease are shown in Table S1.

Antiviral drugs
Four licensed antiviral compounds were evaluated for their efficacy against HHV-6A and HHV-6B. One pyrophosphate analog, foscarnet (AstraZeneca Wilmingotn, DE); an acyclic nucleoside phosphate, cidofovir (Gilead Sciences, Foster City, CA); and two nucleoside analogs, ganciclovir (Roche Laboratories, Nutley, New Jersey) and acyclovir (American Pharmaceutical Partners, Schaumburg, IL) were used in this study.

Assessment of antiviral efficacy against HHV-6A and HHV-6B
Astroglioma and CD4+ T-cell lines were seeded at a density of 2×10^4 cells/ml density in to 6-well plates. Following an overnight incubation, cells were washed twice with phosphate-buffered saline (PBS), infected with HHV-6A or HHV-6B cell-free inocula at a ratio of 1000 viral copies per cell based on real-time TaqMan PCR viral load measurement. Following a 3-h incubation at 37°C, cells were washed three times with PBS and were given fresh medium containing different concentrations of antiviral compounds. Mock infections were performed using the same protocol with virus-free inocula from uninfected T-cell line. For all experiments, cells were subcultivated at day 3 post infection and fresh drug was added to the medium on days 3 and 6. At 7 days post infection, cells were collected for viral load analysis.

DNA extraction and quantitative real-time TaqMan PCR
DNA was extracted from cell pellets using the DNeasy kit as per manufacturers instructions (Qiagen, CA) with a final elution volume of 200 μl. DNA concentration was adjusted to 10 μg/ul and quantitative real-time PCR with primer/probe sets specific for HHV-6A, HHV-6B, and human genomic β-actin was performed as described previously (Nitsche *et al*, 2001). Final viral DNA load per 10^6 cells was calculated by the following formula: (HHV-6 DNA copy number/β-actin copy number/2) $\times 10^6$ = HHV-6 viral load/10^6 cells (Donati *et al*, 2003).

RT-PCR analysis
Total RNA from cells was isolated and DNased using the RNeasy Kit (Qiagen, CA) according to manufacturer's protocol. For amplication of U69 transcript, 1 μg of total RNA from each sample was reverse transcribed into cDNA using Superscript First-Strand Synthesis System for RT-PCR with random hexamers (Invitrogen, CA). Five microliters of the cDNA reaction was used in subsequent PCR amplification using the 2× PCR Master Mix (Fermentas, MD). PCR condition and primers used are listed in Table 1. To ensure that only mRNA transcripts are amplified, samples used for HHV-6 U16/U17 and U12 transcripts were first amplified using MessageAmp *in vitro* transcription system (Ambion, CA). Five hundred nanograms of mRNA were used in the

Table 1 Primers and program cycles used for RT-PCR

ORF	*Nucleotide sequence* 5′ → 3′	*DNA(bp)*	*cDNA(bp)*	*PCR program (35 cycles)*
U16/U17	F-primer: CCTCTCCCCAGACAGAAACA R-primer: TTTAATTGGCCGAAAAGTCG	240	147	94°C: 4 min; 94°C: 30 s, 59°C: 30 s, 72°C: 30 s (35 cycles); 72°C: 7 min
U69	F-primer TGCTTGCGATTGTTCTGCGA R-primer CGGATGACACCGGCTATGAA	449	449	94°C: 2 min; 94°C: 30 s, 60°C: 45 s, 72°C: 30 s (35 cycles); 72°C: 7 min
U12	F-primer CACTGTCATTGAGCTGTCCAA R-primer ACCACATGAGCACAAAATCG	327	202	94°C5 min; 94°C: 30 s, 57°C: 30 s, 72°C: 30 s (35 cycles); 72°C: 7 min
β−Actin	F-primer: CAAGAGATGGCCACGGCTGCT R-primer: TCCTTCTGCATCCTGTCGGCA	340	275	94°C: 4 min; 94°C: 30 s, 60°C: 30 s, 72°C: 30 s (35 cycles); 72°C: 7 min

generation of cDNA as described above. PCR condition and primers used are listed in Table 1.

Flow Cytometery analysis of GFAP and CD46 expression in U251

Adherent U251 astrocytes were trypsinized and washed with PBS, then stained according to the intracellular cellular staining protocol using the Cytofix/Cytoperm Kit (BD Pharmingen, CA). Briefly, 1×10^6 cells were fixed using Fix/Perm Solution (BD Pharmingen) for 20 min. Cells were then washed with 1× Perm Solution 2 times and stained using 1:400 dilution of GFAP antibody (Sigma) in Perm Wash Solution for 30 min in the dark at 4°C. Cells were washed again with Perm Wash and stained with anti-mouse immunoglobulin G–fluorescein isothiocyanate (IgG-FITC) secondary antibody (Jackson Immunolaboratory, PA) for an additional 30 min in the dark at 4°C. Excess secondary antibody was washed two times in Perm Wash before analysis with a FACS Calibur (BD Biosciences, CA). For cell surface staining of CD46, 1×10^6 cells were stained with 5 μl of FITC-conjugated mouse anti-human CD46 (BD Pharmingen) for 30 min in the dark at 4°C. Cells were washed with FACS buffer containing 2% fetal bovine serum (FBS) and 0.01% sodium azide in PBS two times, followed by analysis with FACS Calibur (BD Biosciences).

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