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Effect of (r)-9-[4-hydroxy-2-(hydroxymethyl)butyl]guanine (H2G) and AZT-lipid-PFA on Human Herpesvirus-6B infected cells

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Abstract

Background—Human herpesvirus 6 (HHV-6) has been associated with a wide spectrum of diseases. (r)-9-[4-hydroxy-2-(hydroxymethyl)butyl]guanine (H2G) is an acyclic guanosine analogue that is structurally similar to acyclovir and is in clinical development for treatment of herpesvirus infections. H2G has been found to have activity against HSV type 1, HSV type 2, and HHV-6 in lymphoblast cell lines. A new antiviral duplex drug, 3'-azido-3'-deoxythymidyl- (5' → 2'-O)-3-O-octadecyl-*sn*-glycerol (AZT-lipid-PFA), linking Zidovudine (AZT) and Foscarnet (PFA) via a lipophilic octadecylglycerol residue (lipid) also exhibits anti-viral activities against HIV, HSV type 1 and HCMV.

Objective—To assess the efficacy of H2G and AZT-lipid-PFA conjugate against HHV-6.

Study design—Drug associated toxicity and proliferative response were evaluated. We conducted *in vitro* experiments to determine the efficacy of H2G and an AZT-lipid-PFA conjugate in interfering with expression HHV-6 viral transcript in primary human peripheral blood mononuclear cells (PBMC).

Results—Both H2G and AZT-lipid-PFA were effective at inhibiting expression of HHV-6 gene transcript at comparable concentrations. Additionally, while AZT-lipid-PFA treatment was toxic to cells at concentrations above 5 μ M, H2G treatment was associated with minimal cytotoxicity.

Conclusion—These data suggest the potential application of these anti-viral compounds in controlling HHV-6 infection.

INTRODUCTION

Human herpesvirus-6 (HHV-6) is a ubiquitous β -herpesvirus with a seroprevalence of approximately 90% in the adults. HHV-6 can establish latency following primary infection,

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typically before the age of 2, but can reactivate later in life¹. HHV-6A and HHV-6B are two variants that share high sequence homology, but differ in cellular tropism, antigenic specificity, and clinical manifestations^{2–5}. HHV-6B is the etiological agent of exanthema subitum⁶, while a specific disease association with HHV-6A remains elusive. HHV-6 is also implicated in other conditions such as encephalitis^{7, 8}, rhomboencephalitis⁹, multiple sclerosis^{10–13}, mesial temporal sclerosis^{14, 15}, post-bone marrow transplant (BMT) limbic encephalitis¹⁶, and graft-versus-host disease following hematopoietic stem cell transplant¹⁷. HHV-6 may also play a role as a co-factor in HIV pathogenesis¹⁸.

The association of HHV-6 with a wide spectrum of clinical conditions makes it important to identify effective therapeutic agents with applications in clinical settings. There are a number of anti-herpetic drugs that have shown activity against herpes simplex virus 1, 2 (HSV-1, HSV-2), varicella-zoster virus (VZV) and cytomegalovirus (CMV)³. Some of these compounds, such as ganciclovir (GCV), foscarnet (PFA) and cidofovir (CDV), also exhibit anti-HHV-6 activity¹⁹. GCV is a guanosine analog that requires intracellular phosphorylation by a viral kinase for activation to the phosphorylated product that competes with GTP for the viral DNA polymerase. PFA and CDV are non-guanosine derivatives that do not require enzymatic activation. Another anti-herpetic compound, (r)-9-[4-hydroxy-2-(hydroxymethyl) butyl] guanine (H2G) that is structurally related to acyclovir demonstrated activity against HSV-1, HSV-2, VZV, and CMV in cell lines and primary cells. While efficacy of H2G towards HHV-6 infection in cell lines has been investigated, data based primary human cell infections are lacking^{20–22}. Recently, a novel compound 3'-azido-3'-deoxythymidylyl- (5'→2'-O)-3'-O-octadecyl-*sn*-glycerol (AZT-lipid-PFA), was synthesized by covalently linking 3'-Azido-3'-deoxythymidine (AZT) and PFA via a lipophilic octadecylglycerol residue. This compound demonstrated antiviral activity not only against HIV, but also against drug-resistant strains and clinical isolates of HSV-1 and HCMV²³. AZT is a reverse transcriptase inhibitor used for treating HIV infection that crosses the blood-brain-barrier due to its lipophilic nature²⁴. While previous studies indicated synergistic antiviral effects with PFA and AZT toward HIV and CMV^{25, 26}, the activity of AZT-lipid-PFA towards HHV-6 has not been examined.

Given the potential role of HHV-6 in augmenting HIV pathogenesis and its associations with other diseases we examined the efficacy of AZT-lipid-PFA and H2G to against HHV-6B infection in human peripheral blood mononuclear cells (PBMC).

METHODS

Cell culture and HHV-6 infection of PBMC

SupT1 and PBMC were maintained at 37°C in complete RPMI media containing 10% FCS, 1% penicillin and streptomycin, and 1% L-glutamine (cRPMI). Prior to infection with HHV-6B (Z29), PBMC were activated with Phorbol 12-myristate 13-acetate (PMA, 50 ng/mL) and ionomycin (0.5 µM) (Sigma) for 24 hours with IL-2 (30U/mL) (PeproTech) at 1.0×10^6 cells/mL. After 24 hours, three-quarters of the media was removed and HHV-6B viral supernatant was added at a multiplicity of infection of 100:1 for 3 days with AZT-lipid-PFA (laboratory of Dr. Herbert Schott) and H2G (Epiphany Biosciences). Infection was monitored by cytopathic effect and confirmed by TaqMan PCR.

Fluorescence-activated cell sorting (FACS) analysis

Annexin V and propidium iodine (PI) staining was performed using the Annexin-FITC apoptosis Detection Kit according to manufacture's protocol (BD Pharmingen). Allophycocyanin (APC)-conjugated anti-CD4, Fluorescein isothiocyanate (FITC)-conjugated Ki-67, and Phycoerythrin (PE)-conjugated CD14 antibodies (BD Pharmingen). In a 96-well round bottom plate, 2µl of cell surface antibodies in 30 µl of FACS buffer were added and incubated at 4°C for 30 minutes. Intracellular staining, fixation and permeabilization were performed with Cytofix/CytoPerm (BD Pharmingen) according to manufacture's instructions. Cells were suspended in FACS buffer and analyzed with a FACSCalibur. Isotype antibodies were used as staining controls

Proliferation assay

Proliferation assays were performed with SupT1 cells at 1×10^5 /well for 24 hours and 48 hours at 37°C. Cells were pulsed with ^3H -labeled thymidine for 4 hours, harvested onto fiberglass filters, and measured with a β -scintillation counter (Wallac Instruments). Percent proliferation was calculated as followed: $(\text{CPM}_{\text{treated}} / \text{CPM}_{\text{untreated}}) \times 100\%$.

HHV-6 U67 gene transcript detection

Total RNA was isolated from PBMC using the RNeasy RNA isolation kit (Qiagen) followed by treatment with amplification grade DNase I (Invitrogen). Complementary DNA was synthesized using random hexamer primers using TaqMan Reverse Transcription Reagents according to manufacturer's instructions (Applied Biosystems). PCR amplification was performed as previously described²⁷. Relative expression of U67 transcript was calculated using $2^{-\text{CT}}$ as followed: $2^{-(\text{HHV-6B CT} - \beta\text{-actin CT}) \text{ non-infected} - (\text{HHV-6B CT} - \beta\text{-actin CT}) \text{ infected}}$.

RESULTS

Determination of H2G and AZT-lipid-PFA cytotoxicity

We evaluated the drug-associated cytotoxicity of H2G or AZT-lipid-PFA by assessing levels of Annexin V and PI expression in SupT1 cell line at indicated concentrations for 2 days. Annexin V is a phospholipid binding protein that exhibits highly selective binding affinity to phosphatidylserine. The appearance of phosphatidylserine on the cell surface is often associated with early apoptotic events. PI is a DNA intercalating dye and its binding typically correlates with secondary necrosis of cells. As shown in Figure 1A, no appreciable cytotoxicity was associated with H2G treatment. However, a dose-dependent decrease in percent of viable cells was observed with AZT-lipid-PFA treatment starting at 5 µM. The individual contribution of apoptotic and necrotic processes to the cytotoxicity was investigated. H2G and AZT-lipid-PFA produced comparable levels of necrotic death (Figure 1B). However, a dose-dependent increase of apoptotic cells, as judged by Annexin V staining, occurred with AZT-lipid-PFA at concentrations above 5 µM (Figure 1C). These data suggest that short-term exposure to H2G did not significantly affect cell viability, while exposure to AZT-lipid-PFA was associated with cytotoxicity at higher drug concentrations.

Effect of H2G and AZT-lipid-PFA on cell proliferation

The proliferative capability of SupT1 cells was examined after 24 and 48 hours of incubation with H2G and AZT-lipid-PFA by incorporation of $^3\text{[H]}$ -labeled thymidine. In contrast to H2G-treated cells, SupT1 cells treated with AZT-lipid-PFA showed a decrease in proliferation in a dose-dependent manner (Figures 2A and B). This observed toxicity after exposure to AZT-lipid-PFA (Figure 1) was likely associated with cell death. Based on the results, subsequent experiments were performed using two concentrations, a non-toxic dose of 5 μM for H2G and AZT-lipid-PFA, and higher doses; namely, 40 μM of H2G or 10 μM of AZT-lipid-PFA.

Lymphocyte subsets were not altered by H2G and AZT-lipid-PFA treatments

Due to the cytopathic effect of HHV-6B (Z29), the number of lymphocytes was significantly reduced after 3 days of infection compared to the non-infected control (Figure 3A). Quantification of lymphocyte numbers as a percent of total PBMC is shown in Figure 3B. In addition, no significant changes in the proportion of CD4⁺ or CD4-lymphocytes was observed and addition of H2G or AZT-lipid-PFA did not reverse the cytopathic effect (Figure 3A, middle and bottom panels). Similarly, fewer numbers of CD14⁺ monocytes were detected after HHV-6B infection and anti-viral compounds did not change the number of monocytes (Figure 3C).

Efficacy of H2G and AZT-lipid-PFA in inhibiting HHV-6B infection

The ability of H2G and AZT-lipid-PFA to interfere with *de novo* viral transcription following 3-day drug treatment was assessed by TaqMan PCR ²⁷. At 5 μM of H2G treatment, the relative expression of U67 gene was reduced by approximately 1 log compared to HHV-6B infected PBMC without treatment (Figure 4). This inhibitory effect was further enhanced at a concentration of 40 μM of H2G. In contrast, dramatic reduction in U67 expression was observed at 5 μM and 10 μM concentrations of AZT-lipid-PFA.

DISCUSSION

While recognized as the causative agent of exanthem subitum ⁶, HHV-6B has recently been implicated in various neurologic disorders ^{8, 14–16}, and reactivation has been observed in patients with allogeneic post-BMT or HSCT procedures ^{17, 28, 29}. In non-human primates, HHV-6A infection accelerated HIV progression ¹⁸. While co-infection of HHV-6B and HIV has been described in human cord blood ³⁰, it is unclear whether HHV-6B exhibits similar ability to enhance HIV replication *in vivo*.

In spite of the associations of HHV-6 with various diseases ^{10–17}, there have been only a small number of studies of potential anti-HHV-6 compounds ^{19, 21, 31–34}. Our study evaluated H2G and AZT-lipid-PFA and demonstrated that at the non-toxic concentration of 5 μM of H2G exhibited inhibitory effect on *de novo* gene transcription by HHV-6B, but the same concentration of AZT-lipid-PFA was less effective. Similar to other anti-herpetic compounds, such as acyclovir (ACV), and ganciclovir (GCV), H2G is a guanosine analog that requires the viral thymidine kinase for phosphorylation. However, since HHV-6 lacks a viral thymidine kinase, the observed efficacy suggests that H2G may be phosphorylated by

cellular enzymes²¹. Alternatively, the HHV-6 U69 gene, which encodes a phosphotransferase and is the homolog of the CMV UL97 kinase, may activate H2G¹⁹.

In contrast to the dose-dependent cytotoxicity observed with AZT-lipid-PFA-treated SupT1 cells (Figure 1), the same trend was not observed with HHV-6B infected PBMC. This difference could be related to culture conditions where PBMC were activated with PMA and ionomycin in the presence of IL-2, but mitogenic simulations were not used for SupT1 cells. In addition, H2G and AZT-lipid-PFA also inhibited viral replication in SupT1 cells (data not shown); however, the efficiency was less robust compared to HHV-6B infection in PBMC. Although the 1 log reduction in viral transcript observed with H2G in PBMC from 2 independent donors suggest its potential for controlling HHV-6B replication, the overall reduction was rather modest. In this study, only short term effects relating to anti-viral treatments were examined. Experiments that investigate the effects over time will further shed light on the pharmacokinetics of these compounds. The strong inhibition of HHV-6B viral expression by AZT-lipid-PFA may have potential implications as a therapeutic for HIV and HHV-6 co-infections.

A major challenge with many anti-viral compounds is drug-associated cytotoxicity. We showed that H2G treatment produced minimal effects on cell viability or proliferation. AZT-lipid-PFA exhibited significant cytotoxicity at concentration as low as 5 μ M. However, the overall toxicity as determined by Annexin and PI staining was low and may be attributed to the short duration of the experiment. While PFA was found to be less toxic *in vitro* compared to other viral compounds such as ganciclovir and cidofovir³⁶, while cell toxicity associated with AZT is well documented²⁴. However, since only low concentration of AZT-lipid-PFA was required for effective inhibition of HHV-6 viral replication, the drug-associated toxicity should be minimal. As previously described, the half maximal inhibitory doses (IC₅₀) of AZT-lipid-PFA against highly acyclovir (ACV)-resistant HSV isolates was determined by a plaque reduction assay ranged between 1.87 and 4.59 μ M²³. H2G, while less effective at decreasing viral gene expression, was associated with minimal toxicity even at high concentrations. The broad-spectrum of anti-viral activity^{20–22} and the relative non-toxic nature of H2G make it suitable for long-term administration.

Because HHV-6 infection and reactivation are linked to a variety of neurologic diseases¹², the ability of anti-HHV-6 compounds to cross the blood brain barrier is important. In a normal rodent model, H2G concentrations detectable in the CNS were equivalent to approximately 10–20% of concentrations achieved in blood and muscle³⁷. The achievable concentration may therefore be higher in a diseased model where the blood brain barrier is compromised. AZT is used to treat HIV-associated neurologic complications and it crosses the blood brain barrier at a modest level³⁸, but the ability of AZT-lipid-PFA to penetrate the brain barrier *in vivo* remains to be determined.

Our results indicate that H2G and AZT-lipid-PFA inhibit HHV-6B *in vitro*. Experiments that evaluate the bioavailability of these compounds *in vivo* using animal models, or in clinical trials, and measure their ability to interfere with viral infection or reactivation, are required to determine the benefit of these anti-viral compounds for use in clinical settings.

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Figure 1. Evaluation of H2G and AZT-lipid-PFA treatment associated cytotoxicity

SupT1 cells incubated with various concentrations of H2G or AZT-lipid-PFA for 2 days as indicated. Annexin V and propidium iodine (PI) expression analyzed by FACS. Samples for each concentration were measured in duplicate. **(A)** Percent of live cells following drug treatments was calculated as $[\text{percent Annexin V and PI negative}_{(\text{treated})} / \text{percent Annexin V and PI negative}_{(\text{untreated})}] \times 100\%$. **(B)** Percent PI positive only. **(C)** Percent Annexin V positive only.

Figure 2. Proliferative response of SupT1 cells after anti-viral treatments

SupT1 cells treated with indicated concentration of H2G or AZT-lipid-PFA and proliferative response was measured by ^3H incorporation for 4 hours at 37°C with 5% CO_2 . Triplicate wells were used for each treatment condition. **(A)** Proliferation of SupT1 cells after 1 day of anti-viral treatment. **(B)** Proliferation of SupT1 cells after 2 days of anti-viral treatment.

Figure 3. Analysis of PBMC subsets after HHV-6B infection

Healthy donors PBMC were activated with PMA and ionomycin in the presence of recombinant human IL-2 for 24 hours. In the presence of H2G (5 μ M and 40 μ M) or AZT-lipid-PFA (5 μ M and 10 μ M), cell-free HHV-6B (Z29) inocula were used to infect the activated primary human PBMC on day 3. PBMC subsets were examined by staining for CD4⁺, CD14⁺, and Ki-67 expression followed by FACS analysis. **(A)** FACS profiles CD4⁺ and Ki-67⁺ staining gated on lymphocytes from 2 healthy donors. **(B)** Quantification of percent lymphocytes represented in Figure 3A. **(C)** Average of percent CD14⁺ cells in 2 healthy controls following mitogenic stimulation, HHV-6B infection, and anti-viral treatment.

Figure 4. Comparison of anti-viral treatments in inhibiting viral transcript expression

Healthy donor PBMC infected with HHV-6B and treated with either H2G or AZT-lipid-PFA at the indicated drug concentrations were evaluated for the expression of viral U67 gene transcript. Significantly less U67 transcript was detected in HHV-6B infected PBMC after H2G (untreated vs. 5 μ M, $p = 0.0222$; untreated vs. 40 μ M, $p = 0.0212$; t test) or AZT-lipid-PFA treatment (untreated vs. 5 μ M, $p = 0.0215$; untreated vs. 10 μ M, $p = 0.0220$; t test) for 3 days.