Evaluation of Epstein-Barr Virus, Human Herpesvirus 6 (HHV-6), and HHV-8 Antiviral Drug Susceptibilities by Use of Real-Time-PCR-Based Assays

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Evaluation of candidate antiviral drugs against Epstein-Barr virus (EBV), human herpesvirus 6 (HHV-6), and HHV-8 is hampered by the lack of convenient laboratory assays. We developed real-time quantitative PCR assays performed on supernatants of lymphoma cell lines and determined the 50% inhibitory concentrations (IC$_{50}$) of nucleoside, nucleotide, and pyrophosphate analogues against these herpesviruses.

The Herpesviridae is a large family of DNA viruses, including 8 different human viruses, namely, herpes simplex virus types 1 and 2, varicella-zoster virus, cytomegalovirus, Epstein-Barr virus (EBV), and human herpesvirus 6 (HHV-6), HHV-7, and HHV-8. These ubiquitous viruses differ widely in the pathologies they induce and in their modes of transmission. Immunocompromised patients such as transplant recipients and HIV-infected individuals are by far the most vulnerable hosts. Following a primary infection, these viruses establish lifelong latency and can reactivate under certain conditions.

Primary infections and reactivations of HHV-6, HHV-8, and EBV can cause a variety of clinical syndromes, some of which can be fatal in immunocompromised patients (1, 2, 3). Infection with HHV-6 results in exanthem subitum in infants (4) and is associated with severe diseases (encephalitis and pneumonitis) in immunocompromised patients (3, 5). HHV-8 is mainly associated with Kaposi’s sarcoma, one of the most frequently encountered neoplasms in HIV-infected patients (1). Finally, EBV causes infectious mononucleosis and posttransplant lymphoproliferative diseases and is also associated with nasopharyngeal carcinoma, Burkitt’s lymphoma, and non-Hodgkin B-cell lymphomas (6).

Therefore, there is a need to develop effective antiviral drugs to control the lytic phase of these acute infections especially in immunocompromised patients.

The screening and evaluation of candidate antiviral drugs against HHV-6, HHV-8, and EBV require validated drug susceptibility assays. Due to the absence of virally induced plaque formation in cell culture, most susceptibility assays described so far for these viruses have measured the viral DNA load (7, 8, 9). For HHV-8 and EBV, the lytic viral replication must be first activated by treatment of latently infected B cells with 12-tetradecanoyl phorbol (TPA) and/or sodium butyrate (10, 11). In this study, we developed susceptibility assays based on quantitative real-time PCR for HHV-6 on MT-4 cells and for HHV-8 and EBV induced from latently infected BCBL-1 and P3HR-1 cell lines, respectively. We then tested the activity of four commercially available antiviral agents (acyclovir, ganciclovir, cidovir, and foscarnet) against these viruses in an effort to establish baseline values for evaluation of future candidate drugs.

MT-4 cells were infected with the HST strain of HHV-6 variant B (12) at a multiplicity of infection (MOI) of 0.004 50% cell culture infectious dose (CCID$_{50}$) per cell in RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum (10% FBS) and a mixture of amikacin and vancomycin (both at 20 μg/ml) for 1 h (13). Infected cells were then distributed in a 96-well plate (in a ratio of 2 × 10$^4$ cells to 80 viral particles per well) and incubated with serial dilutions of each antiviral agent for 3 days. HHV-8 was induced from latently infected BCBL-1 cells (0.75 × 10$^6$ cells/ml) with TPA (20 ng/ml) and sodium butyrate (330 μg/ml) in RPMI 1640 plus 10% FBS containing plasmocin (5 μg/ml) and serial dilutions of each antiviral agent for 1 day. Cells were centrifuged, resuspended in fresh culture medium containing the antiviral but without chemical inducers, and incubated for 2 days. The latter procedure was repeated again, and cells were incubated in fresh culture medium containing the antiviral for another 3 days (9). EBV was induced from exponentially growing latently infected P3HR-1 cells (10$^6$ cells/ml; ATCC HTB-62) with sodium butyrate (3.3 mg/ml) in RPMI 1640 plus 10% FBS containing serial dilutions of each antiviral agent for 2 days (10). Cells were centrifuged and resuspended in fresh growth medium containing the antiviral but without chemical inducer for an additional period of 2 days. Cells incubated in the absence of drug were used as controls.

Cell culture supernatants were collected at the end of the incubation period for subsequent DNA extraction with the QIAamp DNA blood minikit as described previously (9). Viral DNA load was quantified by adapted real-time PCR assays with a LightCycler instrument (Roche Diagnostics) using primers and probes designed to amplify the U65-U66 (HHV-6), ORF26 (HHV-8), and EBNA-1 (EBV) genes (14, 15, 16). The 50% inhibitory concentration (IC$_{50}$) was defined as the antiviral concentration that reduces the viral DNA load by 50% compared to the no-drug control.

Cell viability was determined using an MTS (3-(4,5-dimethyl-thiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium) assay as described previously (17). MT-4 cells...
Acyclovir and foscarnet had almost similar CC50 values with those previously obtained with a similar real-time PCR-standard in one amplification reaction. The interrun variations in CT values cloned into the pJET1.2/blunt cloning vector and the HHV-8 28.99, HHV-6 31.58, GCV 0.43, and ACV, acyclovir; GCV, ganciclovir; CDV, cidofovir; FOS, foscarnet. Values are the means ± standard deviations obtained by using 15 replicate samples containing 1,000 copies of each external standard in one amplification reaction. The interrun variations in CT values represent the means ± standard deviations obtained by using 15 replicate samples containing 1,000 copies of each external standard in four different amplification reactions. The linear dynamic ranges and the lower limits of detection of the different assays were determined from three different amplification reactions using serial 10-fold dilutions varying from 1 to 10^10 copies of each external standard.

We also determined the drug susceptibilities of HHV-8 induced from latently infected BCBL-1 cells with TPA/sodium butyrate (Table 2). Cidofovir was again the most active drug followed by ganciclovir, acyclovir, and foscarnet. We also validated the susceptibility assay based on real-time PCR with dot blot analysis performed as described previously (18). The IC50 values determined by real-time PCR and dot blot analysis were 0.28 versus 1.50 μM for cidofovir, 0.97 versus 12.36 μM for ganciclovir, 25.56 versus 33.46 μM for acyclovir, and 38.83 versus 81.44 μM for foscarnet. Thus, the IC50 results followed the same trend using two different assays and were also in agreement with our previously published data (9). The CC50 values for BCBL-1 cells exposed to acyclovir, ganciclovir, and foscarnet for 6 days were almost similar, whereas they were slightly lower for cidofovir. Following treatment with TPA/sodium butyrate for 24 h, the CC50 values remained almost unchanged for acyclovir, cidofovir, and foscarnet, whereas we observed a 4.7-fold decrease for ganciclovir. Cidofovir had the highest SI at 1,000, followed by ganciclovir, acyclovir, and foscarnet at 134, 39, and 17, respectively.

The EBV susceptibility to antiviral agents was measured on P3HR-1 cells induced with sodium butyrate (Table 2). Cidofovir, ganciclovir, and cidofovir had the lowest IC50s (0.51 ± 0.33 μM, 0.57 ± 0.62 μM, and 0.27 ± 0.02 μM, respectively) against EBV compared to foscarnet (2.18 ± 0.65 μM). Although our IC50s were 4- to 7-fold lower than those reported in the literature (7) with a different real-time PCR assay (19), they followed the same trend. These differences may be also related to the fact that we harvested cell culture supernatants for viral DNA load quantification after 4 days of incubation with the antiviral compared to 7 days for the previous assay. The CC50 values for P3HR-1 cells exposed to ganciclovir, foscarnet, and cidofovir for 4 days were lower than that for acyclovir. Following treatment with sodium butyrate for 48 h, the CC50 values remained almost unchanged for foscarnet, whereas a 2.6- to 4.3-fold decrease was observed for the three other antivirals. SI values ranked as 2,078, 703, 456, and 234 for acyclovir, cidofovir, ganciclovir, and foscarnet, respectively.

Among the drugs currently available against herpesviruses, cidofovir appears to be the most active in vitro. By taking into account drug cytotoxicity, cidofovir remains the most potent agent against HHV-6 and HHV-8, whereas acyclovir has the highest SI against EBV. The toxicity profiles of foscarnet and cidofovir and the absence of oral formulations limit their use in clinical practice. Interestingly, the orally bioavailable lipid ester prodrug of cidofovir (i.e., hexadecyloxypropyl-cidofovir), which is evaluated in clinical trials, could avoid the dose-limiting renal toxicity of the parent drug (20).

The drug susceptibility assays for HHV-6, HHV-8, and EBV described here are based on a molecular readout and measure the reduction of the viral DNA load instead of the inhibition of replication-competent viral particles (as in a standard plaque reduc-

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<th>TABLE 1</th>
<th>Validation of HHV-6, HHV-8, and EBV real-time PCR assays using external plasmid standards</th>
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<td>Assay</td>
<td>Intrarun variations in CT (CV) (%)</td>
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<tr>
<td>HHV-6</td>
<td>31.58 ± 0.20 (17.08)</td>
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<tr>
<td>HHV-8</td>
<td>28.99 ± 0.11 (7.35)</td>
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<tr>
<td>EBV</td>
<td>29.08 ± 0.12 (8.60)</td>
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The external standards correspond to the C65-U66 (HHV-6) and EBNA-1 (EBV) genes cloned into the pET1.2/blunt cloning vector and the ORF26 (HHV-8) gene cloned into the pGEM-T Easy vector. CT threshold cycle; CV, coefficient of variation. The intrarun variations in CT values represent the means ± standard deviations obtained by using 20 replicate samples containing 1,000 copies of each external standard in one amplification reaction. The interrun variations in CT values represent the means ± standard deviations obtained by using 15 replicate samples containing 1,000 copies of each external standard in four different amplification reactions. The linear dynamic ranges and the lower limits of detection of the different assays were determined from three different amplification reactions using serial 10-fold dilutions varying from 1 to 10^10 copies of each external standard.

(0.5 × 10^6 cells/ml) and BCBL-1 and P3HR-1 cells (1.0 × 10^6 cells/ml), treated or not treated with TPA and/or sodium butyrate, were incubated with increasing concentrations of each antiviral for 3, 6, and 4 days, respectively. Cells incubated in the absence of drug were used as controls. The antiviral drug concentration that reduces cell viability by 50% compared to the no-drug control (CC50) and the selectivity index (SI; CC50 to IC50 ratio) were calculated.

We first validated each quantitative real-time PCR assay using appropriate external standards. Table 1 shows intrarun and interrun variations in threshold cycle values for all assays. The three assays had a linear dynamic range of 9 logs and a lower limit of detection of 10 copies.

Table 2 shows HHV-6 drug susceptibilities performed on MT-4 cells. Cidofovir was the most active drug with an IC50 of 1.82 ± 0.59 μM, whereas those of ganciclovir, foscarnet, and acyclovir were 2.5-, 5.4-, and 8.9-fold higher, respectively. These results were in agreement with those previously obtained with a similar real-time PCR-based assay (13). Acyclovir and foscarnet had almost similar CC50 values against MT-4 cells, whereas cidofovir and ganciclovir were slightly more toxic. Thus, ganciclovir, foscarnet, and acyclovir ranked almost equally, with respective SI values of 96, 87, and 63, whereas cidofovir had the highest SI at 203.

We also determined the drug susceptibilities of HHV-8 induced from latently infected BCBL-1 cells with TPA/sodium butyrate (Table 2). Cidofovir was again the most active drug followed by ganciclovir, acyclovir, and foscarnet. We also validated the susceptibility assay based on real-time PCR with dot blot analysis performed as described previously (18). The IC50 values determined by real-time PCR and dot blot analysis were 0.28 versus 1.50 μM for cidofovir, 0.97 versus 12.36 μM for ganciclovir, 25.56 versus 33.46 μM for acyclovir, and 38.83 versus 81.44 μM for foscarnet. Thus, the IC50 results followed the same trend using two different assays and were also in agreement with our previously published data (9). The CC50 values for BCBL-1 cells exposed to acyclovir, ganciclovir, and foscarnet for 6 days were almost similar, whereas they were slightly lower for cidofovir. Following treatment with TPA/sodium butyrate for 24 h, the CC50 values remained almost unchanged for acyclovir, cidofovir, and foscarnet, whereas we observed a 4.7-fold decrease for ganciclovir. Cidofovir had the highest SI at 1,000, followed by ganciclovir, acyclovir, and foscarnet at 134, 39, and 17, respectively.

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REFERENCES