JOURNAL OF CLINICAL MICROBIOLOGY, Aug. 2003, p. 3897–3900 Vol. 41, No. 8
0095-1137/03/$08.00+0 DOI: 10.1128/JCM.41.8.3897–3900.2003

Evaluation of Susceptibility of Human Herpesvirus 8 to Antiviral Drugs by Quantitative Real-Time PCR

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Received 14 February 2003/Returned for modification 24 April 2003/Accepted 4 May 2003

A new in vitro system based on real-time PCR was developed for evaluation of human herpesvirus 8 susceptibility to antiviral agents. Cidofovir had the greatest inhibitory activity against HHV-8 (50% inhibitory concentration [IC_{50}], 0.43 μM) followed by ganciclovir (2.61 μM), adefovir (18.00 μM), acyclovir (31.00 μM), and foscarnet (34.15 μM). The potential therapeutic efficacy for HHV-8 (i.e., peak serum drug level/IC_{50}) is highest for cidofovir (167) and foscarnet (22).

The human herpesvirus 8 (HHV-8), also known as Kaposi's sarcoma-associated herpesvirus, is a new member of the γ-*herpesvirinae* subfamily that has been associated with human immunodeficiency virus (HIV)- and non-HIV-related Kaposi's sarcoma (KS) as well as with multicentric Castleman's disease and primary effusion lymphoma (5, 6, 17, 25). Despite the decrease in HIV-related KS with the advent of highly active antiretroviral therapy, there is still an interest in evaluating strategies to inhibit the early stages of HHV-8 replication.

Several in vitro systems have been reported for determination of HHV-8 susceptibility to antiviral drugs (10, 16, 20, 22). Because most B cells are latently infected by the virus, inducing agents such as 12-*O*-tetradecanoylphorbol-13-acetate (TPA) or sodium butyrate have been used to promote lytic viral replication (23). Also, due to the absence of viral plaques in infected lymphoma cell lines, most susceptibility systems were designed to measure viral DNA synthesis. However, the existing assays are very cumbersome, as they require gel electrophoresis and isotopic hybridization. In this report, we describe the development of a rapid and objective in vitro susceptibility system for HHV-8 based on real-time quantitative PCR.

The BCBL-1 cell line, which is a B-cell line latently infected by HHV-8 (23), was kindly provided by Benoît Barbeau (Centre de Recherche en Infectiologie, Ste-Foy, Québec, Canada). The cells were maintained as described previously (16). On day 1, 10 ml of BCBL-1 cells at 2×10^5 cells/ml (in RPMI 1640 medium [Life Technologies, Burlington, Ontario, Canada] supplemented with 10% heat-inactivated fetal bovine serum) were pelleted at 250 × *g* for 10 min and then washed with 2 ml of phosphate-buffered saline. The cell pellet was then resuspended in an equal volume of medium with TPA (without TPA for the negative control) at a final concentration of 20 ng/ml in 25-cm^2 flasks (BD Biosciences, Oakville, Ontario, Canada). Serial concentrations of antivirals, i.e., acyclovir (zovirax; GlaxoSmithKline), foscarnet (Sigma, Oakville, Ontario, Canada), ganciclovir (cytovene; Hoffman La Roche), cidofovir (vistide; Gilead Sciences, Foster City, Calif.), and adefovir (kindly provided by Tomas Cihlar; Gilead Sciences) were made in triplicate and added to culture medium. On day 2, 20 h after TPA stimulation, the cells were repelleted as described above, washed with phosphate-buffered saline, and resuspended in 10 ml of fresh medium containing the same antiviral drugs but without TPA. On day 4 (3 days after the addition of TPA), the same volume of medium containing the same concentrations of antivirals was added to the plates. On the last day (day 7), an aliquot of 1.5 ml of supernatant was removed from the culture for subsequent viral DNA extraction.

The supernatants were centrifuged (2,000 × *g* for 10 min) followed by treatment with 30 U of RNase-free DNase I for 30 min to remove unencapsidated HHV-8 viral DNA. DNA was extracted from 200 μl of treated supernatant using the QIAamp DNA blood mini kit (QIAGEN, Mississauga, Ontario, Canada) and then eluted in 100 μl of sterile water. The HHV-8 DNA load was determined by a previously described quantitative real-time PCR assay (3) using 5 μl of eluted DNA. Briefly, the competitive real-time PCR assay was performed in a LightCycler instrument (Roche Diagnostics, Laval, Québec, Canada) using primers designed to amplify the *orf26* gene (6) and two sets of adjacent fluorogenic probes (one for the target and one for an internal control) to monitor the amplification reaction (3). Fluorescence measurements were performed at each cycle (during annealing) in the F2 (Red-640) and F3 (Red-705) channels, and a threshold cycle (C_t) value for each drug concentration was calculated by determining the point at which the fluorescence exceeded a threshold limit. A standard curve of the C_t values was generated using serial 10-fold dilutions of an external HHV-8 standard from which the C_t values of the different drug concentrations were interpolated. The drug 50% inhibitory concentration (IC_{50}) value was defined as the antiviral concentration that reduced DNA synthesis by 50% compared to TPA-induced controls without drug. Presence of PCR-inhibitory substances was assessed in each sample by verifying that the C_t of the internal control was less than 30.

Under our conditions of TPA stimulation, approximately 1.5×10^6 viral genome equivalents were detected in 5 μl of eluted DNA, which corresponds to 3.0×10^7 copies per 200 μl of initial cell culture supernatant. A typical susceptibility experiment showing the reduction of HHV-8 DNA synthesis by

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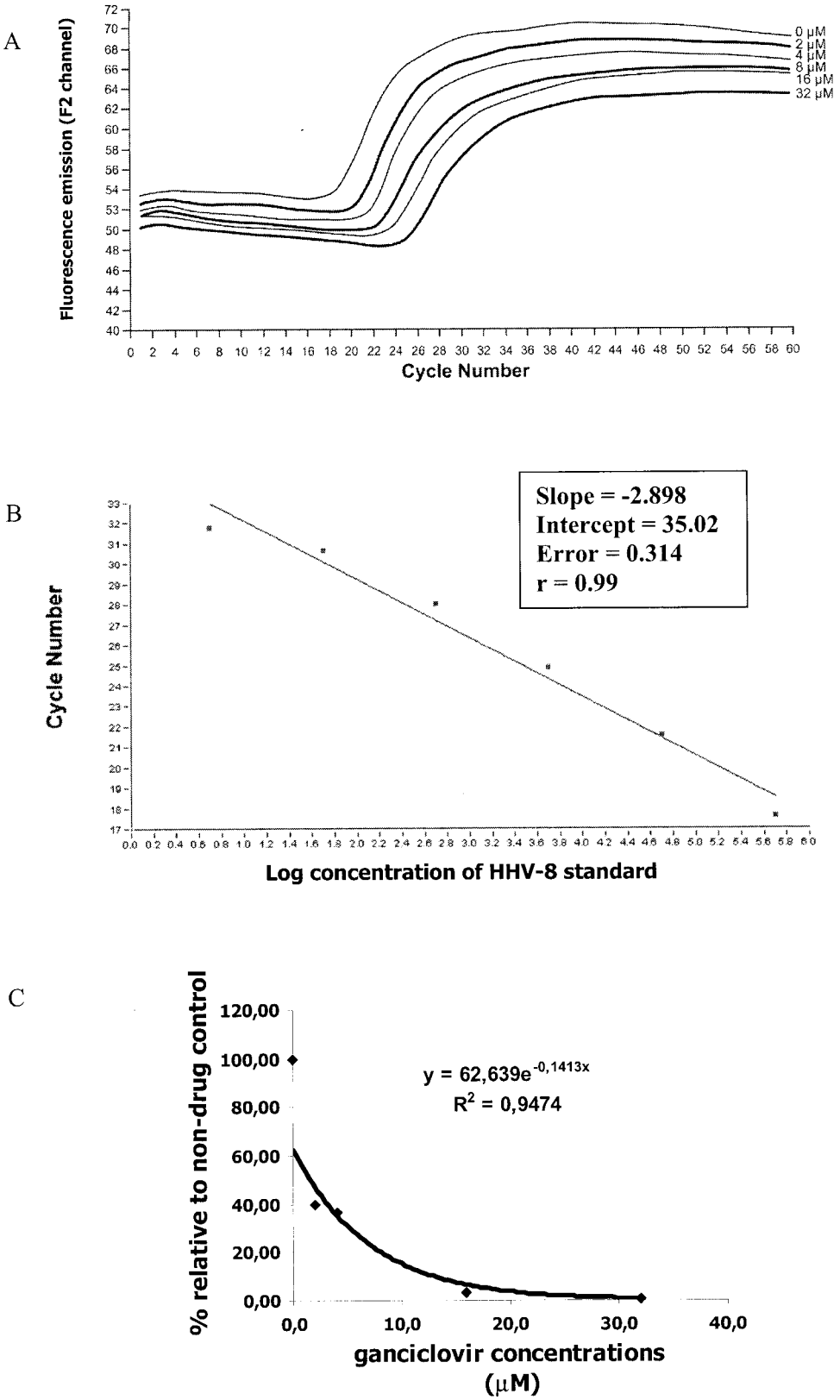

FIG. 1. Real-time PCR assay for evaluation of HHV-8 susceptibility to ganciclovir. (A) Amplification plots showing threshold cycle (Ct) values obtained for the HHV-8 ORF26 gene in presence of serial concentrations (from 0 to 32 μM) of ganciclovir. (B) Standard curve of the real-time PCR assay generated using serial 10-fold dilutions of an external HHV-8 standard and from which the Ct values of the different drug concentrations were interpolated. (C) Calculation of the ganciclovir IC_{50} value based on relative viral load for each drug concentration compared to induced BCBL-1 cells with no drug.

TABLE 1. Susceptibility of HHV-8 to various antiviral agents

Drug	Standard dosing[a]	Peak serum level (μM) (reference)	IC_{50} (μM)[b]	Therapeutic ratio[c]
Acyclovir	10 mg/kg of body weight i.v. tid	90 (9)	31.00 ± 9.00	3
Valacyclovir	2 g p.o. qid	38 (14)		1
Ganciclovir	5 mg/kg i.v. bid	32 (7)	2.61 ± 1.42	12
Valganciclovir	900 mg p.o. bid	24 (4)		9
Adefovir (dipivoxil)	10 mg p.o. od	0.069 (15)	18.00 ± 6.36	0.004
Cidofovir	5 mg/kg i.v. weekly	72 (12)	0.43 ± 0.27	167
Foscarnet	90 mg/kg i.v. bid	766 (1)	34.15 ± 1.87	22

[a] i.v., intravenous; p.o., oral; od, once a day; bid, twice a day; tid, thrice a day; qid, four times a day.
[b] IC_{50} results are means plus standard deviations of two experiments, and each experiment was performed in triplicate.
[c] Therapeutic ratio is defined as the achievable peak serum level for the intravenous (i.e., acyclovir, ganciclovir, cidofovir, and foscarnet) or oral (i.e., valacyclovir, valganciclovir, and adefovir dipivoxil) formulation divided by the drug IC_{50} value.

ganciclovir is shown in Fig. 1. Cidofovir had the lowest IC_{50} value (0.43 ± 0.27 μM), followed by ganciclovir (2.61 ± 1.42 μM), adefovir (18.00 ± 6.36 μM), acyclovir (31.00 ± 9.00 μM), and foscarnet (34.15 ± 1.87 μM) (Table 1). The highest therapeutic ratios (peak serum concentration/IC_{50} value) were seen for cidofovir (167) and foscarnet (22), whereas adefovir (dipivoxil) had the lowest ratio (0.004) (Table 1).

In this study, we report an innovative and reproducible susceptibility assay for HHV-8 based on real-time PCR using supernatant from TPA-induced BCBL-1 cells. Cidofovir and ganciclovir had the lowest IC_{50} values, whereas adefovir had intermediate activity and foscarnet and acyclovir exhibited the highest values (Table 1). Noninduced controls expressed very low levels of viral replication, whereas induced controls without drug expressed peak replication levels at 6 to 7 days after TPA induction (data not shown).

Our IC_{50} values for HHV-8 followed the same trend—although they were not exactly identical—compared to those reported elsewhere using other methodologies (16, 20, 22). For example, although our IC_{50} values for ganciclovir and cidofovir were virtually identical to those reported by Kedes and Ganem (16), values for foscarnet and acyclovir were approximately half of those determined by the same authors. Such differences may be related to cell type, drug uptake and metabolism, and the methodology used to detect and quantify HHV-8. The greatest inhibitory effect on HHV-8 replication was seen with cidofovir (Table 1). This drug is also the most potent anticytomegalovirus (CMV) drug on the market based on in vitro susceptibility testing (12). Adefovir, which is another acyclic nucleoside phosphonate, had notably higher IC_{50} values against HHV-8. More importantly, if we consider the average peak serum level for each drug after intravenous administration (oral administration in the case of adefovir dipivoxil, which is approved for treatment of chronic hepatitis B infection) (15), the most potent inhibitor is cidofovir, with a peak serum level/IC_{50} ratio of 167 compared to 22, 12, 3, and 0.004 for foscarnet, ganciclovir, acyclovir, and adefovir, respectively. However, because the primary use of these drugs is likely to be for prophylaxis of KS in high-risk groups or for preemptive therapy at a time of active viral replication but before the development of HHV-8-associated diseases (8, 11, 13, 21, 26), the convenience of administration and the absence of toxicity are two key factors (in addition to IC_{50} values) that should be considered in the selection of an optimal antiviral agent. In that context, valganciclovir, which is a new bioavailable prodrug of ganciclovir, appears particularly interesting (2, 19). Accordingly, the protective effect of oral ganciclovir (which is 10-fold less bioavailable than valganciclovir) for the development of KS has been previously reported in a CMV study in HIV-infected subjects (18). However, larger clinical trials using KS as the primary endpoint are needed to confirm this finding.

In conclusion, we designed a new in vitro system for evaluation of HHV-8 susceptibility to antiviral drugs which is more rapid, sensitive, and objective than previous assays based on Southern blot analysis. Similar real-time PCR assays could also be used to assess drug susceptibility of other herpesviruses (24).

We thank Tomas Cihlar for careful review of the manuscript.

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