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Evaluation of antiviral activity against human herpesvirus 8 (HHV-8) and Epstein–Barr virus (EBV) by a quantitative real-time PCR assay

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Abstract

A real-time quantitative PCR was developed to assess antiviral activity of molecules against human herpesvirus 8 (HHV-8) and the Epstein–Barr virus (EBV). The antiviral activity of the reference molecules acyclovir, ganciclovir, cidofovir, adefovir and brivudin, as assessed by this methodology, proved very similar to the activity as determined by a DNA–DNA hybridisation method.

Keywords: Human herpesvirus 8 (HHV-8); Epstein–Barr virus (EBV); Antiviral assay; TaqMan; Real-time quantitative PCR

HHV-8 is associated with Kaposi's sarcoma (KS), a common malignancy in patients infected with HIV, primary effusion lymphoma and some forms of Castleman's disease (Chang et al., 1994; Dupin et al., 1999; Cesarman et al., 1996). HHV-8 was reported to be susceptible to anti-herpesvirus agents including ganciclovir and cidofovir (Neyts and De Clercq, 1997; Medveczky et al., 1997; Kedes and Ganem, 1997). A productive infection of EBV results in an acute infectious mononucleosis and the non-productive form is associated with malignancies such as Burkitt's lymphoma, nasopharyngeal carcinomas, and in immunodeficient patients, virus-induced lymphoproliferative disease (EBV-LPD) (zur Hausen et al., 1970; Lucas et al., 1998). Several reports have been published on the effect of antiviral drugs on EBV replication (Meerbach et al., 1998, 2000; Mar et al., 1995; Bacon and Boyd, 1995; Lin et al., 1987; Kira et al., 2000). The aim of the present study was to developed a fast, accurate and convenient quantitative real-time PCR (TaqMan) assay to assess antiviral activity against EBV and HHV-8.

The effects of a selection of anti-herpesvirus nucleoside analogues on the replication of EBV in the producer cell line P3HR-1 and on the replication of HHV-8 in the producer cell line BCBL-1 was determined. Cells were grown in RPMI 1640 medium containing 10% inactivated FBS and antibiotics. Both cell lines were induced by 12-*O*-tetradecanoyl-phorbol-13-acetate (TPA, Sigma) as reported (Meerbach et al., 1998; Neyts and De Clercq, 1997). Exponentially growing BCBL-1 cells were seeded at a density of 3×10^5 cells/ml in the presence of 30 ng/ml TPA. For the EBV experiments 10^6 cells/ml were treated with 20 ng/ml TPA. Cultures were incubated with dilution series of the different drugs for 7 days after which total cellular DNA was extracted using a DNA extraction kit (QIAamp Blood Kit, Qiagen). The blot hybridisation assay was carried out as described earlier (Neyts and De Clercq, 1997; Meerbach et al., 1998).

Real-time detection based on the TaqMan technology was established for both viruses. The PCR primers for EBV detection were designed based on the BNRF1 gene that encodes the membrane protein p140 (Dehee et al., 2001). The forward primer was 5′-CGGCCGTGATGGAGGCTATG-3′, the reverse primer was 5′-AGACAGAGGCCACCACGG-3′, and the TaqMan probe, which was labelled with the reporter dye 6-carboxyfluorescein (FAM) at the 5′ end and the quencher dye 6-carboxytetramethylrhodamine (TAMRA) at the 3′ end, was 5′-TGACCTTTGGCTCGGCCTCCTGC-3′.

PCR primers and the fluorogenic probe used for HHV-8 quantification were generated based on the ORF73 gene sequence (Lallemand et al., 2000). The forward and reverse primer sequences were 5′-CCGAGGACGAAATGGAAGTG-3′ and 5′-GGTGATGTTCTGAGTACATAGCGG-3′, and the probe sequence was 5′-FAM-ACAAATTGCCAGTAGCCCACCAGGAGA-TAMRA-3′. All PCR primers and probes were obtained from PE Applied Biosystems (Foster

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Table 1
Comparison of inhibitory effects of selected compounds on HHV-8 replication by means of TaqMan real-time PCR and a DNA–DNA hybridisation assay[a]

Compound	EC_{50}[b] (μg/ml)		CC_{50}[c] (μg/ml)	SI[d]	
	Real-time PCR	Hybridisation		Real-time PCR	Hybridisation
Acyclovir	21.5 ± 9.6	≥15.7	≥144	≥6.7	≥9.2
Ganciclovir	2.5 ± 0.4	2.3 ± 0.9	90 ± 44	36	39
Cidofovir	1.6 ± 0.3	1.75 ± 0.5	95 ± 31	59	54
Adefovir	12 ± 5	11 ± 8.2	22 ± 3.3	1.8	2
Brivudin	2.4 ± 0.15	8.4 ± 4.2	119 ± 37	50	14

[a] Data are mean values for three or four independent experiments ± standard deviations.
[b] Concentration required to reduces HHV-8 DNA synthesis in TPA-stimulated BCBL-1 cells by 50%.
[c] Cytotoxic concentration (concentration required to reduce the growth of induced BCBL-1 cells by 50%, as evaluated over a 4-day period).
[d] Selectivity index (ratio of CC_{50} to EC_{50}).

City, CA). The PCR reactions were performed in 96-well optical reaction plates with final volumes of 25 μl per well. The TaqMan assay was performed using an ABI Prism 7700 Sequence Detection System. The PCR mixture contained 12.5 μl of TaqMan universal PCR master mix, 300 nM of each primer, 100 nM TaqMan probe and 2.5 μl template DNA; water was added to give a final volume of 25 μl per sample. The PCR conditions for these assay were: 2 min at 50 °C, then 10 min at 95 °C, followed by 55 cycles of 15 s at 95 °C and 1 min at 60 °C each. All assays included two negative controls (water) and a dilution series of the plasmid standard that contained the ORF73 gene for HHV-8 and the BNRF1 gene sequence for EBV. The standard curve of the threshold cycle (C_T) values was constructed for each PCR assay. All PCRs were performed in duplicate. The sample quantity was automatically calculated using the software for data analysis. The 50% effective concentration (EC_{50}) or the concentration required to reduce HHV-8 or EBV DNA synthesis by 50% in TPA-stimulated cells was calculated by regression analysis.

The effects of the anti-herpesvirus drugs on HHV-8 replication, as assessed by Q-PCR or a DNA–DNA hybridisation assay, are presented in Table 1. Ganciclovir, cidofovir and brivudin proved about equipotent in their antiviral activity (with EC_{50} values of 1–2 μg/ml). These values are in the same range, if not almost identical, to the data as obtained by the hybridisation assay. For BVDU, however, the values obtained in the PCR assay were a factor 3–4 lower than in the hybridisation assay. Adefovir and acyclovir were less active; also for these compounds the EC_{50} values generated by both methods were very similar.

Next the effects of the compounds on the replication of EBV in P3HR-1 cells was assessed, by respectively Q-PCR and DNA–DNA hybridisation (Table 2). Cidofovir proved to be the most potent as an inhibitor of EBV replication. Adefovir and ganciclovir exhibited equipotent activity, that was about 3- to 10-fold more pronounced than that of acyclovir. Brivudin conferred the weakest activity. Also for EBV, the EC_{50} values, as obtained by the Q-PCR method, proved very similar to the values obtained by the hybridisation assay. The mechanism of action and the possible reasons for the differences in the antiviral activity of the antiviral drugs against EBV and HHV-8 have been reviewed (De Clercq et al., 2001). The fact that the EC_{50} values for inhibition of HHV-8 and EBV replication by BVDU are somewhat lower than for the hybridisation assay could theoretically be explained by the presence of AmpErase (UNG) in the TaqMan universal PCR master mix. This enzyme has been reported to excise 5 bromo uracil and 5,6-dihydroxyuracil from DNA. If BVDU were incorporated in viral DNA, UNG would preferentially degrade viral DNA templates in the presence of this drug and thus lower the EC_{50} values. It may therefore be advisable to use a master mix without AmpErase.

During the course of this study, Sergerie and Boivin (2003) reported on a real-time Q-PCR methodology for HHV-8 and monitored the antiviral effect of acyclovir (and

Table 2
Comparison of inhibitory effects of selected compounds on EBV replication by means of TaqMan real-time PCR and a DNA–DNA hybridisation assay[a]

Compound	EC_{50}[b] (μg/ml)		CC_{50}[c] (μg/ml)	SI[d]	
	Real-time PCR	Hybridisation		Real-time PCR	Hybridisation
Acyclovir	1.14 ± 0.88	2.3 ± 1.4	88 ± 5.8	77	38
Ganciclovir	0.14 ± 0.07	0.2 ± 0.2	12 ± 1.5	86	60
Cidofovir	0.065 ± 0.036	0.1 ± 0.1	28 ± 3.5	431	280
Adefovir	0.42 ± 0.06	–	27 ± 0.6	64	–
Brivudin	22 ± 9	≥50	75 ± 4.4	3.4	≤1.5

[a] Data are mean values for three or four independent experiments ± standard deviations.
[b] Concentration required to reduces EBV DNA synthesis in TPA-stimulated P3HR-1 cells by 50%.
[c] Cytotoxic concentration (concentration required to reduce the growth of induced P3HR-1 cells by 50%, as evaluated over a 7-day period).
[d] Selectivity index (ratio of CC_{50} to EC_{50}).

valacyclovir), ganciclovir (and valganciclovir), adefovir (dipivoxil), cidofovir and foscarnet. Although a different gene was used to generate the amplicon and a different methodology was employed (Light Cycler instead of Taq-Man in the present study), the overall data obtained were similar to those reported here. In conclusion, the real-time Q-PCR methods reported here allow to rapidly evaluate anti-EBV and HHV-8 activity in a convenient and accurate fashion.

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