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Human Herpesvirus 8 Gene Encodes a Functional Thymidylate Synthase

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We demonstrate that human herpesvirus 8, obtained from the lymphoma cell line BC-3 as well as from Kaposi's sarcoma lesions, carries a gene that encodes a functional thymidylate synthase (TS). The particular characteristics of this enzyme are studied and compared to the characteristics of TSs encoded by other organisms.

Human herpesvirus 8 (HHV-8) is a gammaherpesvirus that is related to herpesvirus saimiri (HVS) and rhesus rhadinovirus (RRV) (17). HHV-8 replication can be selectively inhibited by several antiherpesvirus agents, among which cidofovir proved to be the most effective (19, 6). The HHV-8 genome encodes several enzymes that are involved in nucleoside and nucleotide biosynthesis. These include a thymidine kinase, a ribonucleotide reductase, a dihydrofolate reductase, and a thymidylate synthase (TS) (21). The HHV-8-encoded thymidine kinase (3, 7, 16) and dihydrofolate reductase (5) have been shown to be functional.

TS (5,10-methylenetetrahydrofolate:dUMP C-methyltransferase, EC 2.1.1.45) is an enzyme that catalyzes the reaction of 5,10-methylenetetrahydrofolate and dUMP to dTMP. TS is the only enzyme leading to the de novo synthesis of dTMP, dTDP, and dTTP. The enzyme is a key target for the action of antitumoral drugs (8). HVS and varicella-zoster virus (VZV) have been previously reported to encode a functional TS (9, 22). Human cytomegalovirus, a virus that belongs to the betaherpesvirus family, does not encode a TS but markedly stimulates cellular TS activity in infected human embryonic lung cells (18).

HHV-8 encodes a TS gene (21), and the protein was previously shown to be expressed in the cytoplasm of cells transfected with an expression vector carrying the HHV-8 TS gene, although no proof of functional enzymatic activity was given (5). The gene encoding the TS of HHV-8 is 1,014 bp long, that of HVS is 885 bp long, that of RRV is 1,002 bp long, and that of VZV is 906 bp long. These TSs are markedly shorter than the human counterpart, which is encoded by a gene of 1,533 bp. In Table 1 we present the alignment of the TS of the different herpesviruses with the human TS. This alignment reveals a high degree of amino acid sequence similarity, in particular for the region extending from amino acids 174 to 224 (Table 1). In the region containing the catalytic site, the HHV-8 TS and its human counterpart are 88% identical.

We cloned and expressed the TS gene of HHV-8 by using DNA isolated from the HHV-8-containing BC-3 cell line (ATCC CRL-2277), as well as from biopsies of Kaposi's sarcoma lesions obtained from a Belgian case and from a Hungarian case of classic Kaposi's sarcoma. In addition, the respective TS genes of HVS (C488 strain) (virus kindly provided by H. Fickenscher, University of Erlangen, Erlangen, Germany), VZV (OKA strain) (isolated from fibroblasts infected with the virus), and RRV DNA (DNA kindly provided by Scott Wong, Oregon Health Sciences University, Portland) were cloned and expressed. The forward primers used for PCR amplification of the TS genes contained an *Eco*RI site, and the reverse primers contained a *Sal*I site. The resulting amplicons were cloned in pCR4-TOPO TA vector (Invitrogen), and the cloned fragments were sequenced on both strands by using the Big Dye terminator cycle DNA sequencing kit (ABI PRISM, Applied Biosystems). The HVS, VZV, RRV, and BC-3 HHV-8 TS sequences proved to be 100% identical to the sequences available from the National Center for Biotechnology Information Blast GenBank. HHV-8 DNA from the Belgian patient carried an A-G substitution at nucleotide position 21047 and at nucleotide position 20824. HHV-8 DNA isolated from the Hungarian patient carried a C-T substitution at position 21012, an A-G substitution at position 20776, and a T-A substitution at position 20177 (positions refer to the numbering used in GenBank U75698). The TS genes were subcloned in the bacterial expression vector pGEX4. This vector allows the production of glutathione *S*-transferase fusion proteins (Amersham Biosciences). The resulting plasmids were then used to transform competent *Escherichia coli* BL21 cells. Cultures were induced with 0.1 mM isopropyl-β-D-thiogalactoside (IPTG) (Sigma) for 6 h at 25°C. Cells were pelleted at 10,000 × *g* for 10 min at 4°C and resuspended in BugBuster protein extraction reagent (Novagen). Benzonase nuclease (Novagen) was added for 20 min at room temperature. Clarified lysates were pelleted for 20 min at 15,000 × *g* at 4°C and incubated with glutathione Sepharose 4B (Amersham Biosciences). The recombinant enzymes were eluted with glutathione according to the manufacturer's instructions. The protein concentrations were determined, and the size of the protein was monitored by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. TS activity was assessed by means of the tritium release assay according to a modified version of a method used earlier (1).

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TABLE 1. Alignment of part of the TS gene products from various herpesviruses, as well as from *E. coli* and humans, in the neighborhood of the catalytic site[a]

TS Source	Start position	Amino acid sequence	End position	% Identity with human TS
Human	174	DRRIIMCAWNPRDLPLMALPPCHALCQFYVVNSELSCQLYQRSGDMGLGVP	224	
HHV-8	198	DRRIIMCAWNPADLSLMALPPCHLLCQFYVADGELSCQLYQRSGDMGLGVP	248	88
RRV	194	DRRIVMCAWNPADLARMALPPCHVLCQFYVARGELSCQLYQRSADMGLGVP	244	82
VZV	163	RRMIISSWNPKDIPLMVLPPCHTLCQFYVANGELSCQVYQRSGDMGLGVP	212	78
HVS	155	DRRMLMCAWNVSDIPKMVLPPCHVLSQFYVCDGKLSCQLYQRSADMGLGVP	205	72
E. coli	126	RRIIVSAWNVGELDKMALAPCHAFFQFYVADGKLSCQLYQRSCDVFLGLP	175	64

[a] The catalytic site, PCH, is marked in boldface type.

The appropriate amount of enzyme was incubated with 14.6 Ci of [5-^{3}H]dUMP (Amersham Pharmacia Biotech)/mmol in 50 mM Tris-HCl buffer (pH 7.5) containing 5.0 mM formaldehyde, 15 mM β-mercaptoethanol, 0.1 mM NaF, and (6*R,S*)-5,10-methylene-5,6,7,8-tetrahydrofolate (Schircks Laboratory). According to the particular experimental conditions, various concentrations of dUMP and 5,10-methylene-5,6,7,8-tetrahydrofolate were used. The enzymatic reactions were initiated, following a 2-min preincubation of the reaction mixture at 37°C, by addition of the enzyme and were then incubated in triplicate at 37°C for the appropriate time. The enzymatic reaction was stopped on ice, and 1 ml of active carbon (100 mg/ml in 2% trichloroacetic acid) was added to the tubes. Following a 2-min incubation period on ice, the carbon was pelleted by centrifugation at 3,000 rpm in a Hereaus Minifuge T for 10 min (4°C). Tritium release was measured by determining radioactivity in 200-μl fractions of the supernatant. Enzyme kinetics were calculated by using Graphpad Prism version 3.02 for Windows (GraphPad Software, San Diego, Calif.).

HHV-8 TSs, obtained from three sources as described above, i.e., HHV-8 isolated from the BC-3 lymphoma cell line as well as from two different patients with Kaposi's sarcoma, were expressed. Recombinant HHV-8 TSs obtained from these three sources all proved functionally active. Recombinant TSs that were derived from HVS as well as from VZV also exhibited, as expected, functional TS activity. We failed, however, to detect functional TS activity associated with the RRV TS even though alternative cloning strategies were used and a protein of the correct size was expressed. The question remains whether the RRV TS is indeed functionally active or whether the particular RRV strain that was used here encodes a defective TS.

We next studied and compared the particular kinetics of the TS of HHV-8, HVS, and VZV. K_m values, with dUMP and methylenetetrahydrofolate (CH_2H_4-folate) as a substrate, for the TSs encoded by HHV-8 (DNA obtained from the Kaposi's sarcoma lesion of a Belgian patient), HVS, and VZV are summarized in Table 2. K_m values of *E. coli* TS (enzyme kindly provided by Paola Costi, University of Modena, Modena, Italy) and of human TS (data obtained from the literature) are also listed. K_m values for both substrates (dUMP and CH_2H_4-folate) were very comparable to those of the three herpesvirus enzymes and were in the same range as the K_m values reported for human TS (14).

We then investigated the inhibitory effects of three well-known TS inhibitors, i.e., 5-fluoro-2′-deoxyuridine-5′-monophosphate (FdUMP) (Sigma), 5-iodo-2′-deoxyuridine-5′-mono-phosphate (IdUMP) (Sigma), and 5-(2-bromovinyl)-2′-deoxyuridine-monophosphate (BVdUMP) (kindly provided by P. Herdewijn, Rega Institute, Leuven, Belgium), and of dTMP (Sigma) (which is a product inhibitor of TS) on the three viral enzymes. For all three dUMP analogues studied, the type of inhibition was competitive with respect to dUMP. The TS encoded by HHV-8, HVS, and VZV proved as susceptible to the compounds as the human, murine, and lactobacillus thymidylate synthases (Table 3). This makes it unlikely that HHV-8 is a specific target for antiviral or antitumoral therapy against HHV-8 or tumors associated with HHV-8.

Evidence that cellular TSs from diverse species influence the regulation of p53 expression by decreasing the translational efficiency of p53 mRNA has been provided (11). It is also assumed that inhibition of p53 by the HHV-8 latency-associated nuclear antigen may be involved in HHV-8-induced oncogenesis by suppression of cell death (12). It would be tempting to speculate that HHV-8 TS plays a role in HHV-8-induced oncogenesis. In such a case, the viral TS could be one of the many factors, including the viral G protein-coupled receptor, the viral interferon regulatory factor, viral D-type cyclin, viral interleukin-6, viral bcl-2, viral FLICE-inhibitory protein, laten-

TABLE 2. K_m values for dUMP and CH_2H_4-folate of TSs from different sources[a]

TS source	K_m dUMP (μM)	K_m CH_2H_4-folate (μM)	k_{cat} (s^{-1})	k_{cat}/K_m dUMP (s^{-1} μM^{-1})	k_{cat}/K_m CH_2H_4-folate (s^{-1} μM^{-1})
HHV-8 (Belgian patient)	1.72 ± 0.92	19.6 ± 7.9	4.6 ± 0.6	2.67	0.23
HVS (C488 strain)	2.38 ± 0.57	23 ± 8.5	3.8 ± 0.4	1.59	0.16
VZV (OKA strain)	1.92 ± 0.81	15.2 ± 3.8	3.5 ± 0.2	1.82	0.23
E. coli	3.54 ± 0.41				
Human[b]	1.8 ± 0.7	14 ± 5	3.9	2.2	0.28

[a] Rate constants for the viral TS are based on three independent determinations (K_m dUMP) or two independent experiments (K_m CH_2H_4-folate).
[b] From reference 14.

TABLE 3. Comparison of rates of inhibition of herpesvirus, human, murine, and *Lactobacillus casei* TSs by 5-substituted dUMP analogues and dTMP

Source of TS	K_i (K_I/K_m) with indicated compound			
	FdUMP (nM)	IdUMP (nM)	BVdUMP (nM)	dTMP (μM)
HHV-8 (Belgian patient)	24.5 ± 2.2 (0.014)	1,095 ± 79 (0.64)	3,953 ± 307 (2.3)	12.8 ± 1.1 (7.44)
HVS	25.6 ± 3.4 (0.010)	876 ± 74 (0.37)	3,641 ± 283 (1.53)	33.4 ± 2.3 (14)
VZV	26 ± 1.9 (0.013)	1,301 ± 48 (0.68)	2,099 ± 172 (1.09)	9.1 ± 0.2 (4.739)
Human	6.4[a] (0.0025)		4,500[b] (0.58)	9.6[c] (7.3)
Murine	18.2[d] (0.001)	6,016[d] (3.27)	1,214[e] (0.66)	7.3[c] (3.72)
L. casei	14[e] (0.0027)	1,600[e] (0.30)	2,000[f] (0.66)	22[g] (7.58)

[a] From reference 15.
[b] From reference 13.
[c] From reference 20.
[d] From reference 23.
[e] From reference 1.
[f] From reference 2.
[g] From reference 4.

cy-associated nuclear antigen, K1, and latency-associated membrane protein, that are associated with virus-induced oncogenesis (10).

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Gammaherpesviruses encode functional dihydrofolate reductase activity

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Abstract

We overexpressed and purified from *Escherichia coli* the dihydrofolate reductase (DHFR) of the gammaherpesviruses human herpesvirus 8 (HHV-8), herpesvirus saimiri (HVS), and rhesus rhadinovirus (RRV). All three enzymes proved catalytically active. The K_m value of HHV-8 DHFR for dihydrofolate (DHF) was $2.02 \pm 0.44\,\mu M$, that of HVS DHFR was $4.31 \pm 0.56\,\mu M$, and that of RRV DHFR is $7.09 \pm 0.11\,\mu M$. These values are approximately 5–15-fold higher than the K_m value reported for the human DHFR. The K_m value of HHV-8 DHFR for NADPH was $1.31 \pm 0.23\,\mu M$, that of HVS DHFR was $3.78 \pm 0.61\,\mu M$, and that of RRV DHFR was $7.47 \pm 0.59\,\mu M$. These values are similar or slightly higher than the corresponding K_m value of the human enzyme. Methotrexate, aminopterin, trimethoprim, pyrimethamine, and N(α)-(4-amino-4-deoxypteroyl)-N(δ)-hemiphthaloyl-L-ornithine (PT523), all well-known folate antagonists, inhibited the DHFR activity of the three gammaherpesviruses competitively with respect to DHF but proved markedly less inhibitory to the viral than towards the human enzyme.

Human herpesvirus 8 (HHV-8), the causative agent of Kaposi's sarcoma (KS) and some rare forms of lymphoma, is closely related to herpesvirus saimiri (HVS), a virus of New World primates. Herpesvirus saimiri is a virus of the squirrel monkey that can transform human T lymphocytes. The rhesus monkey rhadinovirus (RRV), a virus of Old World primates, is more closely related to HHV-8 than to any other herpesvirus [1]. HHV-8 replication can be selectively inhibited by several antiherpesvirus agents among which are ganciclovir and cidofovir, the latter compound being the most effective [2–4]. The incidence of KS was shown to be lower in AIDS patients who had received treatment with ganciclovir. In a series of patients who had received either oral or intravenous ganciclovir for ocular CMV infections, the incidence of KS development was reduced by 75% and 93%, respectively, as compared to the placebo group [5]. Cohort studies also reported a reduced incidence of KS among patients receiving ganciclovir [6,7]. This effect can be explained by a reduction in the HHV-8 load and, thus, a diminished chance of developing the virus-induced tumor.

The HHV-8 genome encodes several enzymes that are involved in nucleoside and nucleotide biosyntheses. These include a thymidine kinase (TK), a ribonucleotide reductase, a thymidylate synthase (TS), and a dihydrofolate reductase (DHFR). Dihydrofolate reductase (EC 1.5. 1.3.) is a ubiquitous enzyme that catalyzes the NADPH-dependent reduction of dihydrofolate (DHF) to tetrahydrofolate, which is essential for de novo purine, glycine, and methionine synthesis and for the biosynthesis of thymidylate. Antitumoral and immunosuppressive compounds [such as methotrexate (MTX) and aminopterin (AMT)] are targeted at DHFR [8,9]. DHFR inhibitors such as trimethoprim (TMP) and pyrimethamine (PYR) are potent antibiotics or antiparasitics [10,11].

The HHV-8 encoded TK, TS, and DHFR have been shown to be functionally active [12–14]. Human cytomegalovirus (HCMV) and murine cytomegalovirus (MCMV), viruses that belong to betaherpesviruses, do not encode a DHFR but markedly stimulate cellular DHFR activity in quiescent cells [15,16]. HVS as well as RRV carry a DHFR gene in their genome, but functional activity has so far not been demonstrated for

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these enzymes [17,18]. Epstein–Barr virus (EBV), that does not encode a DHFR, induces a relatively constant level of DHFR mRNA in virus-transformed cells throughout all phases of the cell cycle. This is achieved by suppressing the transcriptional down-regulation common to enzymes responsible for deoxynucleotide metabolism [19]. Since several herpesviruses encode either a DHFR or stimulate the function of the cellular enzyme, it may be surmised that DHFR plays an important role in the replication cycle of herpesviruses. Here, it is demonstrated that also HVS and RRV encode a functional DHFR and the particular characteristics of the DHFR encoded by the gammaherpesviruses HHV-8, HVS, and RRV are investigated.

Materials and methods

Compounds. DHF, NADPH, MTX, AMT, PYR, and TMP were purchased from Sigma. PT523 [20,21] was provided by Andre Rosowsky (Dana-Farber Cancer Institute, Boston).

Biological material. HVS C488 DNA was a gift from Helmut Fickenscher (University of Erlangen, Erlangen, Germany) and RRV DNA (strain 17577) was provided by Scott Wong (Oregon Health Sciences University, Portland). HHV-8 DNA was isolated from the HHV-8 containing BCBL-1 cell line (NIH AIDS Research & Reference Reagent Program). DNA from BCBL-1 cells was isolated using the QIAamp DNA Blood Mini Kit (Qiagen).

Enzyme purification. The HHV-8, HVS, and RRV DHFR genes were amplified by PCR. The forward primer contained an *Eco*RI, and the reverse primer a *Sal*I site. The resulting amplicons were cloned in pCR4-TOPO TA vector (Invitrogen) and the cloned fragments were sequenced on both strands using the Big Dye terminator cycle DNA sequencing kit (ABI PRISM, Applied Biosystems). The HHV-8, HVS, and RRV DHFR sequences proved to be 100% identical to the nucleotide sequences available from the National Center for Biotechnology Information Blast GenBank (U83347, M55264, and AF083501). The DHFR genes were subcloned in the bacterial expression vector pGEX4 (Amersham Biosciences) in the restriction sites *Eco*RI and *Sal*I. The resulting plasmids were then used to transform competent *Escherichia coli* BL21 cells. Cultures were induced with 0.1 mM isopropyl-β-D-thiogalactoside (Sigma) for 6 h at 25 °C. Cells were pelleted at 10,000*g* for 10 min at 4 °C and resuspended in Bug-Buster Protein Extraction Reagent (Novagen). Benzonase Nuclease (Novagen) was added for 20 min at room temperature. Clarified lysates were pelleted for 20 min at 15,000*g* at 4 °C and incubated with glutathione–Sepharose 4B (Amersham Biosciences). The recombinant enzymes were eluted with glutathione according to manufacturer's instructions. The protein concentrations were determined using the Bradford reagent (Sigma) and SDS–PAGE was carried out to control the size and purity of the expressed protein. The eluate was divided in small aliquots and stored at −70 °C until further use.

Enzyme assays and kinetic studies. A modified version of the standard DHFR activity assay was applied [22]. The assay mixture contained 50 μM DHF, 60 μM NADPH, and 14 μM β-mercaptoethanol in MTEN buffer, pH 7.8 [50 mM 2-morpholinoethanesulfonic acid (MES), 25 mM Tris, 25 mM ethanolamine, and 100 mM NaCl]. The reaction was carried out at 25 °C. The enzyme was added to initiate the reaction. The kinetic decrease in absorbance at 340 nm was measured by a Spectramax 190 plate reader (Molecular Devices). Varying concentrations of substrates as well as inhibitors were used for the determination of K_m and K_i values, respectively. Enzyme kinetics were calculated using Graphpad Prism version 3.02 for Windows (GraphPad Software, San Diego).

Results and discussion

We cloned and overexpressed the DHFR of HHV-8, RRV, and HVS in *E. coli*. Recombinant proteins that were obtained from these three sources all proved enzymatically active. We have recently demonstrated that HHV-8 and HVS encode a functional thymidylate synthase [13].

The particular kinetics of the DHFR of HHV-8, HVS, and RRV were investigated. K_m values, with DHF and NADPH as a substrate for the DHFRs encoded by HHV-8, HVS, and RRV are summarized in Table 1. The K_m values of the human DHFR obtained from the literature are also listed. K_m values (for both substrates) proved very comparable for the three herpesvirus enzymes. The K_m value for DHF of the three viral enzymes was invariably 5–15-fold higher than that of the human counterpart, whereas the K_m values for NADPH as a co-enzyme were rather similar to that of the human enzyme.

We next studied the inhibitory effect of five antifolates, i.e.: methotrexate (MTX), aminopterin (AMT), trimethoprim (TMP), pyrimethamine (PYR), and N(α)-(4-amino-4-deoxypteroyl)-N(δ)-hemiphthaloyl-L-ornithine (PT523) on the different viral DHFRs. The three viral DHFRs behaved similarly in their sensitivity to these inhibitors whereby the order of activity ranked as follows: TMP ≪ PYR < AMT < MTX < PT523 (Table 2).

The viral DHFRs, however, proved markedly less susceptible to these inhibitors than the human enzyme. This may indicate that the herpesvirus DHFR is not a

Table 1
K_m values for DHF and NADPH of dihydrofolate reductases from different sources

	HHV-8	HVS	RRV	Human[a]
K_m DHF (μM)	2.02 ± 0.44	4.31 ± 0.56	7.09 ± 0.11	0.4 ± 0.1
K_m NADPH (μM)	1.31 ± 0.23	3.78 ± 0.61	7.47 ± 0.59	1.1 ± 0.1
k_{cat} (s^{-1})	6.45 ± 0.72	7.48 ± 0.83	11.2 ± 2.26	16.8 ± 0.4
k_{cat}/K_m DHF (s^{-1} μM^{-1})	3.17	1.74	1.58	42
k_{cat}/K_m NADPH (s^{-1} μM^{-1})	4.92	1.98	1.5	15.3

Rate constants for the viral DHFRs are based on three independent experiments.
[a] From [14].

Table 2
Inhibition of herpesvirus dihydrofolate reductases by antifolates, as compared to human dihydrofolate reductase

Compound	HHV-8	HVS	RRV	Human
	K_i	K_i	K_i	K_i
MTX (pM)	276 ± 35	656 ± 49	375 ± 38	9 ± 2[a]
AMT (pM)	741 ± 69	1356 ± 298	880 ± 57	14.6 ± 0.1[a]
PT523 (pM)	1.9 ± 0.86	7.15 ± 1.3	6.14 ± 0.35	0.35 ± 0.1[b]
PYR (nM)	32.1 ± 2.7	102 ± 8.4	73.4 ± 9.6	1.0 ± 0.2[a]
TMP (μM)	2.55 ± 0.18	2.74 ± 0.21	2.71 ± 0.14	0.81 ± 0.02[a]

[a] From [14].
[b] From [21].

good target for selective antiviral therapy. Cinquina et al. [14] also reported that the HHV-8 encoded DHFR is less susceptible than the human enzyme to a number of DHFR inhibitors. The genes for HHV-8 DHFR and TS (ORF 2 and 70, respectively) are located in the cytokine-containing segment of the HHV-8 genome and not in locations corresponding to HVS ORFs 2 and 70. On the other hand, the location of the RRV DHFR gene corresponds to that of the HVS DHFR gene. HVS DHFR has a distinct origin and has undergone much less substitution than the HHV-8 DHFR [23]. The HVS DHFR gene is located behind the saimiri transforming protein (STP) which acts as an oncogenic protein [24] and the RRV DHFR is located behind the R1 protein which is also endowed with oncogenic properties [25]. In contrast, the HHV-8 DHFR is located nine reading frames downstream from the oncogenic K1 gene.

The fact that these viruses acquired both enzymes of the TS/DHFR complex may be indicative of an important role in providing the infected cell with sufficient thymidylate for viral DNA synthesis. The alphaherpesvirus, varicella-zoster virus (VZV), also encodes a functionally active TS but does not carry a gene that encodes a DHFR [17]. We have shown earlier [26] that HCMV, a virus that does not encode its own TS, markedly stimulates (up to 20-fold) cellular TS activity. Although HCMV and MCMV do not encode a DHFR, infection with HCMV, as well as with MCMV, results in a prominent increase in cellular DHFR activity in quiescent cells [15,16].

It remains unclear, however, why certain herpesviruses (such as HHV-8, HVS, and RRV) encode their own TS and DHFR, whereas other herpesviruses [such as HSV-1, HSV-2, EBV, HHV-6, and HHV-7] do not. Possibly, DHFR and TS are involved in oncogenesis and play a role in providing the transformed cell with sufficient quantities of thymidylate. The fact that VZV, which is not an oncogenic herpesvirus, encodes a functional TS, does not necessarily argue against this hypothesis, since VZV does not encode the accompanying DHFR. The gammaherpesvirus-encoded TS and DHFR may be expected to increase the virus replication efficiency and may also play a role during persistent viral replication in resting cells, such as lymphocytes, that contain low levels of cellular DHFR.

In conclusion, we have demonstrated that gammaherpesviruses, HHV-8, HVS, and RRV, encode a functional DHFR. These DHFRs have a higher K_m value for DHF and NADPH, but are also significantly less susceptible to folate antagonists than the human counterpart.

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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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