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Human herpesvirus 6 DNA polymerase: enzymatic parameters, sensitivity to ganciclovir and determination of the role of the $A^{961}V$ mutation in HHV-6 ganciclovir resistance

Leen De Bolle [a], Chaysavanh Manichanh [b,1], Henri Agut [b], Erik De Clercq [a], Lieve Naesens [a,*]

[a] *Laboratory of Virology and Chemotherapy, Rega Institute for Medical Research, Katholieke Universiteit Leuven, Minderbroedersstraat 10, 3000 Leuven, Belgium*
[b] *Laboratoire de Virologie, UPRES EA 2387, Groupe Hospitalier Pitié-Salpêtrière, Paris, France*

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Abstract

Human herpesvirus 6 (HHV-6) is latent in the majority of the adult population. Due to its ability of causing opportunistic infections, alone or in concert with the other β-herpesviruses human cytomegalovirus (HCMV) and HHV-7, its importance as a pathogen in immunocompromised patients has increasingly been recognized. We here report the characterization of the main antiviral target, the HHV-6 DNA polymerase, expressed in a eukaryotic in vitro transcription/translation assay. This technique represents a fast and straightforward approach for the study of existing and new inhibitors of HHV-6 DNA polymerase. The present study shows that ganciclovir is less active against HHV-6, as compared to its activity against HCMV, both in cell culture and at the enzymatic (i.e. DNA polymerase) level. Recently, a mutant HHV-6 strain carrying an amino acid substitution in the ganciclovir phosphorylating pU69 kinase has been isolated both from patients and in cell culture. The strain isolated in vitro, however, carried an additional mutation in the viral DNA polymerase. From our experiments presented here, we conclude that the pU69 $M^{318}V$ amino acid substitution rather than the $A^{961}V$ substitution in HHV-6 DNA polymerase, is responsible for the ganciclovir-resistant phenotype.

Keywords: Human herpesvirus 6; DNA polymerase; Resistance; Antiviral

1. Introduction

Human herpesvirus 6 (HHV-6), which exists as two variants, A and B, is a lympho- and neurotropic betaherpesvirus that is closely related to human cytomegalovirus (HCMV) and human herpesvirus 7 (HHV-7). Primary infection with HHV-6B usually occurs in early childhood and results in exanthema subitum, rarely complicated by episodes of seizures or encephalitis (Yamanishi et al., 1988). The primary pathology of the A variant of HHV-6 is unknown. After primary infection, the virus persists in certain tissues (such as peripheral blood mononuclear cells and neural tissue) and establishes latency, presumably in monocytes (Kondo et al., 1991) and early bone marrow progenitor cells (Luppi et al., 1999). Reactivation of latent HHV-6 mainly occurs in immunocompromised adults, such as transplant recipients. The importance of HHV-6 as a (co-)pathogen in viral syndromes following solid organ and bone marrow transplantation has become increasingly apparent. Not only can HHV-6 reactivation or (re)infection cause encephalitis or bone marrow suppression in these patients, it also interacts with the other β-herpesviruses HCMV and HHV-7 and exerts indirect immunologic effects which may lead to allograft rejection or facilitate bacterial or fungal infections (Clark, 2002). Finally, HHV-6 is believed to act as a co-factor in AIDS progression (Lusso and Gallo, 1995).

The HHV-6 DNA polymerase (DNA pol), like all herpesvirus DNA polymerases, is a heterodimer, consisting of a catalytic subunit (encoded by the HHV-6 U38 gene) and a processivity factor (encoded by HHV-6 U27) (Agulnick et al., 1993), which allows synthesis of extended stretches of DNA without dissociation of pU38 from the template. It

* Corresponding author. Tel.: +32 16 33 73 45; fax: +32 16 33 73 40.
E-mail address: lieve.naesens@rega.kuleuven.ac.be (L. Naesens).
[1] Present address: Unité d'Ecologie et Physiologie du Système Digestif, Institut National de la Recherche Agronomique, 78352 Jouy-en-Josas Cedex, France.

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can be differentiated from cellular DNA polymerases on the basis of immunological reactivity (Williams et al., 1989). In the so far single report on the characterization of the HHV-6 DNA pol (Bapat et al., 1989), the enzyme was chromatographically purified from HHV-6-infected cell cultures. However, chromatographic purification represents a laborious procedure, and herpesvirus DNA polymerases are very sensitive to freezing and thawing. We, therefore, chose to implement a eukaryotic in vitro transcription/translation assay as a rapid and reproducible source of fresh enzyme. This technique will prove to be useful in the further characterization of HHV-6 DNA pol and in the evaluation of new nucleoside, or nucleotide, and non-nucleoside analogs for their inhibitory potential. Moreover, it should allow the direct comparison of wild-type and mutant HHV-6 DNA polymerases.

The pyrophosphate analog foscarnet and the deoxycytidine-5′-monophosphate analog cidofovir [after metabolization to its diphosphate by cellular enzymes (Cihlar and Chen, 1996)] target the viral DNA polymerase directly. The deoxyguanosine analog ganciclovir first needs to be activated to its monophosphate derivative by a viral protein kinase (i.e. the HCMV pUL97 phosphotransferase (Littler et al., 1992; Sullivan et al., 1992) or its HHV-6 homolog, pU69 (Ansari and Emery, 1999)). Two subsequent phosphorylations are conducted by the cellular deoxyguanylate (dGMP) kinase and nucleoside diphosphate (NDP) kinase (Gallois-Montbrun et al., 2002; Matthews and Boehme, 1988). Ganciclovir shows reasonable activity against HHV-6 in vitro (De Bolle et al., 2004), although 20-fold lower levels of ganciclovir monophosphate are attained by the HHV-6 U69-encoded phosphotransferase than by its HCMV homolog pUL97 (De Bolle et al., 2002). Foscarnet and cidofovir, and to a lesser extent, acyclovir, also inhibit HHV-6 replication in vitro successfully (De Bolle et al., 2004). A number of case reports support the effectiveness of these compounds against HHV-6 in patients (Bethge et al., 1999; Johnston et al., 1999; Kadakia et al., 1996; Mookerjee and Vogelsang, 1997; Rieux et al., 1998).

Thus far, only one report has been published on the resistance of HHV-6 to antiviral compounds. Notably, a HHV-6 mutant virus obtained after serial passage in vitro under increasing ganciclovir pressure was found cross-resistant to ganciclovir and cidofovir, showing a 24- and 52-fold decreased sensitivity, respectively (Manichanh et al., 2001). Two mutations were identified that yielded amino acid substitutions in the pU69 phosphotransferase ($M^{318}V$, analogous to the $M^{460}V/I$ substitution in HCMV pUL97) and in the DNA pol ($A^{961}V$). Interestingly, the $M^{318}V$ mutant was also detected by PCR in HHV-6-infected PBMCs from an AIDS patient who had received long-term ganciclovir therapy. The $M^{318}V$ substitution was shown to severely impair ganciclovir phosphorylation by pU69 expressed in a baculovirus system (Safronetz et al., 2003). In the following report, we evaluated the importance of both mutations in their respective enzyme assays.

2. Materials and methods

2.1. Compounds

Ganciclovir (GCV, Cymevene™) was purchased from Roche Pharmaceuticals (Basel, Switzerland), cidofovir (CDV, Vistide™) from Gilead Sciences (Foster City, CA), foscarnet (phosphonoformic acid, PFA, Foscavir™) from Astra Zeneca Pharmaceuticals (Brussels, Belgium) and (*E*)-5-(2-bromovinyl)-2′-deoxyuridine (brivudin, BVDU, Zostex™) was from Searle. The triphosphate derivative of ganciclovir was synthesized biochemically and HPLC purified at our laboratory according to a published method (Agbaria et al., 2001); the BVDU 5′-triphosphate was kindly provided by Dr. P. Herdewijn (Rega Institute, Leuven). Deoxy[2,8-^{3}H]adenosine 5′-triphosphate, deoxy[5-^{3}H]cytidine 5′-triphosphate, deoxy[8-^{3}H]guanosine 5′-triphosphate and [methyl-^{3}H]thymidine 5′-triphosphate were from Amersham Biosciences.

2.2. Cloning of the HHV-6 U38 gene

The 3.6 kb U38 gene was PCR amplified from HHV-6B (Z29)-infected cell DNA extracts using Expand™ High Fidelity PCR system (Boehringer Mannheim). The forward primer contained a truncated form of the 5′-UTR of alfalfa mosaic virus (AMV) for enhanced translation (Cihlar et al., 1997). The resulting amplicon, containing terminal HindIII and SacI restriction sites, was cloned into the pGemT and then into the pGem3Z vector (both from Promega) under control of the SP6 promoter, prior to transformation into competent DH5α™ cells (Invitrogen). PCR and sequencing were repeated four times and a PCR-induced mutation was reverted by site-directed mutagenesis to establish a consensus sequence for the HHV-6B (Z29) U38. A plasmid containing the HCMV UL54 gene (encoding the HCMV DNA pol catalytic subunit) was kindly provided by Dr. T. Cihlar (Gilead, Foster City, CA).

2.3. Site-directed mutagenesis

The $c^{2882}t$ mutation (yielding the $A^{961}V$ ganciclovir-resistant phenotype) of U38 was introduced by site-directed mutagenesis using the QuikChange™ site-directed mutagenesis kit (Stratagene), following the manufacturer's instructions. The following primers (at 2.5 μM final concentration) were used: 5′-gaa cac aag att cct ata cat gtg gag aag tat ttc gat cag-3′ and 5′-ctg atc gaa ata ctt ctc cac atg tat agg aat ctt gtg ttc-3′ (synthesized by Invitrogen). After 14 reaction cycles using PfuTurbo™ DNA polymerase (Stratagene), parental DNA template was digested with DpnI (Stratagene) for 1 h and the mutant plasmid was transformed into DH5α™ cells (Invitrogen).

2.4. In vitro transcription/translation assay

We used the TNT™ SP6 Quick Coupled transcription/translation system (Promega). Plasmid DNA, isolated

using the S.N.A.P.™. MidiPrep kit (Invitrogen) and concentrated to 500 μg/ml using the Wizard™ DNA clean-up system (Promega), was added to a ready-to-use mix of rabbit reticulocyte lysate (as a eukaryotic source of ribosomes and cellular enzymes involved in translation processes), NTPs, amino acids and SP6 RNA polymerase. $MgCl_2$ (0.25 mM) and 10 mM potassium acetate were added to the reaction for optimal translation efficiency (as described in Section 3). This reaction mixture was incubated at 30 °C for 3 h. When visualization of proteins was required, biotinylated lysine was incorporated during the translation process using Transcend™ tRNA (Promega).

2.5. RT-PCR analysis

RNA extracts, prepared using the RNeasy mini kit (Qiagen), were treated with 1 U/μl RNase-free DNase (Roche) at 37 °C for 30 min, followed by 10 min incubation at 65 °C to inactivate residual DNase activity. First strand cDNA synthesis from 0.8 μg total RNA was carried out using 0.2 μg of random hexamer primers (Invitrogen), 0.25 mM of each dNTP (Invitrogen), 50 U porcine ribonuclease inhibitor (Amersham Biosciences) and 1.2 U RAV-2 reverse transcriptase (RT) (Amersham Biosciences) in a final reaction volume of 20 μl. The absence of contaminating DNA was certified by a control RT-reaction (to which no RT was added), followed by PCR. Thirty-five PCR cycles were performed using one unit of Taq polymerase (HT Biotechnologies), 100 μM dNTPs and 0.5 μM of the following primers: 5′-acggctagatgtgtggtgtt-3′ and 5′-gcggacataaaatcttctcgaac-3′ (from Invitrogen). PCR products were size-separated on a 2% agarose gel and visualized under UV illumination after ethidium bromide staining.

2.6. Western blot analysis

SDS-PAGE was performed according to standard protocols using 7.5% Tris–HCl polyacrylamide gels. Blotting was done on Hybond™ ECL nitrocellulose membrane (Amersham Biosciences), followed by incubation with alkaline phosphatase-linked streptavidin (Dako) and colorimetric detection using WesternBlue™ substrate (Promega).

2.7. HHV-6 DNA polymerase assay

2.7.1. Determination of enzymatic parameters

Fresh TNT™ reaction product (4 μl) was incubated for 20, 40 and 60 min at 37 °C with a reaction mixture (final reaction volume: 50 μl) consisting of 25 mM Tris–HCl (pH 8.0), 100 mM $(NH_4)_2SO_4$, 0.5 mM dithiothreitol, 10 mM $MgCl_2$, 0.2 mg/ml bovine serum albumin, 5% glycerol, 100 ng/μl activated calf thymus DNA (Amersham Biosciences) and 100 μM of each unlabeled dNTP (Invitrogen). In separate reactions, the rate-limiting tritium-labeled dNTP was added at 0.25–2 μM (specific activity: 10–50 Ci/mmol).

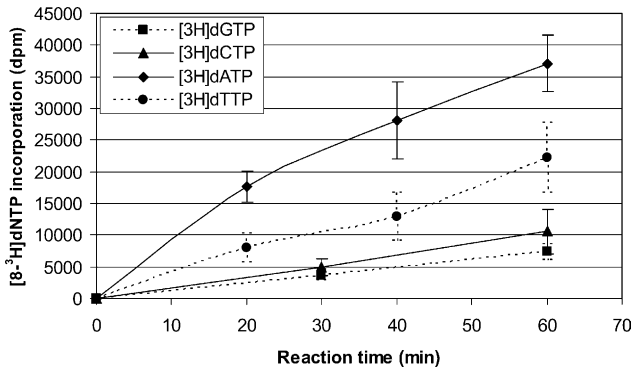

Fig. 1. Time course for incorporation of tritiated dNTPs by HHV-6 DNA polymerase. [8-^{3}H]dNTP was added at 1 μM (specific activity: 10–50 Ci/mmol). Experimental conditions are described in Section 2.

These reaction times fell into the linear range of the incorporation reaction indicating that kinetic parameters were determined under steady-state conditions (Fig. 1). After a 30 min incubation on ice using 5% trichloroacetic acid (TCA) and 20 mM $Na_4P_2O_7$, samples were spotted onto glass microfibre filters (Whatman) and washed using 5% TCA. Filters were then dried overnight and incorporated radioactivity was determined by liquid scintillation counting. One unit was defined as the amount of enzyme that catalyzes the incorporation of 1 pmol [^{3}H]dNTP into acid-insoluble material in 30 min at 37 °C. 4 μl of TNT™ reaction product corresponds to approximately 0.5 units.

2.7.2. Evaluation of compounds

Enzyme assays were performed for 40 min at 37 °C using the reaction mixture described above, containing 0.5 μCi of the radiolabeled dNTP at a concentration approximately twice its K_m value (2 μM) and various concentrations of the test compounds. Amounts of dNTP incorporation were normalized between experiments to an enzyme input of one unit per reaction.

2.8. HHV-6 pU69 kinase assay

The wild-type and a^{952}g mutant U69 genes from HHV-6B (strain HST), as well as a truncated U69 sequence (obtained by insertion of a stopcodon at position 600), were cloned into the pcDNA3.1/Zeo® vector (Invitrogen). Recombinant vaccinia viruses containing these sequences were generated as described earlier (De Bolle et al., 2002). Metabolism studies with ganciclovir and HPLC analysis were performed likewise. Briefly, 143B TK$^-$ cells were infected with the recombinant vaccinia viruses at an MOI of 1 PFU per cell. Two hours post infection (p.i.), 5 μM [8-^{3}H]ganciclovir (specific activity: 20 Ci/mol) was added to the cells. After 24 h incubation, methanolic cell extracts were separated on a Partisphere SAX anion-exchange column (Whatman). The radioactivity

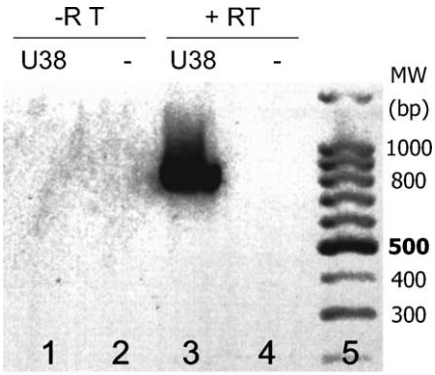

Fig. 2. RT-PCR analysis of HHV-6 U38 mRNA expression in the TNT in vitro transcription system. Lane 3 shows amplification of an 834 bp fragment of U38 mRNA. Lanes 2 and 4 are control reactions to which no U38 plasmid DNA was added. Lanes 1 and 2 are reverse transcriptase control reactions (in which no enzyme was added to eliminate possibility of residual plasmid DNA amplification).

of the fractions containing the mono-, di- and triphosphate metabolites of [8-^{3}H]ganciclovir was determined by liquid scintillation counting.

3. Results

3.1. Verification of HHV-6 DNA pol expression

Expression of HHV-6 DNA pol mRNA expression was verified by RT-PCR analysis of a TNT reaction mixture to which no amino acids were added. Strong and selective amplification of an 834 bp fragment (pos. 1525–2359 of U38) was observed (Fig. 2, lane 3). To ascertain the absence of residual plasmid, PCR amplification was done on the DNase-treated RNA samples and no amplification was detected in the absence of RT (lanes 1 and 2). As no antibody to HHV-6 DNA pol is currently available, we attempted to demonstrate translation products by adding biotinylated tRNA-lysine to the TNT^{TM} reaction mixture. Strong expression of a luciferase control template was observed, both by

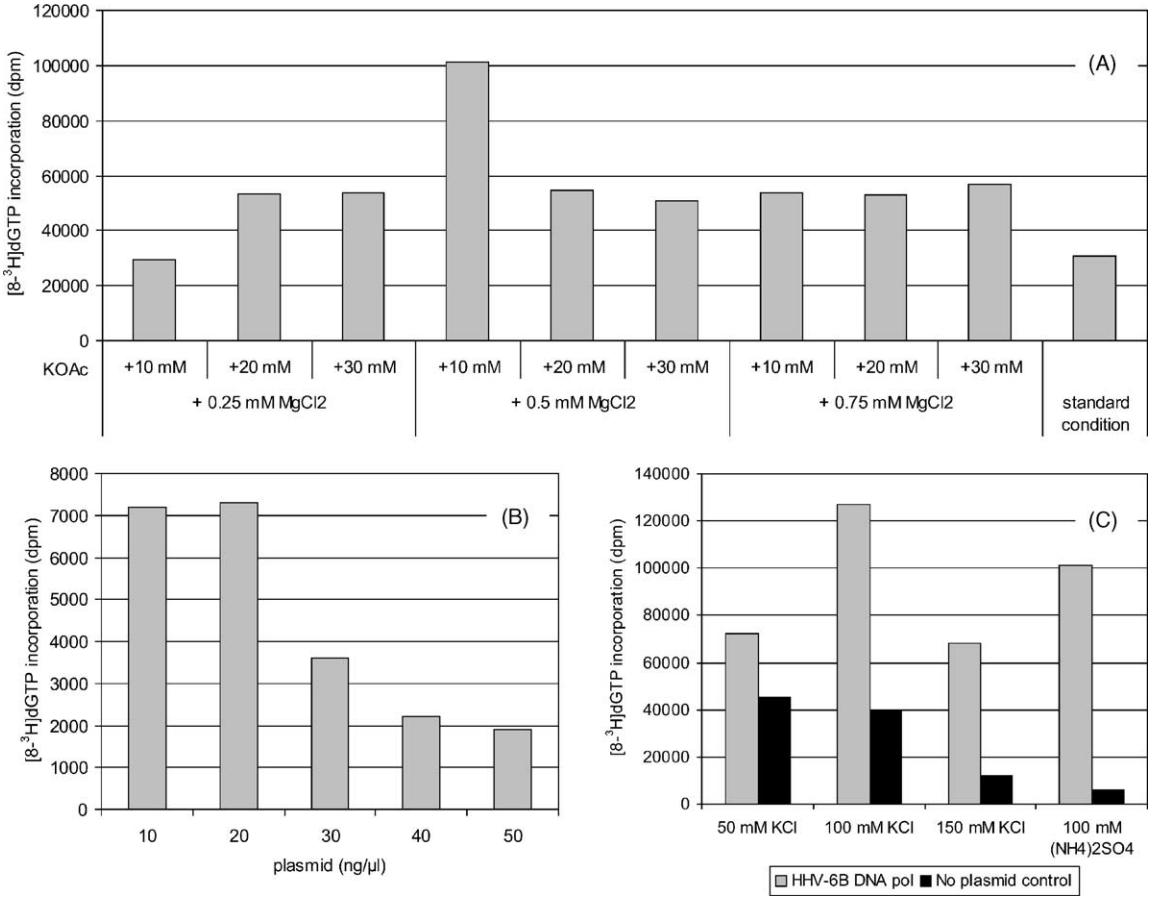

Fig. 3. Optimization of HHV-6 DNA pol expression and activity. Enzyme activity was evaluated by measuring incorporation of [8-^{3}H]dGTP into an activated calf thymus DNA template. Panel A: effect of salt ($MgCl_2$ and KOAc) concentration during in vitro transcription/translation on HHV-6 DNA pol activity levels. Panel B: negative influence of input levels of plasmid template for in vitro transcription on DNA pol activity. Panel C: optimization of salt concentrations during the DNA pol assay. Optimal pU38 activity was attained using 100 mM KOAc. However, residual cellular DNA pol activity (in the no plasmid template control) was more efficiently suppressed using 100 mM $(NH_4)_2SO_4$, resulting in a better signal-to-noise ratio.

detection of the blotted protein after incorporation of biotinylated lysine and by luciferase activity assay. However, the expected 116 kDa band for HHV-6 DNA pol (the slight increase of 112–116 kDa being explained by the incorporation of biotinylated lysine residues) was most probably masked by an non-specific signal from the TNT™ reaction mixture at approximately 120 kDa (data not shown). Although the absence of partial transcription/translation products and the absolute quantity of HHV-6 DNA pol produced could thus not be ascertained, a sufficient level of HHV-6 DNA pol enzyme activity was reached for our further purposes (see below).

3.2. Optimization of HHV-6 DNA pol activity

Given the difficulties in demonstrating HHV-6 DNA pol protein expression, optimization of both in vitro transcription/translation and DNA pol assay conditions was carried out at the level of enzymatic activity. When salt concentrations were varied from 0 to 0.75 mM $MgCl_2$ and from 0 to 30 mM KOAc, we observed that the highest levels of DNA pol expression were obtained by adding 0.5 mM $MgCl_2$ and 10 mM KOAc to the transcription/translation reaction mixture (Fig. 3, panel A). Surprisingly, higher amounts (>20 ng/μl) of plasmid negatively influenced the reaction efficiency (Fig. 3, panel B). We, therefore, used 10 ng plasmid per μl TNT™ reaction mixture throughout our experiments. Finally, reaction conditions for the HHV-6 DNA polymerase assay were optimized by varying the salt concentration between 50 and 150 mM KCl, compared to 100 mM $(NH_4)_2SO_4$ (Fig. 3, panel C). Although the highest activity levels for HHV-6 DNA pol were obtained using 100 mM KCl, a better signal-to-noise ratio was obtained using $(NH_4)_2SO_4$. Apparently, $(NH_4)_2SO_4$ is more effective in suppressing residual cellular DNA pol activity, present in the TNT™ reaction mixture.

3.3. Determination of enzymatic parameters

Enzymatic parameters were derived from Lineweaver–Burk curves, obtained as described in Section 2. K_m values were similar for the four dNTPs, and were to a great extent in the same range as those described for the native enzyme (Bapat et al., 1989) (Table 1). V_{max} values varied between 0.05 and 0.12 pmol/min.

Table 1
Enzymatic parameters for HHV-6 DNA polymerase[a]

Substrate	K_m (μM)	V_{max} (pmol/min)
dATP	1.36 ± 0.67	0.052 ± 0.046
dCTP	1.10 ± 0.49	0.085 ± 0.026
dGTP	1.01 ± 0.36	0.120 ± 0.075
dTTP	1.26 ± 0.16	0.059 ± 0.051

[a] HHV-6B DNA polymerase obtained by eukaryotic in vitro transcription and translation.

Table 2
Activity of selected compounds against HHV-6B and HHV-6B DNA pol

HHV-6B[a]		HHV-6B DNA pol	
Compound	EC_{50} (μM)[b]	Compound	IC_{50} (μM)[c]
PFA	25.4 ± 7.8	PFA	13.2 ± 9.8
GCV	68.6 ± 30	GCV-TP	5.56 ± 2.7
BVDU	NA[d]	BVDU-TP	2.88 ± 2.7

[a] Data obtained in Molt-3 cells as described by De Bolle et al. (2004).
[b] Compound concentration that results in 50% inhibition of virus replication as determined by DNA hybridization, 12 days post infection.
[c] Compound concentration that causes 50% enzyme inhibition.
[d] NA: not active at subtoxic concentrations.

3.4. Evaluation of compounds against wild-type HHV-6 DNA pol

The activity of ganciclovir, foscarnet and BVDU against HHV-6B strain Z29 in cell culture was compared to that of their active metabolites against HHV-6 DNA pol (Table 2). The considerable toxicity of BVDU in the T-lymphoblastoma Molt-3 cell line was such that its antiviral activity could not be determined. For foscarnet, which needs no metabolization prior to targeting the viral DNA pol, 50% inhibitory concentration (IC_{50}) values were similar in the cell culture and enzyme assay. For ganciclovir, the IC_{50} was 68.6 μM in cell culture versus 5.56 μM in the enzyme assay. The IC_{50} values reported here for ganciclovir triphosphate (5.6 μM) and BVDU triphosphate (2.9 μM) correspond well to their published activities against the native enzyme (76% inhibition by 5 μM ganciclovir-TP and 90% inhibition by 6 μM BVDU-TP) (Bapat et al., 1989). The HCMV DNA pol, however, was reported to be much more sensitive to inhibition by both foscarnet and ganciclovir-TP, with IC_{50} values of 0.35 and 0.07 μM, respectively, in agreement with previous reports (Cihlar et al., 1997; Frank et al., 1985).

3.5. Characterization of the ganciclovir-resistant HHV-6 strain

To determine the relative contribution of the two reported mutations to the ganciclovir-resistant HHV-6 phenotype obtained in vitro (Manichanh et al., 2001), we first evaluated the inhibition of wild-type (wt) and $A^{961}V$ HHV-6 DNA pol by ganciclovir triphosphate and, as a control, foscarnet. Both enzymes were equally sensitive to foscarnet and ganciclovir triphosphate (IC_{50} values for ganciclovir-TP: 5.56 and 5.32 μM for wt and $A^{961}V$, respectively) (Fig. 4). Thus, the $A^{961}V$ substitution did not account for the observed ganciclovir resistance at the enzymatic level. We, therefore, investigated the role of the $M^{318}V$ substitution in HHV-6 pU69, using a recombinant vaccinia virus (rVV) assay in which different forms of pU69 from HHV-6B(HST) were expressed (Fig. 5). Although the ganciclovir phosphorylating capacity of wt pU69 was rather limited (4.5 pmol total ganciclovir phosphates per 10^7 cells), phosphorylation

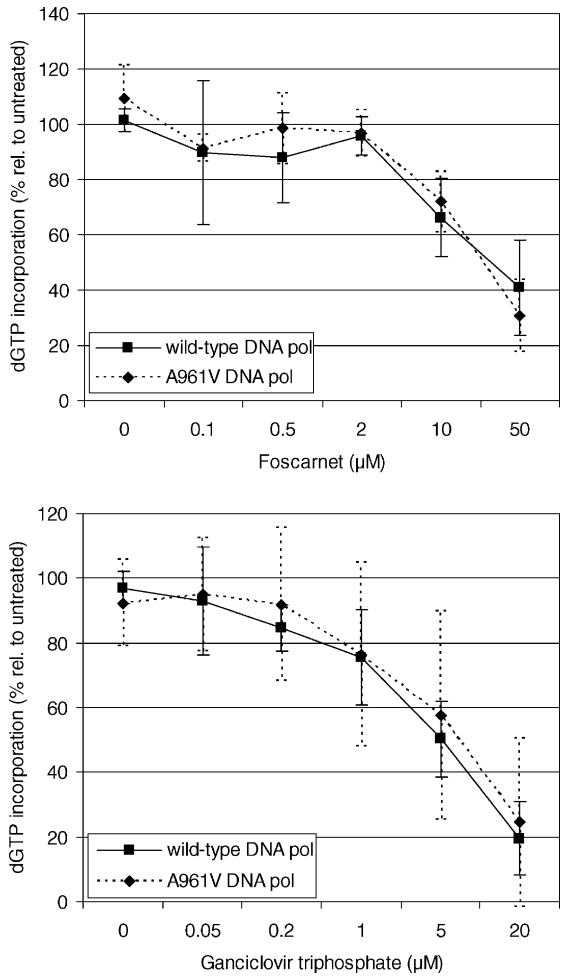

Fig. 4. Inhibitory activity of foscarnet (top panel) and ganciclovir triphosphate (bottom panel) against the wild-type (solid lines) and $A^{961}V$-substituted (dotted lines) HHV-6 DNA polymerase. Data represent the mean ± S.D. for at least three independent experiments.

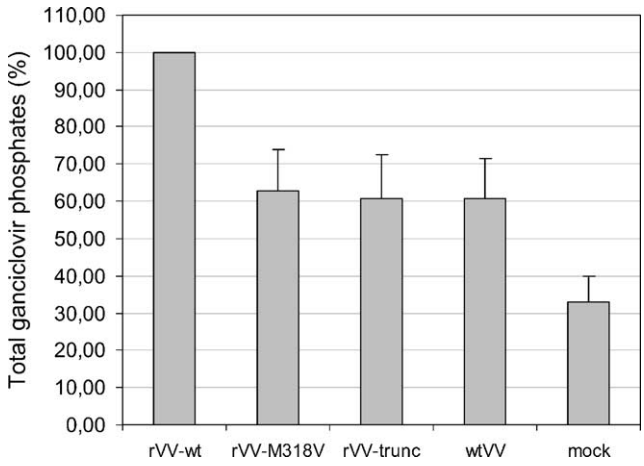

Fig. 5. Phosphorylation of [8-^{3}H]ganciclovir in human 143B cells infected with recombinant vaccinia viruses (rVV), carrying the wild-type, $M^{318}V$ substituted and truncated forms of HHV-6 U69. Wild-type vaccinia virus and mock-infected 143B cells were included as controls. Two hours post infection, cells were pulsed with [8-^{3}H]-labeled ganciclovir (5 μM) for 24 h. Phosphorylated metabolites were measured by anion-exchange HPLC and liquid scintillation counting. Values shown are the mean ± S.D. total ganciclovir phosphates for three independent experiments, and expressed as percentages of the maximal total ganciclovir phosphorylation achieved by wild-type pU69.

levels in cells infected with the rVV encoding the $M^{318}V$ pU69 (2.85 pmol/10^7 cells) were as low as those obtained in cells infected with wild-type VV, rVV encoding a truncated pU69, or mock-infected cells (2–3 pmol/10^7 cells), thus representing a significant decrease in phosphorylating activity. We, therefore, conclude that the $M^{318}V$ substitution in HHV-6 pU69, rather than the $A^{961}V$ substitution in the DNA pol, is responsible for the ganciclovir-resistant phenotype.

4. Discussion

Unlike the DNA polymerases of herpes simplex virus and HCMV, the HHV-6 DNA polymerase has barely been characterized. In the one report available (Bapat et al., 1989), the native HHV-6 DNA polymerase was isolated and chromatographically purified from infected cell cultures, and some of its enzymatic properties were described for the first time. Given the limitations of this traditional isolation method, the eukaryotic in vitro transcription/translation assay described here represents a fast and straightforward approach for the production of the catalytic subunit of the enzyme, encoded by the HHV-6 U38 gene. Our cloning strategy was based on that described for the HCMV DNA polymerase (Cihlar et al., 1997). In these experiments, the UL54 and UL44 genes (encoding the catalytic subunit and accessory protein of the HCMV DNA pol, respectively) were expressed most efficiently when preceded by a truncated alfalfa mosaic virus 5′-UTR. The same 5′-UTR sequence was, therefore, used in our constructs. Optimal conditions for the in vitro production of enzyme may vary between constructs, and were therefore optimized. The observation that the amount of plasmid negatively correlates with the amount of enzyme produced can be explained by the presence of residual salt in the plasmid preparations which negatively influenced translation efficiency. Alternatively, higher amounts of plasmid in the transcription/translation reaction may give rise to truncated mRNAs and thus negatively influence the amount of HHV-6 DNA pol produced, although Cihlar et al. (1997) found an increase in the in vitro production of HCMV DNA pol at plasmid concentrations up to 100 ng/μl.

A characteristic feature of herpesvirus DNA polymerases is that maximum enzymatic activity is achieved in buffers with high salt concentration (Ertl et al., 1991; Huang, 1975; Powell and Purifoy, 1977). For HCMV DNA pol, the optimal salt concentration was shown to be higher in the presence of the accessory subunit pUL44, and thus similar to that for the native enzyme (Cihlar et al., 1997). This is confirmed by our data, as optimal HHV-6 pU38 activity was reached at lower potassium chloride concentrations than observed with

native enzyme (100 mM versus 200 mM KCl) (Bapat et al., 1989). However, background activity of cellular DNA pol α (present in the TNT mixture) was more significantly reduced when using ammonium sulphate, as described earlier (Marcy et al., 1990; Nishiyama et al., 1983). K_m values were found to be very similar to those reported for the native HHV-6 DNA pol (Bapat et al., 1989); they seemed to be somewhat higher than those of the other herpesvirus DNA polymerases (Cihlar et al., 1997; Frank et al., 1985), yet lower than those of cellular DNA pol α (Frank et al., 1985).

Evaluation of inhibitors revealed no major differences between purified and expressed HHV-6 DNA pol. In contrast, the HCMV DNA pol was inhibited by 10- and 100-fold lower concentrations of ganciclovir triphosphate and BVDU triphosphate, respectively (Cihlar et al., 1997; Frank et al., 1985). This would imply that apart from a 10- to 20-fold less efficient ganciclovir phosphorylation by the HHV-6 pU69 phosphotransferase, which is reflected in proportionally lower antiviral activities [EC_{50}: 69 μM against HHV-6 versus 7 μM against HCMV (Smee et al., 1983)], the different sensitivity to ganciclovir of these two viruses is also determined at the level of the viral DNA pol. We also compared the IC_{50} values of foscarnet and ganciclovir triphosphate against the HHV-6 DNA pol with their anti-HHV-6 activities in cell culture (expressed as EC_{50} values). For foscarnet, these were in the same range, whereas for ganciclovir-TP, inhibition at the enzymatic level was about 10-fold more efficient.

As no controlled trials have been conducted on anti-HHV-6 therapy so far, management of HHV-6 infections is mainly based on the experience with HCMV. First-choice therapy for HCMV infections is ganciclovir, often administered prophylactically in immunosuppressed patients. As resistance to ganciclovir develops (especially when used in prophylaxis), foscarnet or cidofovir may be used as an alternative, although both have important side-effects which limit their application. Whereas resistance of HCMV to foscarnet and cidofovir is due exclusively to mutations in the viral DNA pol gene, ganciclovir resistance emerges at two levels. First, and most importantly, it is due to mutations in the HCMV pUL97 phosphotransferase gene (Biron et al., 1986; Stanat et al., 1991), which impairs initial phosphorylation of ganciclovir. More specifically, the $H^{520}Q$ and $M^{460}V$ substitutions (the latter is homologous to the $M^{318}V$ substitution in HHV-6 pU69) were responsible for the lowest levels of residual ganciclovir phosphorylation (Baldanti et al., 2002). Second, additional mutations in the HCMV DNA polymerase may appear after prolonged exposure to ganciclovir, which often give rise to cidofovir cross-resistance (Baldanti et al., 2004; Cihlar et al., 1998a; Smith et al., 1997). Foscarnet resistance-conferring substitutions are largely confined to distinct regions of the HCMV DNA pol than those resulting in ganciclovir and/or cidofovir resistance (Chou et al., 2003), although single mutations conferring multi-drug resistance to ganciclovir, cidofovir and foscarnet have been reported (Chou et al., 2000).

In the ganciclovir-resistant HHV-6 strain (Manichanh et al., 2001), two mutations were observed by genotypic analysis. An $M^{318}V$ amino acid substitution in the pU69 phosphotransferase and an $A^{961}V$ substitution in the viral DNA polymerase were found to appear simultaneously after 9 months of serial cell culture passaging. Only the former mutation was also seen in an HHV-6 isolate from an HCMV-infected patient under prolonged ganciclovir therapy. The $A^{961}V$ DNA pol substitution is situated at the C-terminus of the enzyme, between domain V and the terminal amino acid residues. For HCMV, the last 22 residues of this region were shown to be responsible for the association with the processivity factor encoded by the HCMV UL44 gene (Loregian et al., 2004). This part of the enzyme is clearly distant from the conserved functional domains III, IV, V and δC/ExoIII, which are known to harbor ganciclovir and/or cidofovir resistance-conferring mutations (Chou et al., 2003). Moreover, the termini of HCMV DNA pol have been described to contain multiple sites of interstrain variation (Chou et al., 1999). On the other hand, a mutation in HCMV DNA pol has been described to yield a ganciclovir- and cidofovir-resistant phenotype without altering DNA pol sensitivity to ganciclovir-TP and cidofovir-DP at the enzymatic level (Cihlar et al., 1998b). Thus, assuming that the resistance data for HCMV DNA pol can be extrapolated to HHV-6 DNA pol, the lack of ganciclovir resistance of the $A^{961}V$-substituted HHV-6 DNA pol in our enzyme assay can be interpreted either as a result of interstrain variation or as a lack of correlation between virus and enzyme drug susceptibility. Amino acid substitutions in the region between conserved domain V and the accessory protein binding region of the HSV-1 DNA polymerase have been generated under pressure of HPMPA (9-(S)-[3-hydroxy-2-(phosphonomethoxy)propyl]adenine, the adenine analogue of cidofovir) and have been associated to cidofovir cross-resistance (Andrei et al., 2000). Unfortunately, due to a lack of availability of cidofovir diphosphate, we were unable to investigate the role of the $A^{961}V$ substitution in the cross-resistance of the HHV-6 mutant virus to cidofovir.

Our experiments with the $M^{318}V$-substituted pU69 phosphotransferase, expressed in a recombinant vaccinia virus system, demonstrate that this substitution reduces ganciclovir phosphorylation to background levels. This is also supported by recently published data (Safronetz et al., 2003). We, therefore, conclude that whereas the role of the $A^{961}V$ substitution in HHV-6 DNA pol in the resistance to cidofovir and/or ganciclovir remains undefined, the pU69 $M^{318}V$ substitution is (at least in part) responsible for the ganciclovir-resistant phenotype.

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