Available online at www.sciencedirect.com
ScienceDirect
Antiviral Research 77 (2008) 237–240
Antiviral Research
www.elsevier.com/locate/antiviral

Short communication

# Characterization of a cidofovir-resistant HHV-6 mutant obtained by in vitro selection

Pascale Bonnafous [a,*], David Boutolleau [a], Lieve Naesens [b], Claire Deback [a], Agnès Gautheret-Dejean [a,c], Henri Agut [a]

[a] *Université Pierre et Marie Curie-Paris 6, EA2387, Groupe Hospitalier Pitié-Salpêtrière, 83 boulevard de l'Hôpital, F-75013 Paris, France*
[b] *Laboratory of Virology and Chemotherapy, Rega Institute for Medical Research, Katholieke Universiteit Leuven, B-3000 Leuven, Belgium*
[c] *Laboratoire de Microbiologie, Faculté des Sciences Pharmaceutiques et Biologiques, 75006 Paris, France*

Received 1 October 2007; accepted 10 December 2007

**Abstract**

Cidofovir (CDV) was used for in vitro selection of a human herpesvirus 6 (HHV-6) mutant with decreased susceptibility to this drug. The resulting mutant was highly resistant to CDV as compared to its sensitive counterpart (inhibitory concentration 50% ($IC_{50}$): 213 μM versus 1.8 μM). Its replication fitness was not impaired. Genotypic characterization of the resistant virus revealed a mutation in the U38 gene encoding the viral DNA polymerase. The resulting R798I amino acid change was located in the conserved domain VII close to the highly conserved motif KKRY interacting with the DNA primer–template duplex, and is likely responsible for the high-level resistance to CDV, even though a definite virological and/or biochemical confirmation is required. The possible emergence of such changes in HHV-6 DNA polymerase in patients receiving CDV therapy should be taken into account in the treatment of HHV-6 infections.


*Keywords:* HHV-6; Cidofovir; Resistance; DNA polymerase

Human herpesvirus 6, a β-herpesvirus closely related to human cytomegalovirus (HCMV), has been increasingly recognized as an important pathogen in immunocompromised patients (Zerr, 2006). Two variants of HHV-6, A and B, have been identified (Ablashi et al., 1993) and most symptomatic infections such as exanthema subitum for primary infection or reactivations in transplant recipients are caused by variant B. The drugs clinically used against HHV-6 are the same as those used in HCMV therapy and consist of ganciclovir (GCV), foscarnet (PFA) or cidofovir. These three drugs have proven to be efficient against both HCMV and HHV-6 infections (Janoly-Duménil et al., 2006; Okamoto et al., 1997; Pöhlmann et al., 2007; Rieux et al., 1998). Long-term antiviral treatment can lead to the emergence of drug-resistant HCMV and HHV-6 mutants, both reactivations emerging often concomitantly (Härmä et al., 2006; Razonable et al., 2003). HHV-6B strains with resistance to GCV (Manichanh et al., 2001) or PFA (Bonnafous et al., 2007) have been previously characterized. Several resistant strains were isolated in vitro which exhibited different amino acid changes either in the HHV-6 DNA polymerase or phosphotransferase, encoded by the U38 and U69 genes, respectively. As an example, the M318V change found in the U69 gene product has proven to be responsible for resistance to GCV (Safronetz et al., 2003). CDV is a nucleotide analog which acts, after two phosphorylations performed by cellular kinases, as a competitive inhibitor with regard to dCTP and an alternate substrate for the viral DNA polymerase. Resistance of HCMV to CDV is well known: it is associated with changes located in the conserved domains IV, δC, III and V of the HCMV DNA polymerase (UL54 gene product), and frequently results in cross-resistance to GCV (Gilbert et al., 2002; Scott et al., 2007). In the case of HSV-1, CDV is routinely less used than acyclovir (ACV) and the few changes leading to CDV resistance were also described in the viral DNA polymerase, i.e. in domains II, δC, VI, V and within the region adjacent to the C-terminal

* Corresponding author at: Laboratoire de Virologie, CERVI, Groupe Hospitalier Pitié-Salpêtrière, 83 bd de l'Hôpital, 75651 Paris Cedex 13, France. Tel.: +33 1 42 17 74 18; fax: +33 1 42 17 74 17.
*E-mail address:* pascale.bonnafous@psl.aphp.fr (P. Bonnafous).

0166-3542/$ – see front matter © 2007 Elsevier B.V. All rights reserved.
doi:10.1016/j.antiviral.2007.12.004

part of the conserved domain V (Andrei et al., 2007; Gilbert et al., 2002). Little is known about HHV-6 resistance to CDV. In the study of Manichanh et al. (2001), a GCV-resistant strain, selected in vitro, was found to be cross-resistant to CDV and to contain a single A961V change in the DNA polymerase (Manichanh et al., 2001). This mutation is located in the C-terminal part of the protein, distant from the conserved domains, and its precise role in CDV-resistance has remained unclear. In order to obtain a better insight in resistance of HHV-6 to CDV, we now selected a CDV-resistant mutant strain in vitro by gradually increasing concentrations of CDV. The mutant strain was subjected to phenotypical and genotypical analysis to investigate the influence of amino acid modifications on the HHV-6 susceptibility pattern to CDV, PFA and GCV.

## 1. Selection of a CDV-resistant HHV-6 isolate

The HST strain (HHV-6 variant B), previously adapted to grow in MT4 cells (Manichanh et al., 2000) was used in this study. Ten million MT4 cells were infected with this strain at a multiplicity of infection (m.o.i.) of 0.004 $CCID_{50}$ (cell culture infectious dose 50%) and incubated in the presence of CDV (Vistide™, Pharmacia & Upjohn, St Quentin-en-Yvelines, France) at the initial concentration of 4 μM, equivalent to twice the $IC_{50}$ (Mace et al., 2003). Progress of infection was monitored by immunofluorescence assay (IFA), as previously described (Bonnafous et al., 2007). At weekly intervals, the cell culture was expanded by the addition of uninfected cells and the concentration of CDV was doubled when more than 50% of cells were infected as detected by IFA, until reaching 256 μM (64-fold the $IC_{50}$). Efficient virus propagation was ultimately obtained after 6 months of culture. The emerging mutant isolate, designated as CDVR1, was purified by end-point dilution in the presence of 256 μM of CDV, and a virus stock was prepared under the same concentration of CDV. The infectious titer was determined as $10^3$ $CCID_{50}$ per mL.

## 2. Phenotypic characterization

The CDVR1 strain was characterized for its drug susceptibility pattern and replication fitness. For both studies, virus replication was estimated by determining the intracellular HHV-6 DNA load using a published real-time PCR method (Bonnafous et al., 2005; Mace et al., 2003).

For drug susceptibility assays, MT4 cells infected either with wild-type HST or CDVR1 were incubated for 3 days in the presence of different concentrations of CDV (range: 0.12–256 μM), GCV (range: 1–32 μM) or PFA (range: 2–32 μM) in duplicate wells for each concentration. After cell lysis, HHV-6 load was measured and the values for $IC_{50}$ and RI (resistance index, defined as the ratio of the $IC_{50}$ for CDVR1 to that of the wild-type HST strain) were calculated for each drug. As shown in Table 1, the results obtained from three independent experiments showed that the CDVR1 strain was highly resistant to CDV, exhibiting a RI of 118. Cross-resistance to GCV was also observed, albeit at a moderate level with a RI of 4. CDVR1 was as sensitive to PFA as the wild-type HST strain.

The fitness of HST and CDVR1 strains was compared in infection assays using the same m.o.i. (0.004 $CCID_{50}$ per cell) for both viruses. Virus growth was determined daily for 8 days in the absence of any antiviral drug (Fig. 1A). A 10-fold difference in the numbers of DNA copies on day 0 was observed and likely corresponded to a variable number of non-infectious DNA-containing viral particles in the two virus stocks, possibly due to differences in preparation and storage. Despite this initial difference, the growth curves exhibited the same pattern: an initial decrease corresponding to the degradation of non-infectious DNA on the first day, followed by an exponential growth phase until reaching a plateau on day 5 with approximately $10^4$ HHV-6 DNA copies per cell. The rate of viral growth determined between days 1 and 5 was found to be similar for HST and CDVR1 strains, with values of 0.55 and 0.63 log per day, respectively.

## 3. Genetic analysis and functional characterization of DNA polymerase

The entire genes U38 and U69 of HST and CDVR1 strains were amplified and sequenced using the specific primers and conditions as previously described (Bonnafous et al., 2007). No mutation was observed in the U69 gene when comparing CDVR1 with HST. The only change thus far known to be responsible for HHV-6 resistance to GCV, the M318V change in the U69 gene product (Manichanh et al., 2001; Safronetz et al., 2003), was not found in the CDVR1 strain, despite its decreased

Table 1
Phenotypic and genotypic characterization of HST and CDVR1 strains

| Viral strain | $IC_{50} \pm$ S.D.[a] (μM) [RI[b] of drug] | | | pU38 change |
|---|---|---|---|---|
| | Cidofovir | Ganciclovir | Foscarnet | |
| HST | 1.8 ± 0.9 | 3.1 ± 0.8 | 15.0 ± 7.0 | None (WT) |
| CDVR1 | 213.2 ± 77.3[c] [118.4] | 12.9 ± 4.3[c] [4.2] | 17.8 ± 6.9 [1.2] | R798I |

[a] The inhibitory concentration 50% ($IC_{50}$) was derived from inhibition curves as the concentration of antiviral drug that reduced the marker of virus replication (DNA copy number per cell) by 50% compared with that observed in the absence of the drug. Assays were performed in duplicate and data represent the mean of three independent experiments.

[b] The resistance index (RI) was computed as the ratio of the $IC_{50}$ for CDVR1 to that of HST.

[c] $IC_{50}$ value of CDVR1 was considered significantly different as compared to that of HST if RI ≥ 3. WT, wild-type.

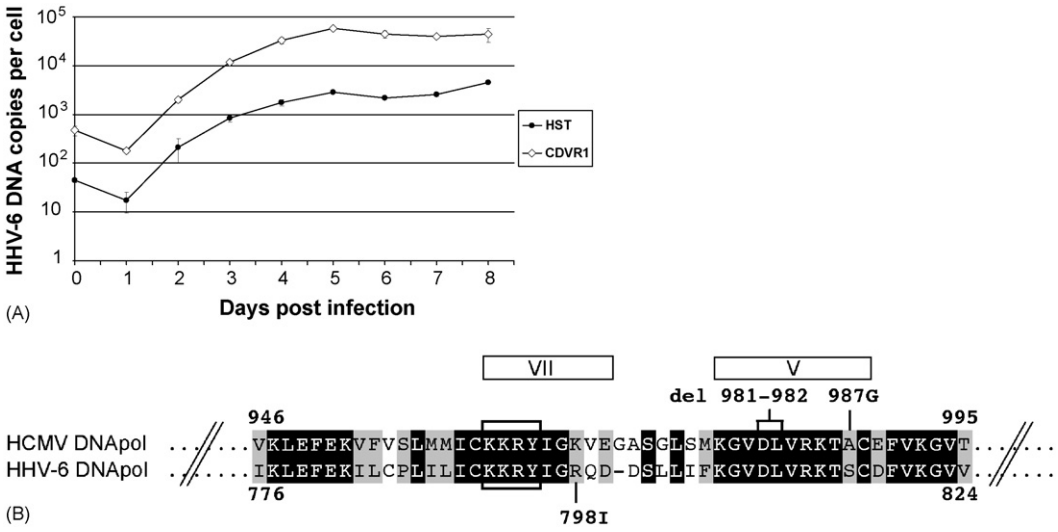


Fig. 1. (A) Replication kinetics of HST and CDVR1 mutant strain. MT4 cells were infected with the HHV-6 strains HST or CDVR1 at the same multiplicity of infection (m.o.i.). The viral growth in the absence of antiviral compound was followed by measuring the viral load, expressed as the number of HHV-6 DNA copies per cell, using real-time PCR detection every day for 8 days. (B) Sequence alignment of the DNA polymerases of HCMV and HHV-6 (partial view). The pUL54 protein of HCMV (strain AD169, GenBank accession number NC_001347) and pU38 protein of HHV-6 (strain HST, GenBank accession number AB021506) were compared by Clustal W (1.83) multiple sequence alignment. Dark gray and clear gray shading indicate identical amino acids and conserved or semi-conserved substitutions, respectively, between the two proteins. The conserved domains VII and V for both viruses are indicated in the top boxes. Amino acid changes identified in CDV-resistant HCMV and HHV-6 strains are indicated in bold font above and under the alignment, respectively. The KKRY sequence motif conserved among the α DNA polymerases is framed and corresponds to the residues 962–965 in HCMV polymerase and 792–795 in HHV-6 polymerase.

sensitivity to GCV. Within the DNA polymerase encoded by the U38 gene, CDVR1 exhibited a single R798I change, which could explain the sensitivity pattern to CDV and GCV (Table 1). For both genes, the electrophoretic sequencing pattern was not ambiguous and no other minority mutation could be detected by this way. This ruled out the hypothesis of minority mutant strains that could contribute to the susceptibility pattern of CDVR1. This is in agreement with the fact that the strain CDVR1 was purified by end-point dilution at the end of the selection process. A longitudinal analysis showed that the nucleotide mutation g2393t, corresponding to the R798I change, became detectable throughout the selection process of CDVR1 when the concentration of CDV reached 64 μM. This R798I change induced a modification of residue function since a polar positively charged amino acid was replaced by a hydrophobic aliphatic amino acid.

A functional test of HHV-6 DNA polymerase has been previously developed to study the impact of different mutations on the susceptibility of the enzyme to antiviral drugs (Bonnafous et al., 2007; De Bolle et al., 2004). In the present study, only PFA could be tested because the GCV triphosphate and the CDV diphosphate, which are the substrates for DNA polymerase, were not available at the time of this study. The concentration of PFA inhibiting the enzymatic activity by 50% was determined and was found to be similar for the CDVR1 and wild-type DNA polymerase (5.1–5.7 μM for HST versus 5.7–7.2 μM for CDVR1), confirming that the R798I change does not alter the susceptibility to PFA.

The sequences of the HHV-6 and HCMV DNA polymerases were aligned and compared (Fig. 1B). The R798I change was found to be located in the conserved domain VII, a region exhibiting a high homology between both enzymes. No equivalent mutation has previously been reported in the case of CDV-resistant and/or GCV-resistant HCMV isolates. However, it is worth to note that changes conferring HCMV resistance to CDV and GCV have been described in the nearby and conserved domain V, namely deletion of amino acids D981-L982 and an A987G change (Chou et al., 2000; Cihlar et al., 1998; Jabs et al., 2001). Of note, as far as HSV-1 is concerned, a mutant strain selected under the pressure of CDV has recently been shown to exhibit a new single change, K960R, within the same conserved domain V (Andrei et al., 2007).

Interestingly, the R798I mutation was adjacent to the motif ICKKRYIG present within the polymerases of HHV-6 and HCMV at homologous positions. The KKRY sequence motif of domain VII is highly conserved among α DNA polymerases, corresponding to residues 705–708 in bacteriophage RB69, 962–965 in HCMV and 792–795 in HHV-6 DNA polymerases, respectively. This sequence interacts with the primer–template duplex next to the polymerisation active site (Braithwaite and Ito, 1993). In RB69 DNA polymerase, Y708 makes a hydrogen bond with the phosphodiester at the primer 3′ terminus, while K705 and R707 interact with template strand phosphates, thereby drawing the primer and template strand backbones together in a B-form conformation of DNA (Franklin et al., 2001). The R798I change in CDVR1 is very close to this motif and the polymerisation active site, and could generate a structural modification, reducing the affinity of the DNA polymerase for CDV and, to a lesser extent, GCV.

This study concerns the first characterization of a strain of HHV-6 highly resistant to CDV. The single change R798I observed in the DNA polymerase is assumed to be responsible for the cross-resistance between CDV and GCV in the absence of

any other change in the U38 and U69 products, but this requires to be definitely proven by means of marker rescue experiments. CDV has been reported to be effective in the treatment of HHV-6 infections (Denes et al., 2004; Pöhlmann et al., 2007). Similar to what was observed after long-term therapy with GCV (Manichanh et al., 2001), treatment with CDV in the context of HCMV or HHV-6 infection could lead to the emergence of an HHV-6 clinical strain resistant to CDV. Indeed, the maximum serum concentration after infusion of 5 mg of CDV/kg (with concomitant administration of probenecid) is 26.1 mg/L corresponding to 93.5 μM (Kendle and Fan-Havard, 1998), which is in the same range as the CDV concentration that we used during the in vitro selection process. Accordingly, clinical failure of CDV characterized by a constant and high HHV-6 viral load in a patient's sample should prompt the search for any potential changes in the HHV-6 DNA polymerase that might explain resistance to CDV.

## References


Ablashi, D., Agut, H., Berneman, Z., Campadelli-Fiume, G., Carrigan, D., Ceccerini-Nelli, L., Chandran, B., Chou, S., Collandre, H., Cone, R., Dambaugh, T., Dewhurst, S., DiLuca, D., Foa-Tomasi, L., Fleckenstein, B., Frenkel, N., Gallo, R., Gompels, U., Hall, C., Jones, M., Lawrence, G., Martin, M., Montagnier, L., Neipel, F., Nicholas, J., Pellett, P., Razzaque, A., Torrelli, G., Thomson, B., Salahuddin, S., Wyatt, L., Yamanishi, K., 1993. Human herpesvirus-6 strain groups: a nomenclature. Arch. Virol. 129, 363–366.

Andrei, G., Fiten, P., Froeyen, M., De Clercq, E., Opdenakker, G., Snoeck, R., 2007. DNA polymerase mutations in drug-resistant herpes simplex virus mutants determine in vivo neurovirulence and drug-enzyme interactions. Antiviral Ther. 12, 719–732.

Bonnafous, P., Gautheret-Dejean, A., Boutolleau, D., Caiola, D., Agut, H., 2005. Persistence of DNA in cell cultures may jeopardize the analysis of human herpesvirus 6 dynamics by means of real-time PCR. J. Virol. Methods 125, 95–98.

Bonnafous, P., Naesens, L., Petrella, S., Gautheret-Dejean, A., Boutolleau, D., Sougakoff, W., Agut, H., 2007. Different mutations in the HHV-6 DNA polymerase gene accounting for resistance to foscarnet. Antiviral Ther. 12, 877–888.

Braithwaite, D.K., Ito, J., 1993. Compilation, alignment, and phylogenetic relationships of DNA polymerases. Nucleic Acids Res. 21, 787–802.

Chou, S., Miner, R.C., Drew, W.L., 2000. A deletion mutation in region V of the cytomegalovirus DNA polymerase sequence confers multidrug resistance. J. Infect. Dis. 182, 1765–1768.

Cihlar, T., Fuller, M.D., Cherrington, J.M., 1998. Characterization of drug resistance-associated mutations in the human cytomegalovirus DNA polymerase gene by using recombinant mutant viruses generated from overlapping DNA fragments. J. Virol. 72, 5927–5936.

De Bolle, L., Manichanh, C., Agut, H., De Clercq, E., Naesens, L., 2004. Human herpesvirus 6 DNA polymerase: enzymatic parameters, sensitivity to ganciclovir and determination of the role of the A961V mutation in HHV-6 ganciclovir resistance. Antiviral Res. 64, 17–25.

Denes, E., Magy, L., Pradeau, K., Alain, S., Weinbreck, P., Ranger-Rogez, S., 2004. Successful treatment of human herpesvirus 6 encephalomyelitis in immunocompetent patient. Emerg. Infect. Dis. 10, 729–731.

Franklin, M.C., Wang, J., Steitz, T.A., 2001. Structure of the replicating complex of a pol alpha family DNA polymerase. Cell 105, 657–667.

Gilbert, C., Bestman-Smith, J., Boivin, G., 2002. Resistance of herpesviruses to antiviral drugs: clinical impacts and molecular mechanisms. Drug Resist. Updates 5, 88–114.

Härmä, M., Höckerstedt, K., Lyytikäinen, O., Lautenschlager, I., 2006. HHV-6 and HHV-7 antigenemia related to CMV infection after liver transplantation. J. Med. Virol. 78, 800–805.

Jabs, D.A., Martin, B.K., Forman, M.S., Dunn, J.P., Davis, J.L., Weinberg, D.V., Biron, K.K., Baldanti, F., Hu, H., 2001. Longitudinal observations on mutations conferring ganciclovir resistance in patients with acquired immunodeficiency syndrome and cytomegalovirus retinitis: The Cytomegalovirus and Viral Resistance Study Group Report Number 8. Am. J. Ophthalmol. 132, 700–710.

Janoly-Duménil, A., Galambrun, C., Basset, T., Mialou, V., Bertrand, Y., Bleyzac, N., 2006. Human herpes virus-6 encephalitis in a paediatric bone marrow recipient: successful treatment with pharmacokinetic monitoring and high doses of ganciclovir. Bone Marrow Transplant. 38, 769–770.

Kendle, J.B., Fan-Havard, P., 1998. Cidofovir in the treatment of cytomegaloviral disease. Ann. Pharmacother. 32, 1181–1192.

Mace, M., Manichanh, C., Bonnafous, P., Précigout, S., Boutolleau, D., Gautheret-Dejean, A., Agut, H., 2003. Real-time PCR as a versatile tool for investigating the susceptibility of human herpesvirus 6 to antiviral agents. Antimicrob. Agents Chemother. 47, 3021–3024.

Manichanh, C., Grenot, P., Gautheret-Dejean, A., Debré, P., Huraux, J.M., Agut, H., 2000. Susceptibility of human herpesvirus 6 to antiviral compounds by flow cytometry analysis. Cytometry 40, 135–140.

Manichanh, C., Olivier-Aubron, C., Lagarde, J.P., Aubin, J.T., Bossi, P., Gautheret-Dejean, A., Huraux, J.M., Agut, H., 2001. Selection of the same mutation in the U69 protein kinase gene of human herpesvirus-6 after prolonged exposure to ganciclovir in vitro and in vivo. J. Gen. Virol. 82, 2767–2776.

Okamoto, T., Okada, M., Mori, A., Saheki, K., Takatsuka, H., Wada, H., Tamura, A., Fujimori, Y., Takemoto, Y., Kanamaru, A., Kakishita, E., 1997. Successful treatment of severe cytomegalovirus retinitis with foscarnet and intraocular injection of ganciclovir in a myelosuppressed unrelated bone marrow transplant patient. Bone Marrow Transplant. 20, 801–803.

Pöhlmann, C., Schetelig, J., Reuner, U., Bornhäuser, M., Illmer, T., Kiani, A., Ehninger, G., Jacobs, E., Rohayem, J., 2007. Cidofovir and foscarnet for treatment of human herpesvirus 6 encephalitis in a neutropenic stem cell transplant recipient. Clin. Infect. Dis. 44, e118–e120.

Razonable, R.R., Rivero, A., Brown, R.A., Hart, G.D., Espy, M.J., van Cruijsen, H., Wilson, J., Groettum, C., Kremers, W., Smith, T.F., Paya, C.V., 2003. Detection of simultaneous beta-herpesvirus infections in clinical syndromes due to defined cytomegalovirus infection. Clin. Transplant. 17, 114–120.

Rieux, C., Gautheret-Dejean, A., Challine-Lehmann, D., Kirch, C., Agut, H., Vernant, J.P., 1998. Human herpesvirus-6 meningoencephalitis in a recipient of an unrelated allogeneic bone marrow transplantation. Transplantation 65, 1408–1411.

Safronetz, D., Petric, M., Tellier, R., Parvez, B., Tipples, G.A., 2003. Mapping ganciclovir resistance in the human herpesvirus-6 U69 protein kinase. J. Med. Virol. 71, 434–439.

Scott, G.M., Weinberg, A., Rawlinson, W.D., Chou, S., 2007. Multidrug resistance conferred by novel DNA polymerase mutations in human cytomegalovirus isolates. Antimicrob. Agents Chemother. 51, 89–94.

Zerr, D.M., 2006. Human herpesvirus 6: a clinical update. Herpes 13, 20–24.