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Short Communication

Effects of mutations on herpes simplex virus 1 thymidine kinase functionality: An *in vitro* assay based on detection of monophosphate forms of acyclovir and thymidine using HPLC/DAD

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ABSTRACT

Discrimination between the mutations responsible for drug resistance and those of *UL23* TK gene polymorphism can be difficult. A non-isotopic method has been developed to assess TK functionality by measuring monophosphate forms of both acyclovir (ACV) and thymidine using HPLC/DAD. Phenotypes of TKs could thus be characterized as TK altered (P84L, A189V, L227F), TK deficient (G200S, L291P) or TK partial (R163H). A reliable link between HSV *UL23* TK mutations and ACV resistance is necessary for developing a powerful genotyping tool to detect ACV resistance quickly in clinical samples.

In immunocompromised patients such as transplant recipients or HIV patients, herpes simplex virus (HSV) types 1 and 2 can be responsible for disseminated infections with potentially very serious outcomes (Strick et al., 2006; Styczynski et al., 2009). Since the 1980s, acyclovir (ACV) has been the first line treatment against HSV. In order to be active, ACV requires phosphorylation by the HSV *UL23* gene encoding thymidine kinase (TK). Resistance to ACV is therefore associated with mutations in *UL23* TK gene in 95% of cases, while 5% are caused by mutations in the *UL30* DNA polymerase gene (Gaudreau et al., 1998; Hill et al., 1991). ACV resistance is currently detected using phenotypic techniques that require virus isolation, but this is time-consuming. The genotypic approach, based on *UL23* TK gene sequencing directly on clinical samples, is an interesting alternative for rapidly detecting resistant HSV mutants by overpassing the viral culture bottleneck (Frobert et al., 2008). In case of resistance, this strategy makes it possible to switch to alternative treatments earlier. Nevertheless, this molecular approach can only be efficient if *UL23* mutations have previously been well-characterized. Except for a few reviews (Piret and Boivin, 2011), no consensual database linking HSV drug resistance mutations to the resulting phenotypes is currently available. Moreover, the considerable variety in *UL23* TK polymorphism and the presence of several mutations in resistant strains can make it difficult to clearly identify whether or not these mutations are directly correlated to ACV resistance. As TK activity affects ACV sensitivity, the aim of this study was to link *UL23* mutations to TK activity using a non-isotopic method with ACV and thymidine (dT) as substrates.

Briefly, a synthetic gene encoding the TK amino acid sequence from HSV1 strain KOS (P17402 UniProtKB/SwissProt) was used as a template to perform site-directed mutagenesis as previously described (primers are available on request) (Frobert et al., 2005). Wild-type and mutant *UL23* TK genes fused in frame with a Strep-tag™ (WSHPQFEK) were over-expressed using the Staby™Codon T7 expression kit (Eurogentec). *Escherichia coli* B SE1 strain (Eurogentec) were grown in ZYP-5052 auto-induction medium containing 10 g/L *N-Z*-Amine AS (Sigma), 5 g/L yeast extract, 1 mM $MgSO_4$, 0.5% glycerol, 0.05% glucose, 0.2% lactose, 25 mM $(NH_4)_2SO_4$, 50 mM KH_2PO_4 and 50 mM Na_2HPO_4 at 37 °C for 7 h before growing to saturation at 20 °C overnight (Studier, 2005). Cells were subsequently harvested and lysed as previously described (Frobert et al., 2005). Mini and midi preparations of recombinant Strep-tag TK proteins were produced by affinity

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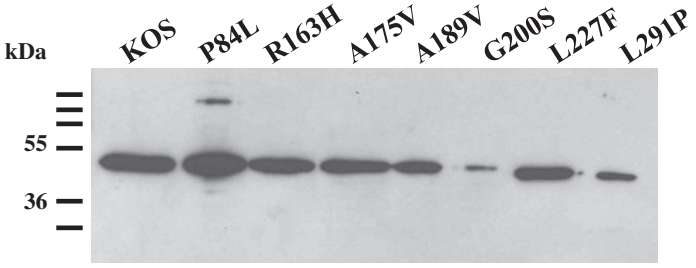
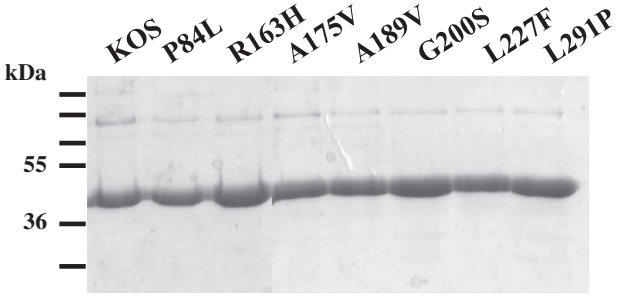
A. Western blot analysis



Western blot of recombinant proteins produced from 3 ml of bacterial culture, purified using Streptatin Magnetic beads (Qiagen), separated on SDS-12% polyacrylamide, transferred on a nitrocellulose membrane and detected using a 1:1000 dilution of a monoclonal mouse antibody directed against StrepTag proteins (Strep-tag R antibody, Qiagen)

B. Purity of Strep-tag TK proteins



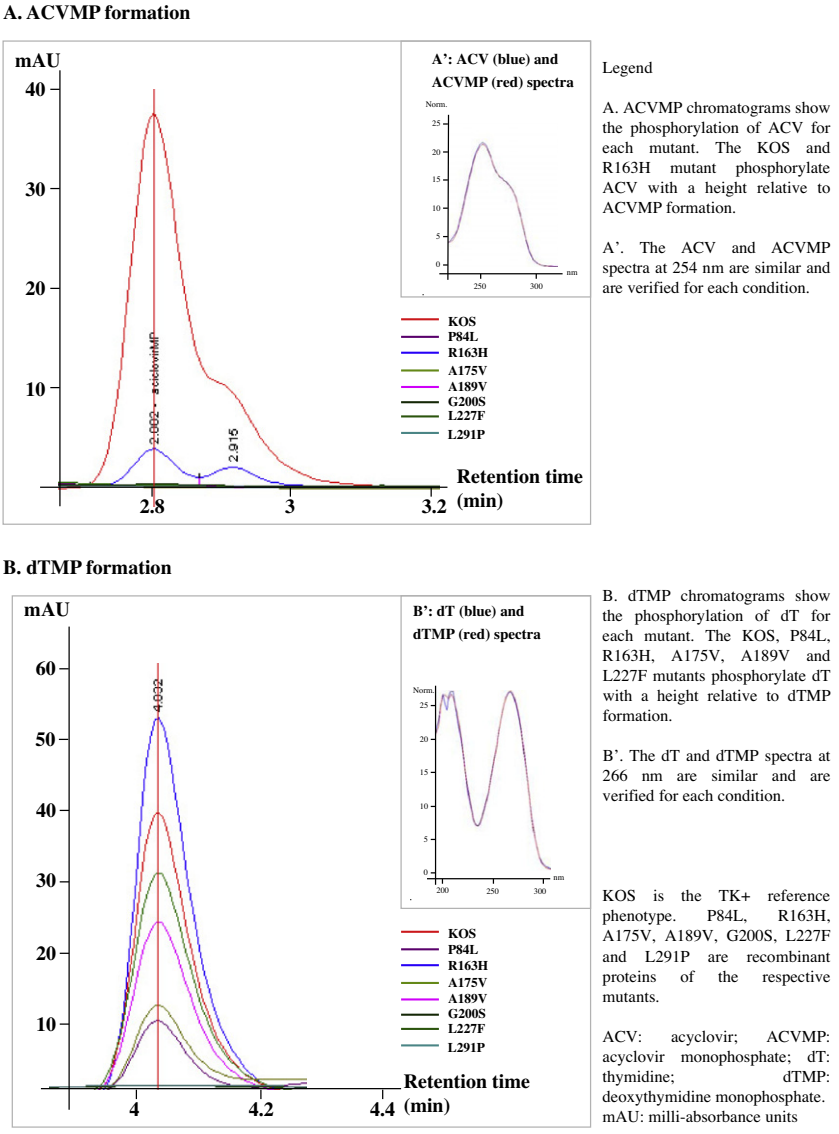
5 μg of recombinant proteins, purified using StrepTrap™ HP column (GE Healthcare), separated on SDS-12% polyacrylamide and stained with Coomassie blue. The purity of each Strep-tag TK proteins is similar.

KOS is the TK+ reference phenotype. P84L, R163H, A175V, A189V, G200S, L227F and L291P are recombinant proteins of respective mutants.

Fig. 1. Analysis of TK proteins.

chromatography using Streptatin Magnetic beads (Qiagen) and StrepTrap™ HP columns (GE Healthcare), respectively, as recommended by the manufacturer's instructions. Strep-tag TK proteins were detected after Western blotting analysis using a 1:1000 dilution of a mouse monoclonal antibody directed against the Strep-tag epitope (Strep-tag R antibody, Qiagen) (Fig. 1A). The purity of the Strep-tag proteins was also checked on a Coomassie gel (Fig. 1B). Phosphorylation of ACV (GlaxoSmithKline) and dT (VWR) by TKs was studied using the method described by Pilger et al. (1999). Briefly, 5 μg of TK proteins were incubated at 37 °C in substrate buffer (50 mM Tris–HCl, pH 7.2, 5 mM $MgCl_2$, 5 mM ATP) containing either 2 mM ACV or 5 mM dT. Each experiment was performed in duplicate. Acyclovir monophosphate (ACVMP) and deoxythymidine monophosphate (dTMP) synthesis were measured after 2, 4 and 7 h of incubation using high performance liquid chromatography (HPLC), with a 150 × 4 mm column packed with Hypersil ODS (3 μM) (Fisher Scientifics) (adapted from Boulieu) (Boulieu et al., 1997). Samples were diluted 1:50 and deproteinized with perchloric acid before testing. The mobile phase consisted of 0.02 M KH_2PO_4 pH 3.5. Separation was performed at a flow rate of 1.4 $ml.min^{-1}$ and DAD (Diode Array Detectors) detection was set at 254 nm for ACVMP and 266 nm for dTMP. Linearity could be considered up to 0.22 mM for both ACV/ACVMP and dT/dTMP, with a quantification limit at 0.4 μM. TK KOS activity was used as 100% of ACVMP and dTMP formation. HPLC/DAD chromatograms measuring ACVMP and dTMP for each mutant are shown in Fig. 2. Substrate phosphorylation rates for the mutant TKs, expressed as a percentage of wild-type KOS, are summarized in Table 1. As widely described in previous works, TKs were characterized as TK deficient (TK^d) [ACV^-/dT^-], TK altered (TK^{alt}) [ACV^-/dT 15–100%] and TK low-producer (TK^{low}) [ACV^-/dT 1–15%] phenotypes (Gaudreau et al., 1998; Hill et al., 1991). Of note, the TK^{low} phenotype can be considered as a TK^{alt} phenotype with less than 15% of dT phosphorylated. The clinical impact of TK^{low} and TK^{alt} could be the same as these ACV resistant strains have the ability to reactivate, whereas TK^d theoretically do not.

When mutations detected by the genotyping approach have not been previously characterized, it is difficult to interpret ACV resistance or sensitivity. Moreover, several mutations can be found in association in *UL23* TK gene and their localization outside active or conserved sites of the enzyme does not exclude them from ACV resistance. In addition, although substitutions in conserved sites are likely to be related to ACV resistance, polymorphism has also been detected in these regions (Kudo et al., 1998; Morfin et al., 2000; Sauerbrei et al., 2010). In this study, we decided to better characterize substitutions that have been previously reported in clinical isolates in a context of ACV resistance but mostly in association (Chibo et al., 2004; Duan et al., 2009; Sauerbrei et al., 2010). Enzymatic activity of TKs showed that A189V and L227F mutations induce a TK^{alt} phenotype (Chibo et al., 2004), whereas



Legend

A. ACVMP chromatograms show the phosphorylation of ACV for each mutant. The KOS and R163H mutant phosphorylate ACV with a height relative to ACVMP formation.

A'. The ACV and ACVMP spectra at 254 nm are similar and are verified for each condition.

B. dTMP chromatograms show the phosphorylation of dT for each mutant. The KOS, P84L, R163H, A175V, A189V and L227F mutants phosphorylate dT with a height relative to dTMP formation.

B'. The dT and dTMP spectra at 266 nm are similar and are verified for each condition.

KOS is the TK+ reference phenotype. P84L, R163H, A175V, A189V, G200S, L227F and L291P are recombinant proteins of the respective mutants.

ACV: acyclovir; ACVMP: acyclovir monophosphate; dT: thymidine; dTMP: deoxythymidine monophosphate. mAU: milli-absorbance units

Fig. 2. HPLC chromatograms of ACVMP and dTMP formation.

Table 1
Characterizing TK mutant proteins by measuring ACVMP and dTMP formation using HPLC/DAD.

TK mutant proteins	Substrate phosphorylation in mM (% of wild-type KOS)[a]						TK phenotype
	dTMP			ACVMP			
	2 h	4 h	7 h	2 h	4 h	7 h	
P84L	0.24 ± 0.01(21)	0.48 ± 0.02 (26)	0.75 ± 0.05 (26)	0	0	0	TK^{alt}
R163H	1.58 ± 0.28 (100)	2.30 ± 0.12 (100)	3.08 ± 0.19 (100)	0.05 ± 0.01 (9)	0.09 ± 0.01 (9)	0.13 ± 0.01 (8)	$TK^{partial}$
A175V	0.35 ± 0.01 (30)	0.65 ± 0.01 (36)	0.95 ± 0.01 (33)	0	0	0	TK^{alt}
A189V	0.52 ± 0.04 (45)	1.27 ± 0.02 (70)	2.02 ± 0.03 (70)	0	0	0	TK^{alt}
G200S	0	0	0	0	0	0	TK^{d}
L227F	0.73 ± 0.03 (63)	1.78 ± 0.04 (97)	2.47 ± 0.11 (86)	0	0	0	TK^{alt}
L291P	0	0	0	0	0	0	TK^{d}
KOS	1.15 ± 0.12 (100)	1.83 ± 0.24 (100)	2.88 ± 0.36 (100)	0.52 ± 0.03 (100)	1.03 ± 0.07 (100)	1.70 ± 0.08 (100)	TK^{+}

Data in parenthesis represent the percentages of ACVMP and dTMP produced by the recombinant mutant proteins relative to the reference KOS TK enzyme.
TK phenotype cut-offs: TK^{d} = ACV^{-}/dT^{-}; TK^{alt} = ACV^{-}/dT 15–100%; TK^{low} = ACV^{-}/dT 1–15%; $TK^{partial}$ = ACV 1–15%/dT 100%.
TK^{alt}, TK altered; TK^{+}, TK positive, TK^{d}, TK deficient. $TK^{partial}$ = TK partial.
[a] Means and standard deviations were calculated from two independent assays.

G200S and L291P mutations induce a TK^{d} phenotype (Table 1) (Duan et al., 2009; Gaudreau et al., 1998). Interestingly, G200S and L291P mutants were produced in a less soluble form than other TK proteins, probably reflecting their harmful impact on TK functionality (Fig. 1A). P84L and A175V mutations in site 2 (Saijo et al., 2002; Sauerbrei et al., 2010) and in the nucleoside binding

site (Frobert et al., 2005; Morfin et al., 2000), respectively, were shown to induce a TK^{alt} phenotype. In this study, the frequency of the TK^{alt} phenotype was higher than expected, as a minority of ACV resistant HSV clinical isolates is normally known to be TK^{alt} (Pottage and Kessler, 1995). This could be explained either by the fact that other studies underestimated this phenotype or by bias due to the low number of substitutions analyzed. As the TK^{alt} phenotype results in ACV resistance but does not preclude reactivation of resistant strains, these phenotypes need to be properly characterized. Interestingly, the R163H mutant (site 3, (Sauerbrei et al., 2010)), which had 100% dT phosphorylating activity associated with only 10% of ACV phosphorylating activity suggests a "TK partial" phenotype that has never been described before. This type of phenotype may imply a level of resistance that depends on ACV regimen. Its clinical impact needs to be assessed through phenotypic assays together with latency and neurovirulence studies (Dambrosi et al., 2010).

The method used here makes it possible to characterize TK activity accurately, and differentiate between functional, deficient, altered and partial TK. The efficiency of KOS TK is reported to be equivalent to other reference strains or to clinical or mutant sensitive strains (Frobert et al., 2005, 2007; Suzutani et al., 2000), making comparison between recombinant proteins and KOS TK activity a good approach for determining the efficiency of mutant TK proteins. Nevertheless, differences in relation to previous reports on TK phenotypes were observed. In particular, A175V mutation was first constructed as the TK^{d} reference mutant (Frobert et al., 2005), whereas it seems more appropriate to designate it as the TK^{alt} phenotype based on the results presented here. The [^{3}H]dT used as substrate associated to a one-hour kinetic in the previous study may in retrospect not be sensitive enough and did not make it possible to distinguish the TK^{alt} from the TK^{d} phenotype. Of note, mutation R176Q, which is also located in the nucleoside binding site, has been linked to a $TK^{alt/low}$ phenotype (Darby et al., 1986). The added value of this technique is the use of ACV and thymidine as both can be used as substrates by HSV TK *in vivo*. Moreover, this method does not require virus-infected TK-deficient cell line (Gaudreau et al., 1998; Hill et al., 1991; Saijo et al., 2002), nor generating recombinant virus (Bestman-Smith and Boivin, 2003; Gierasch et al., 2006) but instead uses recombinant mutant proteins. This technique also shows good reproducibility as the amount of protein used in the assay is defined precisely, which is much more challenging when proteins are synthesized *in vitro* in a rabbit reticulocyte lysate system (Suzutani et al., 2000). Nevertheless, some mutant TKs exhibiting poor solubility may represent a limiting factor in our approach as their large-scale production requires handling large volumes of bacterial cultures.

Therefore, this new method is a good way to make a reliable link between genotype, TK phenotype and "ACV resistance". Such a technique would thus participate in the establishment of a database of *UL23* TK mutations involved in ACV resistance. *UL23* TK mutation studies also need to be associated with structure modeling, for a better understanding of amino acid interactions and the structural impact of the mutations (Wang et al., 2011). *UL30* DNA pol studies also need to be carried out to provide a full overview of antiviral resistance mechanisms (Bohn et al., 2011; Burrel et al., 2010). With a large and complete database of *UL23* TK and *UL30* DNA pol mutations related to antiviral resistance, HSV genotyping could be fast and efficient, as is the case for CMV (Chevillotte et al., 2010; Hantz et al., 2010).

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References

Bestman-Smith, J., Boivin, G., 2003. Drug resistance patterns of recombinant herpes simplex virus DNA polymerase mutants generated with a set of overlapping cosmids and plasmids. J. Virol. 77, 7820–7829.
Bohn, K., Zell, R., Schacke, M., Wutzler, P., Sauerbrei, A., 2011. Gene polymorphism of thymidine kinase and DNA polymerase in clinical strains of herpes simplex virus. Antiviral Ther. 16, 989–997.
Boulieu, R., Gallant, C., Silberstein, N., 1997. Determination of acyclovir in human plasma by high-performance liquid chromatography. J. Chromatogr. B Biomed. Sci. Appl. 693, 233–236.
Burrel, S., Deback, C., Agut, H., Boutolleau, D., 2010. Genotypic characterization of UL23 thymidine kinase and UL30 DNA polymerase of clinical isolates of herpes simplex virus: natural polymorphism and mutations associated with resistance to antivirals. Antimicrob. Agents Chemother. 54, 4833–4842.
Chevillotte, M., von Einem, J., Meier, B.M., Lin, F.M., Kestler, H.A., Mertens, T., 2010. A new tool linking human cytomegalovirus drug resistance mutations to resistance phenotypes. Antiviral Res. 85, 318–327.
Chibo, D., Druce, J., Sasadeusz, J., Birch, C., 2004. Molecular analysis of clinical isolates of acyclovir resistant herpes simplex virus. Antiviral Res. 61, 83–91.
Dambrosi, S., Martin, M., Yim, K., Miles, B., Canas, J., Sergerie, Y., Boivin, G., 2010. Neurovirulence and latency of drug-resistant clinical herpes simplex viruses in animal models. J. Med. Virol. 82, 1000–1006.
Darby, G., Larder, B.A., Inglis, M.M., 1986. Evidence that the "active center" of the herpes simplex virus thymidine kinase involves an interaction between three distinct regions of the polypeptide. J. Gen. Virol. 67, 753–758.
Duan, R., de Vries, R.D., van Dun, J.M., van Loenen, F.B., Osterhaus, A.D., Remeijer, L., Verjans, G.M., 2009. Acyclovir susceptibility and genetic characteristics of sequential herpes simplex virus type 1 corneal isolates from patients with recurrent herpetic keratitis. J. Infect. Dis. 200, 1402–1414.
Frobert, E., Cortay, J.C., Ooka, T., Najioullah, F., Thouvenot, D., Lina, B., Morfin, F., 2008. Genotypic detection of acyclovir-resistant HSV-1: characterization of 67 ACV-sensitive and 14 ACV-resistant viruses. Antiviral Res. 79, 28–36.
Frobert, E., Ooka, T., Cortay, J.C., Lina, B., Thouvenot, D., Morfin, F., 2005. Herpes simplex virus thymidine kinase mutations associated with resistance to acyclovir: a site-directed mutagenesis study. Antimicrob. Agents Chemother. 49, 1055–1059.
Frobert, E., Ooka, T., Cortay, J.C., Lina, B., Thouvenot, D., Morfin, F., 2007. Resistance of herpes simplex virus type 1 to acyclovir: thymidine kinase gene mutagenesis study. Antiviral Res. 73, 147–150.
Gaudreau, A., Hill, E., Balfour Jr., H.H., Erice, A., Boivin, G., 1998. Phenotypic and genotypic characterization of acyclovir-resistant herpes simplex viruses from immunocompromised patients. J. Infect. Dis. 178, 297–303.
Gierasch, W.W., Zimmerman, D.L., Ward, S.L., Vanheyningen, T.K., Romine, J.D., Leib, D.A., 2006. Construction and characterization of bacterial artificial chromosomes containing HSV-1 strains 17 and KOS. J. Virol. Methods 135, 197–206.
Hantz, S., Garnier-Geoffroy, F., Mazeron, M.C., Garrigue, I., Merville, P., Mengelle, C., Rostaing, L., Saint Marcoux, F., Essig, M., Rerolle, J.P., Cotin, S., Germi, R., Pillet, S., Lebranchu, Y., Turlure, P., Alain, S., 2010. Drug-resistant cytomegalovirus in transplant recipients: a French cohort study. J. Antimicrob. Chemother. 65, 2628–2640.
Hill, E.L., Hunter, G.A., Ellis, M.N., 1991. *In vitro* and *in vivo* characterization of herpes simplex virus clinical isolates recovered from patients infected with human immunodeficiency virus. Antimicrob. Agents Chemother. 35, 2322–2328.
Kudo, E., Shiota, H., Naito, T., Satake, K., Itakura, M., 1998. Polymorphisms of thymidine kinase gene in herpes simplex virus type 1: analysis of clinical isolates from herpetic keratitis patients and laboratory strains. J. Med. Virol. 56, 151–158.
Morfin, F., Souillet, G., Bilger, K., Ooka, T., Aymard, M., Thouvenot, D., 2000. Genetic characterization of thymidine kinase from acyclovir-resistant and -susceptible herpes simplex virus type 1 isolated from bone marrow transplant recipients. J. Infect. Dis. 182, 290–293.
Pilger, B.D., Perozzo, R., Alber, F., Wurth, C., Folkers, G., Scapozza, L., 1999. Substrate diversity of herpes simplex virus thymidine kinase. Impact of the kinematics of the enzyme. J. Biol. Chem. 274, 31967–31973.
Piret, J., Boivin, G., 2011. Resistance of herpes simplex viruses to nucleoside analogues: mechanisms, prevalence, and management. Antimicrob. Agents Chemother. 55, 459–472.
Pottage Jr., J.C., Kessler, H.A., 1995. Herpes simplex virus resistance to acyclovir: clinical relevance. Infect. Agents Dis. 4, 115–124.
Saijo, M., Suzutani, T., De Clercq, E., Niikura, M., Maeda, A., Morikawa, S., Kurane, I., 2002. Genotypic and phenotypic characterization of the thymidine kinase of ACV-resistant HSV-1 derived from an acyclovir-sensitive herpes simplex virus type 1 strain. Antiviral Res. 56, 253–262.
Sauerbrei, A., Deinhardt, S., Zell, R., Wutzler, P., 2010. Phenotypic and genotypic characterization of acyclovir-resistant clinical isolates of herpes simplex virus. Antiviral Res. 86, 246–252.
Strick, L.B., Wald, A., Celum, C., 2006. Management of herpes simplex virus type 2 infection in HIV type 1-infected persons. Clin. Infect. Dis. 43, 347–356.
Studier, F.W., 2005. Protein production by auto-induction in high density shaking cultures. Protein Expr. Purif. 41, 207–234.
Styczynski, J., Reusser, P., Einsele, H., de la Camara, R., Cordonnier, C., Ward, K.N., Ljungman, P., Engelhard, D., 2009. Management of HSV, VZV and EBV infections

in patients with hematological malignancies and after SCT: guidelines from the second European conference on infections in leukemia. Bone Marrow Transplant. 43, 757–770.

Suzutani, T., Saijo, M., Nagamine, M., Ogasawara, M., Azuma, M., 2000. Rapid phenotypic characterization method for herpes simplex virus and varicella-zoster virus thymidine kinases to screen for acyclovir-resistant viral infection. J. Clin. Microbiol. 38, 1839–1844.

Wang, Y., Wang, Q., Zhu, Q., Zhou, R., Liu, J., Peng, T., 2011. Identification and characterization of acyclovir-resistant clinical HSV-1 isolates from children. J. Clin. Virol. 52, 107–112.