Antiviral Research 96 (2012) 386–390

Contents lists available at SciVerse ScienceDirect

Antiviral Research

journal homepage: www.elsevier.com/locate/antiviral

Short Communication

Impact of novel mutations of herpes simplex virus 1 and 2 thymidine kinases on acyclovir phosphorylation activity

Sonia Burrel [a,b], Pascale Bonnafous [a], Petr Hubacek [c,d], Henri Agut [a,b], David Boutolleau [a,b,*]

[a] UPMC Univ Paris 06, ER1 DETIV, Paris, France
[b] AP-HP, Service de Virologie, Groupe Hospitalier Universitaire La Pitié-Salpêtrière – Charles Foix, Paris, France
[c] Department of Pediatric Hematology and Oncology, Second Faculty of Medicine, Charles University and University Hospital Motol, Prague, Czech Republic
[d] Department of Medical Microbiology, University Hospital Motol, Prague, Czech Republic

ARTICLE INFO

Article history:
Received 25 July 2012
Revised 29 August 2012
Accepted 24 September 2012
Available online 3 October 2012

Keywords:
Herpes simplex virus
Thymidine kinase
Acyclovir phosphorylation activity
Nonradioactive method

ABSTRACT

The acyclic analogue of guanosine acyclovir (ACV) constitutes the first-line drug for the treatment of herpes simplex virus (HSV) infections. ACV activation requires primophosphorylation by virus-encoded HSV thymidine kinase (TK). In 95% of cases, HSV resistance to ACV is associated with mutations located in TK. The aim of this work was to address the question of the potential involvement of novel HSV-1 and HSV-2 TK mutations in reduced susceptibility to ACV using a novel nonradioactive method, based on luminescent quantitation of ADP, for the evaluation of *in vitro* phosphorylation activity of TK. All recombinant TKs tested exhibited significantly lower ACV phosphorylation activities in comparison with those of reference KOS or gHSV-2 TKs ($p < 0.015$), therefore indicating that amino acid changes Y53D, L170P, R176W, A207P (HSV-1) and S66P, A72S, I101S, M183I (HSV-2) were likely to be involved in HSV resistance to ACV.

Acyclovir (ACV) constitutes the first-line therapy for the management of herpes simplex virus (HSV)-1 and HSV-2 infections (Dignani et al., 2002; Englund et al., 1990; Hill et al., 1991). Long-term prophylactic and curative ACV treatments may result in the emergence of HSV resistance, especially among immunocompromised individuals such as HIV-infected patients and transplant recipients (Danve-Szatanek et al., 2004; Englund et al., 1990; Hill et al., 1991; Morfin et al., 2004, 2000). The prevalence of HSV resistance to ACV has been reported to vary from 3.5% to 10%, with higher rates in hematopoietic stem cell transplant recipients (Danve-Szatanek et al., 2004; Englund et al., 1990; Hill et al., 1991; Morfin et al., 2004). ACV is an analogue of guanosine that requires activation through triphosphorylation. The first phosphorylation is mainly achieved by HSV thymidine kinase (TK), encoded by UL23 gene, whereas subsequent phosphorylations are carried out by host cellular kinases. Then, ACV active form is incorporated by the viral DNA polymerase, encoded by UL30 gene, and disrupts viral genome replication by a chain termination mechanism (Gilbert et al., 2002). In accordance with this mechanism of action, viral mutations conferring resistance to ACV have been mapped both in UL23 and UL30 genes, but 95% of HSV strains exhibiting resistance to ACV harbor mutations within UL23 gene. These mutations lead to the production of TK with deficient or altered phosphorylation activity (Piret and Boivin, 2011). The accurate interpretation of genotypic resistance assays, based on the identification of specific mutations in TK and DNA polymerase, requires the clear distinction between natural polymorphisms and mutations conferring antiviral resistance. Traditionally, the unequivocal assessment of a putative resistance mutation is performed by testing a recombinant viral mutant, generated by marker transfer experiments, for altered drug susceptibility using a standardized phenotypic assay (Piret and Boivin, 2011). However, this method is labor-intensive and relatively tedious to perform. A reliable alternative strategy for the evaluation of the contribution of HSV mutations with regard to antiviral resistance is to test the enzymatic activity of mutant viral enzymes *in vitro*. Regarding HSV TK mutations potentially conferring resistance to ACV, many methods reported so far for the evaluation of TK phosphorylation activity are based on the use of ^{3}H-labeled thymidine as a substrate (Frobert et al., 2007; Piret and Boivin, 2011; Suzutani et al., 2000). But the major drawback of radioactive assay formats lies in the requirement of specialized equipment. We have described eight novel amino acid changes in TK from HSV clinical isolates exhibiting phenotypic resistance to ACV (Burrel et al., 2010). The aim of this work was to address the question of the potential involvement of these unpreviously reported changes in the acquisition of resistance to ACV by the use of a novel *in vitro* enzymatic assay coupled to a nonradioactive measurement method for the evaluation of ACV phosphorylation activity of TK mutants.

* Corresponding author at: AP-HP, Service de Virologie, Groupe Hospitalier Pitié-Salpêtrière, 83 boulevard de l'hôpital, F-75651 Paris Cedex 13, France. Tel.: +33 1 42 17 72 89; fax: +33 1 42 17 74 11.
E-mail address: david.boutolleau@psl.aphp.fr (D. Boutolleau).

0166-3542/$ - see front matter © 2012 Elsevier B.V. All rights reserved.
http://dx.doi.org/10.1016/j.antiviral.2012.09.016

For the construction of wild-type TK plasmids, UL23 genes from KOS and gHSV-2 strains (Gifts from Dr. C. Deback, Antoine-Béclère University Hospital, Clamart, France) were amplified by PCR using the proofreading enzyme Expand High Fidelity® (Roche Diagnostics, Meylan, France) with the primers listed in Supplementary Table 1. The resulting purified amplicons were cloned into the pFC15A HaloTag® Flexi Vector under control of the T7 RNA polymerase promoter, according to manufacturer's instructions (Promega, Charbonnières-les-Bains, France), prior to transformation into competent *Escherichia coli* DE3(BL21)pLysS cells (Promega). TK1_KOS and TK2_gHSV2 plasmids were purified using PureYield plasmid Miniprep System® (Promega) and checked by sequencing using overlapping primer pairs with the Prism Big Dye Terminator Cycle Sequencing Ready Reaction® kit (Applied Biosystems, Courtaboeuf, France) on the automated sequencer ABI PRISM™ 3730 Genetic Analyzer (Applied Biosystems). All sequences were compared with those of reference strains 17 (HSV-1) and HG52 (HSV-2) (GenBank accession numbers X14112 and Z86099, respectively) using Seqscape v2.6 software (McGeoch et al., 1985, 1987). The characterization of TK1_KOS evidenced the following mutations previously associated with natural polymorphism: N23S, K36E, R89Q, V138I, and A265T. The amino acid sequence of TK2_gHSV2 was identical to that of reference strain HG52.

The eight amino acid changes previously identified in TK from phenotypically ACV-resistant HSV clinical isolates were studied (Table 1) (Burrel et al., 2010). In addition, 2 amino acid changes conferring ACV resistance (E83K for HSV-1, R221H for HSV-2) and 2 amino acid changes related to natural polymorphism (L42P for HSV-1, G39E for HSV-2) were used as controls (Bohn et al., 2011; Burrel et al., 2010; Chibo et al., 2004; Frobert et al., 2005; Kudo et al., 1998). TK1_KOS and TK2_gHSV2 plasmids were used as matrix to produce recombinant plasmids harboring the different mutations described above. Mutations were introduced into wild-type plasmids using the QuickChange XL® site-directed mutagenesis kit (Agilent, Massy, France), following the manufacturer's instructions, to produce the following mutant plasmids (one construction per amino acid change): TK1_E83K, TK1_L42P, TK1_Y53D, TK1_L170P, TK1_R176W, TK1_A207P, TK2_R221H, TK2_G39E, TK2_S66P, TK2_A72S, double mutant TK2_S66P + A72S (named TK2_DM), TK2_I101S, and TK2_M183I. All the modified forward and reverse primers used for site-directed mutagenesis are listed in Supplementary Table 2. Similarly to wild-type plasmids, all generated mutant plasmids were transformed and grown into DE3(BL21)pLysS cells, purified using PureYield plasmid Miniprep System®, and checked by sequencing to confirm that no additional change had been introduced during the site-directed mutagenesis step.

Recombinant TKs were synthesized from plasmids using a reticulocyte lysate system (TNT® Quick Coupled Transcription/Translation Systems, Promega), according to the manufacturer's protocol. Newly synthesized TKs, tagged at the carboxy terminus with the HaloTag® included in the pFC15A HaloTag® Flexi Vector, were purified by using the dedicated HaloLink® resin. After translation and purification, protein yields were measured using the Bradford method in order to standardize the quantity of purified TKs used for the phophorylation activity assay. When visualization of synthesized TKs was required, biotinylated lysine was incorporated during the translation process using Transcend™ tRNA (Promega). Recombinant TKs were then subjected to SDS–PAGE followed by transfer onto a nitrocellulose membrane. The biotinylated TKs were visualized by binding with streptavidin–alkaline phosphatase followed by colorimetric detection, according to the manufacturer's instructions (Promega). Fig. 1 shows the HSV-1 recombinant TKs produced in the reticulocyte lysate system in the presence of biotinylated lysines. All the bands had a similar intensity and stood at the expected size (i.e., ~75 kDa), corresponding to the native HSV TK (~42 kDa) fused to the HaloTag® (~33 kDa). Similar results were obtained for HSV-2 TKs (data not shown).

ACV phosphorylation activity of the different purified TKs was determined using the ADP-Glo™ Kinase Assay (Promega), according to the instructions for use. This assay for monitoring kinase activity comprised three main steps: (i) kinase reaction, (ii) depletion of remaining ATP, and (iii) conversion of produced ADP into ATP with luminescence detection dosage. Briefly, after kinase reaction, ADP-Glo™ Reagent was added to terminate the kinase reaction and to allow the complete depletion of remaining ATP. The Kinase Detection Reagent was then added to convert ADP to ATP and to allow the newly synthesized ATP to be measured using a luciferase/luciferin reaction. The luminescence output was recorded using Flex Station 3® reader (Molecular Devices, Saint Grégoire, France). After standardization of the ACV phosphorylation assay, consisting in the determination of the optimal duration of kinase reaction and ACV concentration allowing the identification of TK mutations inducing the impairment of enzymatic activity, the kinase reaction using the ADP-Glo™ Kinase Assay was carried out at 37 °C for 10 min in a reactional mixture containing 50 mM TRIS buffer (pH 8.0), 2 mM ATP, 20 mM $MgCl_2$, 2 mM ACV and 0.1 μg of purified TKs. Of note, the chimioluminescent signal of the TK-negative control tended to increase after 30 min of incubation, and a small quantity of ATP appeared to be consumed in the absence of ACV, indicating that a residual ACV-independent phosphorylation activity was measurable under these experimental conditions (data not shown). The ACV phosphorylation assay was performed with the recombinant TKs harboring the amino acid changes of interest: Y53D, L170P, R176W, A207P for HSV-1; S66P, A72S, I101S, M183I for HSV-2 (Table 1) (Burrel et al., 2010). For comparative purposes, the activities from KOS and

Table 1
Phenotypic and genotypic characteristics of TK mutants.

HSV type	Clinical context[a]	ACV EC_{50} (μM)[b]	TK mutations potentially related to ACV resistance[c]	Localization within TK[d]
1	IC	35	Y53D	ATP-binding site
1	BMT	11	L170P	Nucleoside-binding site
1	BMT	>50	R176W	Nucleoside-binding site
1	BMT	>50	A207P	Outside conserved regions
2	HIV	43	S66P A72S	Outside conserved regions
2	HIV	42	I101S	Outside conserved regions
2	HIV	33	M183I	Outside conserved regions

[a] BMT, bone marrow transplant recipients; IC, immunocompetent individual; HIV: HIV-infected patient; TK: thymidine kinase.
[b] 50% Effective concentration (EC_{50}) obtained for the clinical isolate harboring novel mutations using a plaque-reduction assay. The EC_{50} cut off value for resistance to acyclovir (ACV) was ⩾7 μM (Burrel et al., 2010).
[c] Novel mutations previously reported in TK from phenotypically ACV-resistant HSV clinical isolates (Burrel et al., 2010). Each mutation (or S66P + A72S associated mutations) was identified in a separate HSV clinical isolate.
[d] Functional domains of HSV TKs (i.e., the ATP-binding site and the nucleoside-binding site) are localized between codons 51–63 for both HSV types, and between codons 168–176 for HSV-1, and 169–177 for HSV-2, respectively.

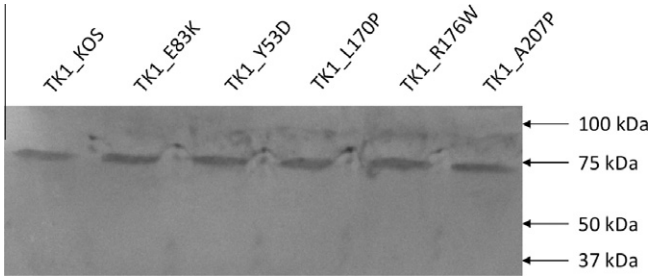

Fig. 1. Western blot analysis of recombinant TKs After translation in reticulocyte lysate in the presence of precharged labeled biotinylated lysine tRNA complex, samples were subjected to SDS–PAGE followed by transfer to a nitrocellulose membrane. Produced TKs, tagged at the carboxy terminus with the HaloTag®, were visualized by colorimetric detection using streptavidin–alkaline phosphatase revelation system at the expected size of ~75 kDa, corresponding to the native HSV TK (~42 kDa) fused to HaloTag® (~33 kDa). TK: Thymidine kinase.

gHSV-2 wild-type TKs were set at 100%, and activities from mutant TKs were expressed as percentage of respective wild-type TK activities. In order to overcome inter-assay variability of the functional assay, controls were included in each experiment: TK-negative control (i.e., pFC15A vector without TK insert), ACV-sensitive and ACV-resistant TKs. For each recombinant TK, ACV phosphorylation activity was measured at least in triplicate and mean values ± standard deviations were calculated. Statistical analysis was carried out to compare the phosphorylation activities of the mutated TKs with those of the references TK1_KOS and TK2_gHSV2 using the Student's *t*-test. As shown in Fig. 2, all mutations, except the known natural polymorphisms L42P (HSV-1) and G39E (HSV-2), induced a significant decrease of recombinant TK activities when compared to reference TK activities ($p < 0.015$).

Antiviral resistance genotypic assays constitute a rapid and efficacious tool that allow obtaining results in a clinically relevant time frame (i.e., 24–48 h) (Burrel et al., 2010). However, the interpretation of these genotypic assays relies on the availability of a continuously updated database for the differentiation of resistance mutations from natural polymorphisms. This work aimed to study the impact of eight novel amino acid changes previously identified in TK from phenotypically ACV-resistant HSV clinical isolates on ACV phosphorylation activity (Table 1) (Burrel et al., 2010), using a novel *in vitro* functional enzymatic assay. Most functional activity assays reported so far to ascertain the role of unknown mutations located within TK in the acquisition of ACV resistance rely on the synthesis of recombinant TKs and the measurement of thymidine phosphorylation activity using radioactive methods (Frobert et al., 2005, 2007; Suzutani et al., 2000). Conversely to previous studies, the phosphorylation activity of recombinant TKs was evaluated towards ACV by the luminescent detection of the ADP newly produced during the kinase reaction. Thus, this novel assay appears to be an attractive alternative to the previous radioactive methods used for the identification of mutations responsible for the decrease of TK phosphorylation activity. However, a weak chimioluminescent signal was measured in the absence of either TK or ACV after 10 min of incubation (data not shown). This phenomenon could be linked to the existence of post-translational processing activities, including phosphorylation activity in rabbit reticulocyte lysate (Walter and Blobel, 1983), and/or TK phosphorylation activity of substrates different from ACV, like purine and pyrimidine triphosphate nucleosides (Deville-Bonne et al., 2010; Wild et al., 1995), and/or a non specific degradation of ATP in the reaction mixture. Therefore, this novel nonradioactive system, measuring the alteration of ACV phosphorylation activity of recombinant mutant TKs, permitted to assume a reduced sensitivity to ACV rather than a resistance to ACV. This latter point requires determining the 50% effective concentration (EC_{50}) of recombinant HSV TK mutants carrying the mutations of interest. Recently, a non-isotopic method has been reported to assess the effects of mutation on HSV-1 TK functionality using High-Performance Liquid Chromatography with Diode–Array Detection (HPLC–DAD) for measuring monophosphate forms of ACV (Malartre et al., 2012).

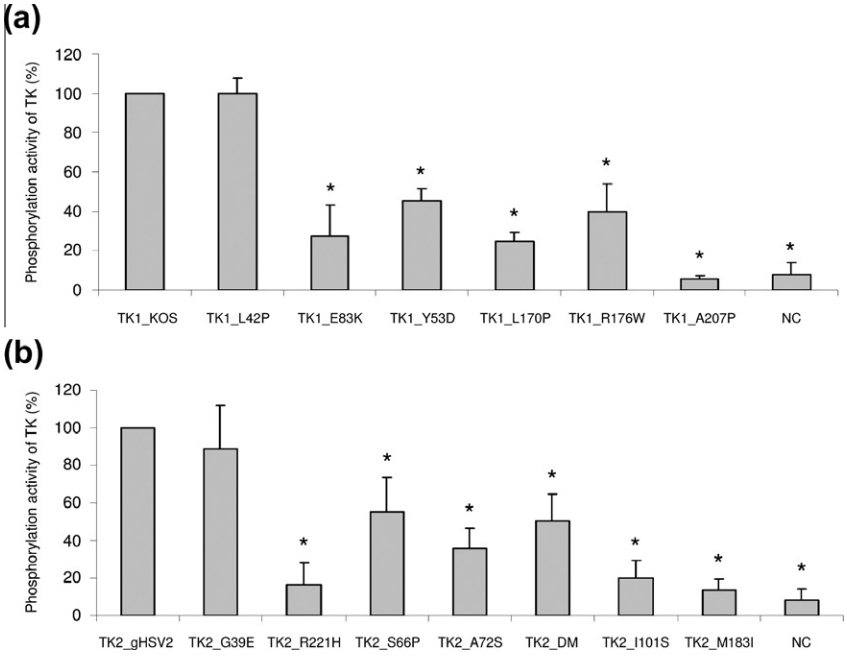

Fig. 2. ACV phosphorylation activities of recombinant TKs ACV phosphorylation activities of HSV-1 (a) and HSV-2 (b) recombinant TKs are shown as the percentage of TK1_KOS and TK2_gHSV2 reference activities (set at 100%), respectively. Error bars represent the standard deviations of the means (ACV phosphorylation activity of each recombinant TK was measured at least in triplicate). *ACV phosphorylation activity of mutant TK statistically lower than that of corresponding reference TK ($p < 0.015$, Student's *t* test). NC: negative control.

All 4 HSV-1 TK mutants studied, harboring Y53D, L170P, R176W, or A207P changes, induced a significant decrease of ACV phosphorylation activity in comparison with wild-type HSV-1 TK (Fig. 2a). These results are in agreement with existing data. Indeed, different amino acid changes located at codon 53 in the TK ATP-binding site, as Y53H and Y53N, were likely implicated in resistance to ACV (Sauerbrei et al., 2010). Changes located in the nucleoside-binding site, close to residues 170 and 176, and conferring resistance to ACV, have been reported: P173L (Suzutani et al., 2003), Y172C and P173R (Sauerbrei et al., 2010), A175V (Frobert et al., 2005). R176Q change has been evidenced in HSV-1 ACV-resistant clinical isolates and laboratory strains (Darby et al., 1981; Gaudreau et al., 1998). Additionally, the R177W change located in HSV-2 TK, the counterpart of R176W in HSV-1 TK, is known to confer resistance to ACV (Chibo et al., 2004; Kost et al., 1993). Furthermore, changes G200C and T201P, located near residue 207, have been described in ACV-resistant clinical isolates (Saijo et al., 2002).

Our results revealed that S66P, A72S, I101S, and M183I changes, located in nonconserved regions, induced a decrease of HSV-2 TK enzymatic activity (Fig. 2b). A72S and S66P changes were initially evidenced in the same HSV-2 ACV-resistant clinical isolate (Burrel et al., 2010). The coexistence of both changes had attracted interest to characterize the effect of each change. S66P and A72S changes were showed to induce separately a decrease of TK activity, and no compensatory effect to restore TK activity could be evidenced when both changes coexisted within the same TK. I101S and M183I changes, located outside conserved sites of TK, led also to a decreased activity of ACV phosphorylation. Other changes located nearby, Q105P and S182D/R, have been described in HSV-2 isolates resistant to ACV (Chatis and Crumpacker, 1991).

In conclusion, this work describes a nonradioactive, reliable and rapid functional enzymatic assay to study the involvement of HSV TK amino acid changes in the phosphorylation level of ACV. The universality of this assay provides the possibility not only to profile HSV TK activity on other nucleoside analogs, like ganciclovir, but also to characterize the activity of kinases from other *Herpesviridae*, such as varicella-zoster virus TK or human cytomegalovirus phosphotransferase. Our results show that the mutations Y53D, L170P, R176W and A207P (HSV-1), S66P, A72S, I101S and M183I (HSV-2) significantly impair the ACV phosphorylation activity of HSV TK and are, therefore, likely to be associated with resistance to ACV. Nevertheless, further studies, as transfer marker experiments, are required to assess formally their role in resistance to ACV in order to complete the database of TK mutations conferring HSV resistance to ACV. These new data might help for the interpretation of genotypic antiviral resistance testing and for the management of antiviral treatments of HSV infections in immunocompromised patients.

Disclosure statement

The authors declare no competing interests.

Acknowledgements

We thank Nathalie Désiré and Angélique Godet for helpful advice.

This work was supported in part by the Association pour la Recherche sur les Infections Virales (ARIV).

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.antiviral.2012.09.016.

References

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 Therapy 16, 989–997.

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. Antimicrobial Agents and Chemotherapy 54, 4833–4842.

Chatis, P.A., Crumpacker, C.S., 1991. Analysis of the thymidine kinase gene from clinically isolated acyclovir-resistant herpes simplex viruses. Virology 180, 793–797.

Chibo, D., Druce, J., Sasadeusz, J., Birch, C., 2004. Molecular analysis of clinical isolates of acyclovir resistant herpes simplex virus. Antiviral Research 61, 83–91.

Danve-Szatanek, C., Aymard, M., Thouvenot, D., Morfin, F., Agius, G., Bertin, I., Billaudel, S., Chanzy, B., Coste-Burel, M., Finkielsztejn, L., Fleury, H., Hadou, T., Henquell, C., Lafeuille, H., Lafon, M.E., Le Faou, A., Legrand, M.C., Maille, L., Mengelle, C., Morand, P., Morinet, F., Nicand, E., Omar, S., Picard, B., Pozzetto, B., Puel, J., Raoult, D., Scieux, C., Segondy, M., Seigneurin, J.M., Teyssou, R., Zandotti, C., 2004. Surveillance network for herpes simplex virus resistance to antiviral drugs: 3-year follow-up. Journal of Clinical Microbiology 42, 242–249.

Darby, G., Field, H.J., Salisbury, S.A., 1981. Altered substrate specificity of herpes simplex virus thymidine kinase confers acyclovir-resistance. Nature 289, 81–83.

Deville-Bonne, D., El Amri, C., Meyer, P., Chen, Y., Agrofoglio, L.A., Janin, J., 2010. Human and viral nucleoside/nucleotide kinases involved in antiviral drug activation: structural and catalytic properties. Antiviral Research 86, 101–120.

Dignani, M.C., Mykietiuk, A., Michelet, M., Intile, D., Mammana, L., Desmery, P., Milone, G., Pavlovsky, S., 2002. Valacyclovir prophylaxis for the prevention of Herpes simplex virus reactivation in recipients of progenitor cells transplantation. Bone Marrow Transplantation 29, 263–267.

Englund, J.A., Zimmerman, M.E., Swierkosz, E.M., Goodman, J.L., Scholl, D.R., Balfour Jr., H.H., 1990. Herpes simplex virus resistant to acyclovir. A study in a tertiary care center. Annals of Internal Medicine 112, 416–422.

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. Antimicrobial Agents and Chemotherapy 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 Research 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. The Journal of Infectious Diseases 178, 297–303.

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

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. Antimicrobial Agents and Chemotherapy 35, 2322–2328.

Kost, R.G., Hill, E.L., Tigges, M., Straus, S.E., 1993. Brief report: recurrent acyclovir-resistant genital herpes in an immunocompetent patient. The New England Journal of Medicine 329, 1777–1782.

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. Journal of Medical Virology 56, 151–158.

Malartre, N., Boulieu, R., Falah, N., Cortay, J.C., Lina, B., Morfin, F., Frobert, E., 2012. 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. Antiviral Research 95, 224–228.

McGeoch, D.J., Dolan, A., Donald, S., Rixon, F.J., 1985. Sequence determination and genetic content of the short unique region in the genome of herpes simplex virus type 1. Journal of Molecular Biology 181, 1–13.

McGeoch, D.J., Moss, H.W., McNab, D., Frame, M.C., 1987. DNA sequence and genetic content of the HindIII l region in the short unique component of the herpes simplex virus type 2 genome: identification of the gene encoding glycoprotein G, and evolutionary comparisons. The Journal of General Virology 68 (Pt 1), 19–38.

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. The Journal of Infectious Diseases 182, 290–293.

Morfin, F., Bilger, K., Boucher, A., Thiebaut, A., Najioullah, F., Bleyzac, N., Raus, N., Bosshard, S., Aymard, M., Michallet, M., Thouvenot, D., 2004. HSV excretion after bone marrow transplantation: a 4-year survey. Journal of Clinical Virology 30, 341–345.

Piret, J., Boivin, G., 2011. Resistance of herpes simplex viruses to nucleoside analogues: mechanisms, prevalence, and management. Antimicrobial Agents and Chemotherapy 55, 459–472.

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 Research 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 Research 86, 246–252.

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. Journal of Clinical Microbiology 38, 1839–1844.

Suzutani, T., Ishioka, K., De Clercq, E., Ishibashi, K., Kaneko, H., Kira, T., Hashimoto, K., Ogasawara, M., Ohtani, K., Wakamiya, N., Saijo, M., 2003. Differential mutation patterns in thymidine kinase and DNA polymerase genes of herpes simplex virus type 1 clones passaged in the presence of acyclovir or penciclovir. Antimicrobial Agents and Chemotherapy 47, 1707–1713.

Walter, P., Blobel, G., 1983. Preparation of microsomal membranes for cotranslational protein translocation. Methods in Enzymology 96, 84–93.

Wild, K., Bohner, T., Aubry, A., Folkers, G., Schulz, G.E., 1995. The three-dimensional structure of thymidine kinase from herpes simplex virus type 1. FEBS Letters 368, 289–292.