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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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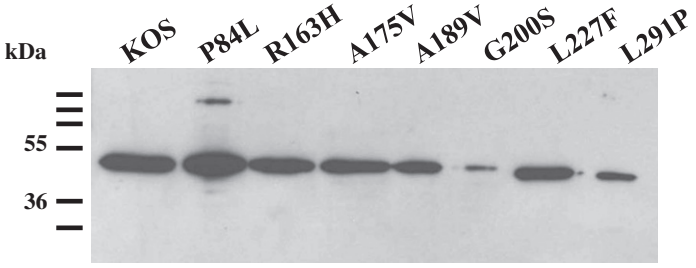
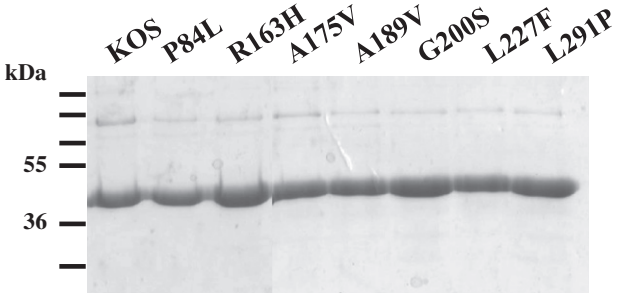
A. Western blot analysis



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