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Phenotypic and genotypic characterization of acyclovir-resistant clinical isolates of herpes simplex virus

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ABSTRACT

Sixteen herpes simplex virus type 1 (HSV-1) and four type 2 (HSV-2) isolates resistant to acyclovir (ACV) were characterized retrospectively for drug resistance. Phenotypic testing was performed by means of tetrazolium reduction assay and genotypic analysis was carried out by sequencing of thymidine kinase (TK) and DNA-polymerase (pol) genes. All strains were characterized as cross-resistant to penciclovir, brivudin and susceptible to cidofovir. In addition, three strains were resistant to foscarnet. Genotypic analysis revealed two to seven non-synonymous mutations in the TK gene of HSV-1 and one to seven non-synonymous mutations in the DNA pol gene of HSV-1 and 2 associated with the gene polymorphism. Seventeen strains contained at least one non-synonymous resistant-related mutation in the TK gene and three strains, which were additionally foscarnet-resistant, revealed one resistance-associated mutation in the DNA pol gene. In most strains, resistant-related mutations in TK gene represented frameshift mutations and single non-synonymous nucleotide substitutions of conserved gene regions. However, numerous amino acid changes could not be interpreted clearly as accounting for resistance. In conclusion, further studies, e.g. site-directed mutagenesis experiments are required to characterize mutations of the TK and DNA pol genes in ACV-resistant viral strains as part of viral gene polymorphism or as cause of drug resistance.

1. Introduction

Acyclovir (ACV), the first selective antiviral agent, is regarded as the drug of choice for the treatment and prophylaxis of infections caused by herpes simplex virus type 1 (HSV-1) and type 2 (HSV-2) for more than 20 years. Intravenous and oral use is indicated for the treatment of the herpes simplex encephalitis, primary and recurrent genital HSV infections and for suppressive treatment of recurrent genital herpes. Acyclovir is often used in immunocompromised patients undergoing transplantations, in whom HSV infections may be associated with chronic, severe and sometimes fatal courses (Dignani et al., 2002; Miller and Dummer, 2007). Furthermore, topical ACV, penciclovir (PCV) and foscarnet (FOS) formulations are available for management of recurrent herpes labialis. The general mode of action of the nucleoside analogues ACV and PCV is through inhibition of viral DNA polymerase (pol) by acting as competitive inhibitors and/or DNA chain terminators. This action requires three intracellular phosphorylation steps to convert the nucleoside analogues into their mono-, di- and triphospates. The initial phosphorylation step is carried out by the viral thymidine kinase (TK), and the two subsequent ones by cellular kinases. Resistance to ACV is mediated in 95% of the cases by mutations in the TK gene leading to a loss of TK activity or an alteration of substrate specificity (Bestman-Smith et al., 2001; Larder et al., 1983; Morfin and Thouvenout, 2003). In 5% of the cases, resistance is conferred by mutations in the DNA pol gene resulting in the alteration of enzyme activity (Larder and Darby, 1985).

To date, HSV isolates resistant to ACV have no clinical relevance in immunocompetent persons. A low prevalence of less than 1% has been reported in several studies (Collins and Ellis, 1993; Danve-Szatanek et al., 2004; Englund et al., 1990). This is probably due to the presence of effective immune response and the low pathogenic potential of the resistant virus variants (Coen et al., 1989). By contrast, in immunocompromised patients, the prevalence of HSV infections with reduced susceptibility to ACV varies between 3.5% and 7.1% (Bacon et al., 2003). The highest prevalence rates of 25% have been reported in patients with allogenic bone marrow transplantation (Chakrabarti et al., 2000). Emergence of resistance can be attributed to the spontaneous development of resistant mutants during viral replication, the selection of these mutants by antiviral treatment and the failure of immune system to eliminate resistant viruses.

In routine diagnosis, analysis of HSV resistance to antiviral drugs such as ACV is mostly performed by the determination of viral phenotype using the plaque reduction assay as a reference technique (Morfin and Thouvenout, 2003) or the dye uptake method (Stránská

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et al., 2004) and DNA hybridization test (Swierkosz et al., 1987). The main advantage is a clear interpretation of laboratory findings on the basis of the inhibitory concentration, which is determined for the antiviral drug tested. Results of phenotypic data are most often available in 7–10 days since phenotypic methods require previous isolation of viral strains in cell cultures and the adaptation of antiviral treatment according to in vitro susceptibility may be delayed. Therefore, genotypic tests have been developed to detect resistant viruses on the basis of resistance-associated mutations in viral genes encoding the two targets of antiviral drugs, namely the TK and the DNA pol, within a shorter delay of few days. The difficulty lies in the fact that numerous nucleotide substitutions may be found which must be identified as mutations responsible for resistance. This interpretation is relatively easy if frameshift mutations are detected resulting in the synthesis of a truncated or non-functional viral enzyme. By contrast, single non-synonymous nucleotide substitutions are difficult to interpret under consideration of the high gene polymorphism especially of the HSV-1-specific TK (Morfin et al., 2000). Therefore, the knowledge of genotypic data of HSV strains with resistant phenotype is of high importance.

The objective of the present study was to characterize retrospectively the complete phenotypic resistance of 20 ACV-resistant HSV isolates against the commercially available anti-herpes drugs. In addition, the genotypic resistance was determined by amplification and sequencing of the viral TK and DNA pol genes. From the results, recommendations were derived for the determination of HSV resistance in virological laboratories.

2. Materials and methods

2.1. Viral strains and cell cultures

In this study, 16 ACV-resistant HSV-1 strains and 4 ACV-resistant HSV-2 strains from 18 different patients were included (Table 1, nos. 1–20). Strains nos. 2–4 were obtained consecutively from one patient. All viral strains were isolated between 2001 and 2008 from immunocompromised patients with recurrent HSV infections of the skin and mucous membranes. The clinical data provided by the clinicians are summarized in Table 1. With suspicion of clinical resistance to ACV, the strains were sent to the German reference laboratory for HSV and varicella-zoster virus (VZV) to verify resistance retrospectively by laboratory methods. There was no detailed information about the duration of antiviral therapy with ACV including the administration of FOS. The inhibitory concentration 50% (IC_{50}) of ACV has been calculated as ≥4.1–8.0 μg/ml in experiments characterizing phenotypic resistance (method described below). As controls served 4 ACV-sensitive HSV-1 strains isolated in 2008 from immunocompetent persons with recurrent HSV infections (Table 1; nos. 21–24). During experiments for the analysis of phenotypic resistance, the IC_{50} of ACV of these strains has been calculated as 0.1–0.3 μg/ml. Furthermore, five TK-positive and two TK-negative HSV reference strains were used as controls (Table 1; nos. 25–31).

The viruses were grown and titrated using monolayers of the permanent African green monkey kidney cells Vero76 (ATCC, CRL 1587) or human embryonic lung fibroblasts (HELF). Vero76 cells were cultured in Eagle's minimum essential medium (EMEM) with Hanks' salts (Cambrex, Verviers, Belgium) plus 2 mM L-glutamine (Cambrex). For cultivation of HELF, EMEM plus 25 mM Hepes (Cambrex) was used. All media were supplemented with 5% fetal calf serum (Invitrogen, Carlsbad, CA), 100 U/ml penicillin (Cambrex), and 100 μg/ml streptomycin sulfate (Cambrex). Cells were incubated at 1% CO_2 (HELF) or 5% CO_2 (Vero76) and 37 °C. For viral propagation, the media were used without FCS. There were titers of viral stocks between $10^{6.0}$ and $10^{8.0}$ cell culture infectious dose 50% ($CCID_{50}$) per ml.

2.2. Antiviral test compounds

The following antiviral compounds were used for the phenotypic characterization of HSV resistance: acyclovir (ACV: GlaxoSmithKline, Uxbridge, UK), brivudin (BVDU; Berlin-Chemie AG, Berlin, Germany), penciclovir (PCV; GlaxoSmithKline, Uxbridge, UK), tri-sodium-phosphonoformate (Foscarnet, FOS; AstraZeneca, Wilmslow, UK), and cidofovir (CDV; Vistide®, Pharmacia & Upjohn, Luxembourg).

2.3. Phenotypic characterization of resistance

Antiviral testing was performed in 96-well flat-bottomed microtitre plates by means of tetrazolium reduction assay (WST-1, Hoffmann La Roche AG, Grenzach-Wyhlen, Germany) according to a method described by Klöcking et al. (1995). Briefly, HELF or Vero76 cells were seeded at a density of 10^5 ml^{-1} and grown for 2 days. After viral infection with about 10^3 $CCID_{50}$ per ml corresponding to a multiplicity of infection of 0.01 and addition of antiviral compounds at a final half log dilution over a range between 0.0625 and 8 μg/ml (ACV, brivudin, PCV, CDV) or between 8 and 128 μg/ml (FOS) the plates were incubated for 5 days. Optical density (OD) was measured at 492 nm (reference wavelength 620 nm) in a microplate reader. In addition, the cytopathic effect was evaluated microscopically. Percentages of antiviral activities of drugs were calculated from the measured OD values according to Pauwels et al. (1988). Each experiment was performed twice and the mean values were calculated. Substance concentrations at half-maximum virus inhibition (IC_{50}) were computed from the dose–response curves by linear regression analysis using the software SigmaStat, Version 1.01 (Jandel Corporation, San Rafael, CA). A viral strain was regarded as resistant if the mean IC_{50} was calculated as five times the mean value of the control HSV strain proved to be susceptible to the corresponding antiviral drug (Morfin and Thouvenout, 2003). On the basis of the mean control values, following cut-off levels for drug resistance were defined: ACV, HSV-1 2.5 ± 1.5 μg/ml (11.1 ± 6.7 μM), HSV-2 2.0 ± 1.5 μg/ml (8.9 ± 6.7 μM); BVDU, HSV-1 1.0 ± 0.5 μg/ml (4.4 ± 2.2 μM); PCV, HSV-1 1.5 ± 1.0 μg/ml (6.0 ± 4.0 μM), HSV-2 1.6 ± 0.8 μg/ml (6.4 ± 3.2 μM); CDV HSV-1/2 4.0 ± 3.0 μg/ml (14.8 ± 11.1 μM). Concerning FOS, HSV strains with IC_{50} ≥100 μg/ml (≥330 μM) were defined as resistant (Safrin et al., 1991).

2.4. Genotypic characterization of resistance

The genotypic analysis of resistance of HSV-1 and 2 was carried out by the amplification of DNA fragments of the viral TK and DNA pol genes as well as subsequent sequencing. The oligonucleotide primers based on the reference strains HSV-1 17 (GenBank Accession no. X14112) (McGeoch et al., 1985) and HSV-2 HG52 (GenBank Accession no. Z86099) (McGeoch et al., 1987) were modified according to studies published previously (Suzutani et al., 2003). The TK gene of HSV-1 and 2 was amplified as one fragment and sequenced in 2 fragments, while the DNA pol gene of HSV-1 and 2 was divided in 4 and 5 fragments, respectively (Table 2).

After isolation of DNA from the supernatant of virus-infected cells by means of QIAamp® Blood Kit (Qiagen, Hilden, Germany), viral DNA was amplified by polymerase chain reaction (PCR). High Fidelity Enzyme Mix (Fermentas, St.-Leon-Rot, Germany), a unique blend of Taq DNA pol and thermostable DNA pol with proofreading activity was used. Standard PCR mixture contained 10 μM of each primer (Table 2) plus approximately 50 ng template DNA in a volume of 50 μl. After an initial denaturation step for 3 min at 95 °C, reaction mixtures were cycled 35 times through denaturation at 94 °C for 60 s, annealing at 55 °C for 60 s, and polymerization at 72 °C for 90 s followed by a final extension step at 72 °C for

Table 1
Phenotypic resistance of acyclovir (ACV)-resistant (nos. 1–20), ACV-sensitive (nos. 21–24) HSV isolates and reference strains (nos. 25–31).

No.	Viral strain	Clinical data (diagnosis)	Inhibitory concentration 50% (μg/ml)				
			ACV[a]	BVDU[a]	PCV[a]	FOS[a]	CDV[a]
1	HSV-1 22/01	HSV pneumonia after BMT	>8.0	>8.0	2.3	13.4	0.1
2	HSV-1 354/02	HSV infections after BMT	>8.0	2.3	>8.0	>128	0.5
3	HSV-1 707/02	HSV infections after BMT	>8.0	>8.0	>8.0	>128	1.0
4	HSV-1 737/03	HSV infections after BMT	>8.0	>8.0	>8.0	>128	1.2
5	HSV-1 2245/03	Oral HSV infections after BMT	>8.0	>8.0	>8.0	41.3	1.9
6	HSV-1 1686/04	Recurrent oral HSV infections under IS	>8.0	>8.0	>8.0	9.3	0.1
7	HSV-1 331/05	Oral HSV infections after BMT	>8.0	>8.0	>8.0	25.1	1.2
8	HSV-1 2016/05	Facial HSV infections after BMT	>8.0	>8.0	>8.0	15.3	1.8
9	HSV-1 164/06	Oral HSV infections after CTX	>8.0	>8.0	>8.0	46.1	1.0
10	HSV-1 1678/06	Oral HSV infections after BMT	>8.0	>8.0	>8.0	46.1	0.2
11	HSV-1 1824/06	HSV pneumonia under IS	>8.0	>8.0	>8.0	43.1	0.7
12	HSV-1 230/07	Not available	>8.0	>8.0	>8.0	38.1	2.2
13	HSV-1 272/07	Not available	>8.0	>8.0	>8.0	22.0	1.8
14	HSV-1 949/07	HSV infections in ALL	>8.0	>8.0	>8.0	41.8	2.5
15	HSV-1 1366/08	Mucositis after BMT	5.8	>8.0	>8.0	40.6	0.1
16	HSV-1 2415/08	HSV infections after BMT	>8.0	>8.0	>8.0	19.0	0.9
17	HSV-2 548/05	Recurrent gluteal HSV infections	4.1	>8.0	3.6	10.8	0.2
18	HSV-2 1080/05	Recurrent herpes genitalis	>8.0	>8.0	2.7	24.4	0.3
19	HSV-2 1541/05	Recurrent anal HSV infections in CLL	>8.0	>8.0	>8.0	18.0	3.6
20	HSV-2 2155/06	Recurrent HSV infection of skin under IS	>8.0	>8.0	>8.0	23.0	4.3
21	HSV-1 308/08	Herpes simplex	0.1	0.5	0.2	14.4	0.7
22	HSV-1 338/08	Eczema herpeticum	0.3	0.8	0.4	15.3	2.8
23	HSV-1 379/08	Herpes simplex	0.2	0.4	0.2	19.1	0.4
24	HSV-1 473/08	Herpes simplex	0.2	0.4	0.3	17.4	1.0
25	HSV-1 Kupka, TK+	–	0.1	0.2	1.1	27.9	0.5
26	HSV-1 Mac Intyre, TK+	–	0.1	0.2	0.2	33.7	0.2
27	HSV-1 KOS, TK+	–	0.2	0.2	0.5	31.2	0.3
28	HSV-1 Cheng, TK-	–	>8.0	>8.0	>8.0	31.5	1.2
29	HSV-1 Fuld, TK-	–	>8.0	>8.0	>8.0	32.7	1.0
30	HSV-2 MS, TK+	–	0.2	>8.0	1.2	12.0	0.3
31	HSV-2 G, TK+	–	0.2	>8.0	0.4	32.5	1.1

ALL: acute lymphoblastic leukemia; BMT: bone marrow transplantation; BVDU: brivudin; CDV: cidofovir; CLL: chronic lymphocytic leukemia; CTX: cardiac transplantation; FOS: foscarnet; IS: immunosuppression; TK: thymidine kinase.

[a] Mean IC_{50} values of the sensitive HSV control strains: ACV, HSV-1 0.5 ± 0.3 μg/ml (2.2 ± 1.3 μM), HSV-2 0.4 ± 0.3 μg/ml (1.8 ± 1.3 μM); BVDU, HSV-1 0.2 ± 0.1 μg/ml (0.6 ± 0.3 μM), HSV-2 >8.0 μg/ml (>24.0 μM); PCV, HSV-1 0.3 ± 0.2 μg/ml (1.2 ± 0.8 μM), HSV-2 0.4 ± 0.2 μg/ml (1.6 ± 0.8 μM); FOS, HSV-1 30.8 ± 5.4 μg/ml (101.6 ± 17.8 μM), HSV-2 34.8 ± 19.8 μg/ml (114.8 ± 65.3 μM); CDV, HSV-1 0.8 ± 0.6 μg/ml (3.0 ± 2.2 μM), HSV-2 0.8 ± 0.6 μg/ml (3.0 ± 2.2 μM).

7 min. Amplified DNA fragments were purified using the QIAquick PCR Purification Kit (Qiagen). Afterwards, viral DNA was quantified spectrophotometrically. An amount of 200 ng DNA/μl was used for sequencing. Sequencing reactions of purified PCR products were performed by means of the cycle sequencing method. First, HSV subgenomic regions were sequenced using DYEnamic ET Terminator Cycle Sequencing Kit (Amersham Biosciences Europe, Freiburg, Germany) and 10 μM oligonucleotide primers (Table 2).

Table 2
Design of primer pairs for genotyping of thymidine kinase (TK) and DNA-polymerase (pol) genes of HSV-1 and 2.

HSV DNA fragment (amplicon in bp)	Function	Sequence 5′ → 3′	Nucleotides of HSV genome
HSV-1 TK	TK-1	TTTTATTCTGTCCTTTTATTGCCGTCA	46,607–46,634
Fragment 1 (701)	TK-R2	GTAGCGGCGGGAGGACACGATGGG	47,308–47,285
HSV-1 TK	TK-2	ACGATGTTTGTGCCGGGCAAGGTC	47,192–47,215
Fragment 2 (716)	TK-R1	CGAGCGACCCTGCAGCGACCCGCT	47,907–47,884
HSV-2 TK	TK-1	TTTTATTCTGTCCTTTTATTGCCGTCA	46,805–46,832
Fragment 1 (699)	TK-R3	TATCGCCTCCCTGCTGTGCTACCC	47,503–47,480
HSV-2 TK	TK-3	ACCAGGTTCGTGCCGGGCGCGGTC	47,387–47,410
Fragment 2 (719)	TK-R1	CGAGCGACCCTGCAGCGACCCGCT	48,105–48,082
HSV-1 DNA pol	HSV-1-pol-1	ATCCGCCAGACAAACAAGGCCCTT	62,655–62,678
Fragment 1 (1041)	HSV-1-pol-R4	CCCCACCCTCGTACTTCTTGATGG	63,695–63,672
HSV-1 DNA pol	HSV-1-pol-2	GTCCGAAGCGGGCGTGTGCTGTCG	63,623–63,646
Fragment 2 (1033)	HSV-1-pol-R2	GGCCGTCGTAGATGGTGCGGGTG	64,655–64,633
HSV-1 DNA pol	HSV-1-pol-3	CCATCTGGAGCTCTCGGCCGTCGC	64,588–64,611
Fragment 3 (1157)	HSV-1-pol-R3	GCTAAAACAGCAGGTCGACCAGGG	65,744–65,721
HSV-1 DNA pol	HSV-1-pol-4	AGATGCTCATCAAGGGCGTGGATC	65,652–65,675
Fragments 4 and 5 (1043/1046)	HSV-1-pol-4a	GTAAGATGCTCATCAAGGGCGTGGATC	65,649–65,675
	HSV-1-pol-R1	GGCTCATAGACCGGATGCTCAC	66,694–66,673
HSV-2 DNA pol	HSV-2-pol-1	CCCGGGCGCGGGTCCGCCGGTAAG	63,124–63,147
Fragment 1 (1046 bp)	HSV-2-pol-R4	GTGGTGGCGTCGACGCCCCCTCG	64,169–64,143
HSV-2 DNA pol	HSV-2-pol-2	GTGCGAAGCGGGGCGCGCGCTGGCC	64,084–64,107
Fragment 2 (1042 bp)	HSV-2-pol-R2	GGATCTGCTGGCCGTCGTAGATGG	65,125–65,102
HSV-2 DNA pol	HSV-2-pol-3	CCACCTGGAGCTTTCCGCCGTCGC	65,049–65,072
Fragment 3 (1176 bp)	HSV-2-pol-R3	GTATCGTCGTAAAACAGCAGGTCG	66,224–66,201
HSV-2 DNA pol	HSV-2-pol-4	CCATCAAGGGCGTGGATCTGGTGCG	66,123–66,155
Fragment 4 (1045 bp)	HSV-2-pol-R1	GGCTCATCGATCGGATGCTGAC	67,167–67,146

Table 3
Non-synonymous mutations of the thymidine kinase gene in acyclovir (ACV)-resistant (nos. 1–20), ACV-sensitive (nos. 21–24) HSV islolates and reference strains (nos. 25–31). Novel mutations are in bold. Mutations with unclear significance are in Italics.

No.	Viral strain	Polymorphism-associated mutations	Resistance-associated mutations
1	HSV-1 22/01	N23S, K36E, R89Q, A265T	**Y53H**, R163H
2	HSV-1 354/02	C6G, N23S, K36E, L42P, G240E, A265T, R281Q	–
3	HSV-1 707/02	C6G, N23S, K36E, L42P, G240E, A265T, R281Q	–
4	HSV-1 737/03	C6G, N23S, K36E, L42P, G240E, R281Q	–
5	HSV-1 2245/03	N23S, K36E, **I78F**, R89Q	Homopolymer G region nt 429–437, insertion of G
6	HSV-1 1686/04	C6G, N23S, K36E, L42P, A265T	**Y172C**
7	HSV-1 331/05	N23S, K36E	Deletion of C nt 665–670
8	HSV-1 2016/05	N23S, K36E, A192V, G251C, A265T, V267L, P268T, D286E	*R41H*, **L315S**
9	HSV-1 164/06	N23S, K36E, **R89H**, A265T	**P84L**, **R163G**, **P173deletion**
10	HSV-1 1678/06	N23S, K36E, **I78F**, R89Q	Homopolymer G region nt 429–437, insertion of G
11	HSV-1 1824/06	N23S, K36E, L42P	Homopolymer C region nt 547–554, deletion of C
12	HSV-1 230/07	N23S, K36E, R89Q, G240E, A265T	**Q104stop**
13	HSV-1 272/07	N23S, K36E, R89Q	Homopolymer G region nt 429–437, insertion of G
14	HSV-1 949/07	N23S, K36E, G240E, A265T, R281Q	**A174P**
15	HSV-1 1366/08	N23S, K36E, R89Q, A265T	**Y83N**
16	HSV-1 2415/08	N23S, R89Q	Homopolymer C region nt 547–554, insertion of C
17	HSV-2 548/05	–	*G39E*
18	HSV-2 1080/05	–	**G25A**, **Y53N**, **R221H**
19	HSV-2 1541/05	*G39E*	Homopolymer C region nt 550–556, deletion of C, **R86P**
20	HSV-2 2155/06	–	**Y133F**
21	HSV-1 308/08	V14D, N23S, K36E, R89Q, A265T	–
22	HSV-1 338/08	V14D, N23S, K36E, R89Q, A265T	–
23	HSV-1 379/08	N23S, K36E, **G61A**, R89Q, G251C, A265T, V267L, P268T, D286E, N376H, **T350S**	–
24	HSV-1 473/08	V14D, N23S, K36E, R89Q, A265T	–
25	HSV-1 Kupka	C6G, N23S, K36E, L42P, A265T, V348I	–
26	HSV-1 Mac Intyre	**S19P**, N23S, K36E, L42P, **I78F**, G251C, A265T, V267L, P268T, D286E, **R293W**, N376H	–
27	HSV-1 KOS	C6G, N23S, K36E, L42P, **I78F**, A265T, V348I	–
28	HSV-1 Cheng	N23S, K36E, R89Q	I143V, **A186P**, **insertion of T after nt 650**
29	HSV-1 Fuld	N23S, K36E, R89Q, A265T	I143V, R256W
30	HSV-2 MS	*G39E*	–
31	HSV-2 G	–	–

Sequencing reaction mixture with a total volume of 10 μl consisted of 1.0–5 μl of the purified viral DNA, 0.5–0.75 μl primers, and 2 μl sequence reaction mix containing DNA pol and labeled ddNTPs. After initial incubation at 95 °C for 3 min to denature the template DNA, the thermal conditions of amplification followed 25 cycles of 95 °C for 20 s, 50 °C for 15 s, and 60 °C for 60 s. A final extension step was done at 60 °C for 10 min. After precipitation and washing with ethanol, the DNA fragments were analyzed on the ABI PRISM™ 310 Genetic Analyzer (Applied Biosystems, Darmstadt, Germany). Sequences of the PCR products were verified on both DNA strands. Primary DNA sequence assembly and analysis were performed using ABI PRISM™ DNA Sequencing Software Version 2.1.2 (Applied Biosystems). Finally, sequencing results were compared with published sequences of the reference strains HSV-1 17 and HSV-2 HG52 using the software MEGA 3.1. All specified sequence positions of single nucleotide polymorphism corresponded to nucleotide positions in the reference strains.

3. Results

In 18 out of 20 ACV-resistant viral isolates, IC_{50} values of >8.0 μg/ml ACV could be calculated (Table 1, nos. 1–20). Two strains, one HSV-1 and one HSV-2 strain had IC_{50} values of 5.8 and 4.1 μg/ml ACV, respectively. In all isolates except of one HSV-1 strain (IC_{50} 2.3 μg/ml), the IC_{50} values of brivudin were also calculated as >8.0 μg/ml. For PCV, 17 strains had an IC_{50} of >8.0 μg/ml and in 3 strains, 1 HSV-1 and 2 HSV-2 isolates, the IC_{50} ranged between 2.3 and 3.6 μg/ml. These results illustrate that all ACV-resistant strains were cross-resistant to brivudin and PCV. Seventeen of these viral strains were sensitive to FOS resulting in IC_{50} values between 9.3 and 46.1 μg/ml. Three HSV-1 strains, which were isolated from one patient, had IC_{50} values of >100 μg/ml FOS. That is why these isolates were considered resistant to this drug. Testing of all ACV-resistant strains to CDV resulted in IC_{50} values between 0.1 and 4.4 μg/ml suggesting susceptibility. In comparison, the four ACV-sensitive HSV-1 isolates had IC_{50} values between 0.1 and 0.3 μg/ml ACV, 0.4 and 0.8 μg/ml brivudin, 0.2 and 0.4 μg/ml PCV, 14.4 and 19.1 μg/ml FOS, and 0.4 and 2.8 μg/ml CDV (Table 1; nos. 21–24). The TK-positive reference strains HSV-1 Kupka, HSV-1 Mac Intyre, HSV-1 KOS, HSV-2 MS and HSV-2 G were also evaluated as sensitive to ACV (IC_{50} 0.1–0.2 μg/ml), sensitive (HSV-1, IC_{50}: 0.2 μg/ml) or resistant (HSV-2, IC_{50}: >8.0 μg/ml) to brivudin, sensitive to PCV (IC_{50} 0.2–1.2 μg/ml), FOS (IC_{50} 12.0–33.7 μg/ml), and CDV (IC_{50} 0.2–1.1 μg/ml; Table 1, nos. 25–28 and 30, 31). By contrast, the TK-negative HSV-1 reference strains Cheng and Fuld were resistant to ACV, brivudin, PCV (IC_{50} >8.0 μg/ml) and sensitive to FOS (IC_{50} 31.5–32.7 μg/ml) and CDV (IC_{50} 1.0–1.2 μg/ml; Table 1, nos. 28 and 29).

The genotypic correlates demonstrated in Tables 3 and 4 were established by sequencing of the TK and DNA pol genes. In the TK gene, all 25 HSV-1 strains included in this study contained two or more polymorphism-associated non-synonymous mutations (Table 3; nos. 1–16, 21–29). The mutation N23S was found in all strains, K36E in 24, A265T in 17, R89Q in 13, L42P in 8, C6G in 6, G240E in 5, I78F and R281Q in 4, V14D, G251C, V267L, P268T and D286E in 3, V348I and N376H in 2 strains and the mutations S19P, G61A, R89H, A192 V, R293W, T350S were observed in 1 strain each. By contrast, only one non-synonymous mutation (G39E), presumably considered polymorphism-associated, was detected in the TK gene out of all ACV-resistant HSV-2 isolates (Table 3, nos. 17–20) and HSV-2 reference strains (Table 3; 30 and 31). Following frameshift and non-synonymous mutations, conferring most likely resistance to ACV and another nucleoside compounds, were detected in the UL23 of the ACV-resistant HSV-1 and 2 isolates

Table 4
Non-synonymous mutations of the DNA-polymerase gene in acyclovir (ACV)-resistant (nos. 1–20), ACV-sensitive (nos. 21–24) HSV islolates and reference strains (nos. 25–31). Novel mutations are in bold.

No.	Viral strain	Polymorphism-associated mutations	Resistance-associated mutations
1	HSV-1 22/01	S33G, **K104Q**	–
2	HSV-1 354/02	S33G, **A330R**, V905M	**D672N**
3	HSV-1 707/02	S33G, **A330R**, V905M	**D672N**
4	HSV-1 737/03	S33G, **A330R**, V905M	**D672N**
5	HSV-1 2245/03	S33G, V905M, P1124H, **A1168V**, T1208A	
6	HSV-1 1686/04	S33G, V905M	–
7	HSV-1 331/05	S33G, **A229T**, **A330R**	–
8	HSV-1 2016/05	S33G, V905M	–
9	HSV-1 164/06	S33G, A566T	–
10	HSV-1 1678/06	S33G, V905M	–
11	HSV-1 1824/06	S33G, P1124H, T1208A	–
12	HSV-1 230/07	S33G, V905M, T1208A	–
13	HSV-1 272/07	S33G, **A330R**, V905M	–
14	HSV-1 949/07	S33G, **A330R**, V905M, **D1103H**, T1208A	–
15	HSV-1 1366/08	S33G, V905M	–
16	HSV-1 2415/08	**A330R**, V905M	–
17	HSV-2 548/05	A9T, P15S, **R41H**, **A232T**, **L995F**, **I1026S**	–
18	HSV-2 1080/05	A9T	–
19	HSV-2 1541/05	P15S, E139K, T801P	–
20	HSV-2 2155/06	A9T, P15S, **Q34H**	–
21	HSV-1 308/08	S33G, **A78V**, P1124H, T1208A	–
22	HSV-1 338/08	S33G, P1124H	–
23	HSV-1 379/08	S33G	–
24	HSV-1 473/08	S33G, P1124H	–
25	HSV-1 Kupka	S33G, V905M, **L1166W**	–
26	HSV-1 Mac Intyre	S33G, **F248L**, T639I, A1203T	–
27	HSV-1 KOS	**A330R**, V905M	–
28	HSV-1 Cheng	**A20V**, S33G, **D72N**, **A78D**	**Deletion of C nt 301**
29	HSV-1 Fuld	**A27T**, S33G, **A330R**, **V743M**, V905M	
30	HSV-2 MS	A9T, **P15R**	–
31	HSV-2 G	**N286Y**, **P880H**	–

(Table 3; nos. 1, 5–20): insertion of G at the nucleotide positions 429–437; insertion of C at the nucleotide positions 547–554; deletion of C at the nucleotide positions 547–554, 550–556 and 665–670; G25A; G39E; R41H; Y53H; Y53N; Y83N; P84L; R86P; Q104stop; Y133F; R163H; Y172C; P173deletion; A174P; R221H; L315S. No resistance-associated non-synonymous mutations were found in the TK gene of the ACV-resistant HSV-1 isolates nos. 2–4, which also had phenotypic resistance to FOS. The TK-negative HSV-1 reference strains Cheng and Fuld (Table 3; nos. 28 and 29) had the non-synonymous mutations I134V, A186P, R256W, and the insertion of T after the nucleotide at position 650, which could have accounted for the ACV resistance.

All HSV-1 and 2 strains included in this study had at least one polymorphism-associated non-synonymous mutation in the DNA pol gene. In 25 HSV-1 strains (Table 4; nos. 1–16, 21–29), the following mutations were detected: S33G in 23, V905M in 15, A330R in nine, P1124H and F1208A in 5, and A20V, A27T, D72N, A78D, A78V, K104Q, A229T, F248L, A566T, T639I, V743M, D1103H, L1166W, A1168V, A1203T in 1 strain each. Likewise, different polymorphism-related mutations were found in the DNA pol gene of six HSV-2 strains (Table 4; nos. 17–20, 30 and 31): A9T in 4, P15S in 3 strains, and P15R, Q34H, R41H, E139K, A232T, N286Y, T801P, P880H, L995F, I1026S in 1 strain each. Three strains of HSV-1 resistant to FOS (Table 4; nos. 2–4), had the additional mutation D672N, which is most likely responsible for the resistance against this drug. Surprisingly, the HSV-1 reference strain Fuld contained a deletion of C at the nucleotide position 301 although this TK-negative viral strain did not show any phenotypic resistance to FOS.

4. Discussion

In this study, the phenotypic and genotypic resistance of 20 ACV-resistant HSV strains was determined. All viral strains were cross-resistant to brivudin and PCV. However, it should be considered that brivudin does not act against HSV-2 because of the missing substrate specificity of HSV-2-specific thymidylate kinase activity (Fyfe, 1982). The cross-resistance of the nucleoside analogues ACV, PCV and brivudin can be explained by the similar mechanism of action, but differences are possible in clinical practice dependent on the bioavailability of drugs. In few studies, HSV isolates resistant to ACV but susceptible to PCV have been reported in connection with either an altered TK (Boyd et al., 1993) or mutation in viral DNA pol gene (Chiou et al., 1995). After high-dose selection with brivudin, different resistance-associated phenotypes against brivudin and ACV have been demonstrated in HSV-1 TK mutants (Andrei et al., 2005) suggesting drug-specific mutations of TK gene. Three sequential ACV-resistant HSV-1 isolates of a single BMT recipient (nos. 2–4) were also resistant to FOS due to IC_{50} values of >128 μg/ml. In general, HSV strains with $IC_{50} \geq 100$ μg/ml were defined as resistant (Safrin et al., 1991). This drug does not show cross-resistance to ACV in most cases. Since the resistant strains were collected from the same immunocompromised patient, the findings indicate that FOS-resistant HSV strains can only be found in rare cases. Because of the different mode of action, this drug does not show cross-resistance to ACV and is, therefore, recommended as alternative compound in ACV-resistant HSV infections (Safrin et al., 1990). For CDV, that may also be considered as alternative in ACV-resistant HSV infections (Tan and Goh, 2006), most strains of this study revealed IC_{50} values between 0.1 and 2.0 μg/ml suggesting sensitivity of this drug. Only few strains had an increased IC_{50} up to the value 4.3 μg/ml. However, considering the results of the sensitive viral controls, these values are in accordance with susceptibility to CDV. Cidofovir resistance of HSV developing in vivo has been described only in rare cases (Wyles et al., 2005). Comparison of IC_{50} for CDV in untreated HSV-1 clinical isolates exhibited a mean value of 2.3 ± 1.5 μg/ml (Wyles et al., 2005), that is comparable with the results of this study. For the determination of phenotypic resistance of HSV, it is a general problem that cut-off levels for antiviral drugs, in particular for ACV, still wait on international standardization.

The genotyping findings confirmed that the TK gene of HSV-1 has an uncommonly high polymorphism (Morfin et al., 2000). Between 2 and 11 polymorphism-associated non-synonymous mutations were found in 4 ACV-sensitive and 20 resistant HSV-1 isolates. Moreover, the HSV-1 reference strain Mac Intyre contained 12 non-synonymous mutations associated with the polymorphism of the TK gene. Altogether, the overwhelming majority of the amino acid substitutions in the TK gene have been reported previously (Chibo et al., 2004; Duan et al., 2009; Frobert et al., 2008; Kudo et al., 1998; Morfin et al., 2000; Morfin and Thouvenout, 2003). However, to our knowledge, the amino acid changes S19P, G61A, I78F, R89H, R293W and T350S, found in the HSV-1 isolates 2245/03, 164/06, 1678/06 and 379/08 as well as the reference strains Mac Intyre and KOS (Table 3), were observed first time in this study. A substitution similar to Q89R has been reported by Kudo et al. (1998). According to the reference strain chosen, the amino acid at the position 89 could be Q (KOS) or R (strain 17). Concerning S19P and I78F, substitutions at the same positions (A19V and D78N) have been reported by Chibo et al. (2004) in ACV-sensitive HSV-2 strains. In contrast to HSV-1 strains, only one HSV-2 isolate contained a polymorphism-related mutation of the TK gene, which was also detected in the reference strain HSV-2 MS. To date, only few polymorphism-related amino acid substitutions have been reported for the TK gene of HSV-2 in previous studies (Chibo et al., 2004). The findings are in agreement with the fact that the variability of the HSV-1 genome is about fourfold higher than that of HSV-2 (Chiba et al., 1998), most likely due to the higher content of guanine and cytosine. Most of polymorphism-associated mutations are located outside active gene centers, but only few of them (e.g. G61A, R89Q, R281Q and D286E) involve conserved gene regions. In conclusion, more HSV-1 as well as HSV-2 isolates have to be examined for the polymorphism of TK gene in further studies to facilitate the interpretation of mutations connected with resistance.

In 8 of 20 ACV-resistant HSV isolates, nucleotide insertions and deletions, responsible for a frameshift and the synthesis of a non-functional TK, were found. These alterations, most often located in homopolymer C regions at nucleotide positions 547–554 and 550–556 as well as homopolymer G region at nucleotide positions 429–437, are well described in the literature (Chibo et al., 2004; Duan et al., 2009; Frobert et al., 2008; Gaudreau et al., 1998; Morfin et al., 2000; Saijo et al., 2002; Stránská et al., 2005; Suzutani et al., 2000; Suzutani et al., 2003). However, the majority of ACV-resistant HSV isolates (9 out of 20) contained one or more non-synonymous nucleotide substitutions in the TK gene, which were not known as part of gene polymorphism. Thus, association with resistance was most likely for the amino acid substitutions Y53H, Y83N, P84L, R163G, R163H, Y172C, A174P (HSV-1) and Y53N, R86P, R221H (HSV-2) located in gene regions conserved among herpesviridae family (Balasubramaniam et al., 1990) or representing ATP and nucleoside binding sites (Graham et al., 1986). With the exception of the substitution R163H that has been reported by Bae et al. (2006), all of these resistance-related single non-synonymous nucleotide substitutions of the TK gene have not been described in the literature to date (Table 3). Similar substitutions such as E83K (Morfin et al., 2000), P84S (Saijo et al., 2002), D162A (Chibo et al., 2004), Y172F (Pilger et al., 1999), and P85C (Chibo et al., 2004) have been reported previously in ACV-resistant strains. In one HSV-1 strain, a novel nucleotide substitution resulted in a stop codon that signals a termination of translation. A considerable part of amino acid substitutions could only be defined as resistance-associated in connection with the phenotypic findings. This is relevant for the mutations L315S (HSV-1), G25A and Y133F (HSV-2), that could not be assigned to active or conserved gene regions and have not been reported in previous studies (Table 3). Furthermore, the amino acid changes R41H and G39E have unclear significance. In this study, the substitution R41H was only found in one ACV-resistant HSV-1 strain together with the novel substitution L315S. In addition, R41H has been described recently as the only substitution most likely associated with ACV resistance in an HSV-1 strain which caused herpes simplex encephalitis (Schulte et al., 2010). In the literature, there are 3 reports on 15 HSV-1 isolates that contain the substitution R41H and have been tested for ACV sensitivity. Eleven of them were ACV-resistant (IC_{50} >3 μg/ml), one showed an IC_{50} <10 μg/ml, and three were ACV-sensitive (Bestman-Smith et al., 2001; Chibo et al., 2004; Duan et al., 2009; Stránská et al., 2004). The substitution G39E could be attributed to one sensitive and two resistant HSV-2 isolates. In one of these resistant HSV-2 isolates (HSV-2 548/05), no other mutation conferring resistance was present. However, several novel mutations were found in the DNA pol gene (R41H, A232T, L995F, I1026S). One of these might be associated to resistance to ACV, without cross-resistance to FOS. In clinical practice, it is a problem to define a questionable resistance of HSV isolates on the basis of these genotyping results alone. The availability of sequential HSV isolates of the same patient may overcome some diagnostic problems of attributing new point mutations either to resistance or polymorphism. In addition, studies by Frobert et al. (2007) suggest that substitutions located outside the TK-conserved sites may be associated with resistance to ACV, but their role needs to be confirmed by further experiments, e.g. site-directed mutagenesis experiments in order to establish a reliable database of TK gene mutations related to ACV resistance.

The HSV TK is involved in about 95% of ACV resistance cases (Morfin and Thouvenout, 2003) and the remaining can be caused by mutations of the DNA pol gene, whose analysis was also included in this study. The results demonstrate that the DNA pol gene of both HSV-1 and HSV-2 shows a considerable dimension of polymorphism. Nearly three quarters of the polymorphism-related non-synonymous mutations in ACV-resistant and sensitive strains have not been described previously (Chibo et al., 2004; Frobert et al., 2008). Most of them are located outside conserved regions (Gilbert et al., 2002). Substitutions similar to A330R (R330A) and P15R (S15P) have been reported by Chibo et al. (2004). Apart from polymorphism, the substitutions A20V, A27T, D72N, and A78D, found in the TK-negative HSV-1 reference strains Fuld and Cheng, might also confer the ACV resistance of these viruses. Three HSV-1 isolates had phenotypic resistance to the nucleoside analogues ACV, brivudin, PCV as well as the pyrophosphate compound FOS. In these strains, the novel substitution D672N, located outside conserved regions and never described as part of polymorphism, has to be considered to be associated to cross-resistance to FOS and ACV.

In immunocompromised patients, resistance of HSV isolates to antiviral drugs is of increasing importance. Thus, virological laboratories have to optimize methods for testing resistance. By the determination of IC_{50} value of antiviral compound, phenotypic methods allow a clear interpretation of laboratory findings, but they are time-consuming because of the necessity to isolate viral strains in cell culture. Using genotyping procedures, resistance can be detected within a shorter delay. However, only frameshift mutations can be interpreted without doubt and numerous amino acid substitutions are diagnostically less conclusive. To improve the significance of testing resistance of HSV to antiviral drugs, genotypic methods should be accompanied by attempts to establish a viral isolate in cell culture. If substitutions of amino acids cannot be assigned clearly to resistance or genotypic findings suggest a mixture of viral strains (Nugier et al., 1992) questionable resistance should be clarified by phenotypic methods.

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