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In vitro selection of drug-resistant varicella-zoster virus (VZV) mutants (OKA strain): differences between acyclovir and penciclovir?

G. Andrei*, E. De Clercq, R. Snoeck

Rega Institute for Medical Research, K.U. Leuven, 3000 Leuven, Belgium

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Abstract

Varicella-zoster virus (VZV) mutants were isolated under the pressure of different classes of antiviral compounds: (i) drugs that depend on the viral thymidine kinase (TK) for their activation, i.e. acyclovir (ACV), brivudin (BVDU), penciclovir (PCV) and sorivudine (BVaraU); (ii) drugs that are independent of the viral TK for their activation, i.e. 2-phosphonylmethoxyethyl (PME) derivatives of adenine (PMEA, adefovir) and 2,6-diaminopurine (PMEDAP); and (iii) drugs that do not require any metabolism to inhibit the viral DNA polymerase, i.e. foscarnet (PFA). Drug-resistant virus strains were obtained by serial passage of the OKA strain in human embryonic lung (HEL) fibroblasts and the different drug-resistant mutants were subsequently evaluated for their in vitro susceptibility to a broad range of antiviral drugs. Virus strains emerging under the pressure of ACV, BVDU and BVaraU were cross-resistant to all drugs that depend on the viral TK for activation, but remained susceptible to the acyclic nucleoside phosphonates (i.e. PMEA, PMEDAP and the 3-hydroxy-2-phosphonylmethoxypropyl derivatives of adenine (HPMPA) and cytosine (HPMPC, cidofovir)) and PFA. In contrast, the virus strains selected under pressure of PCV were resistant to PCV, ACV, PMEA and PFA; but not BVDU, BVaraU, GCV, HPMPC or HPMPA. Similar patterns of drug susceptibility were noted for the virus strains selected under the pressure of PMEA or PFA, pointing to an alteration in the viral DNA polymerase as basis for the resistant phenotype selected by PCV, as well as PMEA and PFA. In contrast, the resistant phenotype selected by ACV as well as BVDU and BVaraU may be attributed primarily to mutations in the viral TK gene. Our data thus indicate that ACV and PCV select in vitro for different drug-resistant VZV phenotypes; whether this is also the situation in vivo remains to be investigated.

Keywords: Varicella-zoster virus; Drug-resistant mutants; Acyclovir; Penciclovir

1. Introduction

Varicella-zoster virus (VZV), a member of the herpesvirus family, is responsible for a primary disease (varicella or chickenpox) as well as for a recurrent disease (zoster or shingles) following reactivation of the virus. In immunocompetent patients, the course of varicella is generally benign, however, in immunocompromised patients (particularly those with AIDS, transplant recipients and cancer patients) VZV infections can be associated with significant morbidity and mortality due to disseminated infections or chronic reactivations (Masaoka et al., 1993; Arvin, 1996; Snoeck et al., 1999). A wide variety of neurological complications may occur in immunocompetent and immunocompromised patients. Post-herpetic neuralgia (PHN) is the most frequent cause of acute and chronic morbidity associated with zoster, particularly in the elderly (Lojeski and Stevens, 2000; Schmader, 2001).

The current treatment of choice for VZV infections in patients at risk is based on the use of acyclovir (ACV) (Snoeck et al., 1999; Wutzler, 1997). The low oral bioavailability of acyclovir has stimulated the development of its oral prodrug form, valaciclovir (VACV). Famciclovir (FCV), the oral prodrug of penciclovir (PCV), is a nucleoside analogue that is closely related to ACV and that has also been approved for the treatment of VZV infections. (*E*)-5-(2-bromovinyl)-2′-deoxyuridine (BVDU, brivudin) and its arabinofuranosyl analogue (BVaraU, sorivudine) are very potent and selective inhibitors of VZV replication in vitro and have been examined in several clinical studies. Brivudin has been licensed in some countries, including Germany (as Zostex®), for the treatment of VZV infections. Both brivudin and sorivudine can be metabolized to (*E*)-5-(2-bromovinyl)uracil, which interferes with the degradation of the anticancer drug 5-fluorouracil by blocking dihydropyrimidine dehydrogenase (DPD) (Desgranges et al.,

* Corresponding author. Fax: +32-16-33-7340.
E-mail address: graciela.andrei@rega.kuleuven.ac.be (G. Andrei).

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1986). Therefore, concomitant treatment with 5-fluorouracil is contra-indicated. In Japan, where sorivudine was initially licensed in 1993 for the treatment of herpes zoster, the compound was withdrawn after several drug-related deaths occurred in patients concomitantly treated with 5-fluorouracil.

Several case reports have appeared on the emergence of ACV-resistant (ACV^r) VZV mutants following long term ACV therapy in immunocompromised patients (Jacobson et al., 1990; Linnemann et al., 1990; Pahwa et al., 1990; Snoeck et al., 1994; Boivin et al., 1994; Talarico et al., 1993; sFillet et al., 1998). Similarly to herpes simplex virus (HSV), most of the ACV^r VZV strains that have been isolated from patients are thymidine kinase-deficient (TK^-) (Linnemann et al., 1990; Snoeck et al., 1994; Talarico et al., 1993; Morfin et al., 1999), and only a few isolates were found to have a TK with altered substrate specificity (Jacobson et al., 1990; Boivin et al., 1994). TK-deficient ACV^r mutants are usually cross-resistant to all other drugs dependent on viral TK activity. Foscarnet has been shown to be alternative treatment for ACV^r VZV infections associated with mutants at the TK gene level (Safrin et al., 1991; Smith et al., 1991). However, resistance to foscarnet associated with mutations in the VZV DNA polymerase gene has been recently described in immunocompromised patients (Fillet et al., 1995; Visse et al., 1998).

The aim of the present study was to characterize phenotypically several VZV mutants selected under the pressure of different classes of drugs: (i) drugs that are dependent on the viral TK for their activation, i.e. ACV, PCV, BVDU, BVaraU; (ii) drugs that are independent of the viral TK for their activation, i.e. the acyclic nucleoside phosphonates (ANPs) and (iii) drugs that do not require any metabolism to inhibit the viral DNA polymerase, i.e. the pyrophosphate analogue foscarnet (PFA).

Human embryonic lung (HEL) fibroblasts (ATCC CCL137) were maintained in minimum essential medium (MEM) supplemented with 10% heat-inactivated fetal calf serum (FCS), 1% L-glutamine and 0.3% sodium bicarbonate. The VZV laboratory strain OKA (ATCC VR-795) was used. Virus stocks were prepared in HEL cells; When 70% cytopathogenic effect was obtained, the cells were trypsinized, resuspended in medium containing 10% DMSO and stored in aliquots at −80 °C. The HSV-1 and HSV-2 reference strains KOS and G were also used.

The sources of the compounds were as follows: acyclovir (ACV, 9-(2-hydroxyethoxymethyl)guanine; MW 237) from GlaxoSmithKline; the pyrophosphate analogues foscarnet (PFA, phosphonoformate sodium salt; MW 300, hexahydrate, trisodium salt) and phosphonoacetic acid (PAA; MW 371) from Sigma Chemicals, St. Louis, MO; PMEA (adefovir, 9-[2-(phosphonylmethoxyethyl)adenine]; MW 293, sodium salt) and HPMPC (cidofovir, (*S*)-1-(3-hydroxy-2-phosphonylmethoxypropyl)cytosine; MW 301, sodium salt) from Gilead Sciences, CA; HPMPA ((*S*)-9-(3-hydroxy-2-phosphonylmethoxypropyl)adenine; MW 325, sodium salt) and PMEDAP (9-(2-phosphonylmethoxyethyl)-2,6-diaminopurine; MW 310, sodium salt) from A. Holý, Institute of Organic Chemistry and Biochemistry, Academy of Sciences of the Czech Republic, Prague, Czech Republic; brivudin (BVDU, (*E*)-5-(2-bromovinyl)-1-(β-D-2-deoxyribofuranos-1-yl-uracil); MW 333) from P. Herdewijn, Rega Institute for Medical Research, Leuven, Belgium; sorivudine (BVaraU, 1-β-D-arabinofuranosyl-(*E*)-5-(2-bromovinyl)uracil; MW 349) from Yamasa Shoyu, Coshi, Japan; ganciclovir (GCV, 9-(1,3-dihydroxy-2-propoxymethyl)guanine; MW 254) from Syntex, Palo Alto, CA; penciclovir (PCV, 9-(4-hydroxy-3-hydroxymethylbut-1-yl)guanine; MW 253) from Antiinfectives Research, Hoechst AG, Frankfurt, Germany.

The drug-resistant virus strains were obtained by serial passage of cell-associated VZV reference OKA strain in the presence of increasing concentrations of the compounds, starting at the IC_{50} values. The initial multiplicity of infection to start the procedure of selection with the different drugs was 0.05. The cell cultures were incubated until virus CPE was about 70%, and the drug concentration was increased by two-fold with every subsequent passage of the virus. After reaching a concentration of 100 μg/ml for PMEA and PFA and of 10 μg/ml for ACV, PCV, BVDU, BVaraU, HPMPC, HPMPA and GCV, a last passage was done in drug-free medium in order to obtain the virus stock. The various drug-resistant VZV strains, denoted ACV^r, $BVDU^r$, $BVaraU^r$, PFA^r, $PMEA^r$, $PMEDAP^r$ and PCV^r, were titrated and subsequently evaluated for their sensitivity in vitro. In the case of PCV and PFA, the selection of drug-resistant strains was performed in duplicate, leading to two independent mutants for each drug, denoted PFA^r/A, PFA^r/B, PCV^r/A and PCV^r/B. The procedure followed for the selection of HSV-1 and HSV-2 drug-resistant strains has been previously reported (Andrei et al., 1995a, 1997).

The drug susceptibilities of drug-resistant VZV mutants were determined by viral plaque reduction assays in HEL cells using cell-associated virus (Andrei et al., 1995b). Confluent HEL cells grown in 96-well microtiter plates (2×10^4 cells/ml to 3×10^4 cells/ml) were infected with the different VZV strains at 20 PFU. After a 2-h incubation period, residual virus was removed and the infected cells were further incubated with MEM supplemented with 2% heat-inactivated FCS, 1% L-glutamine and 0.3% sodium bicarbonate containing serial dilutions of the test compounds (in duplicate). After 5 days of incubation at 37 °C in 5% CO_2 atmosphere, virus plaque formation was determined and the 50% inhibitory concentration (IC_{50}) was defined as the compound concentration required to reduce viral plaque formation by 50%. IC_{50} values for each compound represent the mean IC_{50} from at least three independent experiments.

Drug resistance is defined as a function of the nature of the antiherpetic compound and the probable viral target affected. Thus, drug resistance is defined as an IC_{50} value increase of >10-fold for drugs that depend on viral TK for activation (i.e. ACV, BVDU, BVaraU and PCV) when alterations are supposed to be associated with mutations at the level of the

TK gene, while an increase of 3–10-fold is indicative of mutations at the level of the DNA polymerase gene. For drugs that do not depend on viral TK for activation, drug resistance is defined as an IC_{50} value increase of 8–10-fold for HPMP derivatives, and of 3–5-fold for pyrophosphate analogues and PME derivatives. We have previously used these multi-fold calculations for mutants of HSV and human cytomegalovirus (HCMV) selected under pressure of different classes of antiviral compounds (Andrei et al., 1995a, 1997; Snoeck et al., 1996).

The susceptibility profile for various in vitro isolated drug-resistant VZV strains are shown in Table 1. The number of passages required to obtain the different drug-resistant mutants varied considerably: for the ACV^r, BVDU^r and BVaraU^r strains, lower than 10 passages; for the PCV^r and PFA^r strains, about 30 passages; for the PMEA^r strain, 50 passages and for the PMEDAP^r strain, approximately 75 passages. The selection of GCV^r, HPMPC^r and HPMPA^r has not been successful after about 100 passages over a period of 2.5 years.

Virus strains emerging under the pressure of ACV, BVDU and BVaraU were cross-resistant to all drugs that depend on the viral TK for activation: i.e. ACV, BVDU, BVaraU, GCV and PCV. Thus, for the ACV^r, BVDU^r and BVaraU^r strains, the increase in IC_{50} values for ACV, PCV, GCV, BVDU and BVaraU varied, respectively, from 19- to 27-fold, 10- to 29-fold, 5- to 8-fold, >6300-fold and >26,000-fold. As we have previously shown (Snoeck et al., 1994), resistance to GCV due to alterations at the level of the TK gene are associated with slight increases in IC_{50} values for GCV. The ACV^r, BVDU^r and BVaraU^r strains remained sensitive to the pyrophosphate analogues PFA and PAA and the acyclic nucleoside phosphonates (PMEA, PMEDAP, HPMPC and HPMPA).

Two different PFA^r VZV strains (PFA^r/A and PFA^r/B) proved to be resistant not only to PFA and PAA but also to PMEA and PMEDAP. The increase in IC_{50} values for PFA, PAA, PMEA and PMEDAP varied from 4.4- to 6.8-fold. The PFA^r/A and PFA^r/B strains showed also a 4.6–6-fold increase in the IC_{50} values for ACV, and a three-fold increase in the IC_{50} values for PCV. No significant changes in the susceptibility to GCV, BVDU, BVaraU, HPMPC and HPMPA were noted with the PFA^r strains. Similarly to the PFA^r strains, the PMEA^r strain was resistant to the pyrophosphate analogues, the PME derivatives, ACV and PCV, being the increase in IC_{50} values of, respectively, 13.8-, 8.8-, 5.8-, 3-, >10-, 18-fold for ACV, PCV, PFA, PAA, PMEA and PMEDAP. The strain obtained under the PMEDAP pressure was also resistant to PMEA and PMEDAP (6.7- and 10.6-fold increase in IC_{50}, respectively), while a slight increase in the IC_{50} values for ACV (3-fold), PFA (3.5-fold) and PAA (2.3-fold) was observed. No changes in the IC_{50} values for PCV, GCV, BVDU, BVaraU, HPMPC and HPMPA were detected.

The drug-susceptibility profile of two PCV^r VZV strains differed from that of the ACV^r strain. Thus, the PCV^r VZV strains were not only resistant to PCV but also to PMEA, PMEDAP, PFA, PAA and ACV, suggesting an alteration at the level of the DNA polymerase gene rather than the TK gene level. The increase in IC_{50} values for the PCV^r/A and PCV^r/B were, respectively, of 7.5- and 5.2-fold for ACV, 5.6- and 3.6-fold for PCV, 8.3- and 3.2-fold for PFA, 4.7- and 2.4-fold for PAA, 7.3- and 6.3-fold for PMEA and 9.7- and 5-fold for PMEDAP. The PCV^r strains remained sensitive to BVDU, BVaraU, GCV, HPMPC and HPMPA.

In contrast to the ACV^r and PCV^r VZV mutants, HSV-1 and HSV-2 strains selected under the pressure of ACV and PCV showed a similar pattern of drug-susceptibility profile (Table 2). Thus, two different HSV-1 KOS strains and two different HSV-2 G strains isolated under PCV pressure were resistant to TK-dependent drugs and sensitive to PMEA, PMEDAP, HPMPC, HPMPA and the pyrophosphate analogues, suggesting that mutations at the level of the TK gene were responsible for the drug-resistant phenotype.

Here we report the phenotypic characterization of several VZV strains selected under pressure of different classes of compounds. According to their patterns of cross-resistance, two groups of mutants could be distinguished: (i) mutants that showed resistance to drugs that depend on the viral TK for their activation, i.e. ACV^r, BVDU^r and BVaraU^r strains and (ii) mutants that showed resistance to the pyrophosphate analogues, the PME derivatives, ACV and/or PCV (i.e. the PFA^r, PCV^r, PMEA^r and PMEDAP^r strains).

To our knowledge, this is the first report in the literature of VZV being successfully selected for resistance to PCV in vitro. Our results showed that ACV and PCV are able to select in vitro for different types of VZV mutants. The fact that the ACV^r strain, as well as the BVDU^r and BVaraU^r mutants, showed only resistance to drugs that depend on the viral TK for their activation, points to mutations at the level of the viral TK gene. In contrast, a considerable degree of cross-resistance against the pyrophosphate analogues and the PME derivatives was noted with the PFA^r and PCV^r VZV strains, while these mutants remained fully sensitive to BVDU, BVaraU and GCV. Thus, it can be assumed that the PCV^r and PFA^r strains bear mutation(s) in the DNA polymerase gene, and not in the TK gene. Although the resistant virus strains examined have not been plaque-purified, the fact that the PCV^r strains did not show cross-resistance to BVDU and BVaraU, but have a phenotypic profile similar to the PFA^r, PMEA^r and PMEDAP^r strains, is consistent with no mixture of TK and pol mutants in the PCV^r strains. It should be noted that the level of resistance to PCV and ACV was different depending on whether the TK gene or the DNA polymerase gene was affected. Thus, for the ACV^r, BVDU^r and BVaraU^r strains, the increase in the IC_{50} values for ACV and PCV varied from 19- to 27-fold and 10- to 29-fold, respectively, suggesting an alteration at the level of the TK gene. Whereas, in the case of PCV^r and PFA^r strains, the increase in the IC_{50} values for ACV and PCV varied from 5- to 7.5-fold and 3.0- to 5.6-fold, indicating an alteration at the level of the DNA polymerase gene. It is

Table 1
Drug-susceptibility profiles of drug-resistant VZV OKA strains obtained in vitro

Mutant virus strain[a]	Mean IC_{50} ± S.D. (μg/ml)[b]										
	ACV	PCV	GCV	BVDU	BVaraU	PFA	PAA	PMEA	PMEDAP	HPMPC	HPMPA
Wild-type	0.57 ± 0.36	0.56 ± 0.30	0.32 ± 0.33	0.0022 ± 0.0015	0.000049 ± 0.000027	12.0 ± 4.1	7.1 ± 2.5	4.9 ± 2.6	0.70 ± 0.35	0.067 ± 0.110	0.015 ± 0.023
ACV^r	12.3 ± 4.8	6.8 ± 5.6	2.5 ± 3.4	14.0 ± 10.4	≥13.1 ± 10.4	10.8 ± 3.8	6.4 ± 3.6	5.1 ± 4.0	0.94 ± 1.0	0.027 ± 0.032	0.0045 ± 0.0030
$BVDU^r$	10.7 ± 6.2	5.8 ± 2.8	1.5 ± 1.4	≥16.2 ± 5.2	>20 ± 0	14.8 ± 5.2	8.3 ± 0.9	7.5 ± 2.9	1.32 ± 1.04	0.054 ± 0.036	0.026 ± 0.030
$BVaraU^r$	15.4 ± 5.8	16.4 ± 4.3	1.7 ± 1.4	≥18.5 ± 3.0	>20 ± 0	12.6 ± 1.8	5.8 ± 1.9	7.2 ± 1.9	0.93 ± 0.17	0.032 ± 0.040	0.0034 ± 0.0027
PFA^r/A	3.4 ± 2.8	1.67 ± 0.84	0.33 ± 0.41	0.0039 ± 0.0029	0.00013 ± 0.00001	72.1 ± 20.7	35.6 ± 5.2	23.7 ± 10.2	4.5 ± 2.8	0.14 ± 0.08	0.048 ± 0.055
PFA^r/B	2.6 ± 3.0	1.69 ± 0.82	0.30 ± 0.56	0.0039 ± 0.0028	0.000095 ± 0.00005	82.3 ± 8.5	32.4 ± 5.8	21.8 ± 14.0	4.1 ± 2.6	0.078 ± 0.040	0.048 ± 0.042
PCV^r/A	4.3 ± 3.3	3.16 ± 2.61	0.098 ± 0.072	0.0019 ± 0.0009	0.00012 ± 0.00018	98.0 ± 36.9	33.1 ± 15.8	36.0 ± 20.0	6.8 ± 4.0	0.064 ± 0.049	0.0035 ± 0.0054
PCV^r/B	3.0 ± 1.0	2.0 ± 0.8	0.16 ± 0.06	0.0014 ± 0.0006	0.00019 ± 0.00003	38.6 ± 4.1	17.3 ± 3.2	30.8 ± 2.0	3.5 ± 0.6	0.15 ± 0.03	0.0054 ± 0.0051
$PMEA^r$	7.9 ± 3.2	4.9 ± 3.8	0.36 ± 0.32	0.0039 ± 0.0032	0.00011 ± 0.00006	70.3 ± 8.3	21.5 ± 5.7	>50 ± 0	12.8 ± 8.1	0.24 ± 0.019	0.027 ± 0.019
$PMEDAP^r$	1.7 ± 0.7	0.51 ± 0.32	0.043 ± 0.014	0.00058 ± 0.00027	0.000066 ± 0.000024	42.4 ± 20.7	16.6 ± 14.9	32.9 ± 14.5	7.6 ± 3.1	0.082 ± 0.075	0.014 ± 0.019

[a] Selected for resistance against each of the indicated compounds.
[b] IC_{50}, 50% inhibitory concentration required to reduce viral plaque formation by 50% in HEL cells.

Table 2
Drug-susceptibility profiles of drug-resistant HSV-1 and HSV-2 strains obtained in vitro

Mutant virus strain[a]	IC_{50} (μg/ml)[b]									
	ACV	PCV	GCV	BVDU	PFA	PAA	PMEA	PMEDAP	HPMPC	HPMPA
wt (HSV-1 KOS strain)	0.014	0.02	0.00005	0.0035	32	16	11	2	0.4	0.07
ACV^r	23	>20	7.23	42.5	30	10	16	5.7	0.3	0.11
PCV^r/A	>20	>20	1.75	11	23	12	12	3.3	0.33	0.1
PCV^r/B	>20	>20	0.85	20	20	11	12	3.3	0.36	0.07
wt (HSV-2 G strain)	0.01	0.08	0.002	50	12.5	7.4	3.2	0.8	0.11	0.08
ACV^r	12.5	9	0.37	>20	27	11	8	2.5	0.1	0.011
PCV^r/A	>20	11	0.32	>50	16	6.4	5	1.6	0.11	0.062
PCV^r/B	7.8	6.6	0.08	12.5	10	4.2	3.2	2	0.042	0.025

[a] Selected for resistance against each of the indicated compounds.
[b] IC_{50}, 50% inhibitory concentration required to reduce virus-induced CPE by 50% in HEL cells.

now imperative to perform the DNA sequencing of the DNA polymerase and TK genes to support these assumptions.

ACV, PCV and GCV are analogues of the natural 2′-deoxyguanosine. They are phosphorylated in HSV- and VZV-infected cells by the viral TK to their monophosphate forms and subsequently converted by cellular enzymes to the di- and triphosphate forms. The active (triphosphate) forms inhibit viral DNA synthesis by competing with deoxyguanosine triphosphate as a substrate for viral DNA polymerase and are incorporated into the growing DNA strain. In addition, ACV triphosphate acts as a DNA chain terminator since the acyclic side chain has only one hydroxyl group (Reardon and Spector, 1989). Since PCV has two hydroxyl groups on the acyclic side chain, it can be incorporated into the growing DNA chain and does allow limited chain elongation (Vere Hodge et al., 1989). Although ACV and PCV have identical activation pathways and similar modes of action (Earnshaw et al., 1992; Vere Hodge and Cheng, 1993), the affinities and therefore the molecular interactions of PCV, ACV, and their triphosphates with the viral TK and viral DNA polymerase differ. PCV is phosphorylated much more efficiently than ACV in herpesvirus-infected cells (Vere Hodge et al., 1989; Vere Hodge, 1993). Although PCV-TP reaches higher intracellular concentrations than ACV-TP, PCV-TP is a less potent inhibitor of HSV-1 and VZV DNA polymerase than ACV-TP (K_i, the inhibitory constant, is 100-fold greater for PCV-TP than for ACV-TP). Thus, the high levels of PCV-TP compensate for its diminished affinity for DNA polymerase. PCV-TP is more stable within infected cells than ACV-TP: the intracellular half-life of PCV-TP in cells infected with HSV-1, HSV-2 or VZV is 10, 20 and 7.2 h, respectively, while for ACV-TP the half-life is 0.7, 1.0 h, and below the detection limit in HSV-1-, HSV-2- and VZV-infected cells, respectively (Vere Hodge and Cheng, 1993).

There are at least three mechanisms by which HSV and VZV can acquire resistance to ACV, reduction or loss of viral TK activity, alteration of substrate specificity for viral TK, and mutations affecting the DNA polymerase. Most of the ACV^r HSV and VZV strains that have been isolated from patients are TK-deficient, as are the majority of strains that have been isolated from cell culture upon passage of the virus in the presence of compounds. Subtle differences between PCV and ACV in the interaction with viral TK or polymerase may account for the different types of VZV mutants selected under PCV or ACV. Furthermore, our results indicate that the interactions of PCV with HSV TK and/or DNA polymerase may differ from those with VZV, since PCV^r HSV mutants presented a drug-susceptibility profile similar to the ACV^r HSV mutants (i.e. resistance to all drugs that depend on viral TK for activation (which is suggestive of mutations at the level of the TK genes)). Sarisky et al. (2000) have recently reported that the frequencies with which resistance in HSV arises to PCV and ACV in cell culture are identical. Phenotypic analysis of a total of 48 HSV-1 and HSV-2 isolates selected after passage in the presence of increasing concentrations of ACV or PCV suggested that these isolates were deficient in TK activity (Sarisky et al., 2001). Sequencing of the TK genes from ACV^r mutants identified two homopolymeric G-C nucleotide stretches as putative hot spots. However, mutations identified in PCV^r mutants were generally not in these regions but distributed throughout the TK gene and at similar frequencies of occurrence within A-T or G-C nucleotides (Sarisky et al., 2001).

Previous reports have indicated that PCV is active against most HSV-1 and VZV TK and DNA polymerase mutants that are resistant to ACV (Boyd et al., 1987; Chiou et al., 1995; Pelosi et al., 1998; Hasegawa et al., 1995; Shiraki et al., 1993). This appears also to be the case for the PMEDAP VZV^r strain here described, since this mutant showed clear resistance to PMEA and PMEDAP, reduced sensitivity to PFA, PAA and ACV, although it retained sensitivity to PCV (Table 1). Thus, these reports are in agreement with our results indicating that the interactions between HSV or VZV TK and PCV or ACV, and likewise between the viral polymerases and the triphosphates of PCV or ACV, are distinct and may account for the differences observed between ACV^r and PCV^r VZV strains. Furthermore, Ida et al. (1999) have reported that the emergence frequency of resistant VZV mutants was significantly higher

following ACV exposure than following PCV exposure. In fact, the authors were able to select ACV^r strains after three consecutive passages in 11 out of 12 experiments while no PCV^r strains were selected under the same conditions and the ACV^r strains were shown to be TK-deficient (Ida et al., 1999). These findings are also in agreement with our results, since higher number of passages are required to select PCV^r than ACV^r VZV strains. Selection of ACV^r and PCV^r strains using different clinical strains is warranted to analyze whether virus strain-specific differences could impact differential selection of resistant mutations.

The patterns of cross-resistance here described for the PFA^r, PMEA^r and PMEDAP^r VZV strains are in agreement with those previously reported for HSV-1, HSV-2 and HCMV (Andrei et al., 1995a, 1997; Snoeck et al., 1996). The fact that a high degree of cross-resistance between the PME derivatives and the pyrophosphate analogues PFA and PAA was observed, suggests that the PME compounds may also interact with the DNA polymerase at the pyrophosphate binding site, known to be the site of interaction of PFA and PAA. This is consistent with the finding that the Ser724 → Asn change in the conserved region II of the HSV-1 DNA polymerase confers resistance to PFA, PMEA and PMEDAP (Andrei et al., 2000). Determination of the nucleotide sequence of the DNA polymerase genes of the different VZV mutants seems imperative to correlate resistant phenotype with amino acid changes in the viral DNA polymerase.

BVDU^r and BVaraU^r VZV strains, like the ACV^r VZV mutant, showed a drug-susceptibility profile characteristic of mutants with alteration at the level of the viral TK genes, since a high degree of resistance against those drugs was observed that depend on viral TK for activation. In HSV-1 and VZV-infected cells, BVDU and BVaraU are sequentially phosphorylated by the viral thymidine and thymidylate kinase to the mono- and diphosphate derivatives. The third step of phosphorylation to the active 5′-triphosphate forms is mediated by cellular kinases. BVDU and BVaraU are also monophosphorylated by the HSV-2-induced TK but not further converted onto the diphosphate form, and therefore lacking activity against HSV-2 (Fyfe, 1982).

It should be noted that the VZV-resistant mutants here described remained sensitive to the HPMP derivatives. In addition, selection of HPMPC^r and HPMPA^r strains, as well as GCV^r strains appeared to be very difficult, since no changes in the drug-susceptibility profile were observed after more than 100 passages. Only a restricted number of sites may change in the DNA polymerase gene that result in drug resistance but maintain normal polymerase activity. This may explain why it is difficult to generate DNA polymerase mutants in vitro and why only a few cases of clinical ACV and/or PFA resistance resulting from DNA polymerase alterations have been reported (Fillet et al., 1995; Morfin et al., 1999).

Some hypersensitivity of the resistant viruses to a few of the compounds has been noted (Table 1), i.e. hypersensitivity to HPMPC and HPMPA for the ACV^r and BVaraU^r strains. Previous reports showed evidence that ACV^r HSV strains with altered or deficient TK activity are more susceptible to HPMPC than are wild-type HSV strains (De Clercq et al., 1987; Maudgal and De Clercq, 1991). The difference in susceptibility to HPMPC of wild-type and mutant viruses may be due to mutant viruses inducing a smaller increase than wild-type virus in the intracellular concentration of dCTP in infected cells (Mendel et al., 1995). HPMPC diphosphate is a competitive inhibitor with respect to dCTP for HSV DNA polymerase. Thus, the lower concentration of dCTP in cells infected with the mutant viruses than in cells infected in the wild-type HSV enhances the inhibitory effect of CDV diphosphate against HSV DNA polymerase (Mendel et al., 1995).

In conclusion, we have shown that VZV mutants selected under ACV, BVDU and BVaraU pressure had drug-susceptibility profiles indicative of alterations at the level of the viral TK gene. Conversely, VZV mutants selected by PCV were cross-resistant with the pyrophosphate analogues, the PME derivatives and ACV, suggestive of alterations at the level of the DNA polymerase gene. In contrast to VZV mutants, HSV mutants selected under pressure of ACV and PCV showed a similar pattern of drug-susceptibility profile, i.e. resistance to TK-dependent drugs. Thus, ACV and PCV select phenotypically different VZV mutants in vitro. The genotypic changes that occur in the different mutants remain to be determined and so remains the relevance of our present findings to the in vivo situation.

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