MINIREVIEW

Resistance of Herpes Simplex Viruses to Nucleoside Analogues: Mechanisms, Prevalence, and Management[▽]

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Herpes simplex viruses (HSV) type 1 and type 2 are responsible for recurrent orolabial and genital infections. The standard therapy for the management of HSV infections includes acyclovir (ACV) and penciclovir (PCV) with their respective prodrugs valacyclovir and famciclovir. These compounds are phosphorylated by the viral thymidine kinase (TK) and then by cellular kinases. The triphosphate forms selectively inhibit the viral DNA polymerase (DNA pol) activity. Drug-resistant HSV isolates are frequently recovered from immunocompromised patients but rarely found in immunocompetent subjects. The gold standard phenotypic method for evaluating the susceptibility of HSV isolates to antiviral drugs is the plaque reduction assay. Plaque autoradiography allows the associated phenotype to be distinguished (TK-wild-type, TK-negative, TK-low-producer, or TK-altered viruses or mixtures of wild-type and mutant viruses). Genotypic characterization of drug-resistant isolates can reveal mutations located in the viral TK and/or in the DNA pol genes. Recombinant HSV mutants can be generated to analyze the contribution of each specific mutation with regard to the drug resistance phenotype. Most ACV-resistant mutants exhibit some reduction in their capacity to establish latency and to reactivate, as well as in their degree of neurovirulence in animal models of HSV infection. For instance, TK-negative HSV mutants establish latency with a lower efficiency than wild-type strains and reactivate poorly. DNA pol HSV mutants exhibit different degrees of attenuation of neurovirulence. The management of ACV- or PCV-resistant HSV infections includes the use of the pyrophosphate analogue foscarnet and the nucleotide analogue cidofovir. There is a need to develop new antiherpetic compounds with different mechanisms of action.

Herpes simplex virus type 1 (HSV-1) and type 2 (HSV-2) are responsible for recurrent orolabial and genital infections. In the United States, the seroprevalence of HSV-1 and HSV-2 infections has been estimated to be approximately 50% and 20%, respectively (243). Transmission occurs by contact with secretions from an infected person with either overt infection or asymptomatic excretion of virus. After primary infection, HSV establishes long-term latency in the ganglia of sensory nerves, from which it can reactivate episodically. These reactivations may be accompanied by symptoms or may be clinically silent. The most common clinical manifestations of HSV infections are vesicular lesions affecting the mucous membranes principally of the mouth, nose, or eyes for HSV-1 and the anogenital region for HSV-2. However, an increasing proportion of genital infections are caused by HSV-1 (243). The symptoms associated with both viruses are usually self-limiting, and the goals of treatment are to accelerate lesion healing and to prevent transmission in immunocompetent individuals (61). Both HSV-1 and HSV-2 can also cause infrequent but serious diseases, such as encephalitis and disseminated neonatal infections. HSV infections may be severe in immunocompromised patients, particularly those with defects in cell-mediated im-

munity. In patients with HIV infection and in recipients of solid organ or bone marrow transplants, herpetic lesions can be extensive, tend to persist for longer periods, and have the potential to disseminate. Recurrences tend to be more frequent and may be atypical in appearance (140). In addition to persistent mucocutaneous lesions, HSV can also lead to disseminated visceral infections, such as esophagitis, hepatitis, or pneumonia, as well as meningoencephalitis, in immunocompromised hosts (94, 99, 174).

ANTIVIRAL AGENTS FOR HSV INFECTIONS

Mechanism of action of nucleoside analogues. Since its introduction in the 1980s, acyclovir (ACV) and its derivatives have become the first line drugs for prophylaxis and treatment of HSV infections. ACV [9-(2-hydroxyethoxymethyl) guanine] is a guanosine analogue which must be triphosphorylated to be potent. Upon its entry into infected cells, the first phosphorylation step is performed mainly by the virus-encoded thymidine kinase (TK). This step is followed by two additional phosphorylations carried out successively by host cellular GMP kinase and nucleoside diphosphate kinase (148, 149). ACV-triphosphate (ACV-TP) can serve as a substrate of the viral DNA polymerase (DNA pol) reaction and, hence, be incorporated into DNA at its 3′ terminus (80). The binding of the next required deoxynucleoside 5′-triphosphate to the primer-template leads to the formation of a dead-end complex (166). It has also been suggested that as the 3′-terminal ACV-mono-

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TABLE 1. *In vitro* inhibitory concentrations of antiherpetic drugs in different cell culture systems and assays

Virus	IC$_{50}$ value or range (µg/ml); reference				
	Acyclovir	Penciclovir	Foscarnet	Cidofovir	Adefovir
HSV-1	0.02–0.9; 38	0.04–1.8; 38	24–60; 38	3.5–8.9; 177	7; 154
HSV-2	0.03–2.2; 38	0.06–4.4; 38	24–60; 38	3.5–8.9; 177	7; 154
VZV	0.8–4.0; 38	1.6–8.0; 38	24–60; 38	0.22; 177	10; 154
HCMV	>20; 38	51; 38	30–90; 38	0.1–0.8; 177	25; 154
EBV	1.6–2.8; 8	1.5–3.1; 8	0.6–1.0; 69	0.008; 177	0.3; 154

phosphate residues cannot be excised by the DNA pol-associated 3′→5′ exonuclease (71), they prevent further chain elongation. ACV-TP does not possess a hydroxyl group in the 3′ position and is thus an obligate DNA chain terminator (166). *In vitro*, ACV is most potent against HSV-1, approximately half as potent against HSV-2, a 10th as potent against varicella-zoster virus (VZV) and Epstein-Barr virus (EBV), and least potent against human cytomegalovirus (HCMV) (Table 1). ACV has a favorable safety profile in the clinic (228, 233). Its major limitation resides in its relatively poor oral bioavailability (i.e., 15 to 35%), which led to the development of valacyclovir (VACV), an L-valyl-ester prodrug of acyclovir (13, 22). The absolute bioavailability of ACV following oral administration of VACV is 54% (236). After oral administration, VACV is carried out by the human intestinal peptide transporter and then rapidly converted to ACV by ester hydrolysis in the small intestine (164).

Penciclovir [PCV; 9-(4-hydroxy-3-hydroxymethylbut-1-yl) guanine], an acyclic guanine derivative (which is not commercially available as an oral agent), has a spectrum of activity and a mechanism of action similar to those of ACV (34). However, by virtue of a 3′ hydroxyl group on its acyclic side chain, PCV-triphosphate allows limited chain elongation (short-chain terminator). The *in vitro* inhibitory concentrations of PCV depend on cell type but are usually 2-fold those of ACV for HSV and VZV (Table 1). PCV is very poorly absorbed when given orally, which led to the development of the oral prodrug famciclovir (FCV), which is a diacetylester of 6-deoxypenciclovir (230). The absolute bioavailability of PCV following oral administration of FCV is 77% (230). After oral administration, FCV is rapidly absorbed and efficiently converted to PCV. The first acetate group of FCV is cleaved by esterases found in the intestinal wall, and the second group is removed on the first pass through the liver (119). Thereafter, the conversion of 6-deoxypenciclovir to PCV is catalyzed by the aldehyde oxidase in the liver (53).

Clinical indications for nucleoside analogues. Clinical indications for oral ACV, VACV, and FCV include treatment of primary and recurrent HSV infections, particularly genital HSV (39, 168, 172, 206, 209), as well as chronic suppressive therapy to prevent genital herpes recurrences (72, 169, 216, 234). Oral ACV, VACV, and FCV are also frequently used for prophylaxis and treatment of HSV infections in immunocompromised patients. An intravenous formulation of ACV is used for severe HSV infections (including encephalitis and neonatal herpes), as well as those occurring in immunocompromised patients. Topical formulations of ACV and PCV are available for the management of recurrent herpes labialis (207, 208). An ophthalmic formulation of ACV is also available for the management of HSV corneal diseases (107).

PREVALENCE OF DRUG-RESISTANT HSV

Long-term prophylaxis and treatment with ACV, VACV, or FCV can result in the development of resistance, especially in immunocompromised patients. The prevalence of ACV- or PCV-resistant HSV isolates differs greatly for immunocompetent and immunocompromised patients. This difference is most likely due to two reasons (86). First, virus replication may be prolonged in immunocompromised patients, having a tendency toward true persistence. Second, and most important, the host responses are impaired and less pathogenic viruses that would not survive in the face of normal host responses may continue to replicate. Thus, even if some resistant viruses are less pathogenic, they are capable of producing overt diseases in these patients.

Immunocompetent patients. A low prevalence of HSV resistance to ACV was reported in immunocompetent patients during extensive screening surveys in the United Kingdom (0.7%) and the United States (0.3%) between 1980 and 1992 (59). Most studies since then did not report an increase in the prevalence of resistance (ranging from 0.1 to 0.7%) (7, 9, 33, 51, 68, 171, 212). In general, in this population, drug resistance was not associated with an adverse clinical outcome, due to immune competence. Cases of HSV resistance reported in immunocompetent patients were associated with diagnosis of recurrent genital herpes (81, 109, 125, 126, 153, 219), keratitis (156, 188), disseminated HSV infection (66), and encephalitis (197). Recently, a report documented a relatively high (6.4%) prevalence of ACV-resistant HSV-1 isolates in immunocompetent patients with herpes keratitis, and some of these cases were clinically refractory to ACV therapy (77). Similarly, a survey for HSV resistance to PCV reported a low prevalence of 0.19% in immunocompetent patients (186). Isolates from this study were also included in a larger survey, including 11 worldwide clinical trials. PCV-resistant HSV strains were isolated from a similar proportion (0.22%) of immunocompetent patients (187). In another study, a prevalence of less than 0.3% was reported in persons who used a topical PCV formulation to treat recurrent herpes labialis (200).

Immunocompromised patients. The prevalence of HSV infections with reduced susceptibility to ACV in immunocompromised patients is higher and varies from 3.5% to 10% (9, 51, 82, 156, 212). The prevalence of ACV resistance has ranged from 3.5% to 7% in HIV-positive patients (68, 82, 137, 141, 171, 248) and from 2.5% to 10% in solid organ transplant

recipients (51, 68). However, the highest prevalence rates have been reported in recipients of hematopoietic stem cells, with a range of 4.1% to 10.9% (43, 47, 68, 83, 94, 150, 232, 240). An even higher frequency (36%) of ACV resistance in that population has also been reported (131). Patients receiving either autologous or allogeneic bone marrow transplants had a similar incidence, i.e., 9%, of HSV infection, but resistance occurred only in allogeneic transplants, reaching a prevalence of 30% (151). ACV-resistant HSV isolates were associated with significant morbidity in immunocompromised hosts, including persistent and/or disseminated diseases refractory to antiviral therapy. Most infections with ACV-resistant viruses were detected among AIDS patients and were associated with extensive mucocutaneous lesions but usually not with severe generalized infections. However, a few cases of lethal disseminated visceral HSV infections caused by ACV-resistant mutants were reported in bone marrow transplant recipients (140), and a case of meningoencephalitis was described in an AIDS patient (97). In a survey including 11 worldwide clinical trials, the prevalence of PCV-resistant HSV isolates among immunocompromised patients was 2.1% and this was not associated with treatment failure (187).

MECHANISM OF RESISTANCE TO NUCLEOSIDE ANALOGUES

In agreement with ACV's mechanism of action, viral mutations (deletions/additions or substitutions of nucleotides) conferring resistance to ACV have been found either in the *UL23* gene, which encodes the activating/phosphorylating TK enzyme, and/or in the *UL30* gene, which encodes the viral target DNA pol enzyme (57, 196). Herpes simplex viruses require a functional DNA pol to replicate, whereas the viral TK is dispensable in cultured cells and certain mammalian tissues. Consequently, there is a higher probability of inducing a viable ACV-resistant virus by a mutation in the *UL23* gene than by a mutation in the *UL30* gene. In this respect, *UL23* gene mutations have been reported in 95% of clinical isolates exhibiting ACV resistance (98, 111, 150). HSV resistance to PCV generally maps to mutations in the *UL23* gene, with almost inevitable cross-resistance between the two nucleoside analogues (34, 186, 187). The mutations in *UL23* and *UL30* genes conferring resistance to nucleoside analogues have been extensively described in several reviews (96, 103, 151).

Thymidine kinase mutations. Three different phenotypes of ACV-resistant TK mutants have been identified: TK-negative mutants which lack TK activity, TK-low-producer mutants which express reduced levels of enzymatic activity, and TK-altered isolates which are substrate-specific mutants, i.e., they phosphorylate thymidine but not ACV and/or PCV. Approximately 95% of ACV-resistant HSV clinical isolates are TK-negative and TK-low-producer mutants, whereas a minority consists of TK-altered mutants (165). Moreover, mixtures of wild-type and mutant viruses are also observed.

The HSV-1 thymidine kinase is a 376-amino-acid (aa) protein encoded by the *UL23* gene (147). The most important sites involved in the enzyme activity are the ATP-binding site (aa 51 to 63), the nucleoside-binding site (aa 168 to 176), and the cysteine at codon 336 which maintains the three-dimensional structure of the active site (85). Moreover, six highly conserved
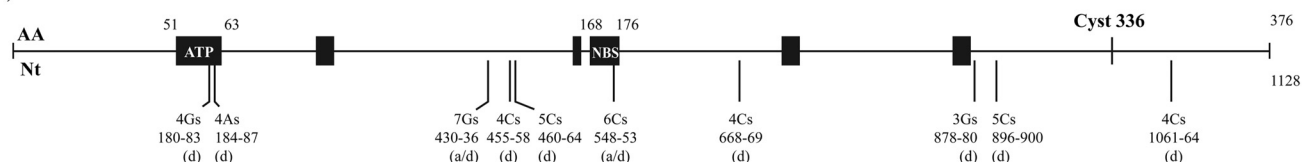
regions have been identified among *Herpesviridae* TK (10). These regions are located at amino acids 56 to 62 (site 1), 83 to 88 (site 2), 162 to 164 (site 3), 171 to 173 (site 4), 216 to 222 (site 5), and 284 to 289 (site 6). The HSV-2 TK consists of a 375-amino-acid polypeptide with the six highly conserved domains located at positions 56 to 62, 83 to 88, 163 to 165, 172 to 174, 217 to 223, and 285 to 290.

The structure of the viral TK consists of an $\alpha\beta$ structure made up of 15 α-helices and 7 β-sheets (36). Five-stranded parallel β-sheets form part of the core of the protein, which contains the active site. The active site consists of the substrate nucleoside-binding pocket, the DRH motif (aa 162 to 164), and the ATP/nucleotide-binding loop and provides the residues that are responsible for coordinating the magnesium counter ion. The ATP molecule binds in the nucleotide-binding site as a complex with Mg^{2+}, which is coordinated by the aspartic acid located at position 162. The LID domain (aa 216 to 222), a region rich in arginine and lysine residues, forms a lid enclosing the active site. For the catalytic reaction, the γ-phosphate of ATP and the 5′-OH deoxyribose need to be activated. Clusters of positive charges from the LID domain and the Mg^{2+} make the phosphorus atom amenable for a nucleophilic attack of the 5′-OH of deoxyribose which is polarized by the two glutamines localized at positions 225 and 83 (239). The substrate binding site is composed of amino acid side chains derived primarily from a set of roughly parallel helices (85). These helices are localized at the N-terminal half of the polypeptide following the glycine-rich loop involved in the binding of the phosphate moiety of ATP. The cysteine at position 336 is located close to the ATP-binding and nucleoside-binding sites and is involved in local domain consolidation.
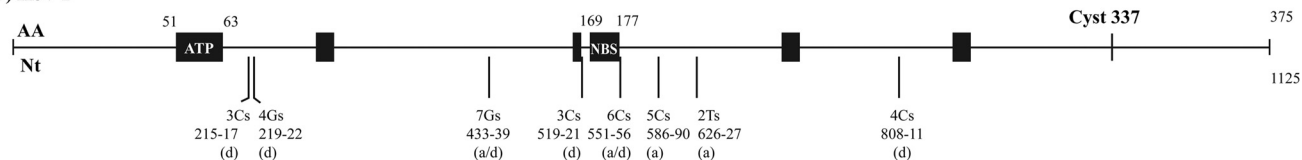
Genotypic characterization of HSV clinical isolates revealed a high degree of polymorphism in the *UL23* gene. Such mutations were not associated with resistance and were located throughout the gene but mainly outside the active site of the enzyme (40, 48, 95, 127, 135, 150, 193). This fact emphasizes the importance of obtaining pretreatment isolates and/or generating recombinant virus to be sure that mutations actually confer resistance.

Half of the cases of ACV resistance are due to additions or deletions of nucleotides in the *UL23* gene, particularly in homopolymer repeats of guanines (G) or cytosines (C), which are considered resistance hot spots (Fig. 1) (98, 150). These additions or deletions can lead to a frameshift reading, resulting in the synthesis of a nonfunctional truncated enzyme (158). The two longest homopolymers, one composed of 7 Gs and one of 6 Cs, are the sites of the most frequently reported mutations in ACV-resistant clinical isolates (98, 122, 150, 190, 192, 193, 195). Other reports of *UL23* gene sequences in TK-negative clinical isolates have identified strains containing C deletion in strings of 3 Cs (122, 159) or G deletion from a 3-G stretch (48). An HSV-1 isolate had a deletion of one A in a string of 4 As that were part of the ATP-binding site (98). HSV-2 resistance to ACV was also mediated by a loss of TK enzyme activity due to a T deletion at nucleotide 927 of the *UL23* gene (158). This mutation caused a phenylalanine-to-leucine change at position 309 and early termination at amino acid 348. A frameshift mutation resulting from a single C deletion from the homopolymer stretch of 4 C residues in the open reading frame

FIG. 1. Mutations identified in the *UL23* gene of HSV-1 (A) and HSV-2 (B) isolates resistant to ACV. The ATP-binding site (ATP), the nucleoside-binding site (NBS), and the six regions of the *UL23* gene that are conserved among *Herpesviridae* are shown by the black boxes. The six highly conserved regions are located at amino acids (AA) 56 to 62 (site 1), 83 to 88 (site 2), 162 to 164 (site 3), 171 to 173 (site 4), 216 to 222 (site 5), and 284 to 289 (site 6) for HSV-1 and 56 to 62, 83 to 88, 163 to 165, 172 to 174, 217 to 223, and 285 to 290 for HSV-2. The additions (a), deletions (d), or both additions and deletions (a/d) reported in homopolymer runs, as well as the nucleotides (Nt) involved, are indicated below vertical bars. Substitutions of amino acids reported in the *UL23* gene that are included in the boxes correspond to those identified in conserved regions, and those outside the boxes are located in nonconserved regions. Underlined mutations correspond to the HSV-2 mutations.

(nucleotides 1061 to 1064) resulted in a TK polypeptide with a longer amino acid sequence (407 aa) (183).

The remaining ACV resistance cases are due to nucleotide substitutions that are usually in the conserved sites of the *UL23* gene (Fig. 1). Several amino acid changes have been mapped in the ATP-binding site (48, 78, 98, 150, 182, 195, 214), the nucleoside-binding site (98, 125, 150, 182), the DRH motif (48, 213), and the LID domain (78, 98, 122, 214) of HSV TK clinical isolates.

A modification of the cysteine at position 336 of HSV-1 or of position 337 of HSV-2 by a tyrosine was reported in clinical isolates resistant to ACV (78, 98, 192). This mutation induced a TK-low-producer/TK-altered phenotype (98). The C336Y mutation primarily affected the ATP-binding site and led to alterations in the binding affinity of nucleoside analogues (167). The mutation seemed to disrupt the three-dimensional structure of the whole active site by shifting the LID domain, resulting in a more open catalytic active conformation (129). Mutations were also located in conserved site 2 (78, 150, 182) and site 6 (66, 98, 113). In addition, several mutations were also shown to confer resistance to ACV despite their location outside highly conserved regions of the *UL23* gene (28, 44, 78, 98, 125, 150, 214, 223).

Two studies reported conflicting results on the mechanism of PCV-selected resistance in HSV strains. In the first study, mutations identified in PCV-selected mutants consisted of nonconservative amino acid changes distributed throughout the *UL23* gene and were never found within homopolymeric G and C nucleotide stretches (190), whereas the second study reported the opposite (218). The nature of the discrepancy between these two studies may be related to the size of the viral inoculum used during serial passages. Indeed, in the first study, a high viral inoculum (7×10^4 PFU), which may contain spontaneous preexisting mutants, may be at the origin of the accumulated mutations developed after serial passages and attributed to PCV resistance. In contrast, in the second study, only a low viral inoculum was used as a starting material. Mutations in the *UL23* gene from PCV mutants consisted of 4% single nucleotide substitutions and 96% frameshift mutations (218).

DNA polymerase mutations. Although less frequently encountered in clinical isolates, mutants with altered DNA pol have also been identified (20, 60, 95, 115, 185, 195, 213). DNA pol is a heterodimer composed of the *UL30* and *UL42* gene products. The *UL30* gene encodes the catalytic subunit, while the *UL42* gene encodes a phosphoprotein that possesses double-stranded DNA-binding activity. DNA pol is a multifunctional enzyme (1,235 and 1,240 amino acids for HSV-1 and HSV-2, respectively [227]) which possesses a polymerase activity for the extension of DNA primer chains (136), an intrinsic 3′→5′ exonuclease proofreading activity (123), and an RNase H activity that could be involved in the removal of RNA
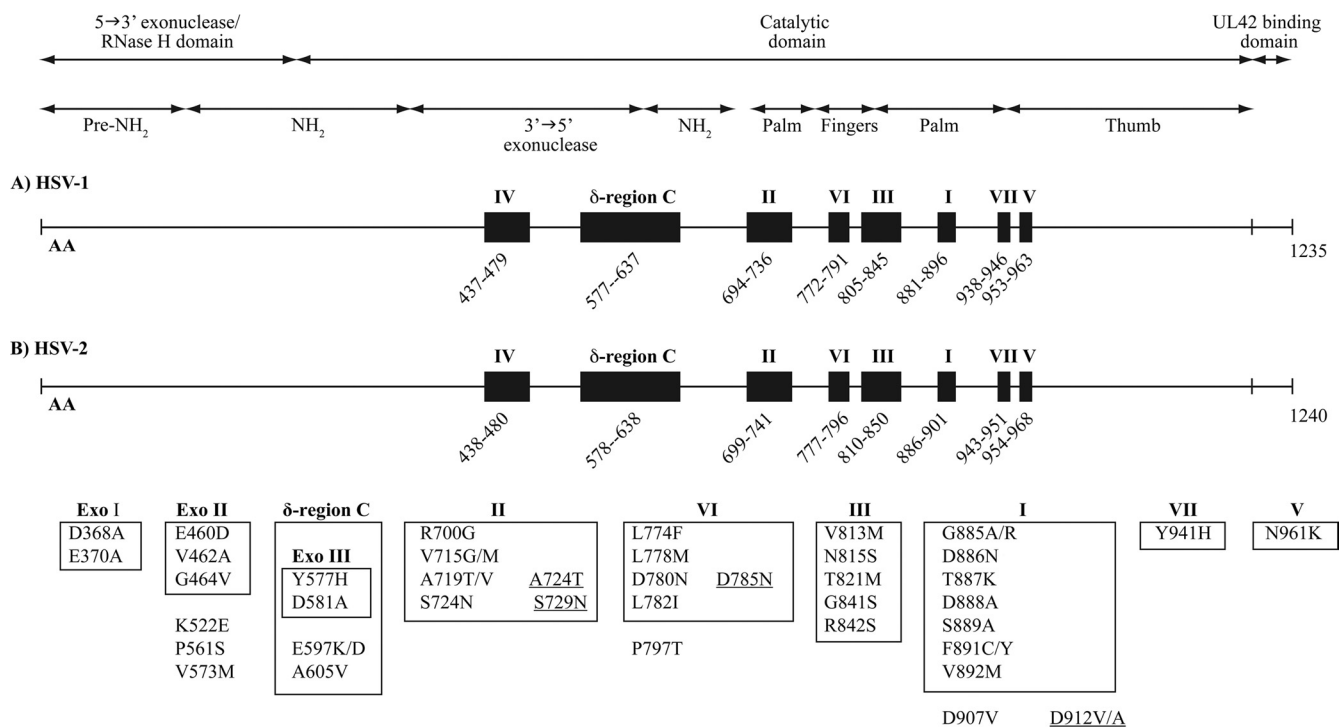
FIG. 2. Mutations identified in the *UL30* gene of HSV-1 (A) and HSV-2 (B) isolates resistant to ACV. Regions conserved among *Herpesviridae* genes are shown by the black boxes. The roman numbers (I to VII and δ-region C) corresponding to each of these regions are indicated above the boxes. Amino acid (AA) locations are noted below each of these regions for HSV-1 and HSV-2. Substitutions reported in the *UL30* gene that are included in the boxes correspond to those identified in conserved regions, and those outside the boxes are located in nonconserved regions. Underlined mutations correspond to the HSV-2 mutations. Mutations E460D, G464V, K522E, and P561S in and outside Exo II are lethal to the virus; mutations Y577H and D581A in the Exo III motif in δ-region C are associated with hypersusceptibility to ACV; and none of the mutations in region I are spontaneously induced.

primers that initiate the synthesis of Okazaki fragments at a replication fork during replication (64). In addition, the carboxyl-terminal region of the catalytic UL30 subunit interacts with the accessory UL42 subunit which acts as a processivity factor (73, 106). HSV DNA pol belongs to the family of α-like DNA polymerases (241) and is also closely related to DNA polymerases δ (247). The α-like DNA polymerase family shares significant amino acid sequence homology in the carboxyl-terminal half of the catalytic subunit. These regions are numbered I to VII on the basis of their degree of conservation among the DNA polymerase genes, with region I being the most conserved. Moreover, HSV DNA pol also contains a δ-region C, which is shared by polymerases related to eukaryotic DNA polymerases δ (247). The relative order of these regions in the polypeptide is IV, δ-region C, II, VI, III, I, VII, and V (Fig. 2). Based on sequence conservation in the α-like polymerase family, the 3′→5′ exonuclease domain of HSV pol contains three highly conserved sequence motifs (Exo I, Exo II, and Exo III) which map to the N-terminal half of the polypeptide (15). The Exo I, Exo II, and Exo III motifs are located, respectively, between positions 363 and 373, in region IV, and in δ-region C.

HSV DNA pol is formed by 6 structural domains, namely, a pre-NH$_2$ domain, an NH$_2$ domain, polymerase palm, fingers, and thumb domains, and a 3′→5′ exonuclease domain (Fig. 2) (138). Regions I, II, and VII are parts of the polymerase palm subdomain, and region V is the base of the thumb subdomain.

These four regions appear to flank the catalytic site in the palm subdomain and contain the catalytic triad of aspartic acid residues (at positions 717, 886, and 888) that are essential for polymerase activity. Regions III and VI are located at the base of the fingers subdomain and may play a role in positioning the template and primer strands.

In clinical isolates, the mutations conferring resistance to nucleoside analogues are single amino acid substitutions located in regions II (95, 185, 195, 213), III (60, 195), VI (195), and VII (115) which are directly or indirectly involved in the recognition and binding of nucleotides or pyrophosphate, as well as in catalysis (Fig. 2). The greatest clusters of mutations in the DNA pol enzyme have been found in conserved regions II and III. Mutations within conserved regions II (95, 185, 195, 213) and VII (115) were frequently associated with cross-resistance to ACV and foscarnet (FOS). Only a few drug-resistant mutations were described within the other conserved regions or outside such regions (20, 115, 195). In this respect, the change of the aspartic acid to valine at position 907, within a nonconserved gene region, induced a low level of resistance to ACV (19).

In laboratory mutant viruses, some mutations in δ-region C (E597K/D and A605V) conferred resistance to ACV (101, 128, 132, 184). The 3′→5′ exonuclease activities associated with replicative polymerases have proofreading functions to improve replication fidelity. Mutant polymerases with defective exonuclease activity can increase the mutation frequency by up

to 3 orders of magnitude (199). Laboratory strains containing mutated residues (Y577H and D581A) within the conserved Exo III motif of the δ-region C of the polymerase gene were shown to be defective in $3' \rightarrow 5'$ exonuclease activity and exhibited extremely high mutation frequencies (116). These mutants also demonstrated higher resistance to phosphonoacetic acid and greater sensitivity to aphidicolin, ACV, and ganciclovir than wild-type virus (117). These results suggest that the conserved Exo III motif of HSV DNA pol may play an important role in maintaining the proper structure of the catalytic site for polymerase activity, in addition to its role in exonuclease activity.

LABORATORY DIAGNOSIS OF ANTIVIRAL DRUG RESISTANCE

The persistence of lesions for more than 1 week after the beginning of therapy without appreciable decrease in size, an atypical appearance of the lesions, or the emergence of new satellite lesions despite antiviral administration is suggestive of treatment failure. After healing of an infection caused by a TK-negative drug-resistant HSV strain and stopping antiviral therapy, recurrences are most often associated with a drug-sensitive strain, although some cases of spontaneous recurrences of ACV-resistant viruses have been reported (1, 78, 100, 113, 125, 126, 142, 146, 152, 153, 178, 201, 205, 232, 246). Laboratory diagnosis of ACV resistance is required to guide clinicians toward different treatment options in cases of therapy failure. ACV resistance can be diagnosed by testing a virus against antiviral agents (phenotypic assays) or by the identification of a specific mutation conferring resistance to antiviral drugs (genotypic assays) (2).

Phenotypic methods. The common basis of phenotypic assays is the measurement of virus growth inhibition in the presence of the antiviral drug. After appropriate periods of incubation with the antiviral, viral replication is measured based on the reduction of virus-induced cytopathic effect or plaque formation, which is evaluated either colorimetrically or microscopically (62).

The gold standard phenotypic method for the evaluation of HSV susceptibility to antiviral drugs is the plaque reduction assay (180). This assay has been the object of most clinical correlation and standardization studies (235) and is approved as a standard protocol by the Clinical and Laboratory Standards Institute (220). In this assay, a controlled viral inoculum (50 to 100 PFU) is inoculated onto a permissive monolayer of Vero cells (African green monkey kidney cells, CCL 81; American Type Culture Collection, Manassas, VA) to allow adsorption. Infected cells are incubated in the presence of serial drug concentrations for 2 to 3 days. Cells are fixed and stained, and the number of plaques is counted. The concentration of drug that reduces the plaque number by 50% (IC_{50}) compared to the number on the virus-infected, no-drug control is then calculated. However, this assay is time consuming and is based on a somewhat subjective read-out.

Virus multiplication can also be evaluated by measurement of antigen expression in cell culture based on enzyme-linked immunosorbent assays (ELISAs), including the sandwich ELISA (181) and the microplate *in situ* ELISA (134). Other currently used antiviral assays include DNA hybridization

(210), flow cytometric analysis (161), and transgenic HSV-inducible reporter cell lines (224). A recombinant HSV-1 expressing the green fluorescent protein has also been described (222). More recently, antiviral drug susceptibility assays of HSV have been developed using real-time PCR quantification of DNA in cell culture supernatants (215) or of intracellular viral DNA load (226). These techniques are both sensitive and accurate. However, the readout must be done early during the exponential phase of virus growth because the long persistence of DNA in cell culture may jeopardize the interpretation of inhibition results at a late stage of infection (32).

In vitro resistance can be defined by an IC_{50} greater than a cutoff value or by showing a greater than 3- to 5-fold increase in the IC_{50} compared to that of a baseline isolate from the same patient. Breakpoint values of ≥ 2 μg/ml (8.8 μM) for ACV and of ≥ 100 μg/ml (333.3 μM) for FOS in the plaque reduction assay are widely accepted (55, 58, 89, 176, 179). There is no suggested consensus breakpoint value for PCV.

Laboratory strains and clinical isolates of HSV that are resistant to ACV naturally constitute a heterogeneous population which contains mixtures of wild-type and resistant virus (81, 156, 160, 174). The proportion of naturally occurring ACV- and PCV-resistant mutants within a virus preparation is in the same order of magnitude (189). It has been estimated that the proportion of ACV-resistant HSV must exceed 20% of the viral population in order to shift the IC_{50} to more than 2 μg/ml (199). The plaque reduction assay measures the overall sensitivity of the viral population. Therefore, an isolate could be considered sensitive based on its IC_{50}, although it may contain a significant proportion of resistant viruses (70, 87). To eliminate this possibility, the mutant virus isolate may be analyzed for evidence of TK heterogeneity by plaque autoradiography. The amount of TK activity in the mutant virus isolate relative to the amount in a wild-type laboratory strain is quantified *in situ* using thymidine labeled to high specific activity with [³H] and storage phosphor technology (108, 113). Infected 143B cells (a human osteosarcoma cell line which is TK negative; American Type Culture Collection) are incubated with a medium containing a semisolid overlay at 37°C. Once plaques reach a reasonable size, the overlay is removed and the cells are incubated with a medium containing [³H]-thymidine for 14 h. Cells are stained, washed, and air dried. Plates are exposed for 6 days to a tritium plate from a phosphorimager and then scanned to obtain images and quantitative data.

Genotypic methods. The objective of genotypic assays is the search for specific mutations in the viral genes encoding the activating/phosphorylating TK enzyme (*UL23*) and the target DNA pol enzyme (*UL30*). The gene of interest is amplified by PCR, and the PCR products are then sequenced. The mutations detected in the gene of interest are then interpreted by comparison with the whole panels of mutations described in the literature. For the mutations that have not been characterized previously, some predictions can be made from the knowledge of the conserved regions implicated in the structural integrity of the enzyme or in its catalytic functions. However, in order for genotypic methods to be helpful in clinical practice, it is essential to be able to discriminate between random variations (polymorphism) and true drug resistance mutations. The generation of recombinant mutant viruses al-

lows the formal assessment of the role of nonconfirmed mutations in antiviral drug resistance.

Methods for generating recombinant mutants. Traditionally, putative resistance mutations have been confirmed by marker transfer experiments. A specific mutation is transferred to a wild-type virus background by homologous recombination (49, 115, 116, 133, 144, 174). The intact viral genomic DNA from a reference strain is cotransfected with a mutated gene in permissive cells, and the resistant recombinant virus is then selected with an antiviral. However, this method is fastidious and inefficient and generally relies on the selection of recombinant mutants through drug pressure, which frequently leads to additional undesired mutations.

The generation of recombinant mutants following cotransfection of overlapping cosmids and plasmids into permissive cells represents an important improvement (19, 52, 65, 198). This procedure leads to the reconstitution of mutant virus only and circumvents the tedious selection procedure against the parental virus. However, it still relies on several recombination events in eukaryotic cells that cannot be controlled. Moreover, this system is inefficient for generating mutations in two or more viral genes.

Recently, several laboratories reported the cloning of HSV-1 genomes into a plasmid as bacterial artificial chromosomes (BACs) (102, 175, 211, 221). This technique allows the stable maintenance of HSV genomes as BACs in *Escherichia coli* and mutagenesis of the viral genome by using the bacterial recombination machinery. The reconstitution of infectious virus is achieved by transfection of the BAC plasmid into permissive mammalian cells. This approach has several advantages. The powerful methods of bacterial genetics allow the introduction of any kind of mutation (deletion, insertion, or point mutation) into the cloned viral genome. Manipulated genomes can be characterized prior to the reconstitution of virus. The transfection of the mutated genomes into permissive cells results in the reconstitution only of viral mutants that are free of contamination with parental virus.

PATHOGENESIS OF DRUG-RESISTANT HSV STRAINS IN ANIMAL MODELS/HUMANS

Among the unique properties of HSV strains are the establishment of latency followed by intermittent reactivations, as well as their neurovirulence potential (14, 121, 237, 245).

HSV TK mutants. The persistence and virulence of a drug-resistant HSV strain depends on its ability to replicate relative to other genetic variants (so-called viral fitness). Although TK is not essential for growth in cell culture, it is important for viral pathogenesis, particularly for reactivation from latently infected trigeminal ganglia in animal models. Depending on the phenotype of the TK mutant, viral replication, establishment of latency, and reactivation can be unaltered or decreased in comparison with wild-type strains. TK-low-producer mutants show some reduction in pathogenicity compared with wild-type strains but are generally able to reactivate from latency (16, 54). In contrast, TK-negative mutants have impaired pathogenicity, establish latency in sensory ganglia with a lower efficiency than wild-type strains, and reactivate poorly in mice (46, 56, 79, 110, 111, 118, 152, 225), in guinea pigs (16), and in rabbits (29, 42, 105, 217, 244). The vast majority of the latter mutants are attenuated for neurovirulence and demonstrate defective replication in the brain and, often, in the peripheral nervous system compared with wild-type strains (30, 50, 74). However, reactivation of some TK-negative HSV clinical isolates has been reported (110, 113, 114, 152, 178, 191). Some of these isolates have been shown to express TK activity at ultra-low levels, demonstrating that the amounts of TK activity required for reactivation are far lower than previously appreciated (18, 54). It has been proposed that mutant viruses with a single G insertion into the 7-G string generated low levels of full-length active TK (less than 1% of wild-type activity) by a net +1 ribosomal frameshift during translation that is sufficient for reactivation (108, 114). In addition, heterogeneous populations (TK competent/TK negative) may coexist in HSV isolates from immunocompromised patients (191).

HSV DNA pol mutants. Much less is known concerning the neurovirulence and potential to establish latency of clinical DNA pol mutants. Viral DNA pol has a crucial role in the replicative cycle, and it is expected that DNA pol mutants would have decreased neurovirulence. It has been shown that HSV DNA pol mutants exhibit different degrees of attenuation of neurovirulence in mice (88, 89, 91, 162, 163, 174, 204). Some HSV DNA pol mutants are clearly attenuated, while others are able to replicate in the central nervous system and cause encephalitis. The degrees of neurovirulence of DNA pol mutants resistant to ACV and/or FOS do not correlate with the *in vitro* levels of resistance to these compounds or with the polypeptide subdomain where the amino acid substitutions are mapped (4). Neuroinvasiveness is expected to correlate with viral fitness, so that highly replicating viruses would be more neurovirulent. Recently, it has been shown that the degree of neurovirulence of several DNA pol mutants could be generally predicted by their *in vitro* replicative capacity (67). However, Pelosi et al. (163) reported a DNA pol mutant (R842S) that could replicate in the central nervous system but, paradoxically, exhibited attenuated neurovirulence. The reason for such discrepancy remains unclear.

MANAGEMENT OF NUCLEOSIDE ANALOGUE-RESISTANT HSV INFECTIONS

When treatment failure is suspected, isolates from the lesions should be submitted for phenotypic or genotypic testing, and a change of therapy should be considered as shown in the algorithm proposed in Fig. 3. It is very unlikely that a patient failing to respond to ACV or VACV therapy will respond to FCV because there will most probably be cross-resistance between ACV and PCV. An initial step in case of treatment failure with oral drugs is to switch to high doses of intravenous ACV (10 mg/kg of body weight every 8 h adjusted for renal function). Most successful treatment of refractory ACV-resistant HSV infections has been achieved with the use of FOS. Lesions that fail to respond to FOS could be treated with cidofovir (5 mg/kg once a week for 3 to 4 weeks).

Foscarnet. FOS (phosphonoformic acid) is a pyrophosphate analogue that inhibits the viral DNA pol by mimicking the structure of pyrophosphate produced during the elongation of DNA (157). FOS, which does not require phosphorylation by viral and cellular kinases, binds to the pyrophosphate binding site on the viral DNA pol and blocks the release of pyrophos-
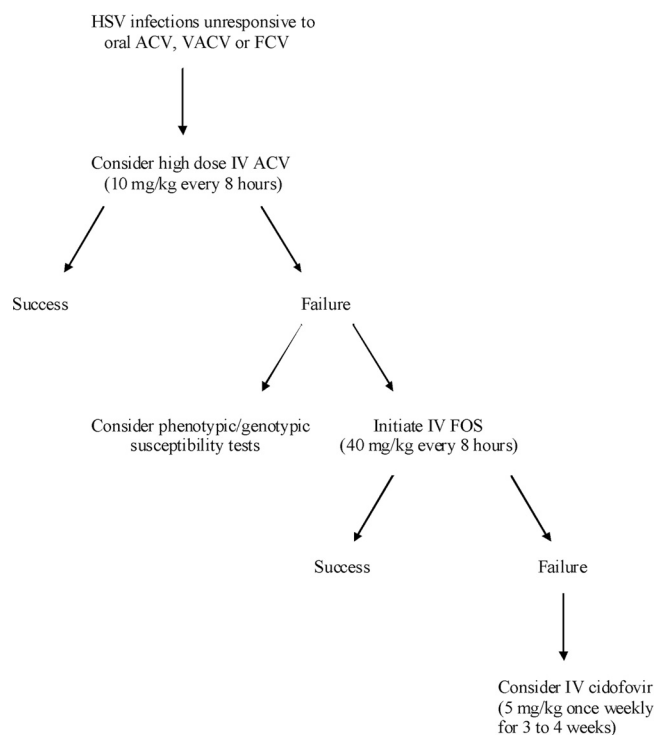
FIG. 3. Management of HSV infections that are unresponsive to nucleoside analogues. IV, intravenous.

phate from the terminal nucleoside triphosphate added onto the growing DNA chain. FOS acts as a noncompetitive inhibitor of DNA pol activity. FOS is potent against all herpesviruses (157) (Table 1). Its principal indication is for HCMV infections, including strains resistant to ganciclovir, but it is also potent against most ACV-resistant HSV and VZV strains. The oral bioavailability of FOS is poor and, thus, it is only available as an intravenous formulation. Foscarnet is nephrotoxic and induces electrolyte disorders. Foscarnet is indicated as second-line therapy for HSV infections that are refractory to nucleosidic drugs. Foscarnet treatment has shown some antiviral efficacy against ACV-resistant infections in AIDS patients (3, 45, 84, 176) and bone marrow transplant patients (170, 229). Few data are available regarding the isolation of FOS-resistant HSV strains in the clinic, mainly from AIDS patients failing therapy (20, 43, 68, 115, 131, 174, 179, 180, 185, 195, 213). Most FOS-resistant clinical HSV isolates contain single base substitutions in conserved regions II, III, VI, or VII and in a nonconserved region (between I and VII) of the DNA pol gene (20, 95, 115, 185, 195, 213). Some of these isolates retained susceptibility or, at the most, borderline levels of susceptibility to ACV and cidofovir (20, 195). However, some mutations, in particular in regions II (95, 185, 195, 213) and VII (115) of the DNA pol, can confer resistance to both ACV and FOS. In this respect, mutations V715G and S724N/S729N (HSV-1/HSV-2) located in region II (185, 195) and Y941H located in region VII (115) have been described in ACV- and FOS-resistant clinical isolates. Mutants with alterations in both HSV TK and DNA pol can also occur, resulting in double resistance to both ACV and FOS.

Cidofovir and adefovir. Acyclic nucleoside phosphonates (ANPs), including (S)-1-(3-hydroxy-2-phosphonyl-methoxy-propyl) derivatives of cytosine (cidofovir) and adenine (adefovir), are converted into active ANP-diphosphates by cellular kinases. Thus, they circumvent the need for activation by the virus-encoded TK and so retain their activity against TK-negative or TK-altered strains of HSV (112). The active forms are competitive inhibitors of the viral DNA pol, and they may act as chain terminators (242). The principal indication for cidofovir is for HCMV infections, including ganciclovir-resistant strains with mutations in the *UL97* gene (112) (Table 1). It is also potent against HSV and VZV strains, including most of the isolates resistant to ACV and/or FOS. Cidofovir is approved for treatment of HCMV retinitis in AIDS patients and is available as an intravenous formulation. It has a particularly long intracellular half-life, and infrequent dosing is possible. Because of its nephrotoxicity, cidofovir is routinely administered with probenecid and requires intravenous hydration. Cidofovir has proven to be effective in the treatment of progressive mucocutaneous infections due to ACV- and/or FOS-resistant HSV in immunocompromised patients (41, 124, 130, 143, 202, 204), but it is not approved for this indication. The safety and efficacy of single topical applications of 1, 3, and 5% cidofovir gels have been evaluated for the treatment of early, lesional, recurrent genital herpes (173), but they are not commercially available. In addition, oral formulations of ether lipid ester prodrugs of cidofovir are under development (23). Mutations associated with cidofovir resistance mapped in DNA pol region II (R700M), region III (G841C and G850I), region VI (L773M), and region VII (Y941H) and in the Exo III motif in δ-region C (V573M) (5). Laboratory-derived HSV strains resistant to cidofovir also contained mutations in nonconserved regions (L1007M and I1028T) of the DNA pol gene (5). Adefovir is effective against hepatitis B virus and also has activity against HSV strains, including most of the isolates resistant to ACV (155) (Table 1). Its oral prodrug adefovir dipivoxil is used for the treatment of chronic active hepatitis B virus infections. Although not approved for that indication, adefovir could be used in the treatment of HSV infections unresponsive to ACV and FOS. The dosage is not clearly established, and the renal function must be monitored. Resistance to adefovir may occur following selection of HSV DNA pol mutants in the laboratory (93, 231). FOS-resistant isolates with DNA pol mutations in regions II and VI and between regions I and VII were also associated with an important decrease in susceptibility to adefovir, suggesting that adefovir-resistant HSV could be selected *in vivo* by FOS therapy (20). In addition, the mutations S724N and L778M which conferred cross-resistance to ACV and FOS also caused reduced susceptibility to cidofovir and adefovir (19).

Other viral or host targets. The helicase-primase complex is composed of 3 proteins encoded by the *UL5*, *UL8*, and *UL52* genes. This complex unwinds double-stranded viral DNA and generates primers for DNA synthesis by the viral DNA pol. Therefore, the helicase-primase complex plays an essential role in HSV DNA replication and thus constitutes an excellent target for antiviral therapy. Helicase-primase inhibitors are nonnucleosidic/nonnucleotidic inhibitors that are extremely potent against HSV in cell culture. The candidate BAY 57-1293 (Bayer) has potent anti-HSV activity *in vitro* and is effec-

tive against ACV-resistant mutants. This compound had a greater efficacy than ACV against genital herpes in a guinea pig model (12), as well as against ocular and lethal infections in both mouse and rabbit models (21, 24, 120). Resistant viruses were rapidly selected by serial passages in cell cultures (25–27). Another helicase-primase inhibitor candidate, BILS 45 BS (Boehringer Ingelheim), was much more potent than ACV against wild-type laboratory and clinical HSV-1 strains, as well as ACV-resistant isolates. Oral administration of BILS 45 BS was very effective against cutaneous infection in nude mice infected with ACV-resistant isolates due to mutations in the TK or the DNA pol gene (63, 76).

BILD 1633 SE (Boehringer Ingelheim) is a peptidomimetic inhibitor of HSV ribonucleotide reductase which catalyzes the synthesis of the deoxyribonucleotide precursors required for DNA synthesis (75, 139). BILD 1633 SE prevents the association between the two subunits that form the protein and thus acts as a nonsubstrate-based antiviral agent. *In vitro*, this compound was more potent than ACV against several wild-type strains and ACV-resistant HSV mutants. This compound was effective in a murine ocular model of HSV-1-induced keratitis.

A novel class of nonnucleosidic inhibitors belonging to the 4-hydroxyquinoline-3-carboxamide derivatives is specific for a broad range of viral DNA pol, including that of HSV-1 and HSV-2. The PNU-183792 derivative has anti-HSV activity in cell culture (35). It also maintains potent antiviral activity against ACV-resistant mutants, suggesting that its mechanism of action is different.

RNA interference (RNAi) is a natural mechanism of posttranscriptional gene silencing that is widely conserved in multicellular organisms. The molecular mediators of RNAi are double-stranded RNAs 21 to 23 nucleotides in length that induce the sequence-specific degradation of homologous RNAs. Synthetic small interfering RNAs (siRNAs) against essential gene products of HCMV could trigger RNAi in infected cells and inhibit viral replication (238), opening the way to a new class of antiviral agents.

A new class of topical drugs has recently been developed that serve as immune response modifiers. These drugs upregulate the natural immune response to infectious organisms, such response being regulated primarily through induced production of interferon and cytokines from activated lymphocytes at the site of application. The Toll-like receptor (TLR)-7/8 agonists imiquimod and resiquimod stimulate the production of cytokines that promote an antigen-specific T helper type 1 (Th1) acquired immune response. A topical formulation of imiquimod is licensed for the treatment of genital and perianal warts caused by human papillomavirus (90). Topical application of imiquimod and resiquimod showed contradictory results in clinical trials evaluating their efficacy in the treatment of herpes labialis (17) and genital herpes (11, 37, 92, 104, 145, 194, 203). The TLR-3 agonist poly(I:C) induced innate-immune-mediated protection against genital HSV-2 infection in mice when administered intravaginally but not when administered systemically (6). In another study, a single intraperitoneal or intranasal pretreatment of mice with poly(I:C) reduced the mortality rate after intranasal challenge with HSV-1, suggesting that this TLR-3 agonist may increase the innate immune mechanism of neuroprotection against HSV (31).

CONCLUSIONS

ACV and PCV, as well as their respective prodrugs VACV and FCV, are the gold standard antiviral agents for the prevention and treatment of HSV-1 and HSV-2 infections. The prevalence of resistance to these drugs is low in immunocompetent individuals. However, immunocompromised patients may develop chronic infections which are more prone to become resistant to currently available drugs. In this respect, the prevalence of resistance in immunocompromised patients reaches levels varying from 3.5% to 10%. With the increased number of transplant and cancer patients, an immunocompromised status is no longer a rare event. Therefore, the problem of drug-resistant HSV infections is not expected to fade. On the other hand, the reduced viral fitness and pathogenicity of most drug-resistant HSV mutants seen in animal models may contribute to the limited impact of resistance in the clinical setting. There is a need to develop new antiherpetic compounds with different mechanisms of action which will be safe and effective against ACV- and/or FOS-resistant HSV isolates.

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