**Penciclovir Susceptibilities of Herpes Simplex Virus Isolates from Patients Using Penciclovir Cream for Treatment of Recurrent Herpes Labialis**

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The antiherpesvirus agent penciclovir (PCV) shares an identical activation pathway and a similar mode of action with acyclovir (ACV). However, since PCV represents a relatively recent treatment option, the clinical resistance profile to PCV is less well known. A susceptibility program was established to assess the resistance profile for serial herpes simplex virus isolates from immunocompetent patients with recurrent herpes labialis obtained throughout a 4-day period of treatment with topical PCV (1% cream) or a placebo. Two isolates (2 of 1,035 [0.19%]), representing 0.34% of the patients (2 of 585), were confirmed to be PCV-resistant (Pcv r) herpes simplex virus type 1 by a plaque reduction assay in MRC-5 cells. These two viruses were highly resistant to PCV (50% inhibitory concentrations [IC50s], >55 μg/ml) and were isolated less than 17 h after the start of patient-initiated treatment. However, subsequent isolates on days 2 and 3 from these patients were completely susceptible to PCV (IC50s, <2.0 μg/ml). Thus, it is not clear whether the resistance to PCV for these two early-treatment isolates was directly associated with the 17 h of PCV treatment; several possible explanations are discussed. In an analysis of the distribution of IC50 differences between the first and last isolates, there were three patients with minor IC50 increases in the PCV-treated population and one in the placebo-treated group, although statistically, only the latter was an outlier. No patients were found to have Pcv r virus at the end of acute treatment, regardless of treatment group. Overall, the prevalence of Pcv r was found to be similar to the 0.3% Acv r reported for immunocompetent, untreated populations.

The use of acyclovir (ACV) and penciclovir (PCV) in treating herpesvirus infections has increased significantly since the introduction of ACV 2 decades ago. These nucleoside analogues of guanine are preferentially phosphorylated to monophosphate by the viral thymidine kinase (TK) and subsequently converted to ACV triphosphate by cellular enzymes. The activated triphosphate inhibits viral DNA polymerase activity and prevents viral DNA elongation (9). Herpes simplex virus (HSV) resistance to these agents typically develops by mutations in the TK gene, although mutations within the viral DNA polymerase can also confer ACV resistance (2–5, 14, 28).

ACV-resistant (Acv r) HSV variants have been readily isolated in culture after sequential passages in the presence of increasing concentrations of ACV (reviewed in reference 19). However, several studies suggest that clinical use of ACV has not been associated with an increased emergence of drug-resistant virus. Sensitivity monitoring surveys have revealed that since the introduction of ACV, the prevalence of resistance in the general population has remained unchanged (1, 2), and little if any impact on the prevalence of resistant virus in the immunocompetent population has been shown (6, 13). Clinically significant resistance to ACV has been limited almost exclusively to the immunocompromised population (7, 10, 11, 21, 30), in which approximately 4 to 10% of patients develop resistance during antiviral treatment (2, 31).

To discern the prevalence of PCV resistance in the immunocompetent population, susceptibility assays were performed on virus isolates from patients participating in two placebo-controlled trials for evaluation of the efficacy of topical PCV for recurrent herpes labialis (29; unpublished data).

**MATERIALS AND METHODS**

Isolate sampling and resistance breakpoint. Topical PCV cream (1%) was evaluated as a treatment for recurrent herpes labialis in two phase III studies conducted according to the same protocol (29). Patients were instructed to apply PCV or placebo cream nine times per day for 4 days within 1 h of the first sign or symptom of a recurrence. Although treatment was initiated by the patient, the patients visited the study center daily for evaluation and virus isolation. Additionally, each patient kept a daily record of treatment time and compliance. To determine the pattern of virus susceptibility to PCV, antiviral assays were performed on the first and last virus isolates obtained from the patients. In some instances, additional isolates obtained at intervals between the samplings for the first and last isolates were obtained and also tested. The first isolate was defined as the virus isolated within 24 h of the start of topical PCV treatment. The last isolate was defined as the final virus-positive culture obtained. In practice these were obtained during the treatment period or after cessation of treatment. Virus isolates were taken during the vesicle to soft-crust stage. The mean interval between first and last isolates was 1.8 days for the PCV group and 1.9 days for the placebo group. In order to determine the pattern of virus susceptibility, antiviral assays were performed on a random, blinded sample of isolates from each study. An in vitro breakpoint for defining ACV resistance has been set at 2.0 μg/ml, since 50% inhibitory concentrations (IC50s) for isolates from individuals failing to respond clinically to ACV therapy are found to be at or above this concentration.
to respond to drug therapy were generally above this level (22). Breakpoints are typically established through a consensus of researchers in the field based on the correlation between clinical treatment failure and in vitro IC_{50} values. Because there is no recognized in vitro breakpoint for defining resistance to PCV, provisional guidelines were set for defining resistance based on the susceptibility of the sensitive control virus strain to PCV, which was determined in each assay. This was to take into account the known variation in IC_{50} values between laboratories. Resistance was considered to be an IC_{50} of ≥2.0 μg/ml or an IC_{90} of >10-fold higher than the IC_{50} for the wild-type sensitive control virus within that particular assay (24).Virological work was performed at two College of American Pathology-approved centers (the clinical virology laboratories of the Children’s Hospital of Philadelphia and the University of San Francisco General Hospital), and each center used wild-type HSV type 1 (HSV-1) as a standard virus for susceptibility assays (either strain SC16 or strain F, depending on the center).

**Statistical analysis.** Analysis of covariance was used to determine whether HSV isolates from PCV-treated patients and placebo-treated patients had different last IC_{50}s after comparison with their respective first IC_{50}s. The analysis was performed using natural logarithms of the IC_{50}s, which provides statistical stringency. It was assumed that within each treatment group, the average difference between last and first isolates was zero, and significant deviation from zero would indicate a trend. Statistically significant differences in the change from the first to the last IC_{50} between PCV-treated and placebo-treated groups would be indicated by a P value of less than 0.05. Similarly, it was assumed that between the two treatment groups, the average difference between first isolates was zero and the average difference between last isolates was zero; a P value below 0.05 would suggest a trend.

**Virus culture.** Lesions were swabbed daily throughout the course of the recurrence, during treatment, and often after treatment had ceased, to maximize the chance of identifying Pcv isolates. Briefly, Dacron swabs moistened with Viral-Chlamydial transport medium (Carr-Scarborough Microbiologicals, Decatur, Ga.) were used to swab the lesions. Swabs were placed in transport medium and either processed immediately for virus isolation or stored at 4°C for up to 3 days at 37°C. After 3 days at 37°C, cells were seeded into 12-well microtiter plates (Costar) and were confluently infected. One hour postinfection, monolayers were stained with crystal violet, fixed with 1.0 ml of 10% formaldehyde or an IC_{50} of 0.25 g/ml. If these isolates were included in the analysis, the average IC_{50} for first isolates would be 1.1 ± 7.4 μg/ml and the IC_{90} range would be 0.07 to 83.2 μg/ml.

**Resistance.** Significance of the average difference between first and last isolates within the treatment group, and for the difference between the average first IC_{50}s for the two treatment groups, was determined with the 95% confidence interval around the mean. The limit of detection for TK activity for HSV-1 SC16 was set at 100%. The limit of detection for HSV-1 SC16 was estimated to be 0.3%, in agreement with a previous report (4).

**RESULTS**

**PCV susceptibilities of paired HSV isolates.** Sensitivity testing was performed on a total of 1,035 isolates from 585 patients, of which 864 (83%) represented paired isolates (first and last isolate obtained from each patient). Of the paired isolates, 358 were from PCV-treated patients and 506 were from the placebo-treated group. The IC_{50} results, including standard deviations and ranges, are summarized in Table 1. The average IC_{50}s for the isolates at the start of treatment were 1.1 ± 7.4 and 0.28 ± 0.28 μg of PCV/ml for the PCV- and placebo-treated groups, respectively. As discussed below, first virus isolates from two patients were resistant to PCV; subsequent isolates from these patients were sensitive to PCV. Analysis of the first-isolate data after removal of the IC_{50}s for these two isolates (55 and 83 μg of PCV/ml) resulted in similar susceptibility profiles overall for the two treatment groups (0.29 ± 0.25 and 0.28 ± 0.28 μg of PCV/ml for the PCV- and placebo-treated groups, respectively). Lastly, similar average IC_{50}s were reported for all last isolates regardless of treatment.
The parameter was utilized to identify alterations or trends in the pattern of susceptibility during the course of therapy. Differences between the IC50 for the last isolate and that for the first isolate are shown as ranges (in micrograms of PCV per milliliter) on the y axis. Numbers of patients analyzed are shown on the x axis. The distributions of susceptibility are similar for the two treatment groups except for four minor populations. Peaks A through C represent three minor populations in the PCV-treated group with a shift to the right in susceptibility, and peak D corresponds to one minor population in the placebo-treated group with decreased susceptibility to PCV.

The trend analysis on data for paired isolates (first and last HSV isolates tested for PCV susceptibility) from this study indicates there are no statistically significant differences between IC50 for last isolates from PCV-treated and placebo-treated patient populations after accounting for any difference in the IC50 for first viral isolates (P = 0.791 by analysis of covariance) (Table 1). Furthermore, monitoring changes in IC50 between first and last isolates can serve as a powerful method to identify alterations in the pattern of susceptibility during therapy. The histogram in Fig. 1 illustrates the differences in IC50 (IC50 for last isolate minus IC50 for first isolate) between the paired first and last isolates for each treatment group, up to an increase of 2.5 μg of PCV/ml. Only two isolates, the two Pcvr variants, had IC50 changes greater than 2.5 μg of PCV/ml.

Several peaks, representing subpopulations with increasing IC50, were apparent in the PCV and placebo treatment groups (Fig. 1). Clearly, for the majority of samples evaluated, similar profiles were apparent for the two treatment groups. However, to the right of the midpoint, which corresponds to no change in the IC50 between first and last isolates, three minor peaks in the PCV-treated population and one minor peak in the placebo-treated group were present. For three PCV-treated samples, the last PCV IC50 was approximately 0.65 to 0.7, 0.75 to 0.80, or 1.0 to 1.5 μg/ml higher than the IC50 for the first isolate (Fig. 1, peaks A, B, and C, respectively). For one placebo-treated last isolate, the PCV IC50 was more than 1.5 μg/ml greater than that for the corresponding first isolate (Fig. 1, peak D). Overall, however, for the majority of isolates, the distribution profiles of the two treatment groups overlap, suggesting that no significant differences in susceptibility are present between treatment groups, although the minor increases in IC50 for four patient samples tested could indicate a trend to increasing resistance or simply represent the inherent variability within the plaque reduction assay.

**PCV susceptibilities of all HSV isolates.** Table 2 summarizes the susceptibility data for all HSV isolates (paired and nonpaired) collected during the two clinical trials. Once again, average IC50s for the two groups of isolates were very similar, as measured by susceptibility to PCV in the plaque reduction assay (0.29 ± 0.28 and 0.28 ± 0.29 μg of PCV/ml for the PCV-and placebo-treated groups, respectively), when IC50 for the two resistant first isolates were excluded. Inclusion of the two Pcvr isolates in the data set results in an average IC50 of 0.60 ± 4.7 μg of PCV/ml for the PCV-treated group. Inclusion of these highly resistant isolates in the test population provides the most accurate representation of the background frequency of Pcvr, approximately 0.34% (2 of 585 patients).

**PCV-resistant clinical isolates.** PCV IC50s for virus isolates taken from two patients (patients 024.028.2586 and 024.023.0495) within 17 h of the start of PCV treatment were 83 and 55 μg/ml, respectively, clearly meeting the criteria defining resistance. However, subsequent isolates from these two patients taken from the same episode were susceptible to PCV (PCV IC50, 1.1 and 0.8 μg/ml, respectively; viruses were isolated on day 3 of treatment in both cases). No other Pcvr viruses were identified from the treatment period or after cessation of treatment with topical PCV.

Only for two other isolates were PCV IC50s equal to or greater than 2 μg/ml; both of these were from patients treated with a placebo. For one isolate, obtained on day 3 from patient 024.027.0424, the IC50 was 3.1 μg/ml, but this value was substantially below 9 μg/ml, the breakpoint defined as 10-fold above the IC50 for the wild type. When the isolate was restested against PCV, an IC50 of 0.74 μg/ml was determined, and therefore this isolate was not considered to be resistant to PCV. For another isolate (obtained on day 1 from patient 024.028.2016), the IC50 was 2 μg/ml. The IC50 for the sensitive control strain ranged between 0.9 and 1.6 μg/ml, and therefore, according to the 10-fold criterion, the test isolate was classified as sensitive; however, based on the standard breakpoint of an IC50 of ≥2.0 μg/ml, this isolate would be labeled as possibly resistant. Unfortunately, this isolate was not available for further analysis or retesting.

**Molecular characterization of Pcvr isolates.** Plaque autoradiography on the two Pcvr patient samples (patients 024.028.2586 and 024.023.0495) confirmed that the resistant
Table 3. Biochemical characterization of Pcv<sup>r</sup> HSV-1 isolates

<table>
<thead>
<tr>
<th>Patient ID&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Phosphorylation&lt;sup&gt;b&lt;/sup&gt; of substrate</th>
<th>Thymidine</th>
<th>ACV</th>
<th>PCV</th>
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<td>024.028.2586</td>
<td></td>
<td>4.0</td>
<td>&lt;0.3</td>
<td>&lt;0.3</td>
</tr>
<tr>
<td>024.023.0495</td>
<td></td>
<td>4.3</td>
<td>&lt;0.3</td>
<td>&lt;0.3</td>
</tr>
</tbody>
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<sup>a</sup> ID, identification number.

<sup>b</sup> Expressed as a percentage of phosphorylation of the given substrate by wild-type HSV-1 (set at 100%).

variants represented the majority of virus present in the virus preparation (data not shown). Plaque-purified isolates from the two patient samples which were confirmed as Pcv<sup>r</sup> (patients 024.028.2586 and 024.023.0495) were evaluated for the ability to phosphorylate <sup>3</sup>H-labeled substrates including thymidine, ACV, and PCV according to previously described methods (23, 26). These isolates were unable to utilize ACV or PCV as a substrate, compared to phosphorylation by wild-type HSV-1 (set at 100%), and they were also highly impaired in thymidine phosphorylation, suggestive of a TK-negative or TK-partial phenotype (Table 3). Lastly, PCV IC<sub>50</sub>s were reduced to below 0.7 µg/ml upon testing in BUHK-TK cells (data not shown), confirming that impaired TK activity was responsible for conferring resistance to PCV. However, the important distinction between TK-negative and TK-partial remains difficult to make based on in vitro TK assays alone.

Sequence analysis of the TK coding region identified the presence of frameshift mutations, consistent with the TK-negative phenotype (Fig. 2). The isolate from patient 024.028.2586 contained two mutations, a deletion of residue 65 (threonine) and a frameshift (cytosine insertion) in the homopolymeric C run at residue 185. The isolate obtained from patient 024.023.0495 contained three nonconservative changes and a frameshift at residue 270 (cytosine deletion). It was not determined whether the nonconservative residues and/or frameshift were directly responsible for conferring drug resistance.

**DISCUSSION**

This study demonstrates that there is no evidence of reduced PCV sensitivity among viral isolates obtained from patients treating a single episode of recurrent herpes labialis with topical PCV. No statistically significant difference between the PCV and placebo treatment groups was identified in the IC<sub>50</sub>s for first isolates (P = 0.796) or the IC<sub>50</sub>s for last isolates (P = 0.757) (Table 1). Furthermore, the average difference between IC<sub>50</sub>s for paired isolates in the PCV group was 0.028, and that in the placebo group was 0.025. Thus, there was no statistically significant difference between the last and first IC<sub>50</sub>s in either the PCV-treated group (P = 0.078) or the placebo-treated group (P = 0.065) (Table 1).

The plaque reduction assay data, along with the histogram analysis presented in Fig. 1, confirm that acute treatment of recurrent herpes labialis in immunocompetent patients with topical PCV did not result in an increase in the prevalence of resistant HSV. Collecting serial isolates represents a powerful method for examining trends in resistance. Since the distribution profiles of changes in IC<sub>50</sub> after treatment significantly overlap for the two treatment groups, acute treatment with PCV cream versus a placebo does not appear to increase the likelihood of selecting for Pcv<sup>r</sup> HSV in immunocompetent patients with recurrent herpes labialis. However, studies monitoring resistance trends during episodic, prolonged treatment need to be performed in order to assess the development of resistance over time with repeated usage.

Two virus isolates for which PCV IC<sub>50</sub>s were 55 and 83 µg/ml were confirmed by biochemical and molecular analyses to be Pcv<sup>r</sup>. Both were first isolates, taken within 17 h of the start of treatment with topical PCV. Spontaneous mutations within the HSV genome are introduced by errors during DNA replication and are independent of the presence of an antiviral agent (15). This natural phenomenon results in the accumulation of 6 to 8 TK-deficient variants per 10<sup>4</sup> plaque-forming viruses in virus populations that have never been exposed to selective pressure (8, 16, 17) and can explain the coexistence of both resistant and sensitive viruses within all clinical HSV isolates. Consistent with this, there is a low prevalence of resistant HSV among individuals who have not been treated with an antiviral agent (1, 2).

Given the replication cycle kinetics of HSV and the fact that both Pcv<sup>r</sup> isolates were obtained within 17 h of initiation of treatment, it seems unlikely, although possible, that they re-

**FIG. 2.** Alignment of mutations within the TK coding sequence. The schematic representation depicts the HSV-1 TK polypeptide and three conserved domains, the nucleotide binding pocket, the thymidine binding site, and the ATP binding site. The homopolymeric hot-spot regions (G7 and C6) are indicated. Below the diagram, the genotypic mutations and resulting residue changes or frameshifts (FS) identified in the two confirmed Pcv<sup>r</sup> isolates are shown.
sulted from selection pressure as the result of antiviral treatment. Since approximately 50% resistant variants are required within a virus preparation to confer such high IC$_{50}$ (12, 24), a preexisting resistant variant would have to represent a substantia
tional proportion of the virus swab and be selectively amplified immediately upon the initiation of treatment. Although the presence of these two resistant isolates from the first isolate swabs may simply reflect the natural heterogeneity of HSV populations (20, 25), isolates taken on day 2 would be expected to also be resistant, yet these IC$_{50}$ were below 2.0 μg/ml. Another possible explanation is that these two isolates result from rare cases where residual topical PCV carried from the viral swab facilitated in vitro selection of resistant virus during the isolation process. If this is true, isolates obtained from these patients on days 2 and 3 would be expected to be completely susceptible to PCV. A third explanation which cannot be ruled out is preferential selection for resistant variants on day 1, followed by subsequent selection against these isolates for fitness. Lastly, patient recording of treatment times indicates that lack of compliance was not a factor in the selection for resistance (data not shown).

Genotypic characterization of the two confirmed PCV-resistant HSV isolates described in this report resulted in the identification of a frameshift mutation at residue 185 in one isolate, which was also found in a previously characterized PCV-resistant isolate (26) and is similar to the mutations routinely found within the homopolymeric region of TK (G7 and C6 hot spots) (27). Moreover, several mutations were also identified in the second isolate characterized, notably with a frameshift at residue 270, which, like the frameshift at residue 185, could be predicted to disrupt the integrity of the ATP/nucleoside binding pocket. Interestingly, although these isolates were plaque purified several times, they were not absolutely defective in the ability to phosphorylate thymidine, whereas phosphorylation of ACV and PCV was below the level of detection. It is not known whether the minor level of thymidine phosphorylation is due to contamination with wild-type virus undetectable by plaque autoradiography, to ribosomal frameshifting (18), or to other factors.

Most recently, the prevalence of ACV resistance was determined to be approximately 0.4% based on data for HSV isolates obtained from 708 immunocompetent, ACV-naïve individuals with genital herpes (1, 2). Furthermore, the proportion of ACV resistance appears to be relatively stable in immunocompetent patients, even after the increased usage of ACV over the past 2 decades. The present study indicates that the overall prevalence of PCV-resistant HSV isolates, for the immunocompetent population examined, was no greater than 0.19%, in agreement with historical data on ACV. Although PCV and ACV share an identical activation pathway and a similar mode of action, suggesting that the mechanisms of resistance are similar, the widespread usage of ACV for treatment has not increased the prevalence of PCV resistance above that reported for ACV.

In conclusion, based on the PCV susceptibilities of sequential isolates from patients with recurrent herpes labialis taken throughout the treatment period in this study, there is no reason to expect a change in the overall prevalence of resistant HSV isolates with use of topical PCV for acute treatment of recurrent herpes labialis in immunocompetent patients.

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