

No Evidence of Pritelivir Resistance Among Herpes Simplex Virus Type 2 Isolates After 4 Weeks of Daily Therapy

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Background. Pritelivir is a novel helicase-primase inhibitor in clinical development for treatment of herpes simplex virus type 2 (HSV-2) infections. In preclinical work, resistance-mediating mutations were identified in the HSV-2 genome at 3 loci in the UL5 gene and 1 locus in UL52.

Methods. To evaluate whether daily pritelivir treatment results in emergence of resistance-mediating mutations, we analyzed HSV-2 strains detected in genital swab specimens from trial participants who were randomly assigned to receive different dosages of pritelivir. We sequenced resistance regions from 87 participants' samples, the UL5 gene in 73 samples from 44 participants, and the UL52 gene in 71 samples from 43 participants.

Results. We found no evidence that pritelivir induced known resistance-mediating mutations or for amino acid variation at other loci. In one participant's HSV-2 isolate, we found a previously unidentified mutation close to the putative resistance-mediating region in UL5 and subsequently determined in vitro susceptibility to pritelivir. We characterized mutations from 32 cultivated HSV-2 isolates previously found to be susceptible to pritelivir in vitro and identified several novel mutations that most likely reflect pre-existing variation in circulating HSV-2.

Conclusions. This study demonstrates evidence of retained susceptibility of HSV-2 to pritelivir in immunocompetent persons following daily therapy for up to 28 days.

Keywords. herpes simplex virus 2; helicase-primase inhibitor; drug resistance; viral genomics pritelivir.

Pritelivir (also known as BAY 57-1293 and AIC316) belongs to a new class of antiviral compounds, the helicase-primase inhibitors (HPIs), that prevent the de novo synthesis of virus DNA through inhibition of the helicase-primase complex [1]. Functionally, both UL5 and UL52, together with the accessory protein UL8, form the helicase-primase enzyme complex, the molecular target of pritelivir [2]. Unlike nucleoside analogues, which are currently the mainstay of therapy for herpes simplex virus (HSV) infections, pritelivir does not require activation by viral thymidine kinase within an HSV-infected cell.

HSV infections resistant to nucleoside analogues are rare in immunocompetent persons, but their frequency is increased in immunocompromised patients [3]. Mutations mediating

resistance to nucleoside analogs are located in the gene encoding the thymidine kinase and/or the gene encoding the DNA polymerase. All resistance-mediating mutations to pritelivir identified so far in vitro are located either at a single amino acid position in the viral UL52 primase (amino acid 906) or within/downstream of the fourth functional motif of the viral UL5 helicase (the conserved helicase motif; amino acids 341-355; Figure 1) [4, 5]. To date, no resistant virus isolate or strain has been identified for which drug resistance against pritelivir or other HPIs was mediated by an amino acid change in UL8.

In a dosage-finding trial in persons with genital HSV type 2 (HSV-2) infection, pritelivir was shown to reduce the level of viral shedding and the recurrence of genital lesions [6]. Here we report the results of a secondary objective of this trial: the monitoring of molecular signals of drug resistance over 28 days of therapy.

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MATERIALS AND METHODS

Study Participants

Written informed consent was obtained from all study participants. Human experimentation guidelines of the Department of Health and Human Services were followed in the conduct of the clinical research. Further details are given in the primary outcomes manuscript [6].

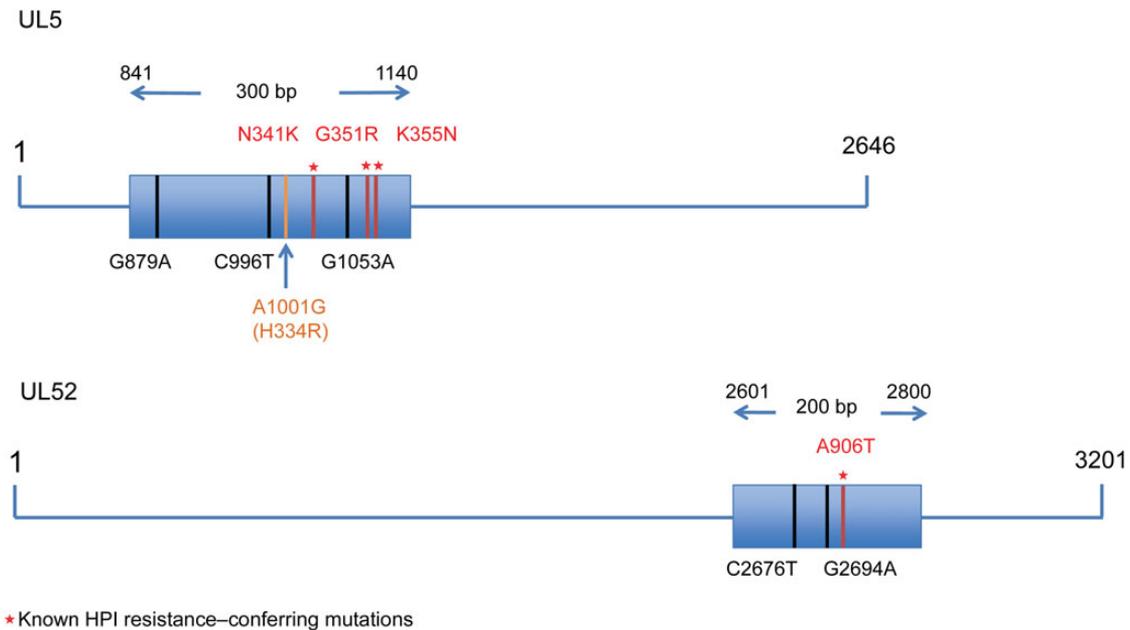


Figure 1. Locations of known resistance DNA mutations in UL5 and UL52 versus mutations identified by DNA sequence analysis of swab specimens from trial participants. These mutations are relative to the consensus. Four different DNA mutations in the region in UL5 associated with helicase-primase inhibitor (HPI) resistance and 2 different mutations in the region in UL52 associated with HPI resistance were identified (black bars), with only 1 nonsynonymous mutation located in UL5 (H334R; orange bar). For comparison, the known HPI resistance–mediating mutations C1023A (N341K), G1051C (G351R), and G1065T (K355N) in UL5 and G2716A (A906T) in UL52 are included in red. However, none of these could be detected in any of the trial participants. Arrows and numbers above the amplified regions indicate the size and location of each region of HPI resistance. This figure is available in black and white in print and in color online.

Samples and Viral DNA

In the dosage-finding trial, persons with genital HSV-2 infection were treated with 5 mg, 25 mg, or 75 mg of pritelivir once daily, 400 mg of pritelivir once weekly, or placebo for 28 days. Participants obtained swab specimens of the genital area daily for detection of HSV DNA by a validated real-time quantitative polymerase chain reaction (PCR) assay, as described previously [7, 8].

Sequencing of Regions Associated With Resistance to Helicase-Primase Inhibitors

For all participants who received at least 1 dose of trial medication (placebo or pritelivir) and from whom an adequate yield of viral DNA was available, we sequenced the regions of UL5 and UL52 genes known to carry mutations mediating phenotypic resistance to HPIs in vitro [9–14]. The sequencing covered a 300–base pair region in UL5 (nucleotides 841–1140) and a 200–base pair region in UL52 (nucleotides 2601–2800). Only samples containing ≥ 5000 copies/mL were selected for sequencing. Ten microliters of each DNA sample was used for high-fidelity PCR. Primers flanking the regions and Platinum TaqDNA Polymerase High Fidelity reagents (Invitrogen, Carlsbad, California) were used to generate PCR products according to the manufacturer's instructions. After column purification by the QIAquick PCR Purification Kit (Qiagen, Valencia, California), the PCR products were sent to Qiagen (Hilden, Germany) for

sequencing by inner primers (Supplementary Table 1), using Big-Dye Terminator cycle sequencing technology [15, 16]. The sequencing data were aligned to those for HSV-2 reference strain HG52 (NC_001798.1 [17]), using the Sequencher 4.10 sequence-analysis application. Sequence results were accepted only if all bases in the region were sequenced at least twice by independent sequencing reactions. Nucleotide sequences were codon aligned and translated into amino acid sequences, using the online bioinformatics tool EMBOSS Transeq [18, 19], and nucleotide and amino acid consensus sequences were computed as references for subsequent analysis.

Whole-Genome Sequencing

Full UL5 and UL52 genes were sequenced from participants with an HSV DNA level of $\geq 50\,000$ copies/mL. In participants with multiple samples, only the first and last samples were selected for sequencing. One primer pair, flanking the entire UL5 gene, was selected to produce 2952–base pair amplicons for sequencing. Two primer pairs, overlapping by 1528 base pairs and with PCR products of 2524 and 2588 base pairs, respectively, were selected to generate PCR products for UL52 gene sequencing (Supplementary Table 2). Eleven primers were used to sequence UL5, and 15 primers were used to sequence UL52. Owing to sequencing artifacts at the boundary of the separately amplified halves of UL52, the analysis excluded nucleotides 1480–1488 and corresponding amino acids 494–496 and

treated as missing data any gaps extending from this region into the adjacent pair of amino acids. The PCR conditions, PCR product purification, sequencing, and sequence analysis were the same as described for sequencing of the region associated with resistance to HPI. Sequence results of each gene were accepted only if all bases within the gene were sequenced at least twice by independent sequencing reactions. The sequences have been submitted to GenBank under accession numbers KP019007–191.

HSV-2 Plaque Reduction Assay

The susceptibility of HSV-2 strains to pritelivir was determined by a plaque reduction assay in Vero cells [12]. Briefly, approximately 100 plaque-forming units of virus were inoculated into 12-well plates (Nunc, Roskilde, Denmark) containing approximately 3×10^5 Vero cells/well. After adsorption for 60 min at 37°C in a humidified atmosphere of 5% CO₂, Dulbecco's modified Eagle's medium overlay (containing 1% newborn calf serum and high-density carboxymethyl cellulose and different concentrations of pritelivir) was added to each well. The plaques were fixed, stained, and enumerated after incubation for 48 hours. The mean number of plaques at each concentration (expressed as the percentage of the mean of the control wells that contained no drug) was plotted against the log₁₀ drug concentration and evaluated with GraphPad Prism 4.

HSV-2 Duplex Droplet Digital PCR (ddPCR)

To confirm the detection of multiple strains of HSV-2 in patients 5d04 and 5d19 by UL5 and UL52 sequencing, 4 different ddPCRs were performed on all positive samples collected from these 2 study participants. The ddPCR reactions were performed on Bio-Rad QX100 Droplet Digital PCR System as described previously [20]. The thermocycling conditions were as follows: 1 cycle at 94°C for 10 minutes, 35 cycles at 94°C for 30 seconds, and 1 cycle at 60°C for 1 minute, followed by 1 cycle at 98°C for 10 minutes and ending at 4°C. Two of the 4 assays detect single nucleotide changes at positions 48034 (T/C) in UL24 and 64473 (G/C) in UL30 of HSV-2 reference sequence JN561323. The other 2 assays detect variations at 2 different regions within UL39. The primers and probes sequences are shown in [Supplementary Table 3](#).

RESULTS

Sequencing of the Regions in UL5 and UL52 Associated with Resistance to Helicase-Primase Inhibitor

During the pritelivir phase 2 trial [6], 8993 genital swab specimens were collected from 155 eligible participants. Of these, 823 swab specimens (median time of collection after treatment initiation, 22 days; range, 1–44 days), collected from 87 subjects (20 in the placebo group and 67 in one of the 4 dosage groups; [Supplementary Table 4](#)), contained an HSV-2 quantity of ≥ 5000 copies/mL and were subjected to resistance-region sequencing. Of the 823 samples, 688 (84%), from 80 patients, underwent

successful sequencing of the UL5 gene, and 775 (94%), from 83 patients, underwent successful sequencing of the UL52 gene (Figure 2). The nucleotide consensus sequences for both UL5 and UL52 were identical to the sequences of these genes from the HG52 reference strain. All changes reported here are variations relative to the consensus; the sequences of these 2 regions are identical across all samples collected from each participant, including samples collected before administration of pritelivir.

Using the nucleotide consensus as the reference sequence, nucleotide changes in the UL5 resistance region were observed in the samples collected from 5 study participants, including 1 placebo recipient and 4 pritelivir recipients in 3 treatment groups (Table 1). All changes were synonymous, except in samples collected from one participant, who received 5 mg of pritelivir once daily. All 23 HSV-2 samples from this participant, including the sample collected at baseline (day 1), before initiating pritelivir therapy, and the sample collected on day 2, after pritelivir initiation, had the same single nucleotide change and corresponding amino acid change relative to the H334R consensus. Since this amino acid change was close to the fourth functional motif of UL5, we evaluated this participant's isolate for potential pritelivir resistance, using a plaque reduction assay [21]. As this isolate was susceptible to pritelivir, the H334R mutation was not regarded to confer resistance (Figure 3). In the UL52 resistance region, a synonymous change to the consensus was observed in 2 participants (1 placebo recipient and 1 participant receiving pritelivir 25 mg once daily); no nonsynonymous changes were found in any of the samples. While these sites were noted previously, the susceptibility of the H334R variant was not previously reported [6].

Whole-Genome Sequencing

We sequenced the entire UL5 and UL52 reading frames from the last PCR-positive swab specimen obtained during treatment and either the pretreatment swab specimen (if positive) or the first swab specimen obtained during treatment, subject to an adequate yield of viral DNA. 582 specimens from 79 participants in the trial were HSV-2 positive at $\geq 50\,000$ copies/mL, the threshold for whole-genome sequencing. We excluded 189 samples that were collected from subjects assigned to the placebo arm and 190 samples that had been collected >1 day after administration of the last dose of study drug. Two additional samples from 1 participant were excluded after they were confirmed as positive for HSV-1. Forty-five participants provided the remaining 203 samples that were eligible for whole-genome sequencing. Among these 45 participants, 15 had only 1 sample, and we sequenced 2 samples from each of the remaining 30. The median time of collection after treatment initiation for these samples (or the latest date, for paired samples), as well as for samples from the 42 participants with successfully sequenced samples after treatment initiation, was 11 days

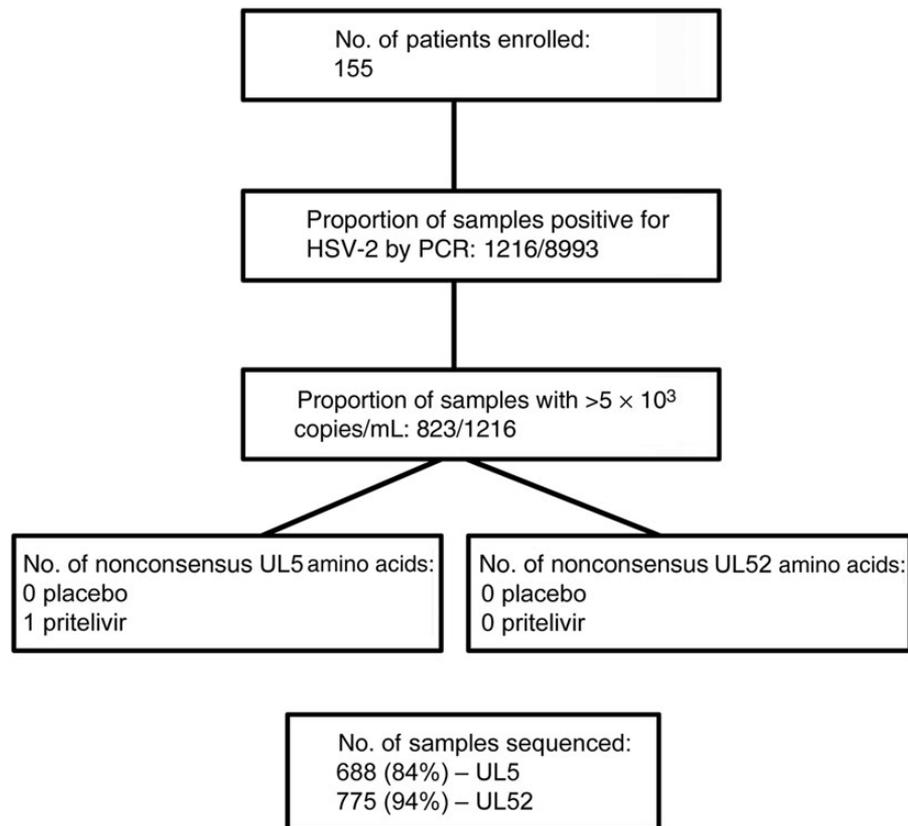


Figure 2. Variation in UL5 and UL52 helicase-primase inhibitor (HPI) resistance–mediating regions. This flowchart shows the fraction of samples from which the regions in each gene associated with HPI resistance were successfully sequenced. Only 1 amino acid mutation was observed in any of the sequences of the HPI resistance regions. All amino acid sequences from any single participant were identical. Abbreviations: HSV-2, herpes simplex virus type 2; PCR, polymerase chain reaction.

(range, 1–29 days). The median number of days between collection of paired specimens was 3 (range, 1–39 days).

UL5

Of the 75 available specimens, 73 (97%) were successfully sequenced for the entire UL5 gene. In all, 15 participants had 1 sample in which HSV-2 UL5 was completely sequenced, and 29 had 2 samples in which HSV-2 UL5 was completely sequenced (Supplementary Tables 5 and 6). Only 2 participants,

both assigned to the 5 mg once daily treatment group, had virus that showed any between-sample variation in the UL5 gene between the first and the last positive swab specimen (Supplementary Table 7). In both cases, the samples were collected after baseline (days 7 and 15 and days 13 and 15, respectively). We used ddPCR to evaluate these samples for polymorphisms unique to different strains of HSV-2. This analysis included single-nucleotide polymorphism analyses of several additional HSV-2 genes, including UL39, and indicated that both of

Table 1. Mutations Relative to the Consensus in the Regions of UL5 and UL52 Associated With Helicase-Primase Inhibitor Resistance Identified in Region-Targeted Sequencing

Participant	pbo13	5d02	5d08	25d05	400w12	pbo07	25d10
Drug cohort	Placebo	5 mg once daily	5 mg once daily	25 mg once daily	400 mg once weekly	Placebo	25 mg once daily
Mutation ^a							
Gene	UL5	UL5	UL5	UL5	UL5	UL52	UL52
Nucleotide	C996T	C996T	A1001G	G879A	G1053A	G2649A	C2676T
Amino acid			H334R				
Sequences, no.							
With variation	10	2	23	10	7	3	2
Total	10	2	23	10	7	3	2

^a Relative to the consensus.

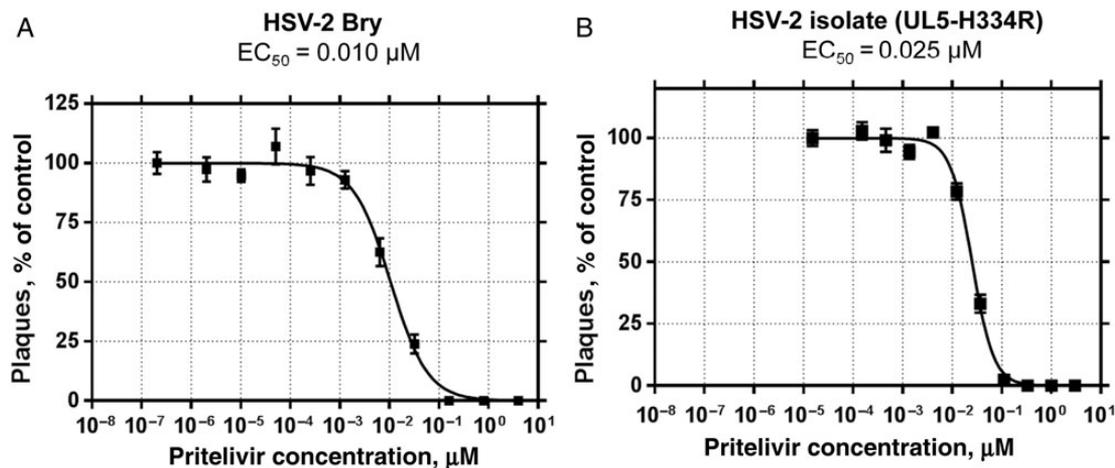


Figure 3. Susceptibility of herpes simplex virus type 2 (HSV-2) laboratory strain Bry (A) and the clinical isolate carrying the amino acid exchange UL5-H334R (B) to pritelivir, as determined by a plaque reduction assay (PRA). The 50% effective doses (EC₅₀) were measured by means of PRA. Plaques were counted after 48 hour incubation. The y-axis shows the number of plaques as compared to control (no drug), as a percentage. The x-axis shows the concentration of pritelivir. Data points represent the means of 6 replicates with standard deviations. These results are from 1 representative experiment.

these participants were dually infected (Supplementary Table 8), supporting the hypothesis that these polymorphisms reflect preexisting sequence variation between the virus strains, rather than drug-induced variation.

Over time, the circulating HSV-2 viral genome is expected to evolve away from the HG52 reference sequence. We found that the consensus of the UL5 nucleotide sequences was identical to the HG52 sequence except at 3 positions with synonymous mutations relative to HG52: C1281T (C in 28% and T in 72%), T1965G (T in 5% and G in 95%), and T2100C (T in 3% and C in 97%). Comparing the trial participants' UL5 sequences from all available samples to the consensus, we identified 8 sites with nonsynonymous mutations, all involving a single nucleotide difference (Supplementary Table 9). One of these, H334R, was also found in the UL5 fragment sequence analysis and was confirmed to be susceptible to pritelivir. Three were observed in >1 person: R415H was observed in 2 participants, A739T in 7, and I763V in 9. All 7 persons with the A739T substitution also had the I763V substitution, as depicted in Supplementary Figure 1A ($P < .0001$, by the Fisher exact test, for independence between the 2 sites). In the data set restricted to 1 sample per person by taking only the participant's latest sequenced sample collected after pritelivir administration, we found 2 amino acid sites—739 and 763—with sufficient variation for analysis of association with pritelivir treatment (ie, at least 10% of the amino acids differed from the consensus at each site). There was no evidence that any of these mutations differed by treatment group ($P > .2$, by the Fisher exact test, for all comparisons; Supplementary Table 10), consistent with the observation that no participants in this randomized trial exhibited sequence variation between first and last available samples (except the 2 whose sequences were of different strains, as discussed above).

We also sequenced the UL5 genes of HSV-2 from 32 samples previously determined to be susceptible to pritelivir by a plaque reduction assay, as previously described [21]. Briefly, these are clinical isolates obtained from cultures of herpetic lesions from pritelivir-naïve persons in 1998–2004, in Seattle. The nucleotide consensus of these samples was identical to that of the trial sequences. We identified 2 amino acid positions—S458G and Y573H—with nonsynonymous mutations relative to the consensus (Supplementary Table 8) that were not found in the sequences of trial participants. Of the 8 amino acid sites with any variation observed in trial participants, the following 4 were not found in the susceptible isolate sequences (including the participant sequence determined to be susceptible): R415H, D513N, S605P, and S689T.

UL52

Only 2 participants exhibited any between-sample variation in the UL52 gene between the first and the last positive swab specimen, and these were the same 2 participants in whom we found multiple strains of HSV-2 (Supplementary Table 7). Owing to the high GC content of the UL52 gene, only 46 of 75 specimens (61%) were successfully sequenced for UL52 (Supplementary Tables 11 and 12). Twenty-five of 75 specimens (33%) were incompletely sequenced, and 4 (5%) failed UL52 sequencing. Among the 33 participants who had at least 1 sample with a complete HSV-2 sequence for UL52, 13 had 2 samples with a complete sequence, and 20 had only 1 sample. To make full use of available data, we analyzed the data set containing 71 sequences (completely and incompletely sequenced) from viruses of 43 participants.

The consensus of the UL52 nucleotide sequences was identical to the HG52 sequence except at 6 positions, the following 3 of which had nonsynonymous mutations relative to HG52:

T169C, corresponding to amino acid variation S57P (T in 3% and C in 92%; 3 sequences with missing data); G430A, corresponding to amino acid variation V144I (G in 3% and A in 91%; 4 sequences with missing data); G653C, corresponding to amino acid variation G218A (C in 94%; 4 sequences with missing data); and an inserted codon at -2140GAC, corresponding to amino acid insertion -714D. The 19 sequences with the codon insertion also had consistent changes in the 2 flanking codons, with all 19 having the mutation GGT—CCC2137GGCGACGAC, corresponding to a synonymous mutation at amino acid position G713 and the substitution-insertion variation P714DD. The other 52 sequences matched HG52 identically at this position. The following 2 positions of the consensus had synonymous nucleotide mutations relative to HG52: A837G (G in 100%) and T2862C (T in 19% and C in 81%). These are given in HG52 coordinates; the latter is T2865C relative to the consensus.

Comparing all available UL52 sequences to the consensus, we identified 20 sites with nonsynonymous variation, including the substitution-insertion mutation, P714DD, which exhibited complete linkage. Of these 20 sites, 5 were observed in viruses from multiple persons. The change T495S, observed in samples from 14 participants, spans the region of low sequencing resolution that we designated to be excluded from the primary analysis. Two of the other changes, S697L and P714DD, involved >1 nucleotide difference. Except for G334S, the nonexcluded variations occurred in mutually exclusive sets of participants ([Supplementary Figure 1B](#)). In the data set restricted to the sequence in the swab specimen collected from each participant at the latest time point after pritelivir administration, we found the following 5 amino acid sites with variation: 334, 495, 697, 714, and 715. As in UL5, we found no evidence that these mutations in UL52 differed by treatment group ($P > .2$, by the Fisher exact test, for all comparisons; [Supplementary Table 10](#)).

We also sequenced the UL52 genes of the 32 susceptible HSV-2 sequences. The nucleotide consensus of these sequences was identical to that of the participant sequences. We identified the following 7 amino acid positions with nonsynonymous variation relative to the consensus ([Supplementary Table 13](#)) that were not found in the sequences of trial participants: E9G, D58N, R119H, R414S, R440C, T518A, and L600P. Of the 20 amino acid sites with variation observed in samples from trial participants, 10 were not observed in the susceptible isolate sequences. One of these, T25A, was found in the sequence from the strain that we confirmed to be susceptible to pritelivir. The remaining 9 were E101K, G312R, R331H, R424M, S459P, A578V, D704G, E719A, and N1020H.

DISCUSSION

Our study is the first to investigate whether mutations consistent with resistance to HPI emerged in vivo in people receiving the drug for treatment of genital HSV-2 infections. Using both full-

gene sequencing of the helicase-primase complex and targeted sequencing, we found no evidence of resistance in HSV-2 strains from persons treated with varying dosages of pritelivir. Instead, the observed variations reflected the preexisting diversity of the HSV-2 strains among the subjects enrolled in the trial. Overall, few mutations relative to the consensus were found [22], and no changes in HSV-2 sequence during treatment occurred in individual patients. None of the mutations fell in or near a putative resistance region, except for one (UL5 H334R), which was found in an HSV-2 isolate obtained from a trial participant in the 5 mg once daily group that, based on results of a plaque reduction assay, we determined to be susceptible to pritelivir.

While these analyses involved a relatively small number of patients, at least 2 dosages in our study were less than optimal for HSV replication inhibition, as shown by persistent viral shedding during dosing [6]. Thus, it is comforting that rapid selection for resistance was not observed even with suboptimal regimens of pritelivir. In human studies, the longest duration of pritelivir administration so far has been 28 days. Thus, whether prolonged use will result in selection for resistance is as yet unclear.

Moreover, studies in immunocompromised patients with poor cellular immunity are lacking. Resistance to nucleoside analogues is rare in immunocompetent individuals with genital herpes who are receiving antiviral therapy, but it arises occasionally in immunocompromised patients [3, 23]. Whether resistance will be eventually observed with HPI therapy is unknown.

One of the interesting findings to emerge in our studies was the population-level genetic diversity in UL5 and UL52, a diversity that heretofore has not been well described. In this study, we sequenced the known drug resistance regions of the UL5 and UL52 genes from 823 HSV-2–positive swab samples collected during the study period. In the UL5 drug resistance region, 4 different DNA mutations were observed (relative to the consensus) in samples collected from 5 different people. One of the mutations was nonsynonymous. This nonsynonymous mutation is not known to cause resistance to HPI, and we experimentally confirmed that the HSV-2 isolate containing this mutation was susceptible to pritelivir. In the UL52 drug resistance region, we detected 2 different mutations in samples collected from 2 different study participants, both of which were synonymous. Treatment with pritelivir did not appear to select for or induce mutations in these 2 drug resistance regions, since all sequences obtained from the same person were identical.

We also completely sequenced the UL5 gene of HSV-2 isolates from 73 samples and the UL52 gene of isolates from 46 samples. In 2 participants, we found differences between samples collected from the same person over time. However, we confirmed that these differences reflect infection with dual HSV-2 strains, rather than variation induced by the treatment. We exhaustively identified all sites exhibiting any variation for

each gene. While many of these variations also appear in sequences of HSV-2 isolates that are known to be susceptible to pritelivir, 4 amino acid sites in UL5 and 9 in UL52 were found in trial participant sequences that were not evaluated for resistance. Since these mutations did not occur near the putative resistance-mediating regions and since they most likely pre-existed in the population of HSV-2 strains infecting the trial participants, it is unlikely that they confer resistance. The sequences may be valuable for future analysis of the UL5 and UL52 genes of HSV-2 as trials evaluate pritelivir and other helicase-primase inhibitors for treatment of HSV infection.

Supplementary Data

Supplementary materials are available at <http://jid.oxfordjournals.org>. Consisting of data provided by the author to benefit the reader, the posted materials are not copyedited and are the sole responsibility of the author, so questions or comments should be addressed to the author.

Notes

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Potential conflicts of interest. P. T. E. reports receiving grant support through his institution from AiCuris. A. B. reports contributing to patents related to galenics and the synthesis of drug substances through AiCuris. C. A. M. and J. J. K. report receiving grant support through their institution from AiCuris. A. B., T. G., B. T., S. S., H. R.-S., and H. Z. are employees of AiCuris. A. B., B. T., H. R.-S., and H. Z. report holding stock options in AiCuris. T. W. reports receiving travel support from AiCuris and Vical and grant support through her institution from AiCuris, Agenus, Genocoea, and Vical. A. W. reports receiving consulting fees from AiCuris and Amgen, travel support from Vical, and grant support through her institution from Agenus, Genocoea, Gilead, and Vical. L. C. reports being a co-inventor on several patents associated with the development of an herpes simplex virus type 2 vaccine and is on the scientific advisory board for and holds stock (<1% of the company) in Immune Design. All other authors report no potential conflicts. All authors have submitted the ICMJE Form for Disclosure of Potential Conflicts of Interest. Conflicts that the editors consider relevant to the content of the manuscript have been disclosed.

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