Brincidofovir is an oral antiviral in development for prevention of cytomegalovirus disease. Cytomegalovirus genotyping results from a phase 2 trial comparing brincidofovir to placebo for prophylaxis against cytomegalovirus infection in hematopoietic cell transplant recipients provided initial data on the clinical resistance profile for brincidofovir. In this study, no known resistance-associated mutations were detected in brincidofovir-treated subjects; identified genotypic substitutions did not confer resistance to cytomegalovirus antivirals in vitro, suggesting that these changes represent polymorphisms unrelated to brincidofovir resistance. Lack of evidence for genotypic resistance during prophylaxis suggests that first-line use of brincidofovir for prevention of cytomegalovirus infection may preserve downstream options for patients.

Keywords. brincidofovir; cytomegalovirus; CMV; stem cell transplantation; CMV phosphotransferase; UL97; CMV (DNA) polymerase; UL54.

Cytomegalovirus (CMV) is an enveloped, double-stranded DNA (dsDNA) virus that infects most people worldwide by adulthood and establishes a latent infection. It is a major cause of morbidity and mortality among immunocompromised individuals, especially those who have undergone allogeneic hematopoietic cell transplantation (HCT) [1]. Prevention of CMV reactivation or new infection with antivirals has been a goal for HCT recipients since proof of concept was established for ganciclovir (GCV); however, neutropenia and other adverse events have prevented the prophylactic use of current antivirals for prevention of CMV infection in HCT recipients [2–5]. Four antiviral drugs are currently available for the prevention and/or treatment of CMV infections: GCV; the GCV prodruk, valganciclovir (vGCV); cidofovir (CDV); and foscarnet (FOS). None are approved for prophylaxis against CMV infection in HCT recipients because of inadequate risk and benefit trade-offs.

Current CMV–associated antivirals target CMV DNA polymerase, encoded by UL54, although GCV is first converted to the monophosphate by CMV-encoded UL97 kinase before it is phosphorylated by cellular enzymes to the active triphosphate [6, 7]. Therefore, CMV resistance to currently available antivirals maps to UL54 or, in the case of GCV, to both UL54 and UL97; mutations in UL97 are detected in approximately 90% of GCV-resistant clinical isolates [6, 7].

Brincidofovir (BCV; CMX001; phosphonic acid, [(S)-2-(4-amino-2-oxo-1(2H)-pyrimidinyl)1-(hydroxymethyl)ethoxy)methyl]monoo[3-(hexadecyloxy)propyl] ester) is an orally bioavailable lipid acyclic nucleoside phosphonate that is converted intracellularly into the active antiviral moiety, cidofovir diphosphate (CDV-PP), that serves as an alternate substrate inhibitor of CMV DNA replication [8, 9]. BCV has demonstrated broad antiviral activity in vitro against dsDNA viruses and is currently in development for the treatment of adenovirus infection, prevention of CMV infection, and treatment of smallpox. While BCV has a spectrum of antiviral activity similar to that of CDV, it is typically 100-fold more potent in vitro [9, 10]. For example, the median concentration that effectively reduced in vitro replication of wild-type human CMV (AD169) by 50% was 1 nM for BCV, 400 nM for CDV, and 3800 nM for GCV in a direct comparison [10].

In vitro, prolonged (10 months) serial passage with BCV selected for a unique mutation (D542E) in UL54 that was associated with slower CMV replication and resistance to BCV and CDV [11]. Mutations in UL97 associated with GCV resistance do not affect in vitro susceptibility to BCV or CDV, but most mutations in UL54 that are associated with resistance to GCV also encode resistance to CDV and BCV [6, 7]. Similarly, data from an expanded-access study that enrolled subjects for treatment of resistant and refractory CMV disease (Study 350) suggest that UL97 mutations have minimal effect on virologic response to BCV, while UL54 mutations associated with CDV resistance diminish virologic response to BCV [12].

In a phase 2 trial (CMX001-201), BCV demonstrated a dose-response relationship for the prevention of CMV infection in adult allogeneic HCT recipients, with BCV 100 mg twice weekly showing statistically significant differences from placebo with...
respect to the proportion of patients with plasma CMV DNA levels of ≥200 copies/mL at the end of treatment assessment (10% vs 37%; P = .002) [5]. To better understand the BCV resistance profile in the context of CMV prevention, we evaluated data from study CMX001-201 to investigate whether substitutions in CMV polymerase were associated with antiviral resistance. Understanding the resistance and cross-resistance profiles of CMV-associated antivirals is critical for optimizing the clinical utility of these agents.

METHODS

Study Population, Clinical Design, and Selection of Samples for Analysis
CMX001-201 (clinical trials registration: NCT00942305) was a phase 2, multicenter, randomized, double-blinded, placebo-controlled, dose-escalation study of BCV in adult allogeneic HCT recipients seropositive for CMV [5]. BCV dosing was distributed over 5 cohorts: recipients of 40 mg once weekly, recipients of 100 mg once weekly or twice weekly, and recipients of 200 mg once weekly or twice weekly; each cohort had an embedded placebo group. Dosing was initiated following engraftment and continued to week 13 after transplantation. CMX001-201 enrolled 171 subjects who received BCV and 59 subjects who received placebo. CMV genotyping (Viracor-IBT 5600) was attempted for plasma samples from 11 of 25 subjects (44%) from the BCV 40 mg once weekly cohort, 8 of 27 (30%) from the BCV 100 mg once weekly cohort, 16 of 39 (41%) from the BCV 200 mg once weekly cohort, 14 of 50 (28%) from the BCV 100 mg twice weekly cohort, 9 of 30 (30%) from the BCV 200 mg twice weekly cohort, and 26 of 59 (44%) from the placebo arm (Figure 1). Among subjects with a confirmed viremia level of ≥200 copies/mL during the treatment phase of the trial, CMV genotyping was attempted for 40 of 41 subjects (98%) from the BCV-treated cohorts, and sequences from 34 of 41 (83%) were obtained. In addition, genotyping was attempted for 20 of 27 subjects (74%) from the placebo-treated cohort, and sequences were obtained from 19 of 27 (70%). Genotyping was also attempted for 18 subjects who received BCV, and 6 who received placebo who did not have a confirmed viremia level of ≥200 copies/mL during the treatment phase of the trial (sequences from 10 and 3 subjects, respectively, were obtained). These latter subjects had an unconfirmed viral load of ≥200 copies/mL during treatment and/or had CMV viremia in the posttreatment phase of the trial. Sequencing failure was primarily attributable to a CMV viremia level of <500 copies/mL. Conduct of CMX001-201 followed the human experimentation guidelines of the Department of Health and Human Services, and informed consent was obtained from all subjects.

CMV DNA Quantification
CMV plasma viral loads (ie, viremia) were determined by a quantitative polymerase chain reaction assay performed by Viracor-IBT Labs (5500), with a lower limit of detection of 100 copies/mL.

CMV Genotype Analysis
Genotyping to assess viral resistance was performed by Viracor-IBT Labs. Sequencing spanned the regions of UL97 and UL54 that contain the sites of recognized resistance-associated mutations (RAMs), including the regions in UL54 expected to be the target of an alternative substrate inhibitor, such as CDV-PP, which is the active antiviral moiety of BCV. The UL97 target was between codons 438 and 658 in UL97. The UL54 target was between codons 252 and 1138 in UL54.

Genotypic changes in plasma CMV DNA from subjects were compared to the wild-type CMV AD169 sequence and the subject’s baseline CMV genotype (when available). Although

![Figure 1. Study and genotypic analysis population. Abbreviations: BCV, brincidofovir; CMV, cytomegalovirus.](image-url)
CMX001-201 enrolled patients in a study of BCV for prevention of CMV reactivation, 50 of 230 enrolled subjects had detectable CMV DNA at baseline.

**CMV Phenotype Analysis**

Candidate RAMs for CMV have typically been evaluated by marker-transfer studies [6, 7]. Here, substitutions associated with virologic rebound or incomplete virologic response that were found in >1 subject or located in a conserved region of UL54 but that have not been proven to confer phenotypic resistance, were phenotyped by marker transfer using published methods [13, 14]. Briefly, mutations were introduced into a bacterial artificial chromosome clone of CMV strain AD169, modified to contain a reporter gene. Susceptibility of the derived live virus was determined by standardized yield reduction assays. Control bacterial artificial chromosome-derived strains included drug-susceptible strains obtained at baseline and mutants with known resistance phenotypes (UL54 mutants A809V and A987G and UL97 mutant C592G). Phenotyping assays included wild-type, CDV-resistant, and BCV-resistant isolate controls and analyses to determine GCV, CDV, and FOS cross-resistance.

**RESULTS**

**Analysis of Genotypic Substitutions During BCV Therapy**

No known CMV antiviral RAMs were detected in BCV-treated subjects in CMX001-201. Forty-three BCV-treated subjects had no known CMV antiviral RAMs were detected in BCV-treated patients. Analysis of genotypic substitutions during BCV therapy showed that substitutions associated with CMV reactivation, 50 of 230 enrolled subjects had detectable CMV DNA at baseline.

The drug-susceptibility phenotypes for the recombinant and control strains are shown in Table 1. Neither M827I nor R1052C conferred decreased susceptibility to BCV, CDV, GCV, or FOS.

**DISCUSSION**

CMV prevention study CMX001-201 provided evidence that BCV may be an effective option for preventing CMV events when administered at 100 mg twice weekly for 9–11 weeks after HCT engraftment, compared with placebo [5]. Analyses of plasma-derived CMV from patients with viral rebound or incomplete suppression in CMX001-201 failed to detect any one of these subjects subsequently initiated CDV therapy, while the other initiated vGCV therapy. The subject receiving CDV had a suboptimal virologic response (decrease in CMV viral load, 0.26 log₁₀ copies/mL after 8 weeks), but the subject receiving vGCV had a complete virologic response.

Another subject had an M827I variant detected in conjunction with 6 polymorphisms (I341T, S655L, N685S, 885 insert S, A885T, and A1122T) after 5 weeks of BCV 40 mg once weekly; 3 weeks after therapy, M827I and the other variants were still present. This subject had a plasma viremia level of 21 000 copies/mL at week 5 and 700 copies/mL at week 4 post-BCV therapy following 4 weeks of GCV therapy (1.5 log₁₀ copies/mL decrease).

**Phenotypic Analysis of Candidate RAMs**

The 2 mutations identified for subsequent in vitro phenotyping (M827I and R1052C) were evaluated by marker transfer. The R1052C mutation was phenotyped in combination with A885T and S897L, which are known polymorphisms that were also present in each of the 3 clinical samples that contained R1052C. The drug-susceptibility phenotypes for the recombinant and control strains are shown in Table 1. Neither M827I nor R1052C conferred decreased susceptibility to BCV, CDV, GCV, or FOS.

**Table 1. Recombinant Phenotypes of Candidate Brincidofovir (BCV) Resistance-Associated Mutations**

| Drug | EC₅₀, Mean±SD | Replicates, No. | EC₅₀ Ratio |
|------|---------------|-----------------|------------|
| Wild-type control strain T3265: UL54 S897L | | | |
| BCV, nM | 0.21 ± 0.06 | 18 | ... |
| CDV, μM | 0.23 ± 0.07 | 24 | ... |
| GCV, μM | 1.29 ± 0.38 | 26 | ... |
| FOS, μM | 51 ± 14 | 21 | ... |
| Strain T3913: UL54 R1052C, A885T, S897L | | | |
| BCV, nM | 0.17 ± 0.07 | 8 | 0.8 |
| CDV, μM | 0.26 ± 0.06 | 19 | 1.1 |
| GCV, μM | 1.13 ± 0.28 | 9 | 0.9 |
| FOS, μM | 51 ± 13 | 8 | 1.0 |
| Strain T4064: UL54 M827I, S897L | | | |
| BCV, nM | 0.17 ± 0.05 | 7 | 0.8 |
| CDV, μM | 0.15 ± 0.05 | 7 | 0.7 |
| GCV, μM | 1.27 ± 0.43 | 11 | 1.0 |
| FOS, μM | 53 ± 7 | 12 | 1.0 |

Abbreviations: BCV, brincidofovir; CDV, cidofovir; EC₅₀, 50% effective concentration; FOS, foscarnet; GCV, ganciclovir.

* Measured over ≥3 setup dates.

* Calculated as the ratio of the specified EC₅₀ to that of wild-type control strain.
known UL97 or UL54 RAMs from subjects receiving BCV, including those who received BCV doses that did not show efficacy, compared with placebo, against the occurrence of CMV viremia (ie, a CMV DNA load of ≥200 copies/mL). These data suggest that BCV is unlikely to select for RAMs when delivered at the 100 mg twice weekly prophylactic regimen that is currently under investigation.

Two CMV UL54 substitutions detected in CMX001-201 (M827I and R1052C) were not previously shown to confer resistance to current antivirals for CMV but merited further investigation because they were located in a region of conserved DNA sequence or were detected in >1 subject. However, these 2 UL54 substitutions did not affect the susceptibility to BCV, CDV, GCV, or FOS when evaluated by recombinant phenotyping, including R1052C in the context of other polymorphisms detected. Given the clinical presentation of these mutations, they may be markers of resistance or require mutations outside of the region sequenced to confer decreased susceptibility in vitro.

One limitation of this study is that the UL54 region sequenced was limited to amino acids 252–1138 in UL54. While this sequence covers all known UL54 RAMs, it is possible that other substitutions outside of this region may be selected by BCV. Resistance analysis in all clinical studies following study CMX001-201 in which CMV is a primary or secondary end point will include sequencing of the entire UL54 gene. The results from this analysis should not be extended to CMV therapy-experienced subjects and/or subjects with a high plasma CMV load at baseline, when preexisting resistance is more likely. Results from CMX001-201 suggest that preventive therapy with BCV is not likely to select for RAMs to BCV, CDV, GCV, or FOS when used as prophylaxis in CMV-seropositive HCT recipients, preserving future options for patients.

Notes

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