Selection and Recombinant Phenotyping of a Novel CMX001 and Cidofovir Resistance Mutation in Human Cytomegalovirus

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CMX001 is an orally available lipid acyclic nucleotide phosphonate that delivers high intracellular levels of cidofovir (CDV)-diphosphate and exhibits enhanced in vitro antiviral activity against a wide range of double-stranded DNA viruses, including cytomegalovirus (CMV). Mutations in the DNA polymerase of CMV that impart resistance to CDV also render the virus resistant to CMX001. Here, we report a novel resistance mutation that arose under the selective pressure of CMX001. The wild-type CMV strain AD169 was propagated in human foreskin fibroblasts under increasing concentrations of CMX001 over 10 months and the resulting strain (CMX001R) was less susceptible to CDV and CMX001 in a plaque reduction assay. Genotypic analysis of CMX001R via conventional sequencing of the genes encoding the CMV DNA polymerase (UL54) and UL97 kinase (UL97) demonstrated one mutation which changed the wild-type aspartate to glutamate at position 542 in UL54. A recombinant virus with this novel D542E mutation was generated via bacterial artificial chromosome-mediated marker transfer experiments. Subsequent phenotypic resistance analysis of the D542E mutant demonstrated reductions in susceptibility of greater than 10-fold to CMX001 and CDV but no resistance to foscarnet (FOS) or ganciclovir (GCV). Analysis of replicative fitness showed that both CMX001R and the D542E mutant viruses demonstrated a smaller plaque phenotype and slower replication kinetics than their respective parent viruses. These data describe the first resistance mutation generated under the selective pressure of CMX001 and suggest that CMX001 may have a unique resistance profile associated with reduced viral replication and maintenance of sensitivity to FOS and GCV.
INTRODUCTION

Infections with human cytomegalovirus (CMV) are highly prevalent, but are typically asymptomatic or self-limited in healthy hosts; however, they are a cause of significant morbidity and mortality in people with compromised or immature immune systems, including transplant recipients, patients with AIDS, and congenitally infected infants (1-4). Currently approved antiviral agents are often limited in their therapeutic utility due to modest antiviral activity, poor bioavailability, resistance, and/or drug toxicities (5, 6). Reports on the incidence and prevalence of resistance vary substantially depending on the patient population and how the drugs are used, but cross-resistance is a common problem with the nucleoside analogs (7, 8). For these reasons, it is important to develop new therapeutic agents for CMV with improved safety, efficacy, and resistance profiles (9).

CMX001 ([phosphonic acid, (S)-2-(4-amino-2-oxo-1(2H)-pyrimidinyl)-1-(hydroxymethyl)ethoxy)methyl]mono[3-(hexadecyloxy)propyl] ester), also referred to as hexadecyloxypropyl cidofovir (HDP-CDV) in previous publication, is an orally available lipid acyclic nucleotide phosphonate that delivers high intracellular level of CDV-diphosphate (CDV-PP) and exhibits enhanced in vitro antiviral activity against a wide range of double-stranded DNA viruses, including herpesviruses, orthopoxviruses, adenoviruses, polyomaviruses, and papillomaviruses (10, 11). CMX001 is designed for delivery into target cells where the lipid side chain is cleaved, thereby releasing CDV, which subsequently undergoes phosphorylation by intracellular kinases to the active antiviral agent, CDV-PP. CMX001 is taken up by cells much more rapidly than CDV and is associated with greater than 100-fold higher intracellular levels of CDV-PP compared
to CDV (12). In CMV-infected cells, CDV-PP acts as an alternate substrate inhibitor of the UL54-encoded CMV DNA polymerase, resulting in decreased DNA synthesis and termination of chain elongation (13, 14).

The pharmacokinetic (PK) profile of CMX001 supports its clinical development due to the potential for both higher antiviral potency and lower toxicity than currently available treatment options. Due to its ability to deliver greater levels of CDV-PP intracellularly, CMX001 exhibits enhanced antiviral activity of up to 1000-fold against CMV compared to CDV in both in vitro and in vivo models, as well as increased activity compared to ganciclovir (GCV) and foscarnet (FOS) (15-18). Data from animal studies and human trials suggests that the dose-limiting nephrotoxicity of CDV has been eliminated with CMX001 (19, 20). This has been mechanistically explained since CMX001, unlike CDV, is not a substrate of the human organic anion transporter 1 (hOAT1) that is located in the proximal renal tubule and is primarily responsible for the toxic accumulation of CDV in the kidney (11, 19). A dose-escalation PK and safety study of CMX001 in healthy adult subjects revealed no significant toxicities at doses of up to 2 mg/kg, the highest single dose tested (20).

In CMV, antiviral resistance to CDV maps to the DNA polymerase gene (UL54). Since CMX001 is converted intracellularly to CDV-PP, an alternate substrate inhibitor of UL54, resistance to this agent would also be expected to map to UL54. The impact of the higher intracellular CDV-PP levels achieved with CMX001 on the development of resistant CMV strains is unknown. To date, there have been no cases of treatment-emergent phenotypic resistance, or mutations known to be associated with resistance, in CMV antiviral treatment-naive patients receiving CMX001. The objective of this study
was to generate and characterize a de novo CMX001-resistant CMV isolate by serially passaging a wild-type laboratory strain of CMV (AD169) in the presence of increasing levels of CMX001.

MATERIALS AND METHODS

Antiviral compounds. CMX001 was provided by Chimerix, Inc. (Durham, NC). CDV and GCV were purchased from the University of Alabama at Birmingham Hospital Pharmacy, and FOS was purchased from Sigma-Aldrich (St. Louis, MO).

Cell culture and virus strains. Human foreskin fibroblast (HFF) cells were prepared and routinely propagated as monolayers in minimal essential medium (Mediatech, Inc., Manassas, VA) supplemented with 10% fetal bovine serum, L-glutamine, penicillin, and gentamicin. The CMV strain AD169 was obtained from the American Type Culture Collection (Manassas, VA), titered at 2 x 10^5 PFU/ml and stored at -80°C.

Resistant strain selection. The CMV strain AD169 was used to infect low passage HFF cells at a low multiplicity of infection (MOI) (0.1 PFU/cell) in the presence of 0.01 µM CMX001. The culture was passaged in increasing concentrations of CMX001 up to a final concentration of 0.5 µM, which is well below the concentration needed (>30 µM) to reduce cell viability by 50% (CC_{50}) (15). Infected monolayers were visually inspected twice a week and the virus was harvested either when significant cytopathology was observed or 3 weeks following infection, whichever occurred first. The total passage time was 10 months, beyond which no virus could be recovered as concentrations of
CMX001 exceeded 0.5 µM. The virus resulting from passaging was titered at 3.5 x 10^4 PFU/ml and designated as CMX001R.

**Plaque reduction assays.** HFF cells were plated in six-well plates and incubated at 37°C. When the cell layer reached confluence, the medium was aspirated from the wells, 0.2 ml of virus was added to each of three wells to yield 20 to 30 plaques per well, and 0.2 ml of medium was added to each of the three uninfected wells to test for drug toxicity. The plates were then incubated for 1 h with gentle rocking every 15 minutes after which the following drug concentration ranges were added to duplicate wells: CMX001 from 1 µM to 0.0003 µM, CDV from 100 µM to 0.03 µM, GCV from 100 µM to 0.03 µM, and FOS from 500 µM to 0.16 µM. After 8 days of incubation, cell monolayers were stained with 1% neutral red solution, the stain was aspirated and plaques were counted using a stereomicroscope. The concentration of drug that reduced plaque formation by 50% (EC50) was determined by comparing drug-treated cultures with untreated cultures. Values from three to five independent experiments were used to calculate the mean and standard deviation values.

**DNA sequencing.** The *UL54* and *UL97* genes were amplified from CMX001R using double-nested polymerase chain reaction (PCR) methods in the following manner. A fragment of *UL97* was amplified using the primers UL97 inner F1 (5'-TCC GCA CTT CGG TCT C-3') and UL97 inner R1 (5'-AAC AGT TGG CGG CAG-3') followed by a second amplification with the primers UL97 short F1 (5'-CTG AGT TCC GTC AGC A-3') and UL97 short R1 (5'-GGT CCT CCT CGC AGA T-3'). Similarly, the *UL54* open reading frame (ORF) was amplified using the primers UL54 For (5'-CGT AAG CTG TCA GCC TCT CA-3') and UL54 Rev (5'-CAG TCT CAG CAG CAT CAT CAC-3'),
followed by primers UL54 Inner For (CTC ACG GTC CGC TAT GTT TT-3’) and UL54 Inner Rev (5’-CGC TGT TTC TCA ACA GCA TTC-3’). All amplifications were done with High Fidelity PCR Master (Roche Applied Science, Indianapolis, IN). The resulting DNA fragments were purified via QIAquick Gel Extraction and PCR Purification Kits (Qiagen, Valencia, CA) and underwent conventional dideoxy chain termination sequencing at the University of Alabama at Birmingham Department of Genetics Core Sequencing Facility to identify any amino acid changes. DNA sequences were analyzed using Informax Vector NTI Contig Express 2003 (Invitrogen, Carlsbad, CA). Consensus DNA sequences were compared to that of the laboratory strain AD169 (GenBank accession number X17403).

**Generation of recombinant viruses.** To further investigate a novel UL54 mutation identified during genotypic resistance analysis, the D542E mutation was reconstructed in AD169 in the HB5 bacterial artificial chromosome (BAC) using methods similar to those previously reported (21). A plasmid containing UL54 with a kanamycin resistance marker (Kan) inserted immediately after the UL54 stop codon was constructed (UL54kan pEXP5 NT, designated as pMP302) to facilitate mutagenesis. The D542E mutation was constructed by quick change mutagenesis (QuikChange II Site-Directed Mutagenesis Kit, Stratagene, La Jolla, CA) with primers UL54 D542E F primer, 5’-CCG TTA CTG TCT GCA G-3’, and a UL54 D542E R primer 5’-CCA ATA CGG CCT CCT G-3’ and pMP302 as a template. The resulting clone, pMP547, was amplified using primers flanking the Kan marker and the UL54 locus and the PCR product was electroporated into the SW102 recombineering strain containing the HB5 BAC and plated on selective medium containing kanamycin and chloramphenicol and the resultant
BAC was designated pMP573. In a similar manner, a wild-type UL54 Kan BAC, designated pMP556, was created from the HB5 BAC using an equivalent PCR product from pMP302, but with no engineered mutations. HindIII restriction digests of all BACs were performed to ensure that no large rearrangements had occurred. UL54 ORFs from each BAC were sequenced to confirm that they had only the engineered mutation. As intended, pMP573 contained only the D542E mutation, while pMP556 contained no UL54 mutations. Recombinant mutant viruses were reconstituted from pMP573 and pMP556 BACs via transfection into HFF cells and were designated RC573 and RC556, respectively. These recombinant viruses underwent plaque reduction assay as described above to determine EC50 values against CMX001 and control compounds. The UL54 Kan wild-type recombinant virus (RC556) was used as a control for phenotypic studies of RC573.

**Replication kinetics.** Monolayers of HFF cells in duplicate 12 well plates were infected at a MOI of 0.01 PFU/cell with CMX001R or the D542E mutant (RC573), and the comparator viruses AD169 and UL54 Kan (RC556), respectively. At 2 hours, and 4, 5, 6, 8, 10, 12, and 14 days following infection the duplicate plates were frozen at -80°C for subsequent evaluation by real time PCR. Total DNA was extracted by lysing the cells in a buffer containing 10 mM Tris pH 7.4, 1 mM EDTA, 1% SDS and incubating with 100 μg of proteinase K for 60 minutes at 37°C. DNA samples were purified using the QIAquick PCR Purification Kit according to the manufacturer’s protocol. Genome copy number was determined on two independent cultures at each time point using primers 5’-AGG TCT TCA AGG AAC TCA GCA AGA-3’ and 5’-CGG CAA TCG GTT TGT TGT
Selection of CMX001-resistant virus. The generation of CMV strains resistant to CDV is typically slow and this proved to be the case for CMX001 as well. Serial passage over a period of approximately 10 months was required to generate a viral isolate that replicated in the presence of 0.5 µM CMX001. This isolate, designated CMX001\textsuperscript{R}, was evaluated for susceptibility to CMX001 and other relevant compounds (Table 1). The resistant isolate exhibited an EC\textsubscript{50} for CMX001 that was 17-fold higher than that of the parent virus. This isolate was also resistant to CDV as expected, yet remained fully susceptible to GCV and FOS.

Genotypic resistance analysis. To identify the molecular determinants of resistance in CMX001\textsuperscript{R}, the genes encoding the CMV UL97 kinase and DNA polymerase were sequenced. No mutations were observed in UL97, but a single nucleotide mutation leading to a change in the inferred amino acid sequence (D542E) was identified in UL54. The D542E mutation is interesting because it has not been previously reported, is located within the conserved UL54 δC/ExoIII domain (7), and is proximal to a known mutation (L545S) that confers resistance to both CDV and GCV (22). Thus, it was considered highly probable that the UL54 D542E mutation would be sufficient to confer reduced susceptibility to CDV and CMX001.
Recombinant phenotyping of the novel mutation. To confirm these data, the D542E mutation was reconstructed in plasmid pMP547, which was used to transfer the mutation to the HB5 BAC using a kanamycin selectable marker and the resulting BAC was designated pMP573. DNA sequencing of the UL54 ORF of pMP573 confirmed the presence of the engineered D542E mutation and no other mutations. This BAC was then used to regenerate a recombinant CMV strain carrying the UL54 D542E mutation. This recombinant virus was also sequenced to confirm that D542E was the only change in the UL54 ORF. This D542E mutant strain was designated RC573. Because RC573 carries the Kan selective marker near UL54, another recombinant virus with the Kan marker in the same locus, but without the D542E mutation in UL54 was engineered from the same HB5 parent BAC (designated RC556) and used as a wild-type control for phenotypic studies of the recombinant D542E mutant virus.

In a series of plaque reduction assays, the D542E mutant virus, RC573, recapitulated the resistance phenotype of the CMX001\textsuperscript{R} isolate, as shown in Table 1, with reduced susceptibility to CDV and CMX001, but no resistance to GCV or FOS. EC\textsubscript{50} values for the D542E mutant strain demonstrated reductions in susceptibility of greater than 30-fold to CMX001 and greater than 10-fold to CDV when compared to the parent wild-type UL54 Kan isolate, RC556.

Assessment of replication kinetics. Both CMX001\textsuperscript{R} and D542E mutant isolates exhibited a small plaque phenotype that is an indicator of impaired replication kinetics in some CMV strains. Further investigations were carried out to evaluate the replicative fitness of each strain in comparison to its respective parent virus (Figure 1). Cells were infected at a low MOI and were evaluated by real time PCR from 2 hours extending out...
to 14 days to assess the replication kinetics of the viruses. For the first several days few
differences in replication were observed, but by day 10 following infection, cells infected
with CMX001<sup>R</sup> exhibited reduced levels of progeny DNA compared to the AD169
progenitor. Levels of CMX001<sup>R</sup> DNA were less than the parent virus on days 10, 12, and
14 and were statistically significant (P < 0.002, paired Student’s t test). Likewise, the
recombinant virus with the D542E mutation produced lower levels of DNA starting at
day 10 after infection compared to the comparator virus RC556 (wild-type UL54 Kan),
which was also statistically significant (P < 0.001). Cells infected with either CMX001<sup>R</sup>
or D542E contained approximately 0.5 log<sub>10</sub> copies/ml lower levels of progeny DNA
compared to the comparator viruses on days 10, 12, and 14. These data indicate that the
UL54 D542E mutation is sufficient to reduce the replicative fitness of viruses that carry
this lesion, resulting in slower replication kinetics that are consistent with the small
plaque phenotype observed for both viruses containing this mutation.

DISCUSSION

CMX001 has the potential to improve upon the efficacy and toxicity profiles of
current CMV antivirals. Initial human studies indicate that it avoids the toxicities of
myelosuppression and renal injury that often limit the use of GCV, CDV, and FOS (20).
Emergence of resistant CMV has not been observed to date in clinical studies of
CMX001 where prior anti-CMV therapy was limited or absent, limiting our knowledge
of de novo resistance to CMX001 and the theoretical associated cross-resistance.

Here, we report the generation of a CMX001-resistant CMV strain under
prolonged selective pressure in cell culture. Genotypic resistance analysis identified the
presence of a single UL54 mutation (D542E) and this mutation was transferred to a control laboratory strain of CMV in order to assess antiviral susceptibility to CMX001 and cross-resistance to other CMV antivirals. These studies indicate that the D542E mutation in UL54 is sufficient to confer resistance to CMX001 and CDV, but does not appear to confer GCV or FOS cross-resistance.

D542E has not been previously identified in clinical or laboratory strains of resistant CMV and is the first resistance mutation described as emerging de novo under CMX001 selective pressure, either in vivo or in vitro. It is located in the δC/ExoIII functional region of UL54, which is a conserved region found within the 3’-5’ exonuclease domain. This exonuclease domain is highly associated with amino acid changes known to confer GCV and CDV cross-resistance, as well as some FOS resistance (7). Since a majority of CDV-resistance associated mutations also confer cross-resistance to GCV and/or FOS, it is somewhat surprising that D542E does not. Taken together, the fact that D542E was not a previously known CDV-resistant mutation and that it varies from the typical cross-resistance profile of mutations in the exonuclease domain indicate that the antiviral pressure of CMX001 may drive the selection of resistance mutations that are distinct from those generated by other antiviral agents. This may be attributable to the substantially higher intracellular levels of the active antiviral achieved with CMX001 as compared to CDV, which results from the lipid-facilitated cellular uptake of CMX001. A similar situation in which differing levels of antiviral drug exposure leads to distinct genotypic profiles has been described for the HIV protease inhibitor, fosamprenavir, where I54L/M is the predominant resistance mutation in
regimens with low trough plasma concentrations, whereas a different mutation (I50V) predominates in those with a high trough plasma concentration (23).

The location of codon position 542 within the CMV DNA polymerase molecule may give insight into the mechanism of resistance for these resistant isolates. Although studies of another resistance mutation (K513N) within the δC/ExoIII region demonstrated that it severely restricted the exonuclease function of UL54 (22), 3-dimensional modeling of the CMV DNA polymerase enzyme suggests that some mutations within this region may actually enhance the proofreading activity of the exonuclease, thereby facilitating the excision of incorporated nucleotide analogs (24). It is theorized that more efficient excision of incorporated nucleoside-based antivirals from the elongating viral DNA chain produces resistance to that antiviral.

Compared to mutations in UL97, UL54 mutations are more likely to be associated with replicative deficits that reduce the growth rate of affected viruses (22, 25, 26). In our studies, the CMX001-resistant CMV strain generated in cell culture (CMX001R) and the recombinant D542E mutant virus (RC573) both exhibited small plaque phenotypes which are often indicative of compromised viral growth kinetics (27). Replicative fitness assays confirmed our observation by showing that CMX001R and RC573 both demonstrated modest growth attenuation in comparison to parent viruses. Not surprisingly, the UL54 Kan strain (RC566) from which RC573 was derived showed a slight decrease in replicative fitness compared to unmodified AD169, likely due to the Kan insert. Increased variation in plaque reduction assays was also noted with both CMX001R and RC573 and we hypothesize this is also associated with the D542E mutation. A growth deficiency of this magnitude would be predicted to lead to a minor replicative
disadvantage in a mixed virus population, but could allow for clonal expansion of the resistant population under selective pressure from an antiviral agent.

In conclusion, we have generated the first de novo CMX001-resistant CMV strain under prolonged in vitro selective pressure, identified a novel UL54 mutation (D542E), and demonstrated with recombinant phenotyping techniques that this mutation confers resistance to CMX001 and CDV, but not to GCV or FOS. No other UL54 mutations and no UL97 mutations were found. As is common with UL54 resistance mutations, D542E is associated with reduced viral growth in cell culture. Clinical trials of CMX001 in high-risk patient populations should continue surveillance aimed at defining the potential for emerging CMX001 resistance, noting that it may be associated with previously unrecognized UL54 mutations.

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REFERENCES


### Table 1. Susceptibility of CMX001<sup>R</sup> and a Recombinant UL54 D542E Mutant Virus

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<th>EC&lt;sub&gt;50&lt;/sub&gt; ± SD (µM)&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Fold Increase&lt;sup&gt;b&lt;/sup&gt;</th>
<th>EC&lt;sub&gt;50&lt;/sub&gt; ± SD (µM)&lt;sup&gt;50&lt;/sup&gt;</th>
<th>Fold Increase&lt;sup&gt;b&lt;/sup&gt;</th>
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<tr>
<td></td>
<td>CMX001&lt;sup&gt;R&lt;/sup&gt; AD169</td>
<td>D542E mutant (RC573)</td>
<td>Wild-type UL54 Kan (RC556)</td>
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<tr>
<td>CDV</td>
<td>8.4 ± 5.5</td>
<td>1.1 ± 0.78</td>
<td>7.6&lt;sup&gt;c&lt;/sup&gt;</td>
<td>2.8 ± 0.62</td>
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<tr>
<td>CMX001</td>
<td>0.017 ± 0.003&lt;sup&gt;d&lt;/sup&gt;</td>
<td>0.001 ± 0.0005&lt;sup&gt;d&lt;/sup&gt;</td>
<td>17&lt;sup&gt;c&lt;/sup&gt;</td>
<td>0.026 ± 0.007</td>
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<tr>
<td>FOS</td>
<td>59 ± 12&lt;sup&gt;d&lt;/sup&gt;</td>
<td>90 ± 21&lt;sup&gt;d&lt;/sup&gt;</td>
<td>0.66</td>
<td>34 ± 36</td>
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<tr>
<td>GCV</td>
<td>4.3 ± 2.0&lt;sup&gt;d&lt;/sup&gt;</td>
<td>3.5 ± 0.55&lt;sup&gt;d&lt;/sup&gt;</td>
<td>1.2</td>
<td>9.6 ± 5.2&lt;sup&gt;e&lt;/sup&gt;</td>
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- Values represent the mean ± standard deviation values from four independent experiments unless otherwise noted.
- The relative resistance compared to the parent virus is shown as the fold increase in EC<sub>50</sub> values.
- P < 0.05
- Mean of three separate determinations with standard deviation value.
- Mean of five separate determinations with standard deviation value.
Figure 1. Replication kinetics. HFF cells infected at an MOI of 0.01 PFU/cell were harvested at the times indicated below. The average DNA copy number of two replicate cultures is plotted below with standard deviation values indicated by the error bars. CMX001<sup>R</sup> (filled black squares) replicated more slowly than the AD169 (open squares) from which it was derived. Similarly, the recombinant virus (RC573) containing the D542E mutation (filled black circles) also replicated more slowly than did the wild-type UL54 Kan (RC556) comparator virus (open circles).