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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 (named CMX001^R) was less susceptible to CDV and CMX001 in a plaque reduction assay. Genotypic analysis of virus strain CMX001^R via conventional sequencing of the genes encoding the CMV DNA polymerase (*UL54*) and *UL97* kinase (*UL97*) demonstrated one mutation that 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 strain CMX001^R 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.

Infections with human cytomegalovirus (CMV) are highly prevalent, but they 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 a 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 1,000-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 of body weight, 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 resis-

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tance or mutations known to be associated with resistance in CMV antiviral treatment-naïve patients receiving CMX001. The objectives of this study were 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), the titers of the virus were determined at 2×10^5 PFU/ml, and virus was stored at -80°C .

Resistant strain selection. The CMV strain AD169 was used to infect low-passage-number 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% (EC_{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 the concentrations of CMX001 exceeded 0.5 μM . The titer of virus resulting from passaging was determined at 3.5×10^4 PFU/ml, and the virus strain was designated CMX001^R.

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 min 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% (EC_{50}) 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 virus strain CMX001^R using double-nested 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 (For for forward) (5'-CGT AAG CTG TCA GCC TCT CA-3') and UL54 Rev (Rev for reverse) (5'-CAG TCT CAG CAG CAT CAT CAC-3'), followed by primers UL54 Inner For (5'-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 the AD169 strain 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 pMP302) to facilitate mutagenesis. The D542E mutation was constructed by quick change mutagenesis (QuikChange II site-directed mutagenesis kit; Stratagene, La Jolla, CA) with primer *UL54*D542E F (F for forward) (5'-CCG TTA CTG TCT GCA G-3') and primer *UL54*D542E R (R for reverse) (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 digested products 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 EC_{50} s 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 an MOI of 0.01 PFU/cell with virus strain CMX001^R or the D542E mutant (RC573), and the comparator viruses AD169 and *UL54* Kan (RC556), respectively. Two 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, and 1% SDS and incubating with 100 μg of proteinase K for 60 min 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 AAA-3', with the probe 5'-(6-carboxyfluorescein [6FAM])-CCG TCA GCC ATT CTC TCG GC 6-carboxytetramethylrhodamine (TAMRA)-3', and the plasmid pMP217 to provide absolute quantification. Data shown represent the average of the log of the genome copy number determined from two independent cultures at each point in time.

RESULTS

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^R, was evaluated for susceptibility to CMX001 and other relevant compounds (Table 1). The resistant isolate exhibited an EC_{50} for CMX001 that was 17-fold higher than that of the parent virus. This isolate was also resistant to CDV as expected, yet it remained fully susceptible to GCV and FOS.

Genotypic resistance analysis. To identify the molecular de-

TABLE 1 Susceptibility of CMV strain CMX001^R and a recombinant *UL54* D542E mutant virus

Antiviral agent	EC ₅₀ ± SD (μM) ^a		Fold increase ^b	EC ₅₀ ± SD (μM) ^a		Fold increase ^b
	CMX001 ^R	AD169		D542E mutant (RC573)	Wild-type <i>UL54</i> Kan (RC556)	
CDV	8.4 ± 5.5	1.1 ± 0.78	7.6 ^c	2.8 ± 0.62	0.23 ± 0.10	12 ^c
CMX001	0.017 ± 0.003 ^d	0.001 ± 0.0005 ^d	17 ^c	0.026 ± 0.007	0.0008 ± 0.0007	32 ^c
FOS	59 ± 12 ^d	90 ± 21 ^d	0.66	34 ± 36	20 ± 15	1.7
GCV	4.3 ± 2.0 ^d	3.5 ± 0.55 ^d	1.2	9.6 ± 5.2 ^e	6.5 ± 8.7	1.5

^a Values represent the mean ± standard deviation values from four independent experiments unless otherwise noted.

^b The relative resistance compared to the parent virus is shown as the fold increase in the EC₅₀.

^c Significantly different ($P < 0.05$).

^d Values (means ± standard deviations) for three separate experiments.

^e Values (means ± standard deviations) for five separate experiments.

terminants of resistance in virus strain CMX001^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/exonuclease III (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 isolate CMX001^R, as shown in Table 1, with reduced susceptibility to CDV and CMX001, but no resistance to GCV or FOS. EC₅₀s for the D542E mutant strain demonstrated reductions in susceptibility of greater than 30-fold to CMX001 and greater than 10-fold to CDV compared to the parent wild-type *UL54* Kan isolate, RC556.

Assessment of replication kinetics. Both the isolate CMX001^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 (Fig. 1). Cells were infected at a low MOI and were evaluated by real-time PCR from 2 h 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 the virus strain CMX001^R exhibited reduced levels of progeny DNA compared to the AD169 progen-

itor. The levels of virus strain CMX001^R DNA were less than those of the parent virus on days 10, 12, and 14 and were statistically significant ($P < 0.002$ by 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 strain CMX001^R or D542E mutant strain contained approximately 0.5 log₁₀ 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 antiviral agents. 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). To date, emergence of resistant CMV has not been observed in clinical studies of CMX001 where prior anti-CMV therapy was

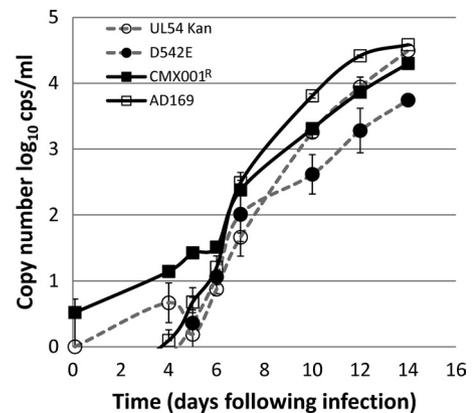


FIG 1 Replication kinetics. HFF cells infected at an MOI of 0.01 PFU/cell were harvested at the times indicated on the x axis. The average DNA copy number of two replicate cultures is plotted with standard deviation values indicated by the error bars. Virus strain CMX001^R replicated more slowly than the AD169 strain from which it was derived. Similarly, the recombinant virus (RC573) containing the D542E mutation also replicated more slowly than did the wild-type *UL54* Kan (RC556) comparator virus.

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 it 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 δ C/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 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 drug concentrations, whereas a different mutation (I50V) predominates in those with a high trough plasma drug 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 δ C/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 (strain CMX001^R) and the recombinant D542E mutant virus (strain RC573) both exhibited small-plaque phenotypes that are often indicative of compromised viral growth kinetics (27). Replicative fitness assays confirmed our observation by showing that virus strains CMX001^R and RC573 both demonstrated modest growth attenuation compared to their parent viruses. Not surprisingly, the *UL54* Kan strain (RC566) from which RC573 was derived showed a slight decrease in replicative fitness compared to un-

modified AD169, likely due to the Kan insert. Increased variation in plaque reduction assays was also noted with both virus strains CMX001^R and RC573, and we hypothesize that 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 it 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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