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Selection and Recombinant Phenotyping of a Novel CMX001/Cidofovir HCMV Resistance Mutation

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Abstract

Background: CMX001 is a novel, orally available lipid ester of the acyclic nucleotide analogue cidofovir (CDV) that exhibits enhanced *in vitro* antiviral activity against a wide range of DNA viruses, including human cytomegalovirus (HCMV), BK virus, and adenovirus. Mutations in the DNA polymerase of HCMV 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. **Methods:** The HCMV strain AD169 was propagated in low passage human foreskin fibroblasts under increasing concentrations of CMX001 over the course of 10 months, with a final CMX001 concentration of 0.5µM. Reduced susceptibility of the resulting strain (CMX001^R) to CDV and CMX001 was demonstrated by standard plaque reduction assays. Genotypic analysis of CMX001^R was performed with Sanger sequencing of the DNA polymerase gene (*UL54*) and the UL97 kinase gene (*UL97*). Recombinant phenotyping of a novel *UL54* mutation was performed via bacterial artificial chromosome mediated marker transfer experiments using a kanamycin selectable marker. **Results:** A resistant isolate (CMX001^R) was generated under the selective pressure of CMX001. Compared to the parent virus, the isolate was resistant to both CMX001 and CDV, with 50% effective concentration (EC_{50}) values that were elevated by approximately 10-fold for each drug. Genotypic resistance analysis of CMX001^R demonstrated a novel *UL54* mutation (D542E) that had not been reported in CDV resistant isolates. There were no other mutations in *UL54* or *UL97*. A recombinant virus with the D542E mutation (RC556) was generated and subjected to further phenotypic analysis that confirmed it conferred approximately 10-fold resistance to both CDV and CMX001. CMX001^R and RC556 isolates both demonstrated a small plaque phenotype. **Conclusion:** This represents the first resistance mutation generated under the selective pressure of CMX001. This mutation arose after an extended period of selective pressure, and affected isolates demonstrate a plaque phenotype consistent with a replicative deficit. D542E was not a previously identified mutation associated with HCMV resistance, which indicates that CMX001 may have a unique resistance profile.

Introduction

- CMX001 is an orally bioavailable lipid acyclic nucleoside phosphonate which is converted inside cells to the active antiviral agent, cidofovir diphosphate (CDV-PP). Compared to CDV, CMX001 demonstrates enhanced antiviral activity against a wide range of dsDNA viruses, while having less risk of dose-limiting nephrotoxicity.[1]
- Antiviral resistance to CDV maps to the HCMV DNA polymerase gene (*UL54*). Since CMX001 is metabolized to CDV-PP, resistance to this agent would be expected to map to *UL54* as well.
- The impact of higher intracellular CDV-PP levels on the development of resistant HCMV strains is unknown.
- The objective of this study was to isolate HCMV mutants resistant to CMX001 and to identify the amino acid changes responsible for the resistance phenotype.

Materials and Methods

Resistant Strain Selection

HCMV strain AD169 was used to infect human foreskin fibroblast (HFF) cells at a low multiplicity of infection (0.1 PFU/cell) in the presence of 0.01 µM CMX001. The culture was passaged 10 times with increasing concentrations of CMX001 up to a final concentration of 0.5 µM. Total passage time was 10 months. After sequential plaque purification, the resultant virus (CMX001^R) underwent plaque reduction assay to determine the EC_{50} values of CMX001 and the control compounds CDV, ganciclovir (GCV), and foscarnet (PFA).

Genotypic Analysis

The *UL97* and *UL54* genes were amplified from CMX001^R using double nested polymerase chain reaction methods and the resulting DNA amplicons underwent Sanger sequencing at the UAB Department of Genetics Core Sequencing Facility to identify any amino acid changes. Polymorphisms were identified by comparing consensus DNA sequences to AD169 (GenBank accession number X17403).

Recombinant Phenotyping

To further investigate a novel *UL54* mutation identified during genotypic resistance analysis, the mutation was reconstructed in AD169 in the HB5 bacterial artificial chromosome (BAC) using methods similar to those previously reported.[2] A kanamycin resistance marker was inserted adjacent to the *UL54* gene segment in the plasmid used as a template for mutagenesis (UL54*kan* pEXP5 NT), allowing for selection of BAC colonies in which recombination correctly occurred. Recombinant virus was reconstituted via transfection into HFF cell culture and underwent plaque reduction assay to determine EC_{50} values against CMX001 and the panel of control compounds. A second recombinant virus (RC556) was engineered with the kanamycin marker in the same locus, but with no *UL54* mutations, was used as a control for phenotypic studies.

Results

- The resistant isolate obtained at the end of 10 months of passage in the presence of CMX001 was designated CMX001^R.
- CMX001^R exhibited a small plaque phenotype indicative of a replicative defect.
- The CMX001^R isolate demonstrated an EC_{50} for CMX001 that was 17-fold higher than that of its AD169 parent virus. CMX001^R was also resistant to CDV (7.6-fold increase over parent virus), but retained susceptibility to GCV and PFA (Table 1).

Table 1. Susceptibility of HCMV Isolate CMX001^R

	EC_{50} (µM)		
	CMX001^R	AD169	Fold Increase
CMX001	0.017 ± 0.003	0.001 ± 0.0005	17
CDV	8.4 ± 5.5	1.1 ± 0.78	7.6
GCV	4.3 ± 2.0	3.5 ± 0.55	1.2
PFA	59 ± 12	90 ± 21	0.66

- To identify the molecular determinants of resistance, the *UL97* and *UL54* genes of CMX001^R were sequenced. No mutations were observed in *UL97*, but a single D542E mutation was identified in the DNA polymerase.
- This novel mutation has not been reported previously in CDV-resistant isolates.
- The D542E mutation was reconstructed in the HB5 BAC, which was used to generate a recombinant virus carrying the D542E mutation (designated RC573). The *UL54* open reading frame of RC573 was sequenced to confirm that it contained the D542E mutation and no other mutations.
- Similar to the resistant isolate from which the mutation was identified, the recombinant virus containing D542E (RC573) exhibited reduced susceptibility to CMX001 and CDV compared to its parent virus, but no cross resistance to GCV or PFA (Table 2).

Table 2. Susceptibility of *UL54* D542E Recombinant Virus

	EC_{50} (µM)		
	D542E (RC573)	UL54*kan* wt (RC556)	Fold Increase
CMX001	0.026 ± 0.007	0.0008 ± 0.0007	32
CDV	2.8 ± 0.62	0.23 ± 0.10	12
GCV	9.6 ± 5.2	6.5 ± 8.7	1.5
PFA	34 ± 36	20 ± 15	1.7

Conclusions

- A CMX001-resistant HCMV strain was generated under prolonged selective pressure in cell culture. The resistant strain exhibited a small plaque phenotype.
- Genotypic resistance analysis of this CMX001-resistant strain identified a novel *UL54* mutation, D542E.
- Based upon recombinant phenotyping studies, we conclude that the HCMV *UL54* D542E mutation is sufficient to confer resistance to both CDV and CMX001.
- D542E has not previously been identified in CDV-resistant HCMV stains. Though it occurs within the conserved δC/ExoIII region of *UL54*, D542E does not confer GCV or PFA cross resistance, as do a majority of other known resistance mutations in this region, indicating that CMX001 may drive the selection of unique resistance mutations.

References

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