Novel Cytomegalovirus UL54 DNA Polymerase Gene Mutations Selected *In Vitro* That Confer Brincidofovir Resistance

Sunwen Chou,^{a,b} Ronald J. Ercolani,^b E. Randall Lanier^c

Division of Infectious Diseases, Oregon Health and Science University,^a and Research Service, VA Portland Health Care System,^b Portland, Oregon, USA; Chimerix, Durham, North Carolina, USA^c

Eight *in vitro* selection experiments under brincidofovir pressure elicited the known cytomegalovirus DNA polymerase amino acid substitutions N408K and V812L and the novel exonuclease domain substitutions D413Y, E303D, and E303G, which conferred ganciclovir and cidofovir resistance with 6- to 11-fold resistance to brincidofovir or 17-fold when E303G was combined with V812L. The new exonuclease domain I resistance mutations selected under brincidofovir pressure add to the single instance previously reported and show the expected patterns of cross-resistance.

Brincidofovir (BCV or CMX001, Chimerix) is an experimental orally bioavailable hexadecyloxypropyl conjugate of the nucleotide analog cidofovir (CDV) (1). A phase 3 clinical trial of BCV for the prevention of human cytomegalovirus (CMV) infection in bone marrow transplant recipients (ClinicalTrials registration no. NCT01769170) was recently completed, and BCV unexpectedly failed to meet its primary efficacy endpoint despite showing a statistically significant antiviral effect during the on-treatment phase (2). These results and those of a prior successful phase 2 trial (3) showed that short-term prophylactic use of BCV did not result in the detection of CMV UL54 DNA polymerase gene mutations conferring drug resistance (4). The genetic pathways and biochemical mechanisms of resistance to BCV are expected to be the same as those of the parent compound CDV (5), into which BCV is metabolized prior to formation of the active antiviral CDP diphosphate (CDV-PP). Intracellular levels of CDV-PP differ markedly after exposure to CDV or BCV (6), as reflected in the much lower BCV concentrations (0.2 to 0.5 nM for BCV versus 200 nM for CDV) required to reduce CMV replication by 50% in cell culture (50% effective concentration [EC₅₀]). *In vitro* exposure to BCV may select for mutations different from those previously reported for CDV or ganciclovir (GCV), as higher intracellular active drug concentrations may select for mutants with higher associated levels of drug resistance at the cost of reduced growth fitness. In one report, the UL54 exonuclease domain III amino acid substitution D542E (7) was detected after many cell culture passages under BCV and shown to confer cross-resistance to CDV but not to GCV or foscarnet (FOS). Eight additional *in vitro* selection experiments under BCV are reported here with characterization of the resistance and relative growth phenotypes of the emergent mutations.

The reference BCV compound was provided by Chimerix and added to the human fibroblast cultures starting with 0.2 nM BCV for all experiments after CMV inoculation at a low multiplicity of infection (MOI), followed by weekly propagation under increasing drug concentrations as permitted by interim viral growth, as previously described (8). We commonly use an error-prone UL54 exonuclease mutant (e.g., D413del, strain T4138) to accelerate the evolution of resistance mutations, but this mutant is already resistant to BCV, CDV, and GCV (Table 1), with EC₅₀s ~5-fold increased over those for the parental wild-type strain T3265 (8, 9),

raising the possibility that additional mutations on this genetic background may not represent evolution in the wild-type virus. Therefore, only 2 selection experiments were performed using the D413del mutant, and 6 others were done with wild-type CMV derived from strain AD169 (BAC clone BD1) (8). Selection cultures were monitored for evolving UL54 mutations by standard dideoxy sequencing as described previously (8), with complete (1,243 codons) UL54 sequences at the end of the experiment and partial (codons 296 to 1,000) sequences at interim sampling points. Amino acid substitutions were defined in relation to the strain AD169 reference sequence.

Of the two selection experiments with the D413del mutant, one evolved no further detectable UL54 mutations after 20 passages and an ending concentration of 4 nM; attempts to escalate the drug concentrations resulted in progressively deteriorating viral growth. The other culture evolved amino acid substitution N408K after escalation to a BCV concentration of 12 nM at 25 passages. N408K is a well-known resistance marker that confers relatively high-level CDV resistance (~20-fold) and GCV cross-resistance (~4-fold) (9). A new N408K recombinant virus (T4221) was constructed on the clone BD1 base to reconfirm the twice previously published CDV, GCV, and FOS susceptibility phenotypes (Table 1) and to document the BCV EC₅₀ at an 11-fold increase over baseline.

Of the six selection experiments with wild-type CMV, only 3 evolved any detectable UL54 mutation after 20 serial passages. Those that evolved no mutation could not maintain ongoing viral growth at BCV concentrations of >2 nM. One experiment evolved substitution D413Y as an ~20% subpopulation at 6 passages (0.8 nM BCV); this evolved to a full population of D413Y in 2 further passages at the same drug concentration. Another exper-

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Address correspondence to Sunwen Chou, chou@ohsu.edu.

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TABLE 1 Genotypes and phenotypes of recombinant viruses

Strain	Genotype	Brincidofovir				Cidofovir				Ganciclovir				Foscarnet			
		EC ₅₀ (nM) ^a	SD	n ^b	Ratio ^c	EC ₅₀ (nM)	SD	n	Ratio	EC ₅₀ (nM)	SD	n	Ratio	EC ₅₀ (nM)	SD	n	Ratio
Control strains																	
T4198	Wild-type control	0.37	0.13	13		0.19	0.05	17		1.1	0.21	17		61	13	29	
T4138	UL54 <i>pol</i> D413del	1.3	0.49	7	5.2	1.4	0.52	10	5.8	4.0	1.0	8	3.8	66	17	8	1.3
T4199	UL54 <i>pol</i> A809V													264	43	26	4.3
Mutants selected under BCV																	
T4302	UL54 <i>pol</i> E303G	4.2	0.82	10	11	3.7	1.2	11	20	8.3	2.4	8	7.3	46	6.4	11	0.8
T4303	UL54 <i>pol</i> E303D	3.6	0.77	11	9.7	2.9	0.92	8	16	7.2	1.7	10	6.3	50	13	9	0.8
T4221	UL54 <i>pol</i> N408K	4.2	0.55	11	11	3.8	1.6	10	21	4.4	0.79	8	3.9	51	17	11	0.8
T4267	UL54 <i>pol</i> D413Y	2.2	0.42	9	6.0	1.8	0.65	8	9.3	5.0	1.3	7	4.4	61	14	7	1.0
T4309	UL54 <i>pol</i> V812L	0.88	0.31	13	2.4	0.58	0.19	8	3.1	2.4	0.69	8	2.1	119	30	12	2.0
T4307	UL54 <i>pol</i> E303G V812L	6.3	0.81	11	17	6.4	1.6	10	34	11.6	1.9	10	10	83	17	13	1.4

^a EC₅₀, mean drug concentration required to reduce culture supernatant secreted alkaline phosphatase (SEAP) growth by 50%.
^b Number of assay replicates (done over at least 4 separate dates).
^c Ratio of the EC₅₀ of the mutant virus to that of the matching wild-type control.

iment evolved substitution E303D, detected at 19 passages (3 nM BCV) and as a fully mutant population 1 passage later. A third experiment evolved substitution E303G (70% mutant sequence) after 13 passages (20 nM BCV). After 7 more passages at the same BCV concentration, an additional substitution V812L (90% mutant sequence) was observed, adding to the fully mutant E303G sequence. V812L was originally identified and characterized 20 years ago as conferring multidrug resistance, including resistance to CDV (10).

Recombinant phenotyping for the newly recognized substitutions D413Y, E303D, and E303G was performed by transfer of individual mutations to the clone BD1 base as previously described (8, 9). E303G was also phenotyped in combination with V812L as observed in the selection experiment, along with the V812L single mutant for comparison. Recombinant viruses were sequence verified and alkaline phosphatase reporter-based yield reduction assays were performed with the replicates, and the results are shown in Table 1, indicating BCV-CDV-GCV cross-resistance for E303D, E303G, and D413Y. V812L had a borderline or low-grade resistant CDV-GCV-FOS phenotype as originally published (10) and augmented the BCV-CDV resistance conferred by E303G as predicted by its evolution *in vitro*. Interestingly the FOS EC₅₀ of the double mutant was lower than that for V812L alone.

Comparative growth curves were obtained for the mutant recombinant viruses as described previously (8, 9) and are shown in Fig. 1. The E303 and D413 mutants were moderately growth attenuated, comparable to the established FOS resistance substitution A809V, while the N408K and V812L mutants had relatively well-preserved growth fitness close to that of the wild type.

The novel UL54 substitutions E303D/G and D413Y reported here add to the considerable number of exonuclease domain mutations associated with dual CDV and GCV resistance (5, 11, 12). The only other drug resistance mutation that has been mapped to CMV exonuclease domain I (codons 300 to 304) is substitution D301N, identified in a single plaque of a clinical isolate picked under GCV (13). That the new instances of domain I mutation involve residue E303 is significant, as both D301 and E303 are

highly conserved residues among herpesvirus DNA polymerases (14). Substitution D413Y confers a drug resistance phenotype similar to those for D413A (15), D413E (13), and D413N (11), all involving another highly conserved exonuclease domain II residue, D413 (14). Together, the three acidic residues D301, E303, and D413 correspond to herpes simplex

FIG 1 Comparative secreted alkaline phosphatase (SEAP) growth curves of recombinant CMV strains. The mutant strains as listed in Table 1 with the indicated UL54 *pol* amino acid substitutions were inoculated at a similar low MOI of ~0.02 as calibrated by mean 24-h supernatant SEAP relative light units (RLU) of 1,313 (range, 1,133 to 1,559). Viral growth was monitored by SEAP RLU at 4 to 8 days postinoculation. The points represent the means $\pm$ SD of 4 replicates. A known growth-attenuated FOS-resistant mutant, A809V, found in clinical isolates (5) was included for comparison.

virus DNA polymerase residues D368, E370, and D471, which crystal structure analysis associates with acidic residues of bacteriophage RB69 DNA polymerase that are involved in the metal ion coordination essential for normal exonuclease activity (14, 16).

Modulation of exonuclease function appears to be the preferred mechanism of BCV/CDV and GCV resistance based on DNA polymerase mutations (5). A recent biochemical study suggests that altered exonuclease function may overcome the dynamic stalling of DNA polymerization (rapid addition and removal of subsequent nucleotides) that occurs when a nonobligate chain terminator nucleotide analog is misincorporated into a growing DNA strand (17). Some exonuclease mutants, such as N408K, have well-preserved growth fitness (Fig. 1 and reference 9), probably accounting for their relatively higher frequency in clinical samples (18), while other exonuclease domain mutants such as D301N (13) and E303G/D are more growth impaired, comparable to polymerase (palm/finger) domain mutants E756K (9, 13) and A809V (Fig. 1).

The emergence of a finger domain substitution, V812L, to augment the level of CDV and GCV resistance conferred by exonuclease substitution E303G has functional and therapeutic implications. V812L was previously described after *in vitro* selection under GCV (10, 19) and in a clinical isolate after GCV and FOS therapy (20). The initial recombinant phenotyping of V812L showed a low-grade (2.5- to 3-fold) increased EC₅₀ for GCV, CDV, and FOS (10). Although a more recent study did not confirm the FOS-resistant phenotype (19), the current data (Table 1) are consistent with the initial report and the general tendency of mutations in this functional domain to confer variable degrees of FOS resistance and low-grade cross-resistance to GCV and CDV (5). This phenotype probably results from altered substrate recognition during polymerization of the incoming base into replicating DNA, based on the structural locus of residue 812 (16). The difference in functional domains plausibly allows for the increased BCV, CDV, and GCV resistance of double mutants such as E303G-V812L (Table 1). The specific example of V812L also shows that GCV and BCV can independently select for the same cross-resistant mutation. In this series of experiments we did not detect mutations in the conserved exonuclease III domain (codons 498 to 546) where several CDV-GCV resistance mutations have been mapped (5), as well as the D542E substitution associated with impaired growth and BCV resistance without GCV cross-resistance (7).

To date, reported *in vitro* selection experiments with CDV (10) or BCV (7), including the current set, have been relatively few in number but have not conveyed the impression of rapid emergence of resistant mutants. Instead, drug resistance as a risk factor for early treatment failure with CDV or BCV may arise mainly from prior exposure to other antivirals, especially GCV, that select for cross-resistant UL54 mutants, as illustrated by clinical case reports of CDV or BCV as salvage therapy (11, 21, 22).

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