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Cytomegalovirus UL97 Mutations Affecting Cyclopropavir and Ganciclovir Susceptibility▽

Sunwen Chou[1,2]* and Terry L. Bowlin[3]

Division of Infectious Diseases, Oregon Health and Science University,[1] and Department of Veterans Affairs Medical Center,[2] Portland, Oregon, and Microbiotix, Inc., Worcester, Massachusetts[3]

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Among the 7 most common UL97 mutations encountered in ganciclovir-resistant clinical cytomegalovirus isolates, the associated cyclopropavir cross-resistance varies from insignificant (L595S) to substantial (M460I and H520Q) as determined by recombinant phenotyping. Mutations M460I and H520Q were preferentially selected *in vitro* under cyclopropavir and conferred 12- to 20-fold increases in 50% effective concentration (EC_{50}) values, while M460V, C592G, A594V, and C603W conferred 3- to 5-fold increases. Uncommon mutations M460T and C603R increased cyclopropavir EC_{50}s by 8- to 10-fold.

As a major opportunistic pathogen in immunosuppressed individuals, especially transplant recipients, human cytomegalovirus (CMV) infection often requires prolonged antiviral prophylaxis or treatment (9). Because the currently marketed drugs ganciclovir (GCV) (or its oral prodrug valganciclovir), foscarnet, and cidofovir can be limited by issues of tolerability and cross-resistance (10), there are ongoing efforts to develop new antiviral options. Several *Z*-isomer methylenecyclopropane nucleoside analogs have shown potent *in vitro* anti-CMV activity (12), particularly the compound cyclopropavir (CPV) (or ZSM-I-62, 5b [11]), which had low cytotoxicity (8) and showed *in vivo* efficacy in an immunodeficient mouse model of human CMV infection (7). CPV is an attractive candidate for clinical trials.

Similarly to GCV, initial phosphorylation by the viral UL97 kinase is required for the antiviral action of CPV (6). Well-characterized (canonical) mutations in UL97 are found in GCV-resistant clinical strains, with one of the seven mutations M460V/I, H520Q, C592G, A594V, L595S, and C603W encountered in close to 90% of cases (10). Other UL97 mutations confer various degrees of GCV resistance, while mutations in the UL54 DNA polymerase gene sometimes evolve to increase

TABLE 1. Genotypes and phenotypes of CMV UL97 mutants

BAC clone name[a]	Virus[b]	UL97 genotype		Cyclopropavir phenotype				Ganciclovir phenotype			
		Mutation	Other change(s)[c]	EC_{50} (μM)[d]	SD	Ratio[e]	No. of assays[f]	EC_{50} (μM)	SD	Ratio[e]	No. of assays[g]
	T2211	None	H587Y	0.26	0.05		18	1.10	0.22		
BA1	T3099	None	H587Y	0.25	0.07		13	1.02	0.30		
BA14	T3185	None	Frt, H587Y	0.23	0.04		11	0.96	0.19		
BA29	T3261	None	Frt	0.21	0.06		23	1.01	0.27		39
BA63	T3326	None	Frt, N68D, L126Q, I244V	0.20	0.06		12	1.11	0.24		22
BA84	T3362	**M460I**	Frt, N68D, L126Q, I244V	**2.44**	0.62	**12**	9	**8.68**	2.35	**7.8**	16
BA77	T3346	**M460T**	Frt	**2.13**	0.43	**10**	10	**9.24**	1.76	**9.3**	
	T2259	M460V		1.05	0.34	4.0	17	**8.52**	1.83	**8.3**	
BA61	T3324	**H520Q**	Frt	**4.24**	0.70	**20**	10	**7.86**	2.14	**7.8**	21
BA27	T3259	C592G	Frt	0.70	0.21	3.3	15	3.00	0.84	3.0	
BA22	T3252	A594V	Frt	0.94	0.22	4.5	14	**8.48**	2.52	**8.6**	
	T2260	L595S		0.33	0.14	1.3	16	**9.35**	3.26	**8.5**	
BA65	T3331	**C603R**	Frt	**1.76**	0.32	**8.4**	13	**8.18**	1.77	**8.3**	
BA64	T3327	C603S	Frt	0.58	0.20	2.8	10	1.85	0.38	1.9	
BA66	T3329	C603W	Frt	0.97	0.18	4.6	11	**7.81**	1.68	**7.9**	

[a] See reference 3 for BAC clone information.
[b] Recombinant virus strain (3, 5).
[c] Other UL97 genetic differences from strain AD169, unrelated to drug resistance. Frt, 34-bp Flp recombinase recognition site upstream of UL97 (3).
[d] EC_{50}, mean value of the number of assays shown, determined by SEAP yield reduction assay (3, 5).
[e] Ratio of mean EC_{50} value to that of matching control with baseline genotype. Items with EC_{50} ratios of >5 are shown in bold.
[f] Number of assays (performed on at least 5 separate dates).
[g] Data without number of assays shown have been published previously (3, 5).

* Corresponding author. Mailing address: VA Medical Center P3ID, 3710 SW U.S. Veterans Hospital Road, Portland, OR 97239. Phone: (503) 273-5115. Fax: (503) 273-5116. E-mail: chous@ohsu.edu.
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TABLE 2. UL97 mutations detected after culture under CPV

Strain	Initial mutation	No. of expts	UL97 mutation(s) detected at passage no. and CPV concn							
			Passage 5		Passage 7		Passage 9		Passages 13-15	
			Mutation (no. of instances)	CPV concn (μM)	Mutations (no. of instances)	CPV concn (μM)	Mutation (no. of instances)	CPV concn (μM)	Mutations (no. of instances)	CPV concn (μM)
T2294	UL54 D413A	12			M460I (3), H520Q (2), C603R (1), none (6)	0.2–0.4			M460I (4), H520Q (5), M460I + H520Q (2), C603R (1)	2–4
T3360	UL54 D413A	6	None (6)	0.8			M460I (6)	2–4		

resistance to GCV and confer cross-resistance to cidofovir and/or foscarnet (10). The extent of CPV cross-resistance conferred by GCV resistance mutations is not well defined. In this study, recombinant viruses containing various UL97 GCV resistance mutations were tested for CPV cross-resistance, and *in vitro* propagation of CMV under CPV was performed to see if mutations showing GCV cross-resistance were selected.

Recombinant phenotyping to determine the effect of specific mutations on drug susceptibility is performed by site-specific mutagenesis of control laboratory strains of CMV followed by assay of the drug concentration required to reduce viral growth by 50% (EC_{50}). Recently this process has been facilitated by the cloning of control strains (e.g., AD169) as bacterial artificial chromosomes (BACs) and incorporation of reporter genes such as the secreted alkaline phosphatase (SEAP) gene for viral quantitation (3, 5). Results from the updated assay system confirm earlier data showing that the canonical UL97 GCV resistance mutations each confer a 5- to 10-fold increase in GCV EC_{50}, except C592G, which confers a 3-fold increase (3, 5, 10). All of the recombinant viruses tested (Table 1) have been phenotyped for GCV susceptibility and published (3, 5), except for T3362 (mutation M460I) and T3324 (mutation H520Q), which were constructed and assayed as previously described (3). The recombinant strains, representing several baseline genotypes, the canonical UL97 mutations, and the uncommon mutations M460T, C603R, and C603S, were assayed for their CPV EC_{50}s by the same SEAP yield reduction assay as that used for GCV (3, 5). CPV was provided by Microbiotix, Inc., and diluted into cell culture medium from a 10 mM stock solution in dimethyl sulfoxide (DMSO).

CPV susceptibility data (Table 1) showed that baseline CMV strains had CPV EC_{50} values of 0.20 to 0.26 μM, which are 4- to 5-fold less than those for GCV (3), consistent with earlier data for CPV (11). Among the GCV-resistant mutants, CPV cross-resistance varied from insignificant (L595S) to substantial (M460I/T, H520Q, and C603R), with the latter mutations conferring 8- to 20-fold increases in CPV EC_{50}. Among the three most common GCV resistance mutations, A594V, L595S, and M460V (10), there was a 4- to 5-fold increase in CPV EC_{50} for two of them.

Previous information on CPV-GCV cross-resistance is hard to evaluate because of its preliminary nature. CPV was reported to have significant activity against GCV-resistant strains (8), but the genotypes of the tested strains were not specified; it is unlikely that mutation M460I or H520Q was represented. A conference abstract reported that a UL97 M460I-C603Y double mutant, selected under a related compound, synadenol, was cross resistant to CPV and that the mutations individually conferred 6- to 8-fold-increased CPV resistance (2). This is comparable to information in Table 1, where different mutations at codon 603 confer various levels of CPV resistance. The M460I-C603Y double mutant apparently showed 13-fold and 11-fold increases in EC_{50} for CPV and GCV, respectively (12). Another conference abstract (1) reported that CPV selected for a frameshift mutation in UL97 that truncated the expressed protein at residue 168, far upstream of residues composing critical kinase domains. Mutations that abrogate normal biological UL97 kinase function are not expected in clinical isolates, based on experience with GCV, because of their associated viral growth defect (10).

Mutations selected *in vitro* under CPV were assessed by propagating an error-prone exonuclease mutant containing UL54 mutation D413A (T2294 or a more recent BAC-cloned strain, T3360). This mutant shows an accelerated evolution of resistance mutations under drug (4). Using the same methods previously described (4), T2294 or T3360 was serially propagated for 15 passages under CPV starting at 0.2 μM and escalating to a final concentration of 2 to 4 μM. Infected cell extracts were checked at intervals for the presence of UL97 mutations. Table 2 shows the outcome of 18 experiments. Mutations became detectable at 7 passages; by passages 13 to 15, a mutation had developed in all of the cultures propagated under CPV. M460I was the most common, appearing in 12 of the cultures, followed by H520Q in 7 cultures, 2 of which contained both M460I and H520Q. In the latter 2 cases, each mutation was present as a mixture with the wild-type configuration. The unusual mutation C603R was selected in one instance. These *in vitro* selection results support the relevance of the cross-resistance data in Table 1, although the exact frequency of specific resistance mutations in posttreatment clinical isolates may differ from those found *in vitro* (10).

In conclusion, UL97 mutations M460I and H520Q have a major role in CPV resistance and GCV cross-resistance. The relative potency and low toxicity of CPV suggest that meaningful antiviral activity may be retained against some common GCV-resistant UL97 mutants (Table 1) that show less than a 5-fold increase in CPV EC_{50}. However, such mutations may favor the subsequent selection of other mutations, including those in the UL54 DNA polymerase gene, that result in higher levels of CPV resistance. This requires further study.

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