Cyclopropavir Susceptibility of CMV UL54 DNA Polymerase Mutants Selected after Antiviral Drug Exposure

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Abstract

Background:
Cyclopropavir (CPV) is a guanosine analog antiviral, activated by the CMV UL97 kinase. Mutations M460I and H520Q in UL97 confer CPV resistance.

Objectives:
1. CMV UL54 *pol* mutants with known patterns of resistance to current antivirals ganciclovir (GCV), foscarnet (FOS) and cidofovir (CDV) were tested for CPV susceptibility by a standardized reporter-based yield reduction assay
2. UL97 and *pol* mutations were monitored over 15 passages of propagation under CPV of an error-prone CMV exonuclease D413A mutant.

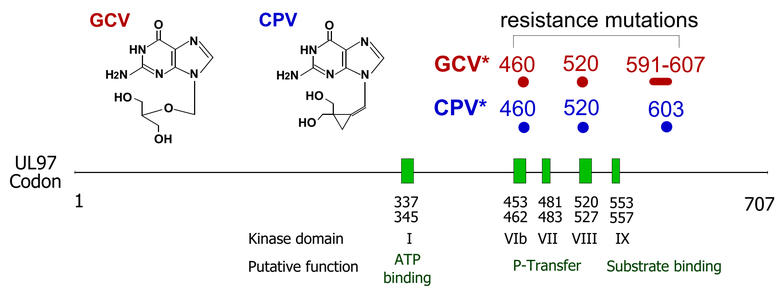
Results:
1. Exonuclease mutations and the region V mutation A987G, which confer dual GCV-CDV resistance, confer no cross-resistance to CPV
2. Catalytic region *pol* mutations known mainly to affect FOS susceptibility show varying CPV cross-resistance with EC50 ratios ranging from 3x-14x. Where tested, CPV EC50 values were further increased about 2x by adding UL97 mutation C592G.
3. Propagation of a *pol* D413A mutant under CPV consistently selected for UL97 mutations within 15 passages (M460I and/or H520Q; C603R once). UL54 *pol* mutations were selected in 3 of 21 experiments: P744T, E756D and M844V.
4. The *pol* P744T mutation did not confer CPV resistance when introduced into a control virus. E756D and M844V are located at residues where other mutations also confer 3x-5x CPV resistance.

Conclusions:
1. UL54 *pol* mutations evolve less often in vitro under CPV than UL97 mutations
1. Unlike GCV and CDV, exonuclease-mediated excision of mis-incorporated CPV does not appear to be a preferred mechanism of resistance
2. Some mutations in and near *pol* region III (codons 805-845), classically associated with FOS resistance, may also confer CPV resistance by affecting its recognition as an incoming base for DNA polymerization.

Introduction

- The second generation Z-isomer methylenecyclopropane nucleoside analog **cyclopropavir** (CPV or ZSM-I-62, Fig. 1) shows potent anti-CMV activity in vitro [1], and antiviral efficacy after oral administration in an immunodeficient mouse model of human CMV infection [2], with low toxicity for host cells. CPV is an attractive candidate compound for future clinical trials.
- Similar to ganciclovir (GCV, Fig. 1), initial phosphorylation by the viral UL97 kinase is required for the antiviral action of CPV [3]. UL97 mutations may confer resistance to either drug. One of seven canonical mutations (Fig. 1) at codons 460, 520 or 590-607 is found in ~90% of GCV-resistant clinical isolates [4]. We recently reported that UL97 mutations M460I and H520Q are most commonly selected under CPV in vitro, and confer 12x and 20x increased resistance to CPV, in addition to their known phenotype of 8x increased GCV resistance [5]. Some GCV-resistant mutants such as L595S show little CPV cross-resistance.
- Viral UL54 DNA polymerase (*pol*) mutations may confer resistance to GCV, CDV, FOS and presumably to CPV as well. Many *pol* mutations have been characterized in relation to current CMV antivirals. Those that confer CPV resistance and the frequency of their selection under drug are not well defined.
- In this study, we tested the CPV susceptibility of CMV strain AD169-derived recombinant strains containing *pol* mutations selected after exposure to current antivirals, to determine which ones showed significant cross-resistance to CPV.
- We also examined the UL97 and *pol* sequences of an error-prone D413A exonuclease mutant after serial propagation under CPV, to study the relative frequency and phenotype of *pol* mutations selected.

Figure 1. Cyclopropavir and ganciclovir structures and UL97 mutation map



*GCV in vivo frequency: A594V(25%), L595S(20%), M460V(13%), C603W(10%), C592G(7%), H520Q(7%), M460I(6%)
*CPV in vitro frequency: M460I (67%), H520Q (43%), C603R (5%)

Materials and Methods

- CPV (ZSMI-62) was provided by Microbiotix and used as 10 mM stock in DMSO. GCV sodium salt (Cytovene, Roche) was diluted in aqueous media.
- Mutant and control recombinant CMV strains based on lab strain AD169 modified with a secreted alkaline phosphatase (SEAP) reporter gene cassette (T2211) were constructed as previously published, by homologous recombination of extracted viral DNA in fibroblast cultures [6], or more recently by recombineering of bacterial artificial chromosome (BAC) clones [7] followed by transfection of mutant BACs into fibroblasts to reconstitute infectious CMV (Fig. 2). Single and double mutants of interest were constructed to define the drug resistance phenotypes conferred.
- All recombinant strains were sequenced throughout the mutagenized gene (UL54 *pol* and/or UL97) to verify the presence of the intended mutation and absence of extraneous changes.
- Drug susceptibility was assayed by the drug concentration required to reduce accumulation of SEAP activity (chemiluminescent substrate) in human foreskin fibroblast (HFF) culture supernatants by 50% at 6 days postinoculation (EC50, see Fig. 2, bottom left) [6]. Criteria for valid assays included an input multiplicity of 0.01 to 0.03 as judged by supernatant SEAP activity at 24 hours, expected EC50 values of known drug-sensitive and –resistant control strains, and a good curve fit of SEAP values observed at various drug concentrations [5, 6]. Based on standard deviations usually ~30% of EC50 values, the minimum criterion for phenotypic resistance was a 2-fold elevation of EC50 values over that of a baseline control strain [4].
- CMV exonuclease mutants (*pol* D413A, strains T2294 and T3360) with error-prone replication that accelerates the emergence of resistance mutations were propagated under CPV to assess the mutations selected in vitro [5]. Either strain was propagated in HFF cultures starting with MOI ~0.1 under 0.2 µM CPV. At weekly intervals, cells were trypsinized and ~30% were dispersed to fresh near-confluent HFF monolayers. As viral cytopathology became less inhibited by CPV, its concentration was increased during propagation, to a maximum of 4 µM (up to 30 µM in 3 cases). Aliquots of infected cell suspensions saved at various passages ranging from 5 to 15 were extracted, PCR-amplified and sequenced for UL97 codons 300-670 and *pol* codons 300-1000.

Results

- Existing *pol* mutants were selected to represent various combinations of decreased susceptibility to current CMV antivirals. Originally, the *pol* mutations were phenotyped because they had emerged after drug exposure [4,7,8]. Observed CPV phenotypes of these mutants are listed in Table 1.
- Exonuclease domain mutations (Fig. 3) commonly associated with GCV-CDV dual resistance, as well as the region V mutation A987G [4], conferred no CPV resistance. EC50 ratios were lower than 1, implying CPV hypersensitivity.
- Various *pol* catalytic region mutations mainly linked to FOS resistance [4], and clustered in region III (codons 805-845), conferred varying degrees of CPV resistance: EC50 ratio ~3x for E756K, V781I, and A809V; 4x-8x for Q578H, T813S, A834P and M844T; 14x for G841A.
- The addition of UL97 mutation C592G to *pol* mutations A809V, T813S, G841A or M844T further increased their CPV EC50s by 1.7x to 2.3x, similar to the effect of UL97-*pol* double mutations on GCV resistance [8].
- In 21 separate experiments including 18 already published [5], serial propagation of an error-prone D413A exonuclease mutant consistently resulted in UL97 mutation: M460I alone (11 cases), H520Q alone (6 cases), both M460I and H520Q (3 cases) and C603R (1 case), all within 15 passages. UL97 mutations were typically detected after 7-10 passages at CPV concentrations of 2-4 µM.
- In the same 21 experiments, a *pol* mutation evolved in only 3 cases, as detailed in Fig. 4. In expt. M89, P744T appeared to be co-selected with UL97 mutation H520Q, whereas in expt. M108, *pol* M844V appeared before UL97 mutations M460I and H520Q; ultimately selecting for an M844V-H520Q double mutant. In the expt. M111, *pol* E756D added to the pre-existing UL97 mutation M460I.
- A P744T recombinant virus showed no CPV resistance (Table 1). Additionally, M844V and E756D recombinants were recently constructed and their CPV EC50 phenotypes are comparable to those of mutants M844T and E756K (Table 1).

Figure 2. Construction of recombinant CMV strains and SEAP assay for drug EC50

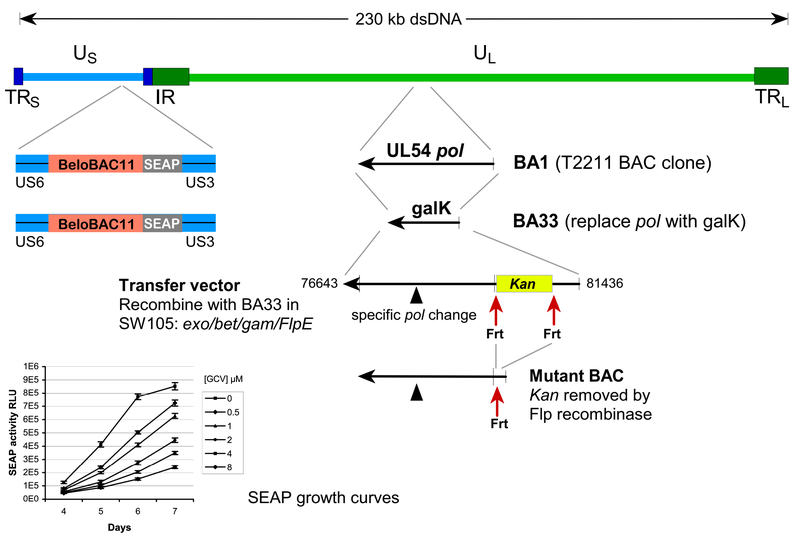


Figure 3. CMV DNA polymerase mutation map [4,7]

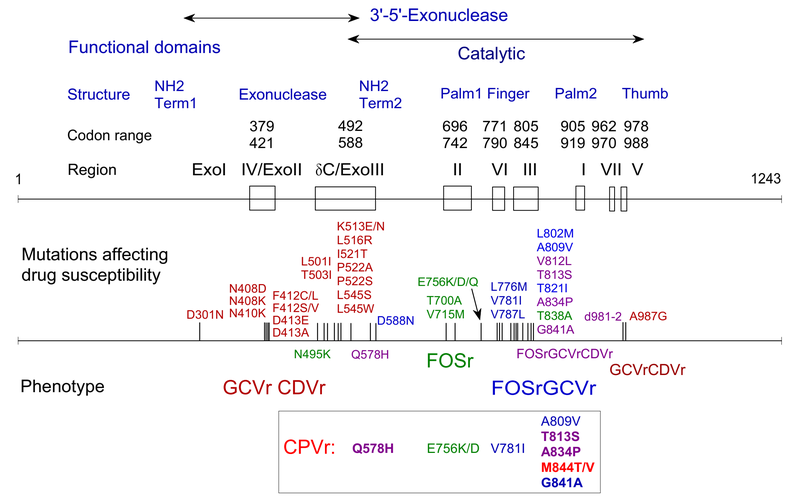


Table 1. Genotypes and phenotypes of CMV strains and recombinants

BAC[1]	Virus[2]	Genotype[3] UL97	Genotype[3] UL54 *pol*	Cyclopropavir phenotype EC50, µM[4]	SD[5]	Ratio[6]	N[7]	EC50 ratio[8] GCV	FOS	CDV
	T2211	baseline	baseline	0.23	0.05		39			
BA31	T3265	baseline	baseline	0.22	0.06		26			
BA27	T3259	**C592G**		0.66	0.17	**3.0**	14	**3.0**		
	T2293		N408K	0.15	0.06	0.7	19	**4.2**	0.7	**21**
	T2311		N408K, **A834P**	0.61	0.25	**2.7**	13	**23**	**7.2**	**19**
BA35	T3267		F412L	0.10	0.04	0.5	13	**4.6**	1.1	**9.4**
	T3005		P522A	0.12	0.05	0.5	7	**3.0**	1.0	**4.1**
BA96	T3400		L545W	0.14	0.05	0.6	15	**4.9**	1.3	**6.3**
BA112	T3426		**Q578H**	1.52	0.15	**6.9**	8	**3.3**	**4.5**	**2.3**
BA103	T3408		D588N	0.41	0.09	1.9	14	**2.0**	**2.8**	1.3
BA143	T3525		P744T	0.25	0.05	1.1	9			
BA189	T3658		**E756D**	0.72	0.11	**3.3**	8	1.2	**3.4**	0.7
BA116	T3430		**E756K**	0.66	0.10	**3.0**	8	1.9	**3.5**	1.7
BA108	T3417		**V781I**	0.69	0.20	**3.1**	9	**2.4**	**3.9**	1.5
	T2417		**A809V**	0.70	0.15	**3.0**	10	**2.4**	**3.3**	1.9
	T2784	**C592G**	**A809V**	1.58	0.52	**6.9**	8	**5.6**	**3.3**	**2.1**
	T2542		**T813S**	1.89	0.59	**8.2**	9	**2.5**	**4.9**	**2.7**
	T2798	**C592G**	**T813S**	3.27	0.48	**14**	7	**6.0**	**5.0**	1.7
	T2291		**A834P**	1.35	0.48	**5.9**	16	**5.4**	**6.4**	**3.0**
	T2420		**G841A**	3.16	0.59	**14**	8	**3.2**	**4.3**	**2.6**
	T2817	**C592G**	**G841A**	6.07	1.74	**26**	11	**7.6**	**5.2**	**3.0**
	T2483		**M844T**	1.03	0.34	**4.5**	10	1.6	**2.0**	1.2
	T2785	**C592G**	**M844T**	1.79	0.44	**7.8**	8	1.9	1.9	1.1
BA187	T3652		**M844V**	1.00	0.11	**4.3**	6			
	T2222		981-2del	0.33	0.08	1.4	8	**4.8**	**3.5**	**4.1**
	T2261	**C592G**	981-2del	0.64	0.19	**2.8**	16	**17**	**4.1**	**5.7**
BA115	T3429		A987G	0.12	0.04	0.5	7	**6.2**	0.9	**5.3**

Genotypes associated with CPV resistance shown in **bold color**, EC50 ratios >1.9 shown in **bold**.

1: Bacterial artificial chromosome clone; 2: Strain name; 3: Mutation(s) introduced into baseline strains by mutagenesis;
4: Mean EC50 value; 5: Standard deviation of EC50 values; 6: Ratio of EC50 value to baseline strain; 7: Number of assays;
8: EC50 ratios are from published data except for M844T±C592G.

Discussion

- UL54 *pol* mutations are much less frequently selected than UL97 mutations as initial genetic pathways to CPV resistance in vitro (1 of 21 experiments). This is similar to the evolution of GCV resistance [4] and probably reflects the relative growth fitness of UL97 and *pol* mutants.
- The level of CPV resistance conferred by individual *pol* mutations (3x-14x) is comparable to that of UL97 mutations. As with GCV [4], the addition of a *pol* mutation to a pre-existing UL97 mutation increases the overall level of CPV resistance, as shown by the effect of combining UL97 mutation C592G with several *pol* mutations in Table 1. Further propagation under higher concentrations of CPV in vitro may reveal additional relevant *pol* mutations. A dual UL97 (H520Q) + *pol* (M844V) mutant was selected under the highest CPV concentration tested.
- Results suggest an absence of CPV resistance based on exonuclease or region V mutations, which are frequent causes of GCV and cidofovir dual resistance. Exo domain mutations are thought to increase the rate of excision of incorporated bases, potentially slowing the rate of DNA synthesis and possibly explaining the tendency to CPV hypersensitivity.
- The *pol* mutations involved in CPV resistance map to residues that are in spatial proximity in or near the finger and palm domains of the polymerase catalytic core (Fig. 5), and probably affect recognition of the incoming base. Such mutations tend to have the phenotype of FOS resistance with borderline or low grade GCV-CDV cross-resistance (Table 1). The exact mutations and the relative level of resistance may differ for each drug. The M844V mutation selected under CPV has not been reported previously, but we had earlier constructed an M844T mutant, which also showed CPV resistance. The CPV-selected E756D mutation was previously reported after FOS therapy [4].
- Mutations *pol* P617H and P744T observed in vitro (Fig. 4) under CPV do not involve conserved residues or structural features. Their transient appearance in 2 drug selection experiments does not suggest a significant role in CPV resistance; P744T did not confer CPV resistance when transferred to a control strain. Most likely this was a spontaneous sequence change in an error-prone exo-mutant strain T2294 that was co-selected with the H520Q marker.

Figure 4. Evolution of UL97 and *pol* mutations in strain T2294 under CPV

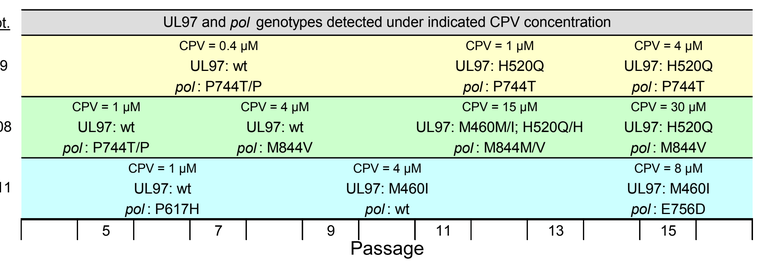
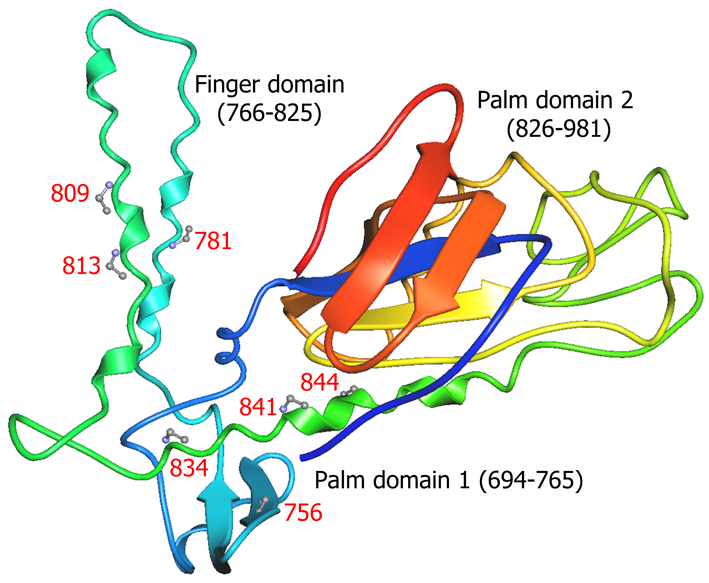


Figure 5. CMV DNA polymerase catalytic region structure



The Pol catalytic region contains Palm and Finger domains as shown, and a Thumb domain extending past residue 981, not shown. Structure is modeled on the published HSV Pol structure (2gv9.pdb) using CPHmodels-3.0. Residues affecting CPV susceptibility are labeled; most are also involved in resistance to FOS ± GCV/CDV, as shown in Figure 3. Mutation Q578H is located in a separate amino terminal domain just left of residue 813 in this view.

Conclusions

- Similar to GCV, *pol* mutations that confer CPV resistance are initially selected much less frequently than UL97 mutations, but mutations at both loci may combine to increase the overall level of drug resistance
- In contrast to GCV, *pol* mutations that confer CPV resistance do not map primarily to exonuclease domains and region V, but to residues at the polymerase catalytic core that are classically associated with FOS resistance
- To date, characterization of UL97 and *pol* CPV and GCV resistance mutations indicates a partial cross-resistance where CPV may retain meaningful antiviral activity against some common GCV-resistant UL97 or *pol* mutants.

References

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