Cidofovir Resistance in Vaccinia Virus Is Linked to Diminished Virulence in Mice

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Cidofovir [(S)-1-(3-hydroxy-2-phosphonomethoxypropyl)cystosine (HPMPC)] is recognized as a promising drug for the treatment of poxvirus infections, but drug resistance can arise by a mechanism that is poorly understood. We show here that in vitro selection for high levels of resistance to HPMPC produces viruses encoding two substitution mutations in the virus DNA polymerase (E9L) gene. These mutations are located within the regions of the gene encoding the 3'-5' exonuclease (A314T) and polymerase (A684V) catalytic domains. These mutant viruses exhibited cross-resistance to other nucleoside phosphonate drugs, while they remained sensitive to other unrelated DNA polymerase inhibitors. Marker rescue experiments were used to transfer A314T and/or A684V alleles into a vaccinia virus Western Reserve strain. Either mutation alone could confer a drug resistance phenotype, although the degree of resistance was significantly lower than when virus encoded both mutations. The A684V substitution, but not the A314T change, also conferred a spontaneous mutator phenotype. All of the HPMPC-resistant recombinant viruses exhibited reduced virulence in mice, demonstrating that these E9L mutations are inextricably linked to reduced fitness in vivo. HPMPC, at a dose of 50 mg/kg of body weight/day for 5 days, still protected mice against intranasal challenge with the drug-resistant virus with A314T and A684V mutations. Our studies show that proposed drug therapies offer a reasonable likelihood of controlling orthopoxvirus infections, even if the viruses encode drug resistance markers.

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introduce other unlinked mutations. This is an important con-
cern, because if drug resistance were inextricably linked to reduced virulence, biodefense strategies could not be readily underminned by the malicious intervention of mutations conferring resistance to nucleoside phosphate drugs.

We have used DNA sequencing and marker rescue methods to show that one or more substitution mutations in the VAC E9L (DNA polymerase) gene suffice to confer an HPMPC\(^{-}\) phenotype. Moreover, the viruses assembled using these meth-
ods still exhibit reduced virulence and can be effectively treated with clinically relevant doses of HPMPC. This work is the first to demonstrate the molecular genetic basis for VAC resistance to ANPs and provides new insights into the enzymology of drug resistance.

MATERIALS AND METHODS

Cell and virus culture. All cells and viruses were purchased from the American Type Culture Collection. Human embryonic lung (HEL) fibroblasts and VAC (strain Lederle) were cultured in minimal essential medium (MEM) supple-
mented with 10% heat-inactivated fetal calf serum, 2 mM L-glutamine, and 0.3% sodium bicarbonate at 37°C in a 5% CO\(_{2}\) atmosphere. Monkey kidney epithelial (BSC40) cells and VAC (Western Reserve [WR] strain) were cultured in MEM containing 5% fetal calf serum, 1% nonessential amino acids, 1% L-glutamine, and 1% antibiotic/antimycotic also at 37°C in a 5% CO\(_{2}\) atmosphere.

Materials. The sources of the compounds were as follows: HPMPC, \(\text{HPMPC}^{-}\), and PMEA (adeovir, 9-[2-(phosphonomethoxy)ethyl] adenine) from Gilead Sciences (Foster City, CA); HPMPA (\(\Sigma\)-9-[3-hydroxy-2-(phosphonomethoxy)propyl]adenine), cyclic HPMPA (cHPMPA), 3-deaza-HPMPA (\(\Sigma\)-9-[3-hydroxy-2-(phosphonomethoxy)propyl]-3-deazadenine), PMEDAP (9-[2-(phosphonomethoxy)ethyl]-2,6-diaminopurine), HPMO-DApy (6-[3-hydroxy-2-(phosphonomethoxy)propoxy]-2,4-diaminopirimidine), and PMEO-DApy (6-[3-hydroxy-2-(phosphonomethoxy)ethoxy]-2,4-diaminopyrimidine) from A. Holy (Institute of Organic Chemistry and Biochemistry, Academy of Sciences of the Czech Republic, Prague, Czech Republic); phosphonoacetic acid (PAA), cyanate \(\beta\)-arabinofuranosidoaracinate (ArAc), and aphidicolin from Sigma Chemical Co. (St. Louis, Mo.); and itinatin-\(\beta\)-thiosemicarbazone (IBT) from Pfaltz & Bauer Inc. (Waterbury, CT). All compounds were diluted to their final concentra-
tion in MEM. “Expanded” high-fidelity DNA polymerase was purchased from Roche Applied Science (Indianapolis, IN).

Isolation of HPMPC\(^{-}\) viruses. Drug-resistant viruses were obtained by serial passage of a single stock of virus in HEL cells in the presence of increasing amounts of HPMPC. Viruses were also serially passed in drug-free media in parallel (wild-type [WT] controls). The starting concentration was ~2 \(\mu\)M, which reduced the cytopathic effect (CPE) by 50% (50\% inhibitory concentration [IC\(_{50}\)]) of WT virus. The infected cells were cultured for 2 or 3 days until a strong CPE was observed. The viruses were then harvested and replated on fresh cells in the presence of more drug, increasing the drug concentration by 2 \(\mu\)M with each subsequent passage. Periodic passage without HPMPC served to increase the titer. Virus capable of replication in the presence of 50 \(\mu\)M HPMPC was cultivated one last time in drug-free media, and then seven plaque-purified HPMPC\(^{-}\) isolates and five plaque-purified WT isolates were obtained from the final viral stocks. All 12 of these isolates were sequenced and further character-
ized.

Cytopathic effect and plaque reduction assays. HEL cells were grown to confluence in 96-well microtiter plates and infected with virus at an input of 50 PFU per well. After 2 h at 37°C, the cells were washed and cultured for 2 or 3 days at 37°C in fresh medium containing serial dilutions of the test compound in duplicate. The CPE was recorded using a 0 to 5 scale (where 5 equals 100\% CPE), and the IC\(_{50}\) was defined as the drug concentration that reduced the CPE by 50\%. The IC\(_{50}\) values represent the mean obtained from two or more inde-
pendent experiments.

Plaque reduction assays were performed in triplicate using 200 to 10,000 PFU per dish. Virus-infected BSC40 cells were cultivated for 2 days and then fixed with 2% formaldehyde and stained with 0.5% crystal violet. The drug concentration causing a 50% reduction in plaque number (50\% effective concentration [EC\(_{50}\)]) was calculated from a nonlinear curve fit using Prism 4.0b software.

Growth curves. BSC40 cells were infected with VAC at a multiplicity of infection (MOI) of 0.03. Following a 1-h adsorption period in phosphate-buff-
ered saline (PBS) at 37°C, the inoculum was replaced with warm medium. Viruses were harvested at different time points postinfection and released by three cycles of freeze-thawing, and yields were determined by plaque assay on BSC40 cells in the absence of drug.

Virus yield reduction assays. HEL cells were grown in six-well microtiter plates and infected with ~200 PFU of virus. After 2 h at 37 °C, the cells were washed and overlaid with drug-containing medium and incubated for 3 days. The viruses were released by freeze-thawing and then titrated by plaque assay in HEL cells.

DNA sequencing. DNA was extracted from virus-infected HEL cells using a QiAamp blood kit according to the manufacturer’s instructions (QIAGEN). The E9L gene was PCR amplified as two overlapping amplicons using primer set 1 (5'-ATAATGTCATACGGCTTCCC-3' and 5'-TGGAGGAATACCTTACCGCTTCC-3') and primer set 2 (5'-AGTCAAGGTTCACTGTTTAA and 5'-GAATAACCGTAAACAAAAGGACCCG-3'). The PCR products were purified and sequenced using E9L-specific primers.

DNA cloning. A high-fidelity DNA polymerase and PCR were used to clone portions of the E9L gene from DNA extracted from HPMPC\(^{-}\) virus (see Fig. 2). Four different primers were used to amplify either the entire 3.1-kb E9L gene (5'-AAATTCTAAAATGTGATTCGGTGTC-3' and 5'-ATTTACTATCTAAAATCAGACCCG-3'), 1.6 kb comprising the left end of the gene (5'-AAATTCTTTAAATGTGATTCGGTGTC-3' and 5'-CTTACCGCTTTA TAAGGAAACTTT-3'), or 2.1 kb comprising the right end of the gene (5'-AC GTTCCGTATATACAAATATGTGACCT-3' and 5'-ATTTACTATCTAAAATCAGACCCG-3'). These DNAs were gel purified, cloned into pCR2.1-TOPO (Invitrogen), and sequenced using E9L-specific primers to confirm the fidelity of gene amplification.

Marker rescue. BSC40 cells were grown to confluence and then infected for 1 h with VAC at 50 PFU/ml in MEM containing 5\% FCS with 100 \(\mu\)M HPMPC. Because 100 \(\mu\)M HPMPC completely inhibited plaque formation by WT virus, therefore, HPMPC\(^{-}\) recombinants were recovered from cells transfected with DNA encoding A314T and A684V mutations through two rounds of passage at low MOIs on BSC40 cells in medium containing 300 to 350 \(\mu\)M HPMPC. Viruses were then plaque purified twice under an agarose overlay in media lacking HPMPC and finally once in the presence of 350 \(\mu\)M drug. Virus recovered from cells transfected with DNA encoding the A314T mutation were subjected to one round of further passage at a low MOI in BSC40 cells in medium containing 300 \(\mu\)M HPMPC. The resulting viruses were then plaque purified twice in media containing 300 or 100 \(\mu\)M HPMPC and finally once in the absence of drug. Viruses recovered from cells transfected with DNA encoding the A684V mutation were purified in the same way as A314T mutant virus except that the agarose overlays contained 100 \(\mu\)M HPMPC. Because 100 \(\mu\)M HPMPC also completely inhibited plaque formation in WT controls, we used an initial 300 to 350 \(\mu\)M dose of drug to eliminate the majority of background WT virus from the marker rescue stocks. For each clone, five independent virus isolates were worked up and retained for further analysis.

Animal studies. Adult NMRI mice (13 to 14 g) were inoculated by an intra-
nasal route with 20 \(\mu\)l of virus (0, 40, 400, or 4,000 PFU) diluted in PBS. Five mice per virus dilution or placebo were used, and body weight was recorded for 30 days postinfection. Animals were euthanized if weight loss exceeded 25\% of initial body mass. To test the effects of drug treatment, HPMPC was adminis-
tered subcutaneously at doses of 10 or 50 mg/kg of body weight/day starting on the day of infection.

RESULTS

Derivation of HPMPC\(^{-}\) vaccinia virus clones. Drug-resistant viruses were obtained by serial passage of the VAC Lederle strain in human embryonic lung cells in the presence of increasing amounts of HPMPC. Following 40 rounds of passage, seven clones were plaque purified from the same pool of virus, and all isolates exhibited a high level of drug resistance. Five WT clones were also plaque purified from the parent stock. Exactly when HPMPC\(^{-}\) viruses appeared is not known with certainty.
However, a retrospective survey of archived stocks detected a >10-fold increase in drug resistance between passages 33 and 38. During this time, the viruses were exposed to a concentration of HPMPC ranging from 40 to 50 μg/ml.

**Phenotypic characterization of HPMPC<sup>R</sup> clones.** The five WT clones and the seven HPMPC<sup>R</sup> plaque-purified clones were tested for sensitivity to a selection of different antiviral compounds, using an assay that measures the inhibition of viral cytopathic effect. Within each of the two groups, the different isolates exhibited similar patterns of drug sensitivity. Figure 1 shows mean IC<sub>50</sub> values calculated for WT versus HPMPC<sup>R</sup> viruses plus the 95% confidence intervals for each group.

The HPMPC<sup>R</sup> clones were significantly (8.8-fold) more resistant than WT virus to HPMPC and showed a similar degree of cross-resistance to several related ANPs. The mean increase in IC<sub>50</sub> values ranged from 6.2-fold for cHPMPC to 12.5-fold for HPMDAP. Interestingly, viruses selected for resistance to HPMPC remained sensitive to 3-deaza-HPMPA. These viruses were also just as sensitive to WT virus as to PMEO-DAPy, the pyrophosphate analog PAA, and the cytosine analog AraC.

**Genotypic characterization of HPMPC<sup>R</sup> clones.** Viruses exhibiting resistance to ANPs often encode mutations in the DNA polymerase gene. We therefore used PCR and the primers targeting sequences flanking the VAC E9L (DNA polymerase) gene to amplify this locus from WT and HPMPC<sup>R</sup> viruses and then sequenced these DNAs in their entirety. The results are summarized in Table 1. The original stock of VAC strain Lederle encodes a polymorphic locus at amino acid 420, which is also found in other WT orthopoxviruses and is thus presumably unrelated to drug resistance. A second polymorphic locus was found at positions 936 to 938 wherein the ancestral sequence in some viruses suffered a small in-frame deletion. However, both types of WT clones proved equally sensitive to all drugs tested (Fig. 1), and thus, this deletion was also unlinked to drug resistance. Finally, all Lederle-derived strains of VAC encode amino acid substitutions at positions 845 and 857, which differentiate this strain from other VAC strains. Again, these loci were not responsible for drug resistance.

Ultimately, only two point mutations were potentially associated with resistance. These mutations create an A314T substitution within the putative 3'-5' exonuclease domain and an A684V substitution within the putative DNA polymerase domain (Fig. 2). Both of these mutations occur at residues that are highly conserved among orthopoxvirus DNA polymerases (Table 1). Analysis of plaque-purified clones isolated from archived virus stocks at passage number 33 showed that in the process of selection of HPMPC<sup>R</sup> mutants, the A684V mutation must have appeared after the A314T mutation (data not shown).

**Marker rescue analysis.** We used marker rescue methods to examine the linkage between A314T and A684V mutations and drug resistance. Plasmids encoding these mutant alleles were transfected into cells infected with VAC strain Western Reserve, and recombinant progeny were screened for growth in media containing 300 or 500 μM HPMPC. The cloned DNA encoded A314T, A684V, or both A314T and A684V mutations (Fig. 2). As a control, VAC-infected cells were also transfected with a plasmid encoding all or part of the WT VAC strain Lederle E9L gene. This provided a test of whether any of the Lederle polymorphisms might contribute in some manner to the resistance phenotype.

**Table 1. Mutations in the E9L gene of HPMPC<sup>R</sup> vaccinia virus (strain Lederle)**

<table>
<thead>
<tr>
<th>Virus</th>
<th>Amino acid present at position(s) (vaccinia virus):&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>HPMPC&lt;sup&gt;R&lt;/sup&gt; clones&lt;sup&gt;b&lt;/sup&gt;</td>
<td>R T S V M R A-N-V</td>
</tr>
<tr>
<td>Clone 1</td>
<td>R A S A M R N-D-G</td>
</tr>
<tr>
<td>Clone 2</td>
<td>R A L A M R N-D-G</td>
</tr>
<tr>
<td>Clone 7</td>
<td>R A S A M R A-N-V</td>
</tr>
<tr>
<td>Clone 8</td>
<td>Q A L A M R A-N-V</td>
</tr>
<tr>
<td>Clone 11</td>
<td>R A S A M R A-N-V</td>
</tr>
<tr>
<td>VAC strains</td>
<td></td>
</tr>
<tr>
<td>Ankara</td>
<td>R A S A T G A-N-V</td>
</tr>
<tr>
<td>Copenhagen</td>
<td>R A L A T G A-N-V</td>
</tr>
<tr>
<td>WR</td>
<td>R A L A T G A-N-V</td>
</tr>
<tr>
<td>Other viruses</td>
<td></td>
</tr>
<tr>
<td>Variola virus India-1967</td>
<td>Q A S A I G A-N-V</td>
</tr>
<tr>
<td>Camelpox virus</td>
<td>Q A S A I G A-N-V</td>
</tr>
<tr>
<td>Monkeypox virus</td>
<td>R A S A T G A-N-V</td>
</tr>
</tbody>
</table>

<sup>a</sup>The amino acid numbering refers to the numbering system for VAC virus E9L gene. The residue numbering differs slightly for homologous residues in other orthopoxvirus genes.

<sup>b</sup>All seven clones were identical.
the acquisition of resistance. The results are shown in Table 2. At higher drug concentrations, HPMPC-resistant viruses were recovered only from cells transfected with DNA encoding both mutations (data not shown), whereas at 300 μM, HPMPC-resistant recombinants were recovered from cells transfected with any of the three different mutant DNAs. The few viruses recovered from cells transfected with WT DNA did not grow when subjected to further passage in drug-containing media and were not pursued further. These data suggested that both mutations were responsible for HPMPC resistance.

Properties of recombinant virus encoding both A314T and A684V mutations. Five independent recombinants were recovered and plaque purified from cells transfected with DNA encoding both A314T and A684V mutations. The E9L genes were cloned and entirely resequenced. Four of the five VAC WR recombinants encoded the two input mutations (A314T and A684V) as well as the Lederle sequence polymorphisms (L420S, T845M, and G857R) present in all of the original HPMPC-resistant viruses. One of these four viruses was designated V-DG1.3 and used for further studies. The fifth isolate contained an additional Y232H mutation (V-DG1.7).

The virus designated V-DG1.3 exhibited a pattern of drug susceptibility comparable to that observed for the original HPMPC-resistant Lederle clones (Fig. 3, gray bars). Thus, compared to the parent VAC WR strain, these recombinant viruses were 13-fold more resistant to HPMPC in an assay measuring CPE. They also exhibited cross-resistance to most HPMPA derivatives while showing near WT levels of sensitivity to PMEODAPy, PAA, and AraC. The actual numerical IC50 values are available from the authors upon request. Plaque reduction assays were also performed on BSC40 cells to gain an independent measure of the degree of HPMPC resistance. Although relatively greater drug concentrations are required to reduce plaque numbers by 50%, compared with an assay that uses CPE as an end point, the mutant E9L gene still greatly increased the degree of drug resistance exhibited by recombinant viruses (Fig. 4A). The recombinant virus was 17-fold more resistant to HPMPC than the parent virus was (EC50 of 890 ± 60 versus 53 ± 3 μM), and the virus encoding an additional Y232H mutation exhibited an even higher level of drug resistance (EC50 of 1,340 ± 50 μM). Yield reduction assays showed that the increased plating efficiency was due to an increase in the number of mutant viruses when grown in the presence of HPMPC (Fig. 4B).

Mutations in DNA polymerases can sometimes affect the replication of the viruses that encode them. However, we used low-MOI growth curves to compare the replication and spread

<table>
<thead>
<tr>
<th>Transfected DNA</th>
<th>Size (kb)</th>
<th>E9L allele(s)</th>
<th>No. of HPMPC-resistant plaques (per 10^5 PFU plated)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mutant DNA</td>
<td>3.1</td>
<td>A314T + A684V</td>
<td>76 ± 9</td>
</tr>
<tr>
<td></td>
<td>1.6</td>
<td>A314T</td>
<td>38 ± 7</td>
</tr>
<tr>
<td></td>
<td>2.1</td>
<td>A684V</td>
<td>23 ± 5</td>
</tr>
<tr>
<td>Wild-type DNA</td>
<td>3.1</td>
<td>ND*</td>
<td>0.3 ± 0.6</td>
</tr>
<tr>
<td></td>
<td>1.6</td>
<td></td>
<td>2.3 ± 0.6</td>
</tr>
<tr>
<td></td>
<td>2.1</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Marker rescue stocks were plated on BSC40 cells under a liquid overlay with or without 300 μM HPMPC. The plaques were fixed and stained 48 h postinfection. Values are means ± standard deviations, and the experiments were performed three times.

**TABLE 2. Marker rescue efficiency in the presence of HPMPC**

![FIG. 2. Vaccinia virus E9L gene and mutational map. The VAC E9L gene encodes 1,006 amino acids and comprises DNA polymerase B exonuclease [DNA pol B exo] and DNA polymerase B [DNA pol B] domains plus six highly conserved sequence elements common to B-family DNA polymerases (I to VI) (32). The gene encoded by VAC strain Lederle bears three preexisting sequence polymorphisms, which differentiate the gene from that encoded by VAC strain WR. All of the viruses isolated by passage in HPMPC-containing media also encoded new A314T and A684V mutations (boxed). The PCR amplimers used for marker rescue studies are shown at the bottom of the top panel. Also shown are the known map sites for mutations conferring resistance to cytosine arabinoside (AraC), phosphonoacetic acid (PAA), and aphidicolin (Aph) (36). The two bottom panels show the sequence context of A314T and A684V mutations and alignments to other B-family DNA polymerases. RB69, phage RB69 gp49; HCMV, human cytomegalovirus; HSV, herpes simplex virus (type 1); EBV, Epstein-Barr virus; Sc, Saccharomyces cerevisiae Pol1; Hs, Homo sapiens polymerase α; T4, phage T4 gp49; AdV, human adenovirus type 5.](downloaded from jvi.asm.org at 26/09/2010)
of the parental and HPMPC<sup>R</sup> recombinant viruses in the absence of drug and saw no detectable differences between these viruses (Fig. 5A). The additional Y232H mutation also had no effect on the growth of V-DG1.7 in culture (data not shown).

The VAC virus WR strain was historically subjected to repeated intracranial passage in mice (24) and is noted for its virulence in murine infection models. We therefore used an intranasal infection route to test the pathogenicity of the parental VAC WR strain compared with recombinant viruses encoding the A314T and A684V mutations. Groups of five mice were challenged with 10-fold serial dilutions of virus and monitored for weight change and other symptoms of infection over the next 30 days. These experiments showed that the introduction of A314T and A684V mutations into VAC WR decreased virus virulence. For example, 4,000 PFU of WT virus caused all five animals to be euthanized by day 11 postinfection (Fig. 6). In contrast, although all five animals showed some morbidity when challenged with the same dose of the V-DG1.3 recombinant virus, no mortality was observed. Collectively, these results show that reduced virulence is genetically linked to drug resistance.

**Properties of recombinant viruses encoding only the A684V mutation.**

Our marker rescue experiments suggested that either mutation alone might suffice to confer HPMPC resistance (Table 2). We transfected VAC WR-infected cells with a 2.1-kb DNA encoding just the A684V mutation (Fig. 2) and plaque purified five recombinant viruses that exhibited growth in the presence of 100 μM HPMPC. (We reduced the drug concentration because, during isolation of these viruses, the putative recombinants grew poorly under top agar containing the 350 μM HPMPC used to isolate V-DG1.3.) DNA sequencing showed that all five of the HPMPC<sup>R</sup> clones had acquired the A684V mutation plus the linked WT polymorphic markers that differentiate strain Lederle from the WR strain of vaccinia virus (L420S, T845M, and G857R). However, none encoded the A314T substitution. An isolate designated V-DG<sub>E9L(R).2</sub> was retained for further study.

Virus encoding just an A684V resistance marker exhibited an intermediary degree of resistance to ANPs compared with virus encoding the A314T and A684V mutations and to WT virus. This is most apparent in the plaque reduction assays where we measured an EC<sub>50</sub> of 140 ± 20 μM for HPMPC (Fig. 4A). These viruses also demonstrated a similar spectrum of resistance to ANPs, although they are relatively more sensitive to HPMPA, cHPMPA, and HPMPDAP than virus encoding both A314T and A684V mutations (Fig. 3). One interesting difference is that the A684V mutation increased PAA resistance threefold relative to the WT parent (Fig. 3). We confirmed this difference using yield reduction assays (Fig. 4C). Because PAA-resistant viruses often show hypersensitivity to aphidicolin, we also tested the effects of this drug on recombinant viruses. As predicted, the V-DGE9L(R).2 virus was approximately twofold more sensitive to aphidicolin than the parental virus was (Fig. 3).

In a side-by-side comparison of low-MOI growth curves, virus encoding the A684V mutation appeared identical to WT virus (data not shown). However, these viruses were still attenuated in vivo. Doses of 400 and 4,000 PFU of V-DGE9L(R).2 caused a transient infection with the animals exhibiting the most mor-

![FIG. 3. Drug resistance properties of HPMPC<sup>R</sup> recombinant viruses. The figure compares the activities of different compounds against recombinant viruses encoding the indicated mutations. The data are presented as a ratio of the IC<sub>50</sub> for the recombinant virus versus the IC<sub>50</sub> for the parent VAC strain WR and are plotted on a log scale to facilitate comparison of a great range of differences in drug resistance (ratios of >1) or hypersensitivity (ratios of <1). Mean IC<sub>50</sub> values were determined using a CPE assay and HEL cells and derive from at least two independent experiments. Additional details are available from the authors upon request.](attachment:figure3.png)
bidity 8 days postinfection (Fig. 6). There were no deaths in any of these cohorts. In contrast, these same doses of virus killed three of five mice and all five mice inoculated with 400 and 4,000 PFU of WT viruses, respectively (Fig. 6).

Properties of recombinant virus encoding A314T and T688A mutations. Initial attempts to isolate virus encoding just the A314T mutation were complicated by the inadvertent selection for virus encoding an additional mutation. VAC-infected cells

![Figure 4](image4.png)

**FIG. 4.** Effects of HPMPC and PAA on vaccinia virus growth. (A) Effects of HPMPC on the growth of different recombinant VAC strains as judged by a plaque reduction assay. About 200 PFU of each virus was plated on BSC40 cells in the presence of various concentrations of drug, incubated for 2 days, and stained to visualize plaques. Each data point was determined in triplicate, and the mean ± standard error (error bar) plotted as a percentage of the number of plaques at zero drug concentration. A nonlinear regression analysis was used to determine EC50 values. The calculated EC50 values were 53 ± 3 (○), 140 ± 20 (□), 240 ± 20 (■), 790 ± 40 (▲), 890 ± 60 (△), and 1,340 ± 50 (×) μM. (B and C) Effects of HPMPC and PAA on the growth of different recombinant VAC strains as judged by virus yield reduction assays. HEL cells were infected with ~200 PFU of each virus in the presence of the indicated drug concentrations and cultured for 3 days, and the yield was determined using plaque assays on HEL cells. The results show the means ± standard deviations (error bars) of two independent experiments.

![Figure 5](image5.png)

**FIG. 5.** Growth properties of HPMPC R viruses. BSC40 cells were infected with the indicated viruses at an MOI of 0.03 and cultured at 37°C, and the yield was determined by plaque assay on BSC40 cells for virus harvested at each of the indicated time points. Each measurement was determined in triplicate; the error bars are approximately the size of the data points.
were transfected with a 1.6-kb DNA fragment encoding the A314T mutation (Fig. 2) and five independent HPMPC-resistant recombinants were plaque purified in the presence of 300 μM HPMPC. All of the recombinant viruses were discovered to encode the input A314T mutation, but they also all encoded a new T688A amino acid substitution in the polymerase domain. One of these isolates, designated V-DG314.5, was used for further study. Like V-DGE9L(L).A, this isolate lacked the L420S VAC Lederle polymorphism and thus, besides the A314T change, should be otherwise isogenic with VAC strain WR.

Like virus encoding only the A684V mutation, virus encoding just the A314T mutation exhibited an intermediary level of HPMPC resistance as judged by CPE and plaque and yield reduction assays (Fig. 3 and 4). These viruses also demonstrated that the high levels of resistance to HPMPA, cHPMPA, and HPMPDAP, which are exhibited by virus encoding both A314T and A684V mutations, are principally due to the A314T allele (Fig. 3). Interestingly, although viruses encoding A314T and A684V mutations showed essentially WT sensitivity to PMEO-DAPy and PAA, on closer examination, it can be seen that this is actually a product of two counteracting phenotypes. The A314T allele creates hypersensitivity to the two drugs, while the A684V mutation creates resistance (Fig. 3). The same situation characterizes aphidicolin resistance, except the two contributing effects are reversed, with the A314T allele responsible for aphidicolin resistance.

The A314T mutation had no detectable effects on virus replication or spread in culture, as judged by growth after plating at a low MOI on BSC40 cells (data not shown). However, like all of the other drug-resistant recombinant viruses, V-DG314.5 exhibited reduced virulence in mice. The highest dose of virus tested (4,000 PFU) caused no deaths and only a transient morbidity comparable to that caused by ~100-foldless WT virus (Fig. 6). In contrast, four of five, three of five, and two of five mice infected with WT virus in parallel died from challenge doses of 4,000, 400, and 40 PFU, respectively.

Virus encoding the A684V mutation exhibits a mutator phenotype. Mutations in DNA polymerases can affect the fidelity of DNA replication. We therefore also examined the rate of forward mutation to isatin-β-thiosemicarbazone resistance in the different virus populations. IBT blocks late gene expression (23), and resistance has been mapped to at least two virus genes (10, 21). We picked six single plaques from each strain and separately expanded the titer of each stock by two rounds of

![FIG. 6. Effects of HPMPC alleles on disease in mice following intranasal infection. Groups of five NMRI mice were infected with 4,000 (□), 400 (△), or 40 (○) PFU of the indicated virus or mock infected with saline (■). For each trial with a recombinant virus, a parallel study was performed using mice infected with the parental VAC strain WR. The mice were then monitored for weight loss over the next 30 days. The percentage of change in total weight for each group of mice [or the surviving member(s)] is plotted. A filled symbol (e.g., ■) denotes a time point where one or more mice in a cohort were euthanized because of weight loss in excess of 25%.](image-url)
passage in drug-free media. We then measured the proportion of virus present in each stock that could grow in the presence of 60 μM IBT. The results are shown in Fig. 7. The proportion of IBT-resistant (IBTR) virus varied greatly from stock to stock as is expected from Luria-Delbruck fluctuation theory (Fig. 7A). However, it was clear that significantly greater numbers of mutants were produced when the virus encoded the A684V mutation (Fig. 7B). The median number of IBTR plaques was elevated fourfold in the V-DG1.3 and V-DG1.4 (R) populations compared to the parent population (185 and 180 IBT R plaques/1,000 PFU plated versus 47 IBT R/1,000 PFU; P < 0.01, Mann-Whitney U test). We also observed increases in the numbers of IBT R viruses in the V-DG314.5 population, but the difference was not statistically significant (P > 0.05, Mann-Whitney U test). These data suggest that the HPMPC R mutations that map to the putative DNA polymerase domain also create a mutator phenotype.

HPMPC-resistant viruses can still be treated with HPMPC. The fact that drug resistance can be readily transferred from virus to virus raises concerns about the impact these mutations might have on antiviral therapy. To examine this question, we infected NMRI mice by the intranasal route with 4,000 PFU of recombinant virus encoding the A314T and A684V mutations (△) or mock infected with saline (○). Animals were treated subcutaneously with saline (△, ○) or HPMPC at a dose of 50 (△) or 10 (○) mg/kg/day for 5 days. The change in total weight (expressed as a percentage) for each group of mice is plotted. A filled symbol (e.g., ■) denotes a time point where one mouse was euthanized.

FIG. 7. Virus encoding the A684V substitution mutation exhibits a mutator phenotype. Six different stocks of each virus were prepared and expanded by two rounds of passage without drug (each starting from a single plaque), virus titers were determined, and ~1,000 PFU was plated on BSC40 cells in the presence of 60 μM isatin-β-thiosemicarbazone (IBT). The proportion of IBTR virus was determined after staining and counting the resulting plaques 2 days later, relative to the number of PFU determined in the absence of drug. (A) Variation in the proportion of IBTR virus for different viral stocks. (B) “Box and whisker” plots showing medians, quartiles, and ranges for each virus group. Statistically significant differences between the median numbers of IBTR plaques between mutants and WT are indicated (*, P < 0.01, Mann-Whitney U test).

FIG. 8. HPMPC treatment of infections caused by HPMPC R virus in vivo. Groups of five NMRI mice were infected with 4,000 PFU of recombinant virus encoding the A314T and A684V mutations (△, ○) or mock infected with saline (○). Animals were treated subcutaneously with saline (△, ○) or HPMPC at a dose of 50 (△) or 10 (○) mg/kg/day for 5 days. The change in total weight (expressed as a percentage) for each group of mice is plotted. A filled symbol (e.g., ■) denotes a time point where one mouse was euthanized.

DISCUSSION

HPMPC was originally granted regulatory approval for treating herpesvirus infections, but it has become increasingly apparent that it may also be used to treat other DNA virus infections, including those caused by poxviruses (14, 19, 20, 22). However, before one would want to adopt HPMPC (or a related ANP) for that purpose, some understanding of the problem of acquired drug resistance is needed. A better understanding of the molecular genetic properties of drug-resistant poxviruses comprises the first step in evaluating potential hurdles in treating these infections.

As a starting point, we have isolated HPMPC R VAC through repeated passage of the virus in the presence of escalating doses of HPMPC. The mutant viruses that emerged...
under this selective pressure are highly resistant to HPMPC as well as cross-resistant to other HPMP derivatives, including the pyrimidine analogs cHPMPC and HPMPO-DAPy and the purine analogs HPMPA, cHPMPA, and HPMPDAP. A similar pattern of cross-resistance has been noted for other HPMPCR herpes- and poxviruses (1, 27–29). However, these HPMPCR viruses remained sensitive to the PME subclass of ANPs represented by PMEA and PMEDAP. HPMPCR HSV type 1 (HSV-1) and HCMV strains are also still sensitive to PMEA and PMEDAP, suggesting that the HPMP and PME ANP analogs interact differently with viral DNA polymerases (1, 29).

Point mutations in the DNA polymerase gene confer drug resistance. All seven of the HPMPCR viruses had acquired two point mutations in E9L consisting of an A314T substitution in the 3′-5′ proofreading exonuclease domain and an A684V substitution in the DNA polymerase catalytic domain. Because all seven of these plaque-purified isolates displayed similar drug resistance properties, had identical E9L sequences, and were isolated from the same final passage stock, they are almost certainly sibling isolates (“sibs”). Other independent screens might well yield other drug-resistant E9L alleles.

Marker rescue methods were used to produce viruses encoding both mutations, and these VAC WR recombinant viruses exhibited a drug resistance profile essentially identical to that measured in the primary isolates. The history of the appearance of these mutations during passage in drug suggested that the A314T mutation appeared first, followed by the A684V mutation. Either mutation alone can confer some degree of drug resistance (Fig. 3 and 4), and this property can explain the history of sequential adaptation to increasingly greater drug challenge. How far one can push this process of selection is uncertain, although other E9L mutations can certainly further enhance drug resistance as illustrated by the fortuitous recovery of viruses encoding the Y232H substitution in addition to the A314T and A684V mutations (Fig. 4A) and a T688A mutation superimposed on an A314T background. It is important to note that the original method of selection selects not only for HPMPCR resistance but also disadvantages any virus in that population where resistance creates a competitive growth disadvantage. Thus, the relatively poor growth of the A314T+T688A virus versus the A314T+A684V virus in culture (Fig. 5) would explain why serial passage selected for the latter virus.

How might the A684V and T688A mutations create drug resistance? VAC E9L belongs to the B-family of DNA polymerases, all of which encode six well-conserved sequence motifs (32). The A684V and T688A mutations affect highly conserved amino acids in one of these motifs called region III (Fig. 2). Region III spans the “finger” and “palm” domains of the DNA polymerase active site and encodes amino acids critical for deoxynucleoside triphosphate binding. Figure 9A shows where the two mutations would likely be located on the basis of sequence homology and the structure of the bacteriophage RB69 polymerase (13). The VAC A684V and T688A mutations can be mapped by homology to the N-terminal end of a long α-helix in RB69 DNA polymerase (Fig. 9A, yellow), which is a well-established hot spot for drug resistance mutations in other virus DNA polymerases. For example, an R842S substitution in HSV DNA polymerase (corresponding to VAC codon R692) confers PAA resistance and aphidicolin hyper-sensitivity (15), and F740I and L741S substitutions in adenovirus polymerase (corresponding to VAC codons S700 and V701, respectively) have been linked to HPMPCR (17).

The VAC A684 and T688 residues likely serve an important role in properly positioning amino acid Y668 (Fig. 9A, orange) in VAC DNA polymerase. This tyrosine residue (Y567 in RB69 DNA polymerase) plays a key role in nucleotide selection, and this function is affected by changes in neighboring amino acids. Yang et al. (39) recently described the properties of an RB69 DNA polymerase encoding a T587A mutation. This is the same mutation as VAC T688A as judged from sequence alignments (Fig. 2). The RB69 T587A mutation caused a fourfold reduction in the dCTP incorporation rate and increased the equilibrium dissociation constant (Kd) for dCTP. We suggest that the VAC T688A substitution acts by also reducing the enzyme’s affinity for HPMP at the cost of a reduced polymerase activity because of poor dCTP binding. This would explain the poor growth of V-DGE9L (L)A virus in culture (Fig. 5B) and the highly attenuated phenotype in vivo. The VAC A684V substitution is likely located just one α-helical turn away from VAC T688 at a site that, in RB69 DNA polymerase, also affects the orientation of VAC Y668 (Fig. 9A). The A684V substitution has a less deleterious effect on VAC replication in vitro than T688A but still negatively affects the fidelity of the viral polymerase (Fig. 8). This may account for the reduced virulence in mice.

A314T mutation. Although mutations in the DNA polymerase domain can greatly enhance virus drug resistance, it is the A314T mutation in the exonuclease domain that appears to be the primary determinant of ANP resistance. The A314T mutation was the first to appear during selection for HPMPCR, it creates a higher level of drug resistance than the A684V mutation in isolation does, and the same A314T mutation was recovered independently during selection for cHPMPC and HPMPA resistance (unpublished data). How this mutation creates HPMPCR is less clear. The mutation maps to the DNA polymerase exonuclease domain on the carboxy side of a conserved “exonuclease II” motif (5) (Fig. 2). In the RB69 structure, the protein element that aligns with this region of VAC DNA polymerase forms an extended β-sheet terminated by a tight hairpin and which, in the editing complex, contacts DNA diverted into the exonuclease active site (25). Mutational studies have shown that the β-hairpin (residues 251 to 262) in RB69 DNA polymerase may be involved in the transfer of DNA between the two active sites (3, 25, 31) with the RB69 R260 residue thought to interact with the penultimate nucleotide at the 3′ end of the primer strand (25) (Fig. 9B). Structure prediction algorithms suggest that poxvirus DNA polymerases likely employ a more positively charged and extended version of this β-sheet (data not shown) with the A314T substitution in some way affecting contact with the DNA. Why this mutation would create HPMPCR is uncertain, although we favor the hypothesis that it would facilitate excision of an HPMPC molecule situated at the penultimate 3′-nucleotide site. Such a gain of function would enhance resistance, because we have previously noted that a molecule of HPMPC cannot be excised, nor can the primer structure be extended, when HPMPC is the penultimate 3′ nucleotide (18). HCMV may also evolve HPMPCR through a similar enhancement of exonuclease activity (8). A notable (and perhaps expected) feature of these mutations is that they are specific for HPMP deriv-
HPMPC<sup>R</sup> alleles mapped onto the structure of RB69 DNA polymerase. (A) Structure of the RB69 DNA polymerase with DNA bound in the polymerase active site (13). Space-filling models and color codes are used to mark the locations of residues that are homologs of the VAC E9L residues A684 and T688 and the associated α-helix (yellow), VAC E9L Y688 (orange), and the incoming deoxynucleoside triphosphates (turquoise). Residue A684 is the uppermost of the two yellow-colored amino acids most clearly seen in panels A2 and A3. (B) Structure of the RB69 DNA polymerase with DNA bound in the exonuclease site (25). An NMY element (mauve) marks the tip of a β-hairpin structure that interacts with the 3′ terminus of the primer strand of the bound DNA and approximates the proposed location of the VAC A314T mutation (Fig. 2). These images were generated using CN3D v4.0 and crystallographic coordinates deposited as mmdbId 16732 and 11301.

The treatment options are as follows:

- **Treatment options.** Several studies have shown that HPMPC provides an effective therapy for treating orthopoxvirus infections including monkeypox in nonhuman primates (34). Sme et al. also showed that HPMPC<sup>R</sup> VAC strains can still be treated with HPMPC, although the greatly reduced virulence (which these authors partially attributed to attenuation caused by long virus passage) complicated the interpretation of the genetic origin of the phenotype. Our study has shown that HPMPC<sup>R</sup> recombinant viruses, isolated after only limited passage in the presence of drug, still exhibit markedly reduced virulence in immune-competent NMRI mice compared with that of the parent VAC strain WR. This suggests that HPMPC<sup>R</sup> alleles like A314T may well be intrinsically linked to reduced virulence in orthopoxviruses. This conclusion is subject to two caveats. First, one cannot exclude the possibility that even brief passage of a virus encoding a mutator allele does not still introduce other mutations. To address this question, we have tried to create a revertant virus encoding a WT VAC Lederle E9L allele in a VAC WR background, plus any other fortuitous mutations, but failed to recover any HPMPC-sensitive recombinants after screening plaques for reversion to WT levels of drug sensitivity. This difficulty precluded testing this question. Second, HPMPC<sup>R</sup> VAC strains replicate poorly in murine cell lines compared to monkey cell lines (27), and it may be that these mutations create intrinsic replication defects that are exacerbated in murine hosts. Thus, the relative degree of attenuation in other animals may not be entirely predicted by effects monitored in mice.
passage in culture) complicated the interpretation of these data (16). Our studies show that even those HPMPCR vaccinia viruses capable of causing significant morbidity can still be treated using ANP-based drugs (Fig. 8). A similar situation characterizes retrovirus treatment where ANP resistance in vivo has been rarely associated with clinical failure in simian immunodeficiency virus-infected macaques (37) and in HIV-infected patients where patients with infections caused by virus encoding the reverse transcriptase K65R mutation may continue to benefit from tenofovir therapy (38). These observations lead to the encouraging conclusions that not only are ANP-resistant orthopoxviruses likely to exhibit an intrinsic reduction in virulence, but the therapies that have been proposed for treating diseases like smallpox or monkeypox are unlikely to be undermined by the malicious release or evolution of HPMPCR strains.

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