Cytomegalovirus UL97 Kinase Mutations That Confer Maribavir Resistance

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The cytomegalovirus (CMV) UL97 kinase inhibitor maribavir (MBV) is undergoing clinical antiviral trials. Two clinical CMV isolates serially passaged in cell culture under MBV showed >20-fold increases in MBV resistance after the development of the UL97 mutation V353A in one of the isolates and of T409M in the other. Marker transfer studies confirmed that the V353A and T409M mutations conferred ~15-fold and ~80-fold increases, respectively, in MBV resistance without significantly affecting ganciclovir susceptibility. The 3 UL97 mutations now known to confer MBV resistance are located upstream of UL97 mutations linked to ganciclovir resistance, closer to kinase domains that are associated with adenosine triphosphate binding and phosphotransfer.

The benazimidazole riboside maribavir (MBV; 1263W94) is an orally bioavailable experimental drug for cytomegalovirus (CMV) infection [1, 2]. On the basis of favorable preliminary data and interim phase 2 clinical trial results, the drug is undergoing phase 3 clinical trials as a less-toxic alternative to drugs currently used to treat CMV infection. MBV inhibits the viral UL97 kinase [1], a mechanism of action distinct from current CMV drugs that target the viral DNA polymerase. Shutoff of UL97 kinase activity severely impairs viral replication through mechanisms that are not fully understood but that could involve defects in encapsidation, nuclear egress, or phosphorylation of replication-related proteins, such as UL44 [3–5]. No cross-resistance has been observed between MBV and current CMV antivirals [6], which is consistent with the difference in antiviral mechanisms.

Well-characterized mutations in CMV UL97 confer varying levels of ganciclovir (GCV) resistance [7], because GCV is a substrate for phosphorylation by UL97 and phosphorylation is necessary for GCV to inhibit the viral DNA polymerase. Early evidence that MBV is an inhibitor of the UL97 kinase included the identification of the UL97 mutation L397R in CMV laboratory strain AD169, which had been serially propagated under a carbocyclic analogue of MBV [1]. This mutation was found to confer high-level MBV resistance. The same experiments also gave rise to several mutations in the viral gene UL27 that conferred mild MBV resistance [8]. To discover additional MBV-resistance mutations, we propagated clinical isolates under drug and studied the mutations that developed by transferring them to reference CMV strains to examine the extent of drug resistance conferred (marker transfer or recombinant phenotyping).

**Materials and methods.** Two clinical CMV isolates (C076 and C194 at passages 9 and 4, respectively) were isolated from transplant recipients who had not received antiviral therapy. MBV was obtained from GlaxoSmithKline. The isolates were propagated in human embryonic lung (HEL) fibroblasts under 0.3 μmol/L MBV and were passed as infected cells under drug weekly. During serial passage, cultures were monitored for a decrease in the abundant refractile nuclear inclusions characteristic of the UL97-deficient cytopathic effect (CPE) [9] induced by MBV. When a change in CPE was noted, DNA was extracted from infected-cell cultures, and the entire viral UL27 and UL97 coding sequences were determined by dye termination DNA sequencing of polymerase chain reaction (PCR) products [8]. The MBV concentration was then increased on subsequent culture passages to a final concentration of 10–15 μmol/L. Acquisition of MBV resistance in the clinical isolates was assessed by plaque reduction assay in HEL fibroblasts in 12-well culture plates [6].

Observed UL97 mutations were examined by recombinant phenotyping, as described elsewhere [10]. Briefly, a segment of the UL97 coding sequence of the mutated clinical isolate was amplified by PCR, cloned, checked for absence of errors in the clone sequence, and then transferred to a reference CMV laboratory strain that had been modified from standard strain AD169 to contain unique restriction sites and to a reporter gene expressing secreted alkaline phosphatase (SEAP) for viral...
quantitation [10]. As a new technical option, to bypass the need for genomic viral DNA for cotransfection, baseline strain T2211 [10] was used to derive an intermediate recombinant strain (T2266) that contained a truncation of the UL97 gene at codon 536. This led to a severely growth-inhibited UL97-deficient phenotype [9] that could be repaired by transfection of T2266-infected cells with cloned UL97 DNA that contained the desired mutation. The recombinant virus outgrew the T2266 parent over a 4-week period and was plaque purified 2–3 times before drug-sensitivity testing. As a control for this process, baseline UL97 amino acid changes present in the clinical isolates before propagation under MBV were cloned and transferred into T2266, to show that the baseline UL97 changes have no effect on drug sensitivity. No drug selection was involved in the construction of recombinant viruses. The previously reported drug-sensitive strain T2233 [10], the GCV-resistant strain T2259 [10], and the MBV-resistant strain T2264 [6] were included as controls for sensitivity testing.

The drug sensitivity of recombinant viruses was assessed on the basis of drug concentrations that reduced the culture supernatant SEAP activity by 50% (IC50) [10, 11]. On days 4–8, aliquots of culture supernatant were frozen daily and later assayed together for SEAP activity [10].

**Results.** After 9 passages under 0.3 μmol/L MBV, clinical isolate C076 showed a change in appearance of infected-cell cultures, with more-rapid progression of CPE and loss of the refractile inclusions seen in UL97-deficient viruses [9]. At this point, sequencing of PCR-amplified viral DNA from infected cells showed no changes from baseline in UL27, and the appearance of UL97 mutation V353A, present at ~60% of the sequence population. After one more passage, the proportion of V353A reached ~90%, and the virus subsequently grew and propagated well on rapid escalation of the MBV concentration to 10 μmol/L. At passage 10, virus was separately propagated without drug, to make infected-cell stock for plaque reduction testing [6]. Results (table 1, top) showed that the isolate had acquired a >20-fold increase in MBV IC50 after acquisition of the V353A mutation. Recombinant phenotyping was performed after cloning a segment of the UL97 DNA of the baseline or mutated isolates and transferring it to reference SEAP-reporter CMV strains, yielding strains T2857 and T2807, respectively. The plaque-purified recombinant viruses had the UL97 genotypes and the drug-resistance phenotypes shown in table 1 (bottom). This confirmed that the V353A mutation (strain T2807) conferred an ~15-fold increase in MBV over baseline strains (T2233, T2856, and T2857), without change in GCV IC50.

With isolate C194, the corresponding change from UL97-deficient CPE to a more-normal CPE was not detected until after 23 passages under MBV, at which point sequencing of infected-cell DNA showed no changes from baseline in UL27.

### Table 1. UL97 genotypes and drug-resistance phenotypes of cytomegalovirus isolates and strains.

<table>
<thead>
<tr>
<th>Assay, isolate or strain</th>
<th>UL97 mutation</th>
<th>UL97 sequence variationa</th>
<th>MBV IC50, μmol/Lb</th>
<th>Passages under MBV, no.</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Plaque reduction assays</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Isolate C076</td>
<td>None</td>
<td>N68D, L126Q, I244V, Q449K</td>
<td>0.31 ± 0.05</td>
<td>0</td>
</tr>
<tr>
<td>Isolate C076</td>
<td>V353A</td>
<td>N68D, L126Q, I244V, Q449K</td>
<td>6.6 ± 1.0</td>
<td>10</td>
</tr>
<tr>
<td>Isolate C194</td>
<td>None</td>
<td>N68D, L126Q, I244V</td>
<td>0.41 ± 0.05</td>
<td>0</td>
</tr>
<tr>
<td>Isolate C194</td>
<td>T409M</td>
<td>N68D, L126Q, I244V</td>
<td>&gt;32</td>
<td>30</td>
</tr>
<tr>
<td><strong>SEAP reduction assays</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Strain T2233</td>
<td>None</td>
<td>None</td>
<td>0.14 ± 0.01</td>
<td>1.2 ± 0.07</td>
</tr>
<tr>
<td>Strain T2857</td>
<td>None</td>
<td>L126Q, I244V, Q449K</td>
<td>0.15 ± 0.01</td>
<td>1.5 ± 0.1</td>
</tr>
<tr>
<td>Strain T2807</td>
<td>V353A</td>
<td>L126Q, I244V, Q449K</td>
<td>2.0 ± 0.12</td>
<td>1.2 ± 0.07</td>
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<tr>
<td>Strain T2856</td>
<td>None</td>
<td>L126Q, I244V</td>
<td>0.11 ± 0.01</td>
<td>1.3 ± 0.1</td>
</tr>
<tr>
<td>Strain T2758</td>
<td>T409M</td>
<td>I244V</td>
<td>10.5 ± 0.5</td>
<td>1.1 ± 0.08</td>
</tr>
<tr>
<td>Strain T2264</td>
<td>L397R</td>
<td>None</td>
<td>&gt;28</td>
<td>1.9 ± 0.2</td>
</tr>
<tr>
<td>Strain T2259</td>
<td>M460V</td>
<td>None</td>
<td>0.054 ± 0.01</td>
<td>8.6 ± 0.5</td>
</tr>
</tbody>
</table>

**NOTE.** Resistant genotypes and phenotypes are shown in boldface type. GCV, ganciclovir; MBV, maribavir; SEAP, secreted alkaline phosphatase.

a Amino acid changes from the laboratory strain AD169 UL97 coding sequence.

b Data for plaque reduction assays are mean ± SE values from at least 4 assays on 4 different setup dates; data for SEAP reduction assays are mean ± SE values from at least 12 assays on 3 different setup dates.
and the new development of mutation T409M in UL97, constituting ~70% of the sequence population. The virus subsequently grew well under 15 μmol/L MBV (passage 30). At this point, the T409M mutation was the only detectable sequence configuration at codon 409; infected-cell stock was grown without drug and tested for MBV IC_{50} by plaque reduction (table 1, top), and a >78-fold increase over the baseline clinical isolate was observed. Similar to experiments done for V353A, the T409M mutation was studied by recombinant phenotyping. UL97 sequences and phenotypes of the recombinant viruses showed that strain T2758, which contained mutation T409M, had an ~80-fold increased MBV IC_{50} compared with that for control strains (T2233, T2856, and T2857), again without affecting GCV sensitivity (table 1, bottom).

Control strain T2259, which contained the GCV-resistance mutation M460V, and control strain T2264, which contained the MBV-resistance mutation L397R, showed the expected drug-resistance phenotypes without evidence of cross-resistance (table 1, bottom). Growth curves of MBV-resistant UL97 mutants (figure 1) showed at most slight growth attenuation relative to that of a wild-type control strain, T2233, in contrast to the severe growth inhibition and abnormal CPE seen with the UL97-defective strain T2266.

Discussion. Propagation of clinical CMV isolates under MBV selected for 2 newly identified viral UL97 kinase mutations that confer relatively high-grade MBV resistance. The 15–80-fold increases in MBV IC_{50} resulting from the V353A and T409M mutations are less than the >200-fold increase observed for the L397R mutation but are likely to be clinically significant, given that the UL97 mutations seen in patient isolates after treatment with GCV typically confer a 2–15-fold increase in GCV IC_{50} [7]. No significant GCV resistance appears to be conferred by any of the UL97 mutations now known to confer MBV resistance, and the mutations do not substantially impair viral growth.

No clinical isolates are yet available from subjects who have received extended treatment with MBV, and the frequency and nature of viral resistance mutations that are selected in vivo remain to be determined. However, during the development of new antivirals, viral propagation under drug in cell culture has given meaningful guidance as to the mutations that develop in clinical practice. For example, in vitro selection of the UL97 mutations V460I and del 590–593 as well as the UL54-pol mutation A987G during the early era of GCV development correctly identified gene loci and mutations that were subsequently encountered in treated subjects, although many other UL97 and UL54-pol mutations were later discovered in clinical isolates, and only then could the relative frequency of various resistance mutations be determined [7, 12].

In the experiments reported here, viral UL27 mutations were not detected, although mutations in this gene have been linked to MBV resistance [8, 13], and we are examining several additional UL27 mutations recently identified in CMV strains and isolates propagated under MBV (authors’ unpublished data). To date, UL27 mutations confer only low-grade (2–5-fold) increases in MBV resistance, the clinical significance and mechanistic basis of which are unclear.

The CMV UL97 kinase attracts considerable research interest as a potential antiviral drug target, because this kinase is required for normal viral growth in cell culture and probably in vivo, given that UL97-deficient CMV clinical isolates have not been reported. However, cellular kinases appear to compensate partially and variably for lack of viral UL97 [11], and the antiviral effect of UL97 inhibition may depend to a degree on cell and tissue factors. Because MBV is a UL97 inhibitor whereas GCV is a substrate for UL97, it is plausible that different functional domains of the kinase are involved in resistance to each drug. Existing data and the new information presented here are consistent with this concept. GCV-resistance mutations, which cluster at codons 460, 520, and especially 590–607 [7], probably involve domains of UL97 that are involved in recognition of GCV as a substrate for phosphorylation. On the other hand, the V353A, L397R, and T409M mutations are close to highly conserved UL97 domains, with easily recognizable homologies to kinase structures involved in ATP binding and phosphorylation. For example, when mapped to homologous residues in the yeast GCN2 kinase structure [14] (Protein Data Bank identifier 1ZDY), these MBV-resistance mutations are probably involved in ATP binding sites in the kinase domain of UL97. The CMV UL97 kinase attracts considerable research interest as a potential antiviral drug target, because this kinase is required for normal viral growth in cell culture and probably in vivo, given that UL97-deficient CMV clinical isolates have not been reported. However, cellular kinases appear to compensate partially and variably for lack of viral UL97 [11], and the antiviral effect of UL97 inhibition may depend to a degree on cell and tissue factors. Because MBV is a UL97 inhibitor whereas GCV is a substrate for UL97, it is plausible that different functional domains of the kinase are involved in resistance to each drug. Existing data and the new information presented here are consistent with this concept. GCV-resistance mutations, which cluster at codons 460, 520, and especially 590–607 [7], probably involve domains of UL97 that are involved in recognition of GCV as a substrate for phosphorylation. On the other hand, the V353A, L397R, and T409M mutations are close to highly conserved UL97 domains, with easily recognizable homologies to kinase structures involved in ATP binding and phosphorylation. For example, when mapped to homologous residues in the yeast GCN2 kinase structure [14] (Protein Data Bank identifier 1ZDY), these MBV-resistance mutations are probably involved in ATP binding sites in the kinase domain of UL97.
Furthermore, it is interesting that the V353A mutation is very close to the invariant lysine (K355 in the case of UL97) that is essential for kinase activity. In fact, the position homologous to residue 353 is encoded as alanine (A) instead of valine (V) in the vast majority of human kinases [15] and could be a factor in the UL97-specific action of MBV. Mapping of UL97 mutations that confer resistance to MBV should be useful in the design of alternative UL97-inhibitor structures that lack cross-resistance.

Acknowledgment

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References