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A Guinea Pig Cytomegalovirus Resistant to the DNA Maturation Inhibitor BDCRB

Amine Ourahmane^a, Anne Sauer^a, Daniel E. Nixon^b, Christine Murphy^a, Melissa Mondello^a, Erin Douglass^a, Stephanie Siegmund^c, Jian Ben Wang^a, and Michael A. McVoy^{a,*}

^aDepartment of Pediatrics, Medical College of Virginia campus of Virginia Commonwealth University, 1101 E. Marshall Street, Richmond, Virginia 23298-0163

^bDepartment of Medicine, Medical College of Virginia campus of Virginia Commonwealth University, 1101 E. Marshall Street, Richmond, Virginia 23298-0163

^cGovernor's School for Government and International Studies, Richmond, Virginia 23230

Abstract

Herpesvirus DNA packaging is an essential step in virion morphogenesis and an important target for antiviral development. The halogenated benzimidazole 2-bromo-5,6-dichloro-1- β -d-ribofuranosyl-1*H*-benzimidazole (BDCRB) was the first compound found to selectively disrupt DNA packaging. It has activity against human cytomegalovirus as well as guinea pig cytomegalovirus. The latter provides a useful small animal model for congenital cytomegalovirus infection. To better understand the mechanism by which BDCRB acts, a guinea pig cytomegalovirus resistant to BDCRB was derived and characterized. An L406P substitution occurred within GP89, a subunit of the complex that cleaves and packages DNA, but transfer of this mutation to an otherwise wild type genetic background did not confer significant BDCRB resistance. The resistant virus also had a 13.4-kb deletion that also appeared to be unrelated to BDCRB-resistance as a virus with a similar spontaneous deletion was sensitive to BDCRB. Lastly, the BDCRB-resistant virus exhibited a dramatic increase in the number of reiterated terminal repeats at both genomic termini. The mechanism that underlies this change in genome structure is not known but may relate to the duplication of terminal repeats that is associated with DNA cleavage and packaging. A model is presented in which BDCRB impairs the ability of terminase to recognize cleavage site sequences, but repeat arrays overcome this impairment by presenting terminase with multiple opportunities to recognize the correct cleavage site sequences that lie within the repeats. Further elucidation of this phenomenon should prove valuable for understanding the molecular basis of herpesvirus DNA maturation and the mechanism of action of this class of drugs.

*Correspondent footnote: Michael A. McVoy, Department of Pediatrics, Medical College of Virginia campus of Virginia Commonwealth University, P.O. Box 980163, Richmond VA 23298-0163, Telephone: 1-804-828-0132, Fax: 1-804-828-6455, michael.mcvoy@vcuhealth.org.

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Keywords

Cytomegalovirus; genome structure; DNA packaging; genome maturation; halogenated benzimidazole; antiviral resistance

1. Introduction

Human cytomegalovirus (HCMV) causes significant morbidity and mortality in HIV patients, recipients of stem cell or solid organ transplants, and congenitally infected newborns (Gandhi and Khanna, 2004). Drugs used to treat HCMV infections target the viral DNA polymerase. Prolonged therapy often results in resistance and limited therapeutic effectiveness and dose-limiting toxicities prevent their use for treating or preventing congenital infections (Biron, 2006). Thus, there is a pressing need for more potent, less toxic anti-HCMV therapeutics.

Herpesvirus DNA replication produces a replicative intermediate composed of genomes linked together in a concatemeric arrangement. A three-subunit viral-encoded terminase complex packages concatemeric viral DNA into capsids, then cleaves the DNA to produce monomeric viral genomes encapsidated within intranuclear capsids that subsequently mature into infectious virions. As these events are critical for completion of the herpesvirus life-cycle yet are of no importance to host cell, terminase is an attractive target for development of novel anti-herpesvirus therapeutics.

The halogenated benzimidazole 2-bromo-5,6-dichloro-1- β -D-ribofuranosyl-1*H*-benzimidazole (BDCRB) was the first compound shown to target herpesvirus DNA maturation (Krosky et al., 1998). Similar activities have since been reported for other halogenated benzimidazoles (Hwang et al., 2007; Krosky et al., 1998; Underwood et al., 2004) and the structurally unique small molecules BAY38-4766 (tomeglovir) (Buerger et al., 2001) and AIC246 (letermovir) (Lischka et al., 2010). Letermovir's performance in phase 3 testing (clinicaltrials.gov NCT02137772) and recent approval by the FDA for use following stem cell transplantation establishes terminase as valid antiviral target.

Resistance to all three classes of terminase inhibitors has been mapped to two subunits of the HCMV terminase, UL56 and UL89 (Chou, 2017a; Goldner et al., 2011; Krosky et al., 1998; Reefsclaeger et al., 2001; Underwood et al., 1998), while a recent report identified additional mutations conferring letermovir resistance in the third terminase subunit, UL51 (Chou, 2017b). That all three classes of terminase inhibitor may interact with the same functional domain of terminase has been proposed based on (i) significant overlap between the subdomains of UL56 and UL89 in which resistance mutations cluster, and (ii) the observation that certain mutations can confer resistance to all three inhibitor classes (Chou, 2017a).

As HCMV cannot replicate in non-human species, animal cytomegaloviruses have been used to investigate *in vivo* aspects of cytomegaloviral disease. In particular, guinea pig cytomegalovirus (GPCMV) can cross the placenta, and thus provides a small animal model of congenital infection (Schleiss, 2002). Prior work has shown that BDCRB is active against

GPCMV *in vitro* (Nixon and McVoy, 2004). To better understand how BDCRB interacts with and inhibits the GPCMV terminase, we generated and characterized a BDCRB-resistant GPCMV. Three genetic alterations were identified: (i) an L406P substitution in GP89, the GPCMV homolog of HCMV UL89; (ii) an 13.4-kb internal deletion that removed non-essential ORFs *GP131-gp143*; and (iii) a dramatic increase in the number of iterations of a 1-kb terminal repeat sequence from zero or one to as many as nine at either genomic terminus. Additional studies determined that the L406P substitution and the 13.4-kb deletion are unlikely to contribute to BDCRB-resistance. We hypothesize that BDCRB impairs the ability of terminase to recognize and correctly cleave concatemeric DNA after a genome has been packaged; therefore, because the terminal repeats contain the *cis*-acting signals that direct terminase where to cleave, the increased number of terminal repeats in the resistant virus may provide terminase with additional opportunities to recognize and cleave appropriately at one of these *cis*-acting signals, thereby mitigating the inhibitory effects of BDCRB.

2. Materials and Methods

2.1 Cell and viral culture

Guinea pig embryo fibroblasts (GEF) were prepared as previously described (McVoy et al., 1997). JH4 clone 1 guinea pig lung fibroblasts (GLF; ATCC CCL-158) and GPCMV strain 22122 (ATCC VR-682) were purchased from the American Type Culture Collection. GEF and GLF were cultured using Minimal Essential Medium supplemented with 10% fetal bovine serum, 50 U penicillin ml⁻¹, and 50 mg streptomycin ml⁻¹ (MEM). Viruses BVD and N2 are variants of GPCMV strain 22122 derived from bacterial artificial chromosome (BAC) clones and have either a 15-kb deletion in *HindIII* E (BVD) or an 18-kb deletion in *HindIII* D (N2) (Cui et al., 2008b). Titers of infectious virus were determined using a 96-well plate method (Cui et al., 2008a). BDCRB was a gift from John Drach and Leroy Townsend (University of Michigan) and was dissolved in DMSO at a stock concentration of 25 mM.

2.2 Southern hybridization

Cells were infected at an MOI of 3–5 and incubated for 4 days. Virion DNAs were extracted from culture supernatants as previously described (McVoy et al., 1997). Concatemeric DNAs were prepared by embedding infected cells in agarose plugs, subjecting the plugs to field-inversion gel electrophoresis to remove monomer DNA, and extracting the concatemeric DNA that remained in the plugs, as described previously (McVoy et al., 1997). Virion or concatemeric DNAs were restricted and the fragments were separated by agarose gel electrophoresis, transferred to Nytran nylon membranes (Schleicher & Schuell), and hybridized using isotopically labeled probes as previously described (McVoy et al., 1997). The following probe DNAs were used: R probe, a gel-purified *MluI/ClaI* fragment from pGP48 (McVoy et al., 1997); O probe, *HindIII*-digested pGP21 (McVoy et al., 1997); and E probe, *HindIII*-digested plasmid pHindIII E, which contains the entire GPCMV *HindIII* E fragment (Gao and Isom, 1984) (a gift from Mark Schleiss).

2.3 PCR and DNA sequencing

PCR products were amplified from extracellular virion DNAs using Easy-A DNA polymerase (Stratagene) and purified using QiaQuick or MinElute PCR purification kits (Qiagen), then either sequenced directly or cloned into plasmids using T/A cloning (Promega) or TOPO XL (Invitrogen) PCR cloning kits. Sanger chain termination sequencing was conducted by either the Biopolymer Laboratory at the University of Maryland at Baltimore or the Nucleic Acids Research Facility at Virginia Commonwealth University. The deletion and the *GP89* mutation were confirmed by direct sequencing of R-75 and wild type GPCMV virion DNA.

2.4 Recombinant virus construction

Two-step galactokinase (*galK*)-mediated recombineering in *E. coli* (Warming et al., 2005) was used to modify BAC clone N13R10r129, which contains the GPCMV strain 22122 genome (McVoy et al., 2016), to include an expression cassette for NanoLuc® luciferase. Sequences for all oligonucleotides used are given in Table 1. In the first step a *galK* cassette encoding galactokinase was inserted into N13R10r129 adjacent to the cre-excisable BAC origin of replication (Cui et al., 2008a). Synthetic oligonucleotides Lox-end-galk-FW and Lox-end-galk-RV were used to PCR-amplify the *galK* cassette in plasmid pGalK (Warming et al., 2005) with 50-bp flanking GPCMV targeting homologies and recombination into BAC N13R10r129 was accomplished using positive selection for *galK* as previously described (Warming et al., 2005). In the second step, negative selection (Warming et al., 2005) was used construct BAC N13R10r129-loxNanoLuc in which the *galK* cassette was replaced by a NanoLuc® expression cassette, which was PCR-amplified from plasmid pNL1.1.CMV[Nluc/CMV] (Promega) using synthetic oligonucleotides Lox-end-NanoLuc-FW and Lox-end-NanoLuc-RV.

Similar methods were used to insert the *galK* cassette into *GP89* in N13R10r129-loxNanoLuc following PCR amplification of pGalK DNA using oligonucleotides GP89-406-galk-FW GP89-406-galk-FW and GP89-406-galk-RV. Negative selection was then used to replace the *galK* insertion with *GP89* sequences that either restored a wild type *GP89* sequence or encoded a mutant *GP89* containing the L406P mutation. Two pairs of synthetic oligonucleotides containing 20-bp complementary 3' overlaps were designed. GP89-406(WT)-FWAN and GP89-406(WT)-RVAN contained wild type *GP89* sequences, while GP89-406(P)-FWAN and GP89-406(P)-RVAN contained mutant *GP89* sequences. The two primer pairs were PCR amplified without additional template DNA to generate 180-bp products containing wild type or mutant *GP89* sequences. BAC clones N13R10r129-NanoLuc_WT and N13R10r129-NanoLuc_exon89_L406P were derived by recombination with the respective 180-bp PCR products followed by negative selection against *galK*, as described above. *GP89* exon 2 sequences in N13R10r129-NanoLuc_exon89_L406P, N13R10r129-NanoLuc_WT, and parental BAC N13R10r129-loxNanoLuc were confirmed by Sanger sequencing of PCR products generated with primers GP89-406-Flank FW and GP89-406-Flank RV.

Infectious viruses were reconstituted by transfection of GLF cells with BAC DNA as described previously (Cui et al., 2008b). Viruses designated GPCMV-NanoLuc-WT and

GPCMV-NanoLuc-L406P were reconstituted from BACs N13R10r129-NanoLuc _WT or N13R10r129-NanoLuc _exon89_L406P, respectively. For reconstitution of viruses GPCMV-NanoLuc-WT, GPCMV-NanoLuc-L406P, BVD(cre), and N2(cre), the appropriate BAC DNAs were cotransfected with plasmid pCre to excise the BAC origin by Cre-mediated recombination. As this reaction is sometimes incomplete GFP-negative viruses (lacking the BAC origin) were isolated by limiting-dilution in 96-well plates (Cui et al., 2008a). Viruses designated N2 and BVD were reconstituted without pCre DNA and consequently retained the BAC origin. *GP89* exon 2 sequences in viruses GPCMV-NanoLuc-WT and GPCMV-NanoLuc-L406P were confirmed by PCR amplification of viral DNA followed by Sanger sequencing as described above.

2.5 Luciferase-based assays for antiviral activity and cytotoxicity

Clear 96-well plates containing confluent monolayers of GLF cells were infected with viruses GPCMV-NanoLuc-WT or GPCMV-NanoLuc-L406P at an MOI of 0.006. Eight threefold serial dilutions of BDCRB were prepared in MEM and added in triplicate to wells containing infected cells. Final concentrations ranged from 100 μ M to 15.2 nM. Uninfected wells and wells that were infected but contained no BDCRB were included as controls on each plate. After incubation for nine days, 50 μ L of culture supernatants from each well were transferred to wells of a black-wall/clear-bottom 96-well plate containing uninfected confluent GLF monolayers. After incubation for 48 h luciferase activities in each well were determined by removal of 100 μ L of culture medium from each well and addition of 100 μ L/well Nano-Glo® Luciferase Assay Reagent (Promega). After incubation for 10 min. at room temperature, relative luminosity units (RLU) were measured using a Biotek Synergy HT Multi-Mode Microplate Reader.

Cytotoxicity was measured in parallel using confluent uninfected GLF cultures in black-wall/clear-bottom 96-well plates incubated with medium containing matching final concentrations of BDCRB. After nine days 100 μ L of culture medium was removed from each well and replaced with 100 μ L/well CellTiter-Glo® reagent (Promega). After 10 min. of incubation RLU were measured using a Biotek Synergy HT Multi-Mode Microplate Reader.

Prism 5 (GraphPad Software) was used to determine best-fit four-parameter curves for RLU (means of triplicate data) vs. log (BDCRB concentration). Antiviral 50% effective concentrations (EC_{50}) were calculated from the inflection points of the four-parameter curves and reported in μ M $\pm$ one standard error as determined by Prism 5. For graphical presentation RLU were normalized to % maximum RLU for each data set.

3. Results

3.1. Generation of a BDCRB-resistant GPCMV

Previous work (Nixon and McVoy, 2004) demonstrated that BDCRB inhibits GPCMV with an EC_{50} of 4.7 μ M. To generate a BDCRB-resistant GPCMV, wild type strain 22122 GPCMV was serially propagated in the presence of increasing BDCRB concentrations until ultimately passing several times at a final concentration of 75 μ M BDCRB. When efficient

replication became evident the stock was subjected to several rounds of limiting-dilution cloning in 96-well plates in the absence of BDCRB to isolate a clonal virus designated R-75. Growth curves in the presence or absence of 50 μ M BDCRB (approximately 10-fold the EC_{50} of wild type GPCMV) confirmed BDCRB-resistance of virus R-75 (Fig. 1A). R-75 exhibited no significant impairment in the absence of BDCRB, reaching a peak titer on day seven similar to that of wild type, while in the presence of 50 μ M BDCRB peak titer was only reduced 5-fold. In contrast, BDCRB reduced the peak titer of wild type virus by over four logs (Fig. 1A).

3.2. An L406P amino acid substitution in GP89 does not confer significant resistance to BDCRB

The HCMV terminase is composed of three protein subunits, UL51, UL56, and UL89 (Borst et al., 2013; Neuber et al., 2017; Wang et al., 2012). Sequence homologs in GPCMV are GP51, GP56, and GP89, respectively (Schleiss et al., 2008). In HCMV, BDCRB-resistance mutations have been confirmed in genes encoding UL56 (Q204R) and UL89 (D344E, A355T) (Krosky et al., 1998; Underwood et al., 1998). To identify potential resistance mutations in R-75, the GPCMV genes encoding terminase subunits (*GP51*, *GP56*, and *GP89*) and the capsid portal protein (*GP104*) were sequenced from wild type virus and R-75. No mutations were identified in *GP51*, *GP56*, or *GP104*; however, R-75 had a single nucleotide change in *GP89* that conferred a predicted L406P substitution. While this mutation lies within the GPCMV homolog of HCMV UL89, in the linear amino acid sequence it is over 50 residues distant from D344E and A355T mutations that confer BDCRB resistance in HCMV (Krosky et al., 1998; Underwood et al., 1998).

To determine if the L406P mutation is responsible for BDCRB resistance, two recombinant GPCMV s were constructed. Both contain an expression cassette for NanoLuc® luciferase. GPCMV-NanoLuc-WT contains a wild type genome, while GPCMV-NanoLuc-L406P encodes the L406P substitution. A luciferase-based yield reduction assay determined the EC_{50} of BDCRB for GPCMV-NanoLuc-WT to be $2.90 \pm 1.13 \mu$ M, while that of GPCMV-NanoLuc-L406P was not significantly different at $4.95 \pm 1.27 \mu$ M (Fig. 1B). These EC_{50} measurements are similar to the EC_{50} of 4.7 μ M that was previously determined for wild type GPCMV using titer reduction (Nixon and McVoy, 2004). Cell viability remained greater than 70% at BDCRB concentrations as high as 100 μ M (Fig. 1B). These results indicate that the L406P mutation in GP89 is unlikely to contribute significantly to the BDCRB resistance of R-75.

3.3. The genome of R-75 has a large deletion in HindIII E

To determine if R-75 has significant genomic alterations, viral DNAs were purified and subjected to restriction analysis. Comparison of *HindIII*- and *XbaI*-restricted virion DNAs revealed an apparent lack of *HindIII* M, *HindIII* R, *XbaI* L, *XbaI* M, and *XbaI* N fragments in R-75 (Fig. 2A). *HindIII* R and *HindIII* M derive from the left and right termini of the GPCMV genome, respectively (Fig. 2B). *XbaI* N also derives from the left terminus (not shown), while *XbaI* L and *XbaI* M are located internally within the *HindIII* E region (Fig. 2C). Southern hybridization using a *HindIII* E probe (not shown) confirmed loss of *HindIII* E as well as *XbaI* L, M, j, and n fragments contained in *HindIII* E (see map, Fig. 2C) from

R-75 DNA and acquisition of a novel ~6-kb *HindIII* fragment. Additional hybridization experiments (not shown) using restriction enzymes that cleave frequently within *HindIII* E confirmed the above results and narrowed the region containing the apparent deletion. PCR reactions were then conducted on each side and progressively closer to the expected break point until reactions were observed that failed to amplify R-75 DNA. Primers 13F and 9R, from the two closest successful reactions (Fig. 2C) amplified a 2.0 kb PCR product from R-75 DNA but not wild type DNA (not shown) and were presumed to span the breakpoint of the deletion. Cloning and sequencing of this PCR product revealed the exact breakpoints of the deletion, which spans 13.4 kb of viral sequence (nucleotides 197,671 to 211,005 in accession KC503762.1). These results were confirmed using R-75 virion DNA directly as the template for sequencing reactions.

The deletion removes or disrupts annotated ORFs *GP131* to *gp143* (Fig. 2C). However, a previously described (Cui et al., 2008b) GPCMV mutant designated BVD contains a similar spontaneous deletion and lacks the same set of ORFs (Fig. 2C). That BVD has a similar deletion yet is inhibited by 50 μ M BDCRB to an extent similar to that of wild type GPCMV (not shown) suggests that the absence of these genes from R-75 is unlikely to contribute to BDCRB resistance of R-75.

3.4. The genome of R-75 has extensive reiterations of the terminal repeat

The restriction analyses described above suggested that restriction fragments from both ends of the R-75 genome were missing or greatly under-represented. Wild type GPCMV virions contain a roughly equal mixture of two genome types having either a single copy of a 1-kb terminal repeat at each end, or one copy at the left end and none at the right end (Gao and Isom, 1984). To specifically detect genomic termini, Southern hybridization probes were designed containing unique sequences proximal to the terminal repeats. Thus, as illustrated in Figs. 2D and 2E, when virion DNA is analyzed by Southern hybridization genomic termini from the left end of the genome can be detected using the R probe while those from the right end can be detected using the O probe.

At the left end of the genome wild type virion DNA exhibited the expected preponderance of R fragments containing one repeat but also present were lesser amounts of fragments larger by one or two kb (R^{+1} , R^{+2} ; Fig. 2D). As the terminal repeat is 1 kb in length we interpret these R^{+1} and R^{+2} fragments as reflecting low levels of termini having one or two additional repeats, as illustrated in Fig. 2D. R-75 DNA contained a reduced amount of R and a ladder of larger fragments increasing in size with a periodicity of approximately 1 kb (Fig. 2D). Upon overexposure fragments with up to eight additional repeats could be detected (not shown). At the right end wild type virion DNA contained the expected O and M fragments (Fig. 2E), reflecting the expected preponderance of genomes having zero or one repeat, respectively. In addition, lesser amounts of fragments larger by one or two kb (M^{+1} , M^{+2}) were also detected. R-75 virion DNA contained diminished levels of O and M fragments, while a ladder of larger fragments was detected, again having a size periodicity of approximately 1 kb (Fig. 2E). Similar results were obtained when virion DNAs were restricted with *HindIII* or *KpnI* (data not shown). From these results we conclude that while

some R-75 genomes retain normal R, M, and O termini, the majority of R-75 genomes have additional terminal repeats present in variable numbers.

Although approximately half of wild type GPCMV genomes have one repeat at each end, concatemeric junctions contain predominantly one copy of the terminal repeat, suggesting that repeats are duplicated during concatemer packaging (Nixon and McVoy, 2002). It was therefore of interest to determine whether R-75 concatemer junctions also contain a large number of repeats similar to the genomic termini or relatively small number of repeats, which would suggest that repeats at R-75 concatemer junctions undergo extensive duplication during concatemer packaging. Concatemeric DNA was restricted with *ClaI* and hybridized with the R probe. As R terminal fragments are not present on concatemer ends (McVoy et al., 2000), this probe should detect only junction fragments in concatemeric DNA. As previously observed (Nixon and McVoy, 2002), wild type concatemer DNA contained predominantly single repeat-containing OR junctions and a lower amount of double repeat-containing MR junctions, although trace amounts of MR^{+1} and MR^{+2} were present (Fig. 2F). In contrast, reduced levels of OR and MR junctions were detected in R-75 concatemers while a ladder of larger fragments were detected having a periodicity of approximately 1 kb (Fig. 2F). The absence of R, R^{+1} , and R^{+2} clearly demonstrates that concatemer DNA was not contaminated with genomic DNA; hence, the larger fragments that were detected must represent junctions rather than terminal fragments derived from contaminating genomes. These results indicate that the repeat reiterations on the termini of R-75 genomes are derived from cleavage of arrays of reiterated repeats at concatemer junctions. Therefore, although repeat duplication must play a role in the initial accumulation of the repeat arrays, once these arrays have been generated extensive duplications are not required during each cleavage event.

3.5 Additional terminal repeats do not arise spontaneously on under-length genomes

Given that herpesviruses have an optimal size range for efficient genome packaging (Bloss and Sugden, 1994), it is possible that the deletion in R-75 resulted in subsequent accumulation of terminal repeats in order to restore normal genome length. To test this hypothesis, GPCMV deletion mutants with genomes predicted to be under-length by 6 to 18 kb were evaluated by Southern hybridization after 35 serial passages. The resulting restriction patterns from genomic termini of viruses with under-length genomes were similar to wild type virus and not like R-75, in that R and M fragments predominated and only low levels of $M+1$ and trace amounts of $R+1$ fragments were present (Fig. 3). Therefore, an under-length genome, *per se*, does not give rise to accumulation of additional terminal repeats, and the extensive terminal repeats observed on R-75 genomes are unlikely to have resulted spontaneously during serial passage of the R-75 genome rendered under-length by the deletion in *HindIII* E.

4. Discussion

The halogenated benzimidazoles, including BDCRB, were the first compounds discovered that inhibit herpesvirus DNA maturation (Underwood et al., 1998). Despite their nucleosidic structure they do not inhibit DNA synthesis, but rather, affect the formation of monomeric

genomes from concatemeric replicative intermediate DNA (Krosky et al., 1998; Underwood et al., 1998). In the case of HCMV, the amount of monomeric DNA formed is reduced and a supergenomic HCMV DNA species named “monomer-plus” is formed at low levels (Underwood et al., 1998). We have hypothesized that monomer-plus is formed because BDCRB causes the HCMV terminase to skip the normal cleavage site and to continue to package DNA until a second cleavage site is encountered 30 kb further along the concatemer (McVoy and Nixon, 2005). In GPCMV the response to BDCRB is somewhat different. Monomer length genomes are abundantly produced but they are truncated slightly at the left end (Nixon and McVoy, 2004). Thus, in both viruses BDCRB modifies the way in which terminase recognizes and cleaves DNA, but in slightly different ways: the GPCMV terminase is rendered less selective and cleaves prematurely, whereas the HCMV terminase is rendered less efficient at recognizing its proper cleavage site and prone to cleavage site skipping. This suggests that HCMV and GPCMV terminases may differ in their interactions with BDCRB.

To gain additional insights into these interactions we derived a GPCMV that has acquired the ability to replicate efficiently in the presence of BDCRB. The resistant virus has three notable genetic changes: (i) a mutation resulting in a single amino acid change (L406P) in the GP89 terminase subunit, (ii) a large deletion in the *HindIII* E region, and (iii) a substantial increase in the number of terminal repeats present at both ends of the genome and at concatemer junctions.

In linear sequence the L406P mutation in GP89 is over 50 residues distant from the positions of confirmed resistance mutations (D344E, A355T) in UL89 (Krosky et al., 1998; Underwood et al., 1998). Moreover, when transferred to an otherwise BDCRB-sensitive genetic background the L406P mutation did not confer significant resistance to BDCRB. We cannot exclude the possibility that the L406P mutation serves a compensatory function, perhaps improving replication of the resistant virus by facilitating genomic cleavage at cleavage sites containing multiple repeats. The deletion in *HindIII* E is also unlikely to directly contribute to BDCRB resistance because a similar deletion in virus BVD did not result in resistance to BDCRB. This leaves the intriguing possibility that the third genetic feature -- the presence of multiple repeats at genomic termini and concatemer junctions -- might explain the BDCRB-resistance of virus R-75. In GPCMV, previous studies (Nixon and McVoy, 2004) showed that BDCRB promotes abnormal concatemer cleavages just prior to entry of the repeat. Shortening of the unique region of the genome (due to the deletion) might shift the point at which such aberrant cleavages occur to allow terminase to reach and encounter the repeat arrays. Multiple cleavage sites within these arrays could then give terminase additional opportunities to engage the *cis* elements that direct it where to cleave, hence mitigating the impairment to cleavage site recognition induced by BDCRB.

How R-75 acquired additional terminal repeats is not known. In wild type GPCMV concatemeric DNA the junctions between genomes predominantly contain one copy of the terminal repeat, yet about half of the genomes produced have two terminal repeat copies (one at each end), suggesting that certain cleavage events result in duplication of terminal repeats (Nixon and McVoy, 2002). However, that additional repeats do not normally accumulate suggests that extensive reiterations are not favored, perhaps due to a restriction

imposed by the packaging capacity of the capsid. This, in turn, might predict that viral genomes rendered under-length by internal deletions would gradually accumulate additional repeats in order to restore normal genome length. However, such accumulation of additional repeats was not observed following serial passage of GPCMV with genomes under-length by 6 to 18 kb. On the other hand, spontaneous deletions in *HindIII* E (very similar to the one in R-75, Fig. 2C) frequently occur when the viral genome is over-length, presumably to restore the genome to optimal length (Cui et al., 2008b). These observations support a model for generation of the R-75 genome in which terminal repeats accumulated first, possibly in response to BDCRB selection, and the resulting increase in genome length caused a compensatory deletion in *HindIII* E.

Additional studies are clearly needed to understand how herpesvirus terminal repeats are duplicated, what regulates their copy number, and how this may relate to resistance to BDCRB or other terminase inhibitors. This in turn will enhance our understanding of terminase function, a novel and important antiviral target.

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Highlights

- Herpesvirus DNA packaging is an important target for developing novel antiviral interventions
- The halogenated benzimidazole BDCRB is active against human cytomegalovirus and guinea pig cytomegalovirus
- In human cytomegalovirus, BDCRB resistance maps to two terminase subunits, UL56 and UL89
- BDCRB resistance in guinea pig cytomegalovirus is associated with an increase in the number of repeats at genomic termini
- This suggests a novel mechanism of resistance to the halogenated benzimidazole DNA packaging inhibitors

Fig. 1. BDCRB inhibition of GPCMV

(A) Cells were infected with wild type GPCMV (wt) or virus R-75 at an MOI of 0.01 in the presence or absence of 50 μ M BDCRB. The titers of the culture supernatants (pfu/ml) were determined on days 3, 5, and 7 post infection. (B) Cells were infected with viruses GPCMV-NanoLuc-L406P (carrying an L406P mutation in GP89) or GPCMV-NanoLuc-WT (wild type GP89) at an MOI of 0.006, then incubated for nine days in the presence of increasing concentrations of BDCRB. Culture supernatants were then transferred to fresh cell cultures and assayed for luciferase activity 48 hours later. RLUs were converted to percent maximum RLUs for each virus. Uninfected cells were incubated in parallel with the same concentrations of BDCRB and after eight days assayed for cell viability using CellTiter-Glo® (red).

Fig. 2. Restriction analysis of the R-75 genome

(A) Virion DNAs from wild type GPCMV (wt) and R-75 (75) were restricted with *Hind*III or *Xba*I, separated by agarose electrophoresis, stained with ethidium bromide, and visualized with UV light. Gray arrows indicate restriction fragments that are underrepresented or missing from R-75 DNA. The sizes of molecular weight markers (mk) are indicated. (B) Schematic illustrating the *Hind*III map of the GPCMV genome as derived by Gao and Isom (Gao and Isom, 1984). (C) The *Hind*III E region is expanded to indicate relevant *Xba*I fragments and their sizes. The locations of PCR primers 13F and 9R (lines) or sequences that are deleted from the genomes of viruses R-75 (dark grey box) or BVD (light grey box) are indicated. Black arrows represent open reading frames that are removed or disrupted by the deletions in R-75 and BVD. (D) Virion DNAs from wild type (wt) or R-75 (75) were restricted with *Cl*aI and separated by agarose electrophoresis, transferred to a nylon membrane, and hybridized with the left end-specific R probe. (E) Virion DNAs were digested with *Cl*aI and hybridized with the right end-specific O probe. (F) Concatemeric DNAs were digested with *Cl*aI and hybridized with the R probe. In panels D–F the positions of molecular weight markers are indicated; gray arrows connect hybridizing *Cl*aI fragments with schematics illustrating termini or concatemeric junctions. 1-kb terminal repeats are represented by gray boxes and the predicted sizes of *Cl*aI restriction fragments are indicated in parentheses. Fragments are designated with a superscript +*n*, where *n* = the number of additional repeats that they contain (for simplicity, fragments with more than three or four

repeats are not illustrated). Back bars indicate the sequences contained in the hybridization probes used.

Fig. 3. GPCMV with large spontaneous deletions do not accumulate reiterated terminal repeats
 The genomes of spontaneous deletion mutants BVD(cre) and N2(cre) are predicted to be under-length by 15 kb or 18 kb, respectively (Cui et al., 2008b), and by retaining the 9-kb BAC origin additional variants were generated with genomes predicted to be under-length by 9 kb (N2) or 6 kb (BVD). After 35 serial passages virion DNAs were analyzed by *HindIII* digestion and Southern hybridization as described in Fig. 2D. Wild type (wt) virion DNA was included as a control. The probe used (pGP48) contains terminal repeat sequences and is predicted to hybridize only to fragments containing one or more terminal repeat (Nixon and McVoy, 2004). The locations of DNA size markers (kb) are shown on the left.

Table 1

Oligonucleotides used for BAC construction and sequencing

purpose	name	Sequence (5' - 3' μ)
<i>galK</i> insertion to be replaced by NanoLuc cassette	Lox-end-galk-FW	<u>TTTCTTTCTCTCGGACTATTACTTCTTCTCTTTTCTTAACATCATCATCAGGACTCACTATAGGGCGGAATTGG</u>
	Lox-end-galk-RV	<u>CGGCCCGCCCTTAATTAATAACTTCTGTATAGCATATATACGGAAGTTATGCTATGACCATGATTACGCCAAGC</u>
replace <i>galK</i> with NanoLuc cassette	Lox-end-NanoLuc-FW	<u>TTTCTTTCTCTCGGACTATTACTTCTTCTCTTTTCTTCTAACATCATCATCTCAATATTGGCCATTAGGCCATATT</u>
	Lox-end-NanoLuc-RV	<u>CGGCCCGCCCTTAATTAATAACTTCTGTATAGCATATATACGGAAGTTATGCTATGACCATGATTACGCCAAGC</u>
<i>galK</i> insertion in <i>GP89</i>	GP89-406-galk-FW	<u>CCACGTTTCATCACCATCAGCTCGGAGGTGCGGAGGACCGCCAAACATGTTTACGACTCACTATAGGGCGGAATTGG</u>
	GP89-406-galk-RV	<u>TCCGAGATCTTGTGTGTGCCCCCCTATCATCTCGTCCATGAAGGAACCCGCGCTATGACCATGATTACGCCAAGC</u>
replace <i>galK</i> with <i>GP89</i> wild type	GP89-406(WT)-FWAN	<u>GGACGCCACGGCCCTGCCCGTGTACCGGCTGCACAAGCCCCACGTTTCATCACCATCAGTCTGGAGGTGCGGAGGACCGCCACATGTTTCTTCTGGCGGGTTCC</u>
	GP89-406(WT)-RVAN	<u>AACTCCTCGCGGCCGTCGTCGTGATCAGCACTGTCTCTCCGAGATCTTGTGTGCCCCCCTATGATCTCGTCCATGAAGGAACCCGCGGAAACATGT</u>
replace <i>galK</i> with <i>GP89</i> L406P	GP89-406(P)-FWAN	<u>GGACGCCACGGCCCTGCCCGTGTACCGGCTGCACAAGCCCCACGTTTCATCACCATCAGTCTGGAGGTGCGGAGGACCGCCACATGTTTCTCCCGCGGGTTCC</u>
	GP89-406(P)-RVAN	<u>AACTCCTCGCGGCCGTCGTCGTGATCAGCACTGTCTCTCCGAGATCTTGTGTGCCCCCCTATGATCTCGTCCATGAAGGAACCCGCGGAAACATGT</u>
sequencing <i>GP89</i> codon 406 region	GP89-406-Flank FW	TGCTTTCTGACCAGACTGAGCAAC
	GP89-406-Flank RV	GCTGATTTTGTACGTCCCCCACC

^a underline indicates homologies to pGalK, pNL1.1.CMV[Nluc(CMV)], or each other; lower case indicates mutations to encode L406P mutation