Antiviral Research 154 (2018) 44–50

Contents lists available at ScienceDirect

Antiviral Research

journal homepage: www.elsevier.com/locate/antiviral

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 Chiu[a], Stephanie Siegmund[c], Jian Ben Wang[a], Michael A. McVoy[a,*]

[a] Departments of Pediatrics, Medical College of Virginia Campus of Virginia Commonwealth University, 1101 E. Marshall Street, Richmond, VA 23298-0163, USA
[b] Departments of Medicine, Medical College of Virginia Campus of Virginia Commonwealth University, 1101 E. Marshall Street, Richmond, VA 23298-0163, USA
[c] Governor's School for Government and International Studies, Richmond, VA 23230, USA

ARTICLE INFO

Keywords:
Cytomegalovirus
Genome structure
DNA packaging
Genome maturation
Halogenated benzimidazole
Antiviral resistance

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 re-iterated 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.

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 cells, 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,

* Corresponding author. Department of Pediatrics, Medical College of Virginia campus of Virginia Commonwealth University, P.O. Box 980163, Richmond VA 23298-0163 USA.
E-mail address: michael.mcvoy@vcuhealth.org (M.A. McVoy).

https://doi.org/10.1016/j.antiviral.2018.04.006
Received 1 December 2017; Received in revised form 2 April 2018; Accepted 6 April 2018
Available online 09 April 2018
0166-3542/

2017a; Goldner et al., 2011; Krosky et al., 1998; Reefschlaeger 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) and 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 artifical chromosome (BAC) clones and have either a 15-kb deletion in *Hin*dIII E (BVD) or an 18-kb deletion in *Hin*dIII D (N2) (Cui et al., 2009). Titers of infectious virus were determined using a 96-well plate method (Cui et al., 2008). 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, *Hin*dIII-digested pGP21 (McVoy et al., 1997); and E probe, *Hin*dIII-digested plasmid pHindIII E, which contains the entire GPCMV *Hin*dIII 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., 2008). 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., 2009). 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., 2008). Viruses designated N2 and BVD were reconstituted without pCre DNA