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Cidofovir Diphosphate Inhibits Molluscum Contagiosum Virus DNA Polymerase Activity

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TO THE EDITOR

Molluscum contagiosum virus (MCV) produces smooth, flesh-colored papules with central umbilication. In HIV-infected individuals, extensive and recalcitrant MCV lesions are a therapeutic challenge. Meadows *et al.* (1997) first described therapeutic success with topical or intravenous cidofovir (CDV), a nucleoside analog of deoxycytidine monophosphate, for otherwise recalcitrant MCV lesions in three AIDS patients. Topical 1–3% CDV cream has been highly efficacious in treating generalized MCV lesions, with successful treatment of a boy with Wiskott–Aldrich syndrome (Davies *et al.*, 1999), two HIV-infected children (Toro *et al.*, 2000), and two otherwise healthy children (Zabawski and Cockerell, 1999).

Because MCV cannot be propagated in tissue culture and does not infect animals, overexpression of MCV DNA polymerase is necessary to evaluate the effects of CDV on viral DNA polymerase activity. However, identifying a successful overexpression system for this enzyme has been difficult. Dorsky and Crumpacker (1988) reported high expression of herpes simplex virus-1 (HSV-1) DNA polymerase in *E. coli*, but the recombinant protein was insoluble and enzymatically inactive. Haffey *et al.* (1988) successfully expressed HSV-1 DNA polymerase in the yeast *Saccharomyces cerevisiae*, but the extracts also contained the yeast α DNA polymerase. McDonald and Traktman (1994) found that vaccinia virus DNA polymerase cannot be expressed in *E. coli* due to extreme toxicity and proteolysis or in *S. cerevisiae* due to transcriptional termination within the polymerase gene. To overcome these technical issues, we selected an *in vitro* transcription–translation system to express viral DNA polymerases, similar to the successful expression systems for HSV-1 DNA polymerase (Dorsky and Crumpacker, 1988) and equine herpesvirus 1 (Loregian *et al.*, 2006).

We cloned the viral DNA polymerase genes of MCV (*MC39L*), cowpox virus (CPV) (*CPXV75*), and HSV-1 (*UL30*) into the T7 expression vector pGEM-3Z (Promega, Madison, WI). Of these, *MC39L* and *CPXV75* are the most closely related, with 53% identity and 72% similarity (Figure 1a). For *MC39L*, a 3,159-bp *Acc*III–*Bst*PI fragment spanning the complete *MC39L* from 12-bp upstream of the ATG to 133-bp downstream of the TAG was inserted into pGEM-3Z. For *CPXV75*, a PCR product from 6-bp upstream of the ATG to 381 bp 5′ of *CPXV75* was generated using primers 5′-GGTACCTAGAAATG GATGTTCGGTGC-3′ (*Kpn*I site, underlined) and 5′-TCGTCCAACGAGTAACA TCC-3′, shortened to 362 bp by *Kpn*I–*Eco*RV digestion, and cloned into the *Kpn*I–*Bam*HI sites of pGEM-3Z along with a 4,591-bp *Eco*RV–*Bam*HI fragment containing 2,664 bp 3′ of *CPXV75*. For *UL30*, 524 bp 5′ of *UL30* was amplified with primers 5′-GAATTCATGTTTTCCG GTGGCGGCGG-3′ (*Eco*RI site, underlined) and 5′-ATGGCGTCCATAAACCG CGC-3′, shortened to 482 bp by *Eco*RI–*Sph*I digestion, and inserted into the *Eco*RI–*Sph*I site of pGEM-3Z along with a 3,760-bp *Sph*I fragment containing 3,227 bp 3′ of *UL-30*.

The cloned DNA polymerase genes were transcribed with T7 RNA polymerase and translated in rabbit reticulocyte lysates (Promega). Polymerases synthesized in the presence of [^{35}S]methionine were visualized by electrophoresis (Figure 1b). DNA polymerase assays were performed in 100 μl volumes containing 10 μg of activated calf thymus DNA, 100 mM ammonium sulfate, 50 mM Tris–hydrochloride (pH 8.0), 50 μg of BSA, 0.5 mM dithiothreitol, 7.5 mM $MgCl_2$, 5 μM each of dCTP, dGTP, and dTTP, and 2.5 μCi of [^{32}P] dATP (3,000 Ci mM^{-1}) as described (Dorsky and Crumpacker, 1988). The incorporation of [^{32}P]dATP by the programmed reticulocyte lysates increased linearly with time up to 20 minutes (Figure 2). In contrast, the endogenous polymerase activity of the control reticulocyte lysates without mRNA remained near the filter background level (data not shown).

Once taken up by cells, CDV is converted to a diphosphate (CDVpp) and acts as a competitive inhibitor of viral DNA polymerases. The effects of CDVpp (Trilink Biotechnologies, San Diego, CA) on viral DNA polymerases were examined using 30 μl of reticulocyte lysates programmed with 8 μg of transcripts for each viral DNA polymerase gene. MCV, CPV, and

Abbreviations: CDV, cidofovir; CDVpp, cidofovir diphosphate; CPV, cowpox virus; HSV, herpes simplex virus; MCV, molluscum contagiosum virus

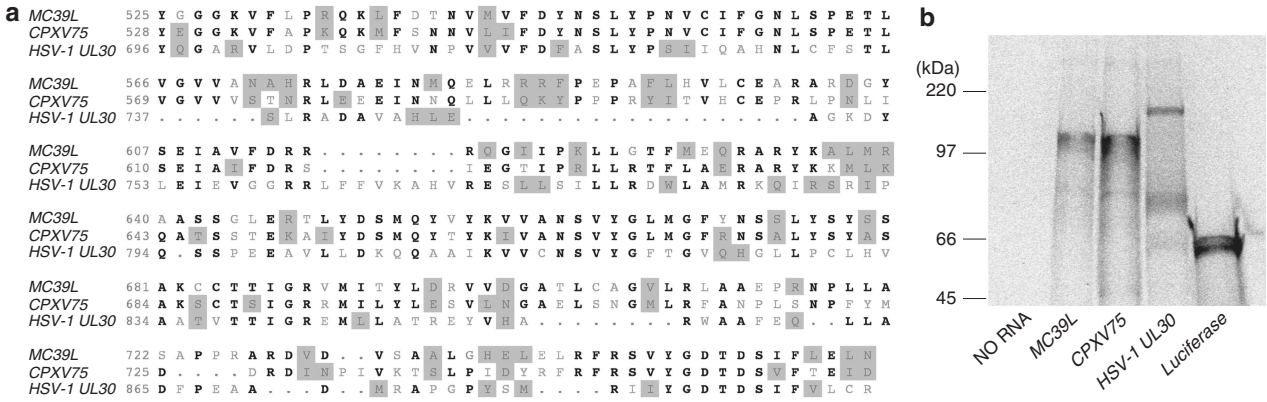

Figure 1. Primary structure comparisons and expression of viral DNA polymerase genes. (**a**) Alignment of the deduced amino-acid sequences of *MC39L* (GenBank accession number U60315), *CPXV75* (DQ066528), and HSV-1 *UL30* (AB231455). Boldface letters indicate identical amino acids. Gray boxes highlight identical and conserved amino acids. Dots indicate gaps in the sequence to allow optimal alignment. Numbers on the right indicate the amino-acid position. (**b**) Rabbit reticulocyte lysates were programmed with mRNA in the presence of [^{35}S]methionine and then visualized by SDS-PAGE. The apparent molecular weights of the three viral DNA polymerases were 116 kDa (MCV and CPV) and 140 kDa (HSV-1), consistent with the predicted sizes and corresponding to the sizes of expressed or purified polypeptides in previous studies (Dorsky and Crumpacker, 1988; Haffey *et al.*, 1988; McDonald and Traktman, 1994). The luciferase gene was expressed as a positive control. The positions and masses of protein markers are on the left.

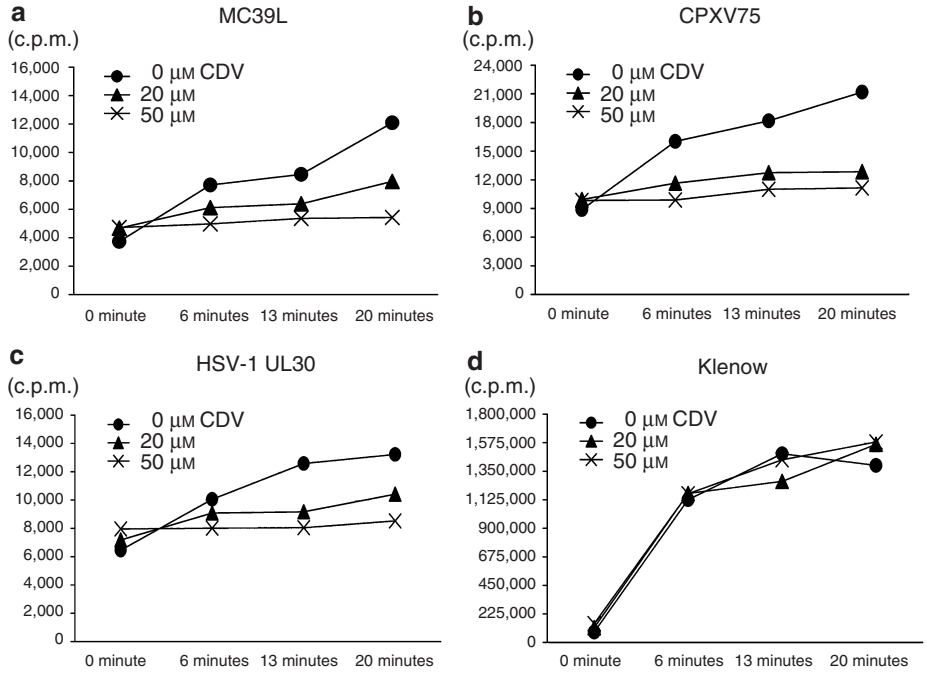

Figure 2. CDVpp inhibits *in vitro* translated DNA polymerase. DNA polymerase inhibition assays were performed as described in the text at the noted CDVpp concentrations. One representative assay out of three is shown for each viral DNA polymerase. The rabbit reticulocyte lysates were programmed with (**a**) MC39L, (**b**) CPXV75, and (**c**) HSV-1 UL30 mRNA and used for DNA polymerase assays. (**d**) For *E. coli* DNA polymerase assay, Klenow fragment (Takara Bio, Shiga, Japan) was used instead of programmed lysates.

HSV-1 DNA polymerase activities were completely inhibited by 50 μM CDVpp (Figure 2a–c). These observations are consistent with the plaque reduction assay results, showing that the minimum inhibitory concentration required to inhibit virus-induced cytopathogenicity by 50% (IC_{50}) is 31–62 and 12.7–31.7 μM for CPV and HSV-1, respectively (Safrin *et al.*, 1997). Since the maximum plasma concentration (C_{max}) of CDV is 26–72 μM (Hitchcock *et al.*, 1996), intravenous administration of CDV would inhibit these viruses. In contrast, CDVpp did not inhibit *E. coli* DNA polymerase activity elicited by Klenow fragment (Figure 2d), suggesting that CDV derives its specificity from a higher affinity for viral DNA polymerases than for bacterial or human DNA polymerases in cell culture (Safrin *et al.*, 1997).

Unlike acyclovir and other nucleoside analogs, CDV is not dependent on

phosphorylation by a virally encoded thymidine kinase to exert its antiviral effect. This mechanism of action would be advantageous as a therapy for MCV, which does not possess a functional thymidine kinase (Gubser *et al.*, 2004). In fact, acyclovir and foscarnet are ineffective against CPV because the virally encoded thymidine kinase does not catalyze acyclovir or foscarnet (Baker *et al.*, 2003). In contrast, several studies have unequivocally shown that CDV is a highly effective prophylactic and therapeutic drug for lethal CPV infection in mice (Neyts and De Clercq, 2003).

There is increasing interest in effective antiviral agents because of several concerns: increasing risk of bioterrorism with variola virus as a biologic weapon, recent outbreak of monkeypox disease in humans, occasional orf (sheep pox) infections, and smallpox vaccination complications, such as vaccinia gangrenosa and eczema vaccinatum (Neyts and De Clercq, 2003). CDV is one of the most promising antiviral agents with high efficacy against the poxvirus family. In a case of orf, the ecthyma infectiosum lesion completely disappeared following topical application of 1% CDV that otherwise would have led to amputation of the affected finger (Geerinck *et al.*, 2001). In another study, antiviral treatment with CDV was more effective than smallpox vaccination after lethal infection of monkeys with monkeypox (Stittelaar *et al.*, 2006). We showed for the first time that 20–50 μM of CDVpp inhibited MCV DNA polymerase activity, providing biochemical support for CDV as a treatment for MCV lesions.

CONFLICT OF INTEREST

The authors state no conflict of interest.

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See related commentary on pg 1067

IL-4 Suppresses the Recovery of Cutaneous Permeability Barrier Functions *In Vivo*

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TO THE EDITOR

Limited information has been reported concerning the effects of immune responses, especially Th2 type, on barrier dysfunction *in vivo*. A recent study reported that IL-4, a Th2 cytokine, suppresses the enhancement of ceramide synthesis and cutaneous permeability barrier function induced by tumor necrosis factor-α and IFN-γ in human epidermal sheets or in the living skin equivalent, which is a model of reconstructed skin *in vitro*. In addition, IL-4 blocks the recovery of barrier function and enhancement of ceramide synthesis after barrier disruption by acetone treatment in living skin equivalent (Hatano *et al.*, 2005, 2007). However, living skin equivalent seems inadequate to observe cutaneous permeability barrier function, since the