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Biochemical Basis for Increased Susceptibility to Cidofovir of Herpes Simplex Viruses with Altered or Deficient Thymidine Kinase Activity

DIRK B. MENDEL,* DAVID B. BARKHIMER, AND MING S. CHEN

Department of Biochemistry and Virology, Gilead Sciences, Foster City, California 94404

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It has been observed that herpes simplex virus mutants with deficient or altered thymidine kinase activity are more susceptible to Cidofovir {CDV; 1-[(*S*)-3-hydroxy-2-(phosphonomethoxy)propyl]cytosine dihydrate} in tissue culture than are the parental strains. During infection of cells, the elevation of the dCTP pool by thymidine kinase mutant viruses is less than that induced by the wild-type virus. The competition between CDV diphosphate and dCTP at the viral polymerase is therefore changed in favor of CDV diphosphate, enhancing its activity.

Cidofovir (CDV) is an acyclic phosphonate analog of CMP (or dCMP) with potent in vitro and in vivo activity against a broad spectrum of herpesviruses (3, 4, 12). The acyclic nucleoside phosphonates differ from most other nucleoside analogs in that they do not contain a sugar ring and, because of the phosphonate group, they need undergo only two additional phosphorylations in order to be metabolically equivalent to the nucleoside triphosphates. CDV is phosphorylated by cellular enzymes (8), and therefore, its metabolism is not affected by herpes simplex virus (HSV) infection (15). As a result, CDV is active against acyclovir (ACV)-resistant HSV in vitro and in animal models (1, 20). More recently, CDV has been used successfully to treat ACV-resistant HSV in patients (19, 24).

In a recent case study (19), viral isolates were obtained from a single ACV-resistant perirectal HSV lesion in a patient with AIDS prior to and during intravenous administration of CDV. The initial isolate contained virus which was approximately 10 times as susceptible to CDV as the virus isolated from the same lesion during CDV therapy. However, the susceptibility to CDV of the latter isolate was similar to that observed for wild-type laboratory strains of HSV, raising the possibility that the virus from the initial isolate was hypersusceptible to CDV. The initial isolate was also resistant to ganciclovir (our unpublished results), suggesting that the virus contained a mutation within the thymidine kinase (TK) gene.

ACV resistance in patients due to TK-altered or TK-deficient viruses has been reported (11, 14, 18). ACV-resistant HSV type 2 (HSV-2) in AIDS patients has also been reported (14). A review of the literature revealed anecdotal evidence suggesting that other ACV-resistant HSV clinical isolates might be hypersusceptible to CDV (12, 20). However, no study had been performed to critically evaluate this question. Furthermore, an increased susceptibility to CDV for the mutant viruses was not anticipated since CDV metabolism is not affected by HSV infection (15). In this report, families of well-characterized, plaque-purified viruses were studied to determine if acyclovir-resistant HSV strains with altered or deficient TK activity have an increased susceptibility to CDV. In addition, the biochemical basis for this increase in the susceptibility to CDV of the mutant viruses was determined.

Cells and viruses. MRC-5 human embryonic lung fibroblast cells (American Type Culture Collection, Rockville, Md.) and MA-104 rhesus monkey kidney epithelial cells (BioWhittaker, Walkersville, Md.) were grown at 37°C in a humidified atmosphere containing 5% CO_2. Growth medium consisted of minimum essential medium containing Earle's salts and supplemented with 10% fetal bovine serum. Three families of plaque-purified HSV were obtained from Jack Hill (Wellcome Research Laboratories, Burroughs Wellcome, Research Triangle Park, N.C.). SC16 (TK-positive parent) and SC16-S1 (TK-altered mutant) have been described previously (10). SC16-TkDM21 (TK-deficient mutant) is derived from SC16 and has been genetically altered to delete the TK gene. BW-S (TK-positive parent) and BW-R (TK-deficient mutant) have been described previously (23). 8702 (TK-positive parent), 8708 (TK-altered mutant), and 8713 (TK-deficient mutant) have been described previously (13). Clinical isolates (19) were provided by Larry Drew (Mt. Zion Medical Center, University of California, San Francisco, San Francisco). HSV-1 and HSV-2 reference strains, H^{mc} and MS, respectively, were from the American Type Culture Collection.

Antiviral assays. Two different assays were used to assess the antiviral activities of CDV and ACV. Cell killing assays were performed as previously described (2) in 96-well microtiter plates containing confluent monolayers of MRC-5 cells (3×10^4 to 4×10^4 cells per well). CDV or ACV was added to the cells just prior to the addition of virus (100 PFU per well). The infection was allowed to continue until approximately 90% of the cells in the untreated wells were killed (3 to 4 days). The relative number of viable cells in each well was determined at the end of the assay by using XTT (21). Standard plaque reduction assays were performed in six-well tissue culture plates containing confluent monolayers of MRC-5 cells or MA-104 cells. CDV or ACV, at various concentrations, was added to the cells the night prior to infection. At the start of the experiment, cells were infected with virus (100 PFU per well) in growth medium. After 1 h the virus inoculum was removed and replaced with medium containing CDV or ACV at the desired concentration and 0.1% Sandoglobulin (human immune globulin; Sandoz). After 2 or 3 days, the cell monolayer was stained with 0.1% crystal violet in 20% methanol to visualize the plaques.

* Corresponding author. Mailing address: Department of Biochemistry and Virology, Gilead Sciences, 353 Lakeside Dr., Foster City, CA 94404. Phone: (415) 573-4839. Fax: (415) 573-4890.

TABLE 1. Susceptibilities of virus strains to CDV and ACV in MRC-5 cells[a]

Virus (genotype)	ED_{50} (μM)[b]	
	CDV	ACV
HSV-1		
H^{mc} (wild type)	6.5 ±2.5	4.5 ± 1.5
SC16 (TK positive)	9.3 ± 1.8	5.2 ± 3.8
SC16-S1 (TK altered)	1.4 ± 0.6	87.5 ± 12.5
SC16-TkDM21 (TK deleted)	0.6 ± 0.3	500.0 ± 100.0
BW-S (TK positive)	8.5 ± 1.0	4.5 ± 0.5
BW-R (TK deficient)	1.1 ± 0.9	77.5 ± 2.5
HSV-2		
MS (wild type)	9.1 ± 1.8	3.8 ± 0.5
8702 (TK positive)	8.3 ± 0.3	5.3 ± 0.4
8708 (TK altered)	0.4 ± 0.1	86.7 ± 23.9
8713 (TK deficient)	2.2 ± 0.7	126.7 ± 23.5
Clinical isolates		
Initial	0.4 ± 0.1	525.0 ± 275.0
Final	8.0 ± 1.5	81.0 ± 4.0

[a] MRC-5 cells were infected with the indicated virus and the relative number of viable cells in each well was determined by using XTT.

[b] Data are the means ± standard deviations from at least two independent experiments.

Measurement of dNTP pool sizes. Deoxynucleoside triphosphate (dNTP) pool sizes were measured by a minor modification of the procedure of Sherman and Fyfe (22). Briefly, confluent monolayers of MA-104 cells in 75-cm^2 tissue culture flasks (approximately 4×10^6 cells per flask) were infected with the different viruses at 3 to 5 PFU per cell. After 1 h the virus inoculum was removed and the cells were refed with 10 ml of medium. Unless otherwise indicated, cells were harvested by trypsinization 5 h later, counted with a hemocytometer, and extracted with 60% methanol as previously described (2, 6). The methanol-soluble fraction was dried, resuspended in TE buffer (10 mM Tris [pH 7.5], 1 mM EDTA) at 2×10^6 cells per ml, and combined with an equal volume of ice-cold 0.8 N perchloric acid. Samples were neutralized and each of the dNTPs was quantitated as described previously (22) by using the Klenow fragment of DNA polymerase I (2 U/100 μl; Worthington Biochemical, Freehold, N.J.). Samples were taken at 40, 60, and 80 min to ensure that the reactions had reached completion.

The concentrations of CDV and ACV necessary to reduce by 50% the amount of cell death in MRC-5 cells infected with each of the indicated viruses (ED_{50}s) are shown in Table 1. These results confirm, as reported by Lalezari et al. (19), that the ACV-resistant clinical isolate taken prior to intravenous CDV treatment of an AIDS patient (initial) is more susceptible to CDV than the isolate taken after CDV treatment (final). The susceptibility of the initial clinical sample to ACV (ED_{50} = 525 μM, compared with <5 μM for both H^{mc} and MS) confirms that this sample contains virus which is resistant to ACV. The ED_{50} of ACV for the final clinical sample was still elevated (81 μM); however, these viruses have not been plaque purified and therefore may reflect a mixed population of susceptible and resistant viruses.

The data from Table 1 also indicate that for each of the three families of related viruses the mutant viruses with altered or deficient TK activity have lower ED_{50}s for CDV than do the wild-type (TK-positive) viruses from which they were derived. The mutant viruses exhibit as much as a 20-fold decrease in their ED_{50}s for CDV, in agreement with results obtained with the clinical isolates. These data were confirmed in a plaque reduction assay using MRC-5 (fibroblastoid) and MA-104 (epithelial) cells, indicating that the increased susceptibility to CDV of the ACV-resistant HSV strain is not dependent on the type of host cell used.

There is less of a difference between the relative susceptibilities to CDV of the parental and mutant viruses in the plaque reduction assay than in the cell killing assay, probably reflecting a difference in the assay systems. It is likely that the cell killing assay amplifies the differences observed in the plaque reduction assay. This interpretation is consistent with the higher ED_{50}s observed for CDV in the cell killing assay than in the plaque reduction assay.

The TK encoded by HSV-1 and -2 viruses, unlike the cellular enzyme, is a nonspecific nucleoside kinase which phosphorylates both deoxycytidine and thymidine (16, 17) as well as ACV and ganciclovir. HSV TK also phosphorylates thymidylate (5). HeLa cells have shown an increase in dCTP pool size after infection with Rolly strain 11 of HSV (7). We therefore tested the effect of HSV wild-type and mutant virus infection on the intracellular concentration of the dNTPs. Following infection with the wild-type virus there is a roughly sevenfold increase in the dTTP pool size during the first 5 h after infection, with no further change over the period studied (Fig. 1). During the same period the dCTP pool size increases four- to fivefold relative to that in uninfected cells. The increases in dTTP and dCTP pools follow a similar time course, which would be expected if both are due to the activity of the viral TK. The dGTP pool size also increases, but only two- to threefold. There is no apparent change in the concentration of dATP.

We next investigated whether the dCTP pool size differs in cells infected with wild-type virus or with virus containing altered or deficient TK. The results shown in Fig. 2 demonstrate that cells infected with the wild-type virus (SC16) contain roughly two to three times the amount of dCTP found in cells infected with virus containing a TK with altered specificity (SC16-S1) or with virus deficient in TK activity (SC16-TkDM21). Cells infected with the mutant viruses do have more dCTP than uninfected cells (150 pmol/10^6 cells, compared with 70 pmol/10^6 cells for uninfected cells [Fig. 1]). The elevated dCTP level following infection with the mutant viruses is prob-

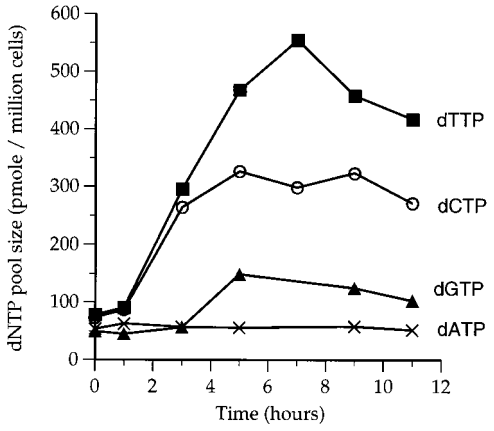

FIG. 1. Time course of changes in intracellular dNTP pool sizes following HSV infection of MA-104 cells. Confluent monolayers of MA-104 cells were infected with the wild-type SC16 virus (3 to 5 PFU per cell). At the indicated times, cells were harvested from a single 75-cm^2 flask and the dNTP pool sizes were determined. The 0-h values were obtained from cells which were not infected. The 1-h values were obtained at the end of the period during which the cells were exposed to virus.

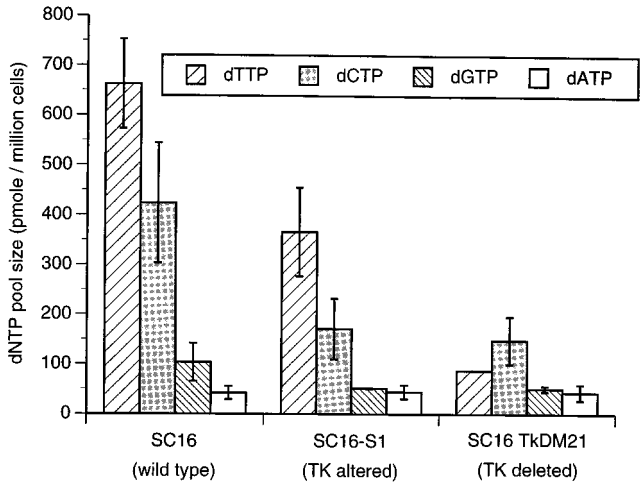

FIG. 2. Intracellular dNTP pool sizes of MA-104 cells infected with wild-type or acyclovir-resistant HSV. Confluent monolayers of MA-104 cells were exposed to the indicated virus (3 to 5 PFU per cell) for 1 h. Cells were harvested 5 h after the end of the exposure to virus, and the pool sizes for each of the dNTPs were determined. Results are presented as the averages (error bars, standard deviations) for two separate experiments.

ably due to the ability of the viral ribonucleotide reductase, which should be functional in the wild-type and mutant viruses, to reduce CDP to dCDP (9).

The results of this study demonstrate that ACV-resistant HSV strains with altered or deficient TK activity are more susceptible to CDV than are wild-type HSV strains. The difference in susceptibility to CDV of wild-type and mutant viruses is not due to viral effects on CDV metabolism, as is the case for ACV. Rather, the difference may be due to mutant viruses inducing a smaller increase than wild-type virus in the intracellular concentration of dCTP in infected cells. CDV diphosphate is a competitive inhibitor with respect to dCTP for HSV DNA polymerase (15). Thus, the lower concentration of dCTP in cells infected with the mutant viruses than in cells infected with wild-type HSV enhances the inhibitory effect of CDV diphosphate against HSV DNA polymerase.

CDV is a member of a new class of therapeutic agents which does not require viral gene products for activation. CDV has been shown to be effective against ACV-resistant HSV (1, 20, 24). CDV has also been shown to have a prolonged antiviral effect, enabling it to be administered infrequently (20, 25). An additional advantage of CDV as a therapeutic agent for ACV-resistant HSV is that HSV strains with altered or deleted TK activity, which make up the vast majority of ACV-resistant HSV strains, have an increased susceptibility to CDV. The ability to use CDV topically for localized lesions, or as an infrequent intravenous administration for disseminated disease, combined with the increased susceptibility of TK-deficient HSV mutants to this agent, makes CDV an attractive potential therapy for the treatment of infections caused by wild-type and ACV-resistant HSV strains.

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