

Ether Lipid Ester Derivatives of Cidofovir Inhibit Polyomavirus BK Replication In Vitro

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Polyomavirus BK is a significant pathogen in transplant recipients, but no effective antiviral therapy is available. We show that cidofovir can inhibit BK virus replication in vitro. Esterification of cidofovir with hexadecyloxypropyl, octadecyloxyethyl, and oleyloxyethyl groups results in up to a 3-log lowering of the 50% effective concentration and an increased selectivity index.

The polyomavirus BK virus (BKV) is associated with hemorrhagic cystitis following bone marrow transplantation. More recently, BK virus nephropathy has emerged as an infectious complication that is seen in 1 to 10% of kidney transplant recipients (11). Currently, there is no satisfactory treatment available for clinical management of BKV infections. It is customary to reduce immunosuppression, but this strategy is only effective if the diagnosis can be made early, before significant tissue damage has occurred.

Based on in vitro studies (2), leflunomide and cidofovir have been empirically used in the treatment of viral nephropathy (20), but the antiviral effect of these drugs is modest (9). This study demonstrates that the addition of ether lipid ester side chains to the cidofovir molecule results in a substantial increase in anti-BKV effect, analogous to previously reported effects on cytomegalovirus, cowpox virus, and vaccinia virus (16).

Testing for anti-BKV activity of drugs in vitro has been difficult because this virus grows very slowly in culture. Thus, a well-developed cytopathic effect occurs only in low-passage embryonic kidney cells after incubation periods of 4 to 6 weeks (18). To overcome this problem, we have previously published a 7-day quantitative PCR assay in which antiviral testing is done using the BK virus, Gardner strain (ATCC VR837), grown in WI-38 cells (ATCC CCL-75) (5). The virus was expanded in 12-well culture plates by seeding 2×10^6 BKV particles for 10^5 WI-38 cells. Cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum and L-glutamine, incubated at 37°C under 5% CO₂, and harvested on day 7 using 0.25% trypsin–1 mM sodium EDTA digestion at 37°C for 10 to 20 min.

DNA extraction of the cell lysates was performed with a commercially available kit (QIAamp DNA minikit; QIAGEN, Valencia, CA). BKV capsid protein VP-1 DNA was amplified by a TaqMan quantitative PCR performed in an ABI Prism 7700 sequence detector (ABI, Foster City, CA) (17). Analysis of dose-response curves allowed determination of the 50% effective concentration (EC₅₀). Simultaneous quantitation of the housekeeping gene aspartoacylase (ACY) DNA permitted monitoring of host cell rep-

lication. The 50% cytotoxic concentration (CC₅₀) was determined both by the standard neutral red assay and calculated by our PCR assay as the drug concentration that reduced the yield of cellular ACY DNA by 50%.

We used this assay to measure the antiviral activity of cidofovir ([S]-9-(3-hydroxy-2-phosphonylmethoxypropyl)cytosine; Vistide) (Gilead Sciences, Foster City, CA), and its esters octadecyloxyethyl (ODE)-cidofovir, oleyloxyethyl (OLE)-cidofovir, and hexadecyloxypropyl (HDP)-cidofovir. The chemical structures and methods of synthesis of these compounds have been published previously (4, 16, 21).

The CC₅₀, EC₅₀, and selectivity index values for these drugs are shown in Table 1 (means \pm standard errors of three or more experiments). It can be observed that the esterification of cidofovir with ether lipid side chains causes an up to 3-log reduction in the EC₅₀ of cidofovir. The CC₅₀s measured by the neutral red and PCR assays were essentially similar for cidofovir and HDP-cidofovir. However, for OLE-cidofovir and ODE-cidofovir the PCR assay generated a value 1 log lower than that of the neutral red assay. Lowering of CC₅₀ by the ether lipid esters of cidofovir was not of the same order of magnitude as the reduction of EC₅₀. This resulted in an up to 120-fold increase in the selectivity index of these compounds. The multiplicity of infection in all experiments summarized in Table 1 was 20:1. This corresponds to our clinical observation that BKV load in kidney transplant recipients with viruria is 28.8 ± 12.8 viral copies per cell (17).

In latent infection in tissue the viral load is 3.4 ± 1.8 copies per cell. Hence, we also performed two experiments with HDP-cidofovir at an MOI of 2:1, and obtained an EC₅₀ of 0.12 ± 0.04 μ M, which is essentially the same as that observed at the higher multiplicity of infection (0.13 ± 0.03). It has been reported previously that quantitative PCR drug sensitivity assays are not very sensitive to changes in multiplicity of infection (6, 14).

As pointed out earlier, the CC₅₀ and selectivity index for ODE-cidofovir and OLE-cidofovir are significantly lower in the PCR assay than in the neutral red assay. The exact explanation is not clear. However, it should be recalled that the PCR method relies on the inhibition of cellular replication by the drug of interest (Fig. 1). In contrast, the neutral red assay is a cellular toxicity assay dependent on uptake of the drug and subsequent accumulation in lysosomes of healthy cells. Dis-

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TABLE 1. Effect of cidofovir analogs on BKV replication in vitro^a

Drug	Concn range tested (μM)	Mean CC_{50} (μM) \pm SE		Mean EC_{50} (μM) \pm SE	Mean SI	
		N	P		N	P
Cidofovir	10–500	299.9 \pm 12.3	202.6 \pm 54.5	115.1 \pm 37.1	2.6	1.8
ODE-cidofovir	0.001–10.0	8.7 \pm 1.7	0.8 \pm 0.2	0.083 \pm 0.02	104.8	9.6
OLE-cidofovir	0.001–10.0	10.5 \pm 3.8	0.7 \pm 0.3	0.23 \pm 0.13	45.6	3.0
HDP-cidofovir	0.001–10.0	15.6 \pm 1.9	14.7 \pm 3.0	0.13 \pm 0.03	120.0	113.0

^a All concentrations are in μM and are presented as means of three experiments. CC_{50} and selectivity index (SI) have been calculated both by the neutral red assay (N) and by the PCR assay (P).

crepancies between CC_{50} values calculated by neutral red and other methods of measuring cytotoxicity have been noted before. Thus, in their work with herpes simplex virus, Kern et al. (13) describe methylenecyclopropane analogs of nucleosides which have essentially no toxicity in neutral red assays but moderate toxicity in cell proliferation assays. Similar observations have been made during evaluation of anti-orthopox virus compounds (3).

The best method for measuring CC_{50} values for antiviral compounds is still a matter of discussion and interpretation. However, this issue is rendered somewhat moot by data already collected in animal models, which show that the toxicity of these compounds is not a major limiting factor in the further advancement of these compounds for possible use in human subjects (5, 7, 16).

Cidofovir is Food and Drug Administration approved for the therapy of cytomegalovirus retinitis. Hence, in the absence of a better alternative, it is already being used empirically in treatment of BKV nephropathy. However, due to its nephro-

toxicity, clinicians are hesitant to administer this drug in full dose to kidney transplant recipients, who frequently have significant renal parenchymal injury at the time antiviral therapy is initiated. In an attempt to circumvent this dilemma, cidofovir is given at low doses (0.25 to 1 mg/kg of body weight intravenously, which is more than 10-fold lower than the doses given for cytomegalovirus therapy). Unfortunately, pharmacokinetic data indicate that doses greater than 3 mg/kg are needed to achieve plasma levels of $>10 \mu\text{g/ml}$ (31.7 μM) (8). This presumably explains the inability of cidofovir to completely eradicate BKV from the urine of patients with BKV nephropathy.

Our data provide a rationale to pursue the development of ether lipid esters of cidofovir for use in the clinical arena. The potential advantages of these derivatives over the unmodified cidofovir are that these compounds can be administered orally because of their high bioavailability, the EC_{50} is several orders of magnitude lower and more likely to result in therapeutic plasma concentrations, and these compounds tend not to be concentrated in the kidney. It is possible that the reduced renal uptake of

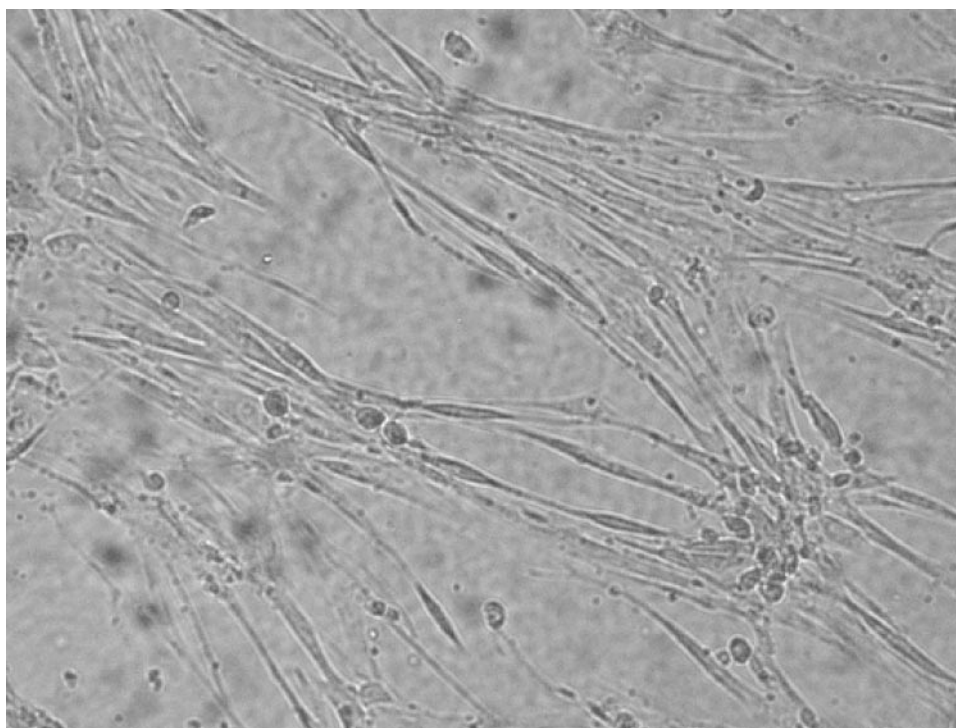


FIG. 1. Cultured WI-38 cells exposed to 5 μM of HDP-cidofovir for 7 days. Compared to control cells (not shown), which were completely confluent, the drug-treated cells covered only about 40% of the tissue culture plate. A few cells are rounded up and in the process of detaching from the surface.

lipid derivatives may compromise the efficacy of these drugs in patients with BKV nephropathy. However, one could also argue that the effect of reduced renal uptake could be compensated for by the 100-fold-increased efficacy and higher selective index of HDP-cidofovir and the other compounds. A definitive answer to this question cannot be provided on the basis of our *in vitro* data. It will be necessary to perform animal and human studies to resolve this issue. Nonetheless, it deserves mention that reduction in mean renal vaccinia virus titers after HDP-cidofovir therapy has been shown in mice inoculated through the intranasal route (16, 19).

The mechanism by which cidofovir and its derivatives mediate anti-BKV activity is unclear. In the case of cytomegalovirus, cidofovir is believed to exert its effect by inhibiting viral DNA polymerase (12, 15), but the BKV genome does not code for such an enzyme. However, it is worth mentioning that the viral T antigen does possess a functional domain with DNA polymerase activity, although it is not known if cidofovir or its active form, cidofovir diphosphate, can inhibit the activity of this domain. Alternatively, since these drugs are nucleoside analogs, the antiviral effect may reflect direct inhibition of viral DNA synthesis by cellular DNA polymerases.

The esterification of cidofovir with ODE, OLE, and HDP side chains leads to increased cellular uptake of drug, more rapid phosphorylation to cidofovir diphosphate (1), or more effective competition with dCTP in the process of DNA synthesis. The mechanisms are clearly not virus specific, since ether lipid analogs of cidofovir are active against not only BKV but also other viruses, including adenovirus, cytomegalovirus, herpes simplex virus types 1 and 2, varicella-zoster virus, human herpesviruses 6 and 8, Epstein-Barr virus, and orthopoxviruses (10, 16, 21, 22). Finally, we cannot exclude that cidofovir has another, as yet uncharacterized, mechanism of antiviral action which does not involve inhibition of DNA synthesis.

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