Hexadecyloxypropyl-Cidofovir (CMX001) Suppresses JC Virus Replication in Human Fetal Brain SVG Cell Cultures

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JC virus (JCV) is a polyomavirus that infects human oligodendrocytes, leading to development of progressive multifocal leukoencephalopathy (PML), an often fatal demyelinating disease occurring in immunocompromised individuals. Currently there are no effective therapies for the treatment of PML that result in clearance of JCV from the brain. Cidofovir (CDV) is an acyclic nucleoside phosphonate that inhibits DNA polymerases and has been used for the treatment of PML. However, CDV demonstrated little efficacy as a treatment for PML and causes substantial side effects to patients. To improve efficacy and reduce the toxicity of CDV, a lipid-ester derivative, CMX001, was generated by Chimerix and is currently in multicenter phase II clinical trials for the prevention or control of cytomegalovirus infection in hematopoietic stem cell transplant recipients and of BK virus in the urine of stem cell or renal allograft recipients. CMX001 caused minimal cytotoxic effects in human fetal brain SVG cells when used at concentrations between 0.01 μM and 0.1 μM. CMX001 resulted in a dose-dependent decrease in the number of JCV-infected cells during initial infection and nearly eliminated JCV-infected cells during an established infection. In addition, CMX001 treatment resulted in a 60% reduction in JCV DNA copy number during initial infection, which suggests that suppression of JCV infection by CMX001 is likely due to inhibition of virus DNA replication. This study demonstrates that CMX001 suppresses JCV infection at concentrations that have limited toxicity to human brain cells, indicating its potential use to limit JCV replication in infected patients.

JC virus (JCV) is a human polyomavirus that causes the fatal demyelinating disorder progressive multifocal leukoencephalopathy (PML) as a result of its productive replication in oligodendrocytes within the brain. The JCV genome is a circular, double-stranded, supercoiled DNA that encodes the multifunctional early proteins large T antigen and small t antigen and the capsid proteins VP1, VP2, and VP3 (23). JCV infects a large portion of healthy individuals in the absence of clinical symptoms (3, 39). In immunocompromised individuals, JCV can reactivate from latency and traffic to the brain, where it replicates, leading to the development of PML (24, 25). PML presents with a variety of clinical symptoms, including visual impairment, motor dysfunction, and cognitive defects (6). Currently, there is no effective treatment for PML (27), although immune reconstitution followed by inflammatory reaction at the sites of PML lesions may limit or clear infection (12). Although agents that target the immune system, such as interferons, significantly inhibit JCV gene expression in cultured human fetal brain cells (13), as with other drugs, poor penetration of the blood-brain barrier (BBB) limits their efficacy in the patient.

JCV can infect several human cell types, including hematopoietic progenitor cells, B lymphocytes (29), fetal Schwann cells (4), and stromal cells (29, 30); however, substantial productive multiplication occurs only in glial cells. Due to the inaccessibility of human brain tissue to most laboratories, SVG cells were developed as a human-brain-derived cell line in which virus replicates to high levels similar to those observed in primary cultures (26), and they remain the only relevant human cell line available that is uniformly susceptible to JCV infection. The SVG cell line resulted from expansion of primary human brain cells (PHBC) that had been transfected with an origin-defective mutant of simian virus 40 (SV40), and maintenance of the continuous cell line is dependent upon expression of SV40 T antigen in these cells. The presence of SV40 T antigen accelerates JCV infection in these cells compared to that in PHBC. JCV progeny virions remain cell associated and are not released into the cell culture supernatant. Progeny virus is not released via lysis of infected cells; rather, JCV-infected cells die by a necrotic cell death pathway (42). As a result, the success of JCV infection can be quantified at the level of DNA by in situ DNA hybridization, which measures the number of cells actively replicating viral DNA as well as the level of DNA in these cells (22). In SVG cells, JCV DNA can be detected as early as 4 days after viral exposure, and its replication exceeds the maximal amount of replication detected at 21 days after viral exposure in PHBC by 14 days postexposure. Importantly, infection of SVG cells with JCV is reproducible, allowing for consistent results between experiments, which is essential for accurate measurement of the effect of drug treatment on virus infection. The SVG cell culture system has been successfully used in previous screenings of anti-JCV drugs, such as cytosine β-D-arabinofuranoside (Ara-C) and cidofovir (21) and mefloquine (7).
The majority of therapies for the treatment of PML seek to target different steps in viral DNA replication. JCV is a member of the human polyomaviruses, which are absolutely dependent upon the host viral DNA polymerase, as evidenced by the addition of the DNA polymerase α-primase complex from a permissive cell line allowing viral replication in a nonpermissive cell line (31). Strategies that target viral DNA replication have been successful in tissue culture models; however, these have had limited success for PML therapy in patients. Nucleoside analogs, which terminate DNA replication, have been used for treatment of PML but have not shown a reproducible ability to alter the course of disease progression. For example, the nucleoside analog Ara-C successfully limits JCV replication in an in vitro human fetal brain cell line (21) but demonstrated no benefit to PML patients in a clinical trial (19). Importantly, Ara-C was reported to be unable to penetrate the BBB (5). Antiviral agents that have been clinically evaluated as treatments for PML include acyclovir (ACV), cidofovir (CDV) (14, 18, 32, 35, 45), and zidovudine (AZT) (43). As with patients treated with Ara-C, patients treated with CDV show various responses to treatment (14, 16, 18, 41). The major reasons for the limited effectiveness of CDV in PML therapy are its limited ability to pass the BBB and its high toxicity to host cells. In addition, CDV is administered intravenously and is associated with significant nephrotoxicity (34). A lipid-ester derivative of CDV, CMX001 (hexadecylpropandiol-CDV), developed for oral prophylactic treatment of smallpox (36), has not demonstrated nephrotoxicity in healthy individuals or patients to date (20, 33).

CMX001 is formed by linking 3-hexadecyloxy-1-propanol to the phosphonate group of CDV, resulting in a lipid derivative that mimics a naturally occurring lipid, lyssolecithin. CMX001 crosses the intestinal wall and circulates as the lipid derivative in the periphery. Following penetration of cells, it is cleaved to the free form of cidofovir. CMX001 has been shown to have improved potency compared to that of CDV for orthopoxviruses (e.g., variola and vaccinia viruses), herpesviruses (e.g., cytomegalovirus [CMV], herpes simplex virus [HSV], varicella zoster virus [VZV], and Epstein-Barr virus [EBV]), adenovirus, and BK virus in preclinical cell culture assays (20). Clinical trials are under way for the use of CMX001 in the treatment of CMV and BK virus infection (8, 9).

In this study, we investigated the effect of CMX001 in comparison to that of CDV on the replication of JCV in the human fetal brain cell line SVG. Limited toxicity of CMX001 to SVG cells was observed for concentrations between 0.01 and 0.1 μM. CMX001 caused a dose-dependent decrease in the number of JCV-infected cells during initial infection and nearly eliminated JCV-infected cells during a previously established infection, which appeared to be due to a defect at the level of viral DNA replication. Suppression of JCV infection at concentrations that do not show significant toxicity to human brain cells combined with diminished potential for nephrotoxicity and oral dosing suggests a potential use of CMX001 to limit JCV multiplication in PML patients.

**MATERIALS AND METHODS**

**Cells and virus.** SVG cells were previously generated by transfecting human fetal brain cultures with an origin-defective SV40 mutant and by growing the resultant culture of cells, which are immortalized by stable expression of SV40 T antigen (26). SVG cells were maintained in minimal essential medium (MEM) supplemented with 10% fetal bovine serum (FBS), 2 mM l-glutamine, and penicillin-streptomycin.

There is no laboratory-adapted strain of JCV used for study in culture. The Mad-4 isolate of JCV was chosen because it was isolated from the brain of a PML patient and is considered representative of other isolates obtained from the brains of PML patients. Mad-4 and another isolate typically used in experiments, Mad-1, show no significant difference in levels of replication in tissue culture models. The Mad-4 isolate of JCV was grown in and purified from human-fetal-brain progenitor-derived astrocytes (28). Virus concentration was determined by hemagglutination of human type O erythrocytes and is expressed as hemagglutinin units (HAU) (26). One HAU of Mad-4 virus contains 1 × 10^5 infectious particles.

**JCV virus infection.** SVG cells were seeded at densities of 1×10^4 to 2×10^4 cells per well in 96-well plates or 3×10^4 cells per well in 6-well plates. Cells were grown overnight at 37°C. The culture medium was then removed, and cells were washed 3 times with phosphate-buffered saline (PBS). Cells were exposed to a minimal volume of PBS containing Mad-4 JCV at a concentration of 10 HAU per 5×10^4 cells (2 infectious particles per cell) for 90 min. Culture medium was added to each well to the nominal volume of the culture plate. The noninfected control cultures were incubated with PBS for 90 min in the absence of virus. After overnight exposure to JCV, the culture medium was replaced with drug-containing media.

**Maintenance of JCV-infected SVG cultures.** Infected cultures of SVG cells were generated by exposing SVG cells to Mad-4 JCV at a concentration of 10 HAU per 5×10^4 cells (2 infectious units per cell) for 90 min. Culture medium was added to the nominal volume of the culture plate, and cells were fed with new medium as needed. When cultures became confluent, the cells were trypsinized and passed at an appropriate density. Infected cultures were carried for 7 to 11 passages. After 7 passages, the culture was considered to be an established infection and was used in drug treatment experiments. Passage 8 contained infected cells that had been grown in culture for 56 days, and passage 12 contained infected cells that had been grown in culture for 114 days. Maintenance of JCV infection during cell passages was determined by in situ DNA hybridization to a JCV DNA-specific probe.

**Drug preparation and dilutions.** Cytosine β-arabinofuranoside (Ara-C) was obtained from Sigma-Aldrich (St. Louis, MO) and was stored as a 5-mg/ml stock in PBS at −20°C. Ara-C was diluted directly into cell culture medium at 5- and 20-μg/ml concentrations. Cidofovir (CDV) was obtained from Gilead (Foster City, CA) and was stored as a 1.2 M stock as an aqueous solution at room temperature. CDV was diluted directly into cell medium at 0.01, 0.03, 0.07, 0.1, and 1 μM concentrations. Hexadecyloxypropyl-cidofovir, CMX001, was obtained from Chimerix Inc. (Durham, NC) and was stored as a 1.8 mM stock in methanol-water-ammonium hydroxide (50:50:0.2, vol/vol/vol) at 4°C for 9 months without loss of bioactivity. CMX001 was diluted directly into cell culture medium at concentrations of 0.01, 0.03, 0.07, 0.1, and 1 μM. The chemical structures of the drugs used in this study are diagramed in Fig. 1.

**In situ DNA hybridization.** Replication of viral DNA in JCV-infected SVG cells was detected by in situ DNA hybridization using a subgenomic JCV biotin-
yaled-DNA BioProbe over the full genome (Enzo Life Sciences, Inc., New York, NY) as previously described (22). Calf thymus DNA was used as a nonspecific control for the JCV probe. A brown precipitate in the nuclei of cells is indicative of a positive signal for JCV DNA. The total number of JCV-positive cells was counted per sample from the 18-mm by 18-mm coverslip.

**MTS assay.** Quantification of cell viability using the MTS [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2(4-sulfophenyl)-2H-tetrazolium] assay is based on the bioactivity of mitochondrial dehydrogenase in living cells, which converts colorless tetrazolium salt to a colored formazan. MTS assays were performed according to the manufacturer's instructions (Promega, Madison, WI). Briefly, culture medium was removed from cells, and 50 μl of PBS was added into each well of a 96-well plate. Ten microliters of MTS reagent was added to wells of cells and to a well without cells to determine background. Mixtures were incubated at 37°C for 2 h, and the absorbance was measured at 490 nm. Trypan blue staining was performed on identical cultures to correlate MTS values with cell viability. The final values indicated are the means of results from 3 replicates. The value for the nontreated control was set to 100%, and all other values are represented as percentages of the control value.

**AB assay.** Quantification of cell viability using the AlamarBlue (AB) assay was performed according to the manufacturer's instructions (Invitrogen, Gaithersburg, MD). AB is a ready-to-use reagent enabling quantitative, scalable, and rapid assay of cell viability and proliferation. As with MTS, resazurin, a nonfluorescent indicator dye in this assay, is converted to bright-red-fluorescent resoru-fin via the reduction reactions of metabolically active cells. Briefly, 10 μl of AB reagent was added to each well of a 96-well dish containing cells or lacking cells to determine background. Mixtures were incubated at 37°C for 6 h, and the absorbance was measured at 570 nm, using 600 nm as a reference wavelength. The value for the nontreated control was set to 100%, and all other values are represented as percentages of the control value.

**Semiquantification of hematoxylin intensity.** Because cell density in cultures processed for in situ DNA hybridization could not be measured by the MTS or AB assay, cell density was approximated in cell cultures processed by quantifying hematoxylin staining intensity. Coverslips containing cells were prepared for in situ DNA hybridization and costained with hematoxylin. Subsequently, each coverslip was scanned and the hematoxylin intensities were quantified using ImageJ (38). To determine the total cell number per coverslip, images were acquired at 10 random positions at a ×100 magnification. Cells were counted throughout each image, and the average cell number per slide was generated by averaging counts from the 10 images for each experimental group. Exactly 900 images at a ×100 magnification cover the surface of an 18-mm by 18-mm coverslip.

**DNA extraction.** DNA was harvested from noninfected and JCV-infected SVG cell cultures using the DNeasy blood and tissue kit according to the manufacturer's instructions (Qiagen, Valencia, CA). DNA was quantified using a NanoDrop ND-8000 spectrophotometer (Thermo Scientific, Wilmington, DE) and was stored at 4°C before use.

**qPCR.** Quantification of JCV viral-genome copy number in JCV-infected SVG cultures was performed using a quantitative real-time PCR assay (qPCR) with primers of JCV Mad1-specific primers and a probe targeting the nucleotide sequences of the N terminus of the viral T antigen as previously described (40). A standard curve for cell viability was generated by plotting MTS values (optimal densities) versus viable cell counts as determined by trypan blue staining, as shown in Fig. 2C. As expected, there was a linear relationship between MTS values and cell viability, as observed by regression analysis. This standard curve was used to determine cell number based on MTS values in subsequent experiments. To determine the viability of SVG cultures over time, noninfected or JCV-infected SVG cells were seeded into 96-well plates at 2 × 10^3 per well, and MTS values were monitored at days 1, 2, 3, 4, and 7 postplating. The MTS values for each day (data not shown) were converted to cell number based on the regression analysis in Fig. 2C and were plotted versus time in days, where day 0 is the time of plating. Figure 2D demonstrates that the cell number at day 1 was similar to the seeding amount of 2 × 10^3 per well, indicating a lag period of 1 day for cell growth after cell plating. Cells grew exponentially from day 1 through day 3.

Because a previous study examining the effect of Ara-C on JCV replication showed that Ara-C is capable of reducing JCV infection in SVG cells over 6 days of treatment (21), we sought to reproduce these results in ourSVG cultures to validate the use of the cells to measure the effect of drug treatment on JCV replication. SVG cells were exposed to Mad-4 JCV at a concentration of 10 HAU per 5 × 10^5 cells (2 infectious particles per cell) or to PBS alone for the noninfected control. Approximately 24 h after JCV exposure, noninfected and JCV-infected cells were treated with 5 and 20 μg per ml (21 and 82 μM) of Ara-C. The culture medium was replaced with new Ara-C-containing medium on day 4. On day 7 after JCV exposure, cell viability was measured by MTS analysis and active viral infection was measured by in situ DNA hybridization. JCV-positive cells, stained brown, were present in the JCV-infected culture and not in noninfected culture, as shown in Fig. 3A. Duplicate plates of noninfected and JCV-infected SVG cells were also hybridized with a nonspecific DNA probe and showed no background signal (data not shown). The total number of JCV-positive cells per sample was counted, and the percentage of JCV-positive cells was expressed as a percentage of the no Ara-C control value (Fig. 3B). Ara-C treatment caused a statistically significant, dose-dependent decrease in the percentage of cells containing JCV DNA of 25% for 5 μg per ml and 83% for 20 μg per ml (P < 0.05). The effect of Ara-C on the cell viability was also determined (Fig. 3C). MTS analysis determined that the 5-μg/ml Ara-C treatment did not

**RESULTS**

**SVG cells as a tissue culture model to measure the activities of drugs upon JCV infection.** Because JCV productively replicates in brain cells of the central nervous system (CNS), SVG cells have been used for studies of the effects of drug treatment on JCV replication (7, 21). SVG cells are a heterogeneous culture that resulted from immortalization of primary human fetal brain cultures with an origin-defective mutant of SV40 (26). They are immortalized by stable expression of SV40 T antigen. SVG cells maintain the morphology of astrocytes, with large flat cell bodies that are irregular in shape and contain a large nucleus (Fig. 2A and B). An MTS assay was used to determine the growth kinetics and cell viability of SVG cultures. Cells were seeded at a range of densities, and 24 h after plating, cell viability was determined by MTS assay and trypan blue staining. A standard curve for cell viability was generated by plotting MTS values (optimal densities) versus viable cell counts as determined by trypan blue staining, as shown in Fig. 2C. As expected, there was a linear relationship between MTS values and cell viability, as observed by regression analysis. This standard curve was used to determine cell number based on MTS values in subsequent experiments. To determine the viability of SVG cultures over time, noninfected or JCV-infected SVG cells were seeded into 96-well plates at 2 × 10^3 per well, and MTS values were monitored at days 1, 2, 3, 4, and 7 postplating. The MTS values for each day (data not shown) were converted to cell number based on the regression analysis in Fig. 2C and were plotted versus time in days, where day 0 is the time of plating. Figure 2D demonstrates that the cell number at day 1 was similar to the seeding amount of 2 × 10^3 per well, indicating a lag period of 1 day for cell growth after cell plating. Cells grew exponentially from day 1 through day 3.

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cause cytotoxicity, but 20 μg/ml decreased cell viability by 21% (P < 0.01). To determine whether the reduction in the percentage of JCV DNA-positive cells after the 20-μg/ml Ara-C treatment was due to an inhibitory effect of Ara-C on JCV rather than an indirect effect via reducing the total cell number through cytotoxic effects, the percentages of cells containing JCV DNA were normalized to the number of viable cells present in the culture using the MTS reading (Fig. 3D). Normalization to cell viability showed that Ara-C treatment of 5 and 20 μg per ml reduced the percentage of JCV DNA-containing cells by 27% and 78%, respectively (P < 0.05), which is similar to the values shown in Fig. 3B. These results demonstrate that Ara-C inhibits JCV replication in SVG cells. Therefore, SVG cells are a valid model in the present study to examine the effects of drug treatment on cell viability and JCV replication in vitro.

Limited toxicity of CMX001 to SVG cells. Treatment of PML with CDV has been associated with a positive prognosis in rare cases; however, it is not considered an efficacious treatment due to the inability to pass the BBB and high cytotoxicity (14, 18). CMX001 is a modified derivative of cidofovir that has demonstrated a higher level of potency than CDV for suppression of many viruses (20, 33). Previous studies showed that antiviral drugs were capable of limiting JCV infection as early as 5 days after treatment for CDV (21) and 3 days after treatment for mefloquine (7). Therefore, to determine the effect of CMX001 on cell viability, SVG cells were treated with CMX001 or CDV at concentrations ranging from 0.01 to 1 μM for 4 days. CDV did not elicit any visible changes in cell density, as detected by microscopy (Fig. 4A), or viability, as measured by AB staining (Fig. 4B), at a concentration range of 0.1 to 1 μM. Cell viability was quantified with AB staining because it is a more sensitive analysis than MTS. Importantly, cytotoxicity has been associated with high concentrations of CDV of 20 to 50 μg per ml (63 and 159 μM) (2), which doses are effective in suppressing virus in vitro. Treatment with CMX001 caused visible reduction in cell density and some morphological changes at 0.1 and 1 μM concentrations (Fig. 4A). In addition, treatment with CMX001 at all concentrations tested caused a reduction in cell viability, including 6% at 0.01 μM, 11% at 0.1 μM, and 32% at 1 μM (P < 0.01), as shown in Fig. 4B. From these results, we conclude that a concentration range between 0.01 and 0.1 μM is suitable for comparison of CMX001 and CDV for their effects on JCV due to their limited effects on cell viability at these concentrations.

CMX001 suppresses JCV replication in SVG cells. Confluent cultures of SVG cells were exposed to 10 HAU per 5 × 10⁴ cells (2 infectious particles per cell) of Mad-4 JCV in a 6-well plate. After overnight JCV exposure, cells were treated with CMX001 or CDV at concentrations of 0.01, 0.03, 0.07, and 0.1 μM or with drug diluent as a nontreated control for 4 days. JCV DNA replication was measured in the cultures by in situ DNA hybridization (Fig. 5A). The total number of JCV-positive cells per sample was counted, and the percentage of JCV-positive cells was expressed as a percentage of the nontreated-control value (Fig. 5B). CMX001 caused dose-dependent reductions in the percentages of JCV DNA-containing cells to 54%, 43%, and 29% of the nontreated-control value for the
concentrations of 0.03, 0.07, and 0.1 μM, respectively. In contrast, CDV at the same concentrations did not elicit any significant reduction in JCV DNA-containing cells. Because CMX001 affects cell viability at the concentrations tested, the total cell number was determined by semiquantification of hematoxylin intensity from the coverslips used for quantification by in situ DNA hybridization. As illustrated in Fig. 5A, the density of cells did not change in the CDV-treated samples. However, CMX001 treatment resulted in a dose-dependent reduction in cell density (Fig. 5C). To determine if the reduction in the percentage of JCV DNA-positive cells upon CMX001 treatment was due to an inhibitory effect of the drug on JCV rather than an indirect effect of the reduction of the total cell number through cytotoxic effects, the percentages of cells containing JCV DNA were normalized to the total cell number on the coverslips (Fig. 5D). CMX001 treatment at 0.01 μM did not result in a significant reduction in the total cell number, whereas the higher concentrations resulted in a dose-dependent decrease in the total cell numbers to 64%, 73%, and 48% of the nontreated-control value with 0.03, 0.07, and 0.1 μM, respectively. CDV did not cause cytotoxicity at any concentration tested, similar to the results measured by AB staining (Fig. 4B). Normalization of the percentages of JCV DNA-containing cells to the total cell number showed that CMX001 caused a dose-dependent reduction in the amount of cells containing JCV DNA, with a maximal reduction to 48% at a concentration of 0.1 μM (Fig. 5D). The reduction in the percentages of JCV DNA-containing cells between the nontreated group and the groups treated with CMX001 was significant (P < 0.0001).

**CMX001 reduces JCV DNA replication in SVG cells.** CMX001 is a derivative of CDV, which disrupts DNA viruses by inhibiting DNA polymerase function (15). Therefore, the suppression of JCV multiplication by CMX001 is likely caused by blockage of viral DNA replication by the host DNA polymerase. To determine if JCV DNA replication is affected by CMX001, quantitative real-time PCR (qPCR) was used to measure the total viral DNA present in CMX001-treated JCV-infected SVG cells. Confluent cultures of SVG cells were exposed to 10 HAU per 5 × 10⁴ cells of Mad-4 JCV. After overnight JCV exposure, JCV-infected cells were treated with CMX001 or CDV at different concentrations or with drug diluent (nontreated control) for 4 days. Cells were harvested, and the total DNA was extracted. Equal quantities of DNA from replicate samples were used in a JCV-specific qPCR assay. The JCV genome copy number in the nontreated controls was given a value of 100%, and all other values are reported as percentages of the control value. CDV treatment had no effect on the copy number of JCV genomes present in SVG cells at any concentration tested (Fig. 6). In contrast, CMX001 treatment caused a dose-dependent decrease in the number of JCV genome copies present in infected cells by 57%
The viability of JCV-infected cells ($P < 0.01$). This trend is consistent with viability determinations from the initial infections (Fig. 4).

**CMX001 treatment nearly eliminates JCV-infected cells from an established infection.** To determine the effect of CMX001 on JCV multiplication during an ongoing or established infection, noninfected and infected cultures of SVG cells were treated with CMX001 at 0.1 μM or with drug diluent for a non-treated control for 4 days. CMX001 treatment had minimal effects on cell density or morphology in noninfected or JCV-infected cells, as shown by the results of phase-contrast microscopy (Fig. 8A), which is consistent with the determination of cell viability in Fig. 7. The presence of JCV DNA in the CMX001-treated cultures was determined by *in situ* DNA hybridization, and cell density was determined by the intensity of hematoxylin staining. JCV DNA-containing cells were observed in the non-drug-treated, infected cultures and rarely in the CMX001-treated culture (Fig. 8B). The total number of JCV DNA-positive cells per sample was counted, and values are expressed as percentages of the value for the non-treated control (Fig. 8C). CMX001 had a modest effect on cell viability, as shown in Fig. 8D. The percentages of JCV DNA-containing cells were normalized to total cell number (Fig. 8E), demonstrating that CMX001 treatment caused 94% elimination of JCV-positive cells from an established infection of SVG cells ($P < 0.05$).

**DISCUSSION**

In this study, we demonstrated that the lipid-linked derivative of hexadecyloxypropyl-cidofovir, CMX001, suppresses JCV multiplication in the human fetal brain cell line SVG. Modest levels of cytotoxicity were observed for CMX001 in SVG cells for concentrations between 0.01 and 0.1 μM. CMX001 caused a dose-dependent decrease in the number of JCV-infected cells during initial infection and a significant elimination of JCV-infected cells during an established infection. Quantitative PCR analysis revealed that CMX001 interrupts the ability of JCV to replicate DNA by up to 60%. Suppression of JCV infection at concentrations that have no significant toxicity to human brain cells, combined with decreased nephrotoxicity and oral administration, suggests a potential use of CMX001 to limit JCV multiplication in PML patients.

A combination of *in situ* DNA hybridization and qPCR is a reliable approach to determine JCV DNA replication and virus multiplication. To understand whether CMX001 directly affects JCV replication or indirectly reduces the copy number of JCV by its cytotoxicity, measurements of viral replication were normalized against cell viability. To ensure the accuracy of measuring cell viability, different methods were employed in this study, including the MTS assay (Fig. 7), AlamarBlue staining (Fig. 4), and semiquantification of hematoxylin intensity (Fig. 5 and 8). All three methods of determining cell viability demonstrated the same trend, namely, that CMX001 was most toxic to cells at a concentration of 1 μM. Normalization to cell viability clearly demonstrated that CMX001 suppresses JCV infection in the SVG cell model during initial infection (Fig. 5) as well as during an established infection (Fig. 8). These results suggest that CMX001 has the ability to interfere with JCV
replication during active infection and could be an appropriate candidate for treatment of PML in patients. CDV at the tested concentration range from 0.01 to 0.1 μM did not show any effect on JCV replication, whereas CMX001 demonstrated a more potent activity in suppressing JCV at these concentrations (Fig. 5). It has been shown that CDV is active only at a concentration range from 20 to 50 μg per ml (63 to 159 μM) in the suppression of primate polyomaviruses (2). JCV multiplication appears to be very sensitive to CMX001 treatment. The effective concentration that produces

FIG. 5. CMX001 suppresses JCV replication in SVG cells. SVG cells were exposed to 10 HAU of Mad-4 JCV per 5 × 10⁵ cells overnight. Cells were then treated with drug diluent or 0.01, 0.03, 0.07, or 0.1 μM CMX001 or CDV. (A) JCV DNA in infected SVG cells was detected by in situ DNA hybridization. The scale bar represents 50 μm. (B) The total number of JCV DNA-containing cells was quantified for each concentration of drug tested and is expressed as a percentage of the value for the nontreated control. (C) The total cell number for the SVG cells processed for in situ DNA hybridization was determined by semiquantification of hematoxylin intensity and is expressed as a percentage of the value for the nontreated control. (D) The number of JCV DNA-containing cells was normalized for total cell number and is expressed as a percentage of the value for the nontreated control. These results were generated from two independent experiments. Error bars represent standard deviations. A single asterisk indicates a P of <0.05, and two asterisks indicate a P of <0.01.

FIG. 6. CMX001 reduces JCV DNA replication in SVG cells. SVG cells were exposed to 10 HAU of Mad-4 JCV per 5 × 10⁵ cells overnight. Cells were then treated with 0, 0.01, 0.03, 0.07, and 0.1 μM CMX001 or CDV. Total DNA was isolated 4 days after drug treatment, and JCV DNA was detected by quantitative real-time PCR. JCV genome copy number is expressed as a percentage of the values for the nontreated control from 2 replicates. Error bars represent standard deviations. Two asterisks indicate a P of <0.01.

FIG. 7. Limited cytotoxicity of CMX001 in an established JCV infection of SVG cells. JCV infection was initiated in SVG cells and maintained over 12 passages in culture. Cells were subsequently treated with 0, 0.01, 0.1, and 1 μM CMX001 for 4 days in culture. Cell viability was measured by MTS assay. Cell viability is expressed as a percentage of the values for the nontreated control from 6 replicates. Error bars represent standard deviations. Two asterisks indicate a P of <0.01.
a 50% maximal response (EC$_{50}$) for CMX001 for BK virus infection, another related polyomavirus, has been reported at 0.13 μM (33, 37). The results described in this study demonstrate that the EC$_{50}$ of CMX001 for JCV is 0.045 μM, which suggests that CMX001 may be a highly effective drug for the treatment of JCV infection.

CMX001 is a derivative of CDV, which has been reported to inhibit viral DNA polymerases from herpesviruses to CMV by acting as a chain terminator that blocks DNA polymerase (15, 20). However, DNA viruses such as polyoma- and papilloma-viruses do not encode a viral DNA polymerase but utilize the host’s DNA polymerase for replication of viral DNA (17, 31, 46). CMX001 is active in vitro against both the human polyomavirus BK (37) and human papillomavirus (HPV) (1).

Quantitative PCR for the JCV genome in CMX001-treated, JCV-infected SVG cultures demonstrated that CMX001 reduces the level of viral DNA produced by up to 60% (Fig. 6). This result suggests that CMX001 suppresses viral multiplication at the level of DNA replication. The mechanism of action of CMX001 against DNA viruses may be due to inhibition of host DNA polymerase and warrants further examination during virus infection. Regardless of the mechanism, without viral DNA replication, there would not be adequate template to produce virion structural proteins or to encapsidate, resulting in a severe reduction in the capacity of cells to produce infectious progeny.

In this study, 1 μM CMX001 caused a level of toxicity to JCV-infected cells that was slightly detectable above the toxicity that it caused to noninfected SVG cells (Fig. 7). Three possible mechanisms have been proposed to explain the preferential killing of infected cells over noninfected cells when the virus lacks a DNA polymerase. The first is that virus infection enhances cell proliferation and that more rapidly proliferating cells are more susceptible to toxicity by cidofovir-diphosphate (CDV-PP) (1). The second is that interactions between cellular proteins and viral proteins (e.g., E6/E7/Tag with p53/retinoblastoma [Rb]) sensitize cells to CDV-PP (1). Recently, mutations in the SV40 large T antigen associated with resistance to CDV have been delineated; the primary mutation found in all resistant clones was V505A near the ATP binding site in the helicase domain (44). The third putative mechanism invokes alterations in the anabolism of CDV following infection. Fol-
lowing infection with HPV type 16, primary keratinocytes produce higher levels of CDV-PP than prior to infection (1). Similar results were observed in human lung fibroblasts infected with CMV, where infection enhanced the activities of enzymes responsible for CDV-PP anabolism and led to much higher levels of CDV-PP than before infection (11). These proposed mechanisms are not mutually exclusive.

Currently, it is difficult to predict the required length of treatment for patients. In a patient, the diagnosis of PML most frequently occurs after an extended period of time in which the virus was capable of replicating unchecked in the brain, as evidenced by the demyelinating lesions visible by magnetic resonance imaging (MRI). Because experiments evaluating the effect of CMX001 on JCV infection initiated close to the administration of the drug do not mimic the course of infection in patients, we measured the effect of CMX001 on an established infection from which the virus was maintained over multiple passages in culture. Results presented in Fig. 8 suggest that CMX001 is able to reduce the number of virally infected cells in an established infection over the course of 4 days of drug treatment, which suggests that the effect on JCV multiplication in the host may be rapid and requires a shorter time period of treatment than that of drugs like cidofovir. The more relevant information in determining treatment duration for PML therapy is available from a phase 1 clinical study conducted by Chimerix Inc., where CMX001 was administered orally to 84 healthy volunteers and the safety and pharmacokinetics of single and multiple doses of CMX001 were evaluated (10). The result demonstrated that CMX001 was well tolerated at all doses.

This study strongly demonstrates the superior efficacy of CMX001 over CDV as a suppressor of JCV replication in a cell culture model. In addition, CMX001 also has many other advantages over CDV, such as oral administration and reduced nephrotoxicity (20, 33). Future studies are required to determine the dose necessary to be effective against JCV multiplication in humans; however, based on the efficacy of CMX001 in reducing JCV infection observed in this study, CMX001 may be an appropriate drug to evaluate for PML therapy.

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