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Short Communication

The efficacy of nucleoside analogs against JC virus multiplication in a persistently infected human fetal brain cell line

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The effectiveness of nucleoside analogs in blocking viral multiplication was evaluated using an immortalized human neuroglial cell line capable of sustaining a persistent JCV infection, SVG-JC. Results from *in situ* DNA hybridization and hemagglutination assays performed on drug treated cultures were used as a measure of viral DNA replication and multiplication, respectively. Of the three drugs tested, Ara-C (cytosine arabinoside), AZT (3′-azido-3′-deoxythymidine), and cidofovir (S)-1-[3-hydroxy-2-(phosphonyl-methoxypropyl] cytosine), only Ara-C showed a significant effect in decreasing active JCV replication and multiplication. *In vitro* data, using different cell types and virus strains have shown that specific drugs can indeed modulate viral infection. However, such modulation has not previously been demonstrated in those cells of the CNS which are specifically targeted by JCV. The SVG-JC cells represent a unique system with which further studies can be conducted on the effects of drugs on brain derived cells that are susceptible to viral infection.

Keywords: drug; antiviral; JC virus; nucleoside analogs

Progressive multifocal leukoencephalopathy (PML) has become an increasingly common neurological disease that is essentially exclusive to immunodeficient individuals (Major and Ault, 1995). The etiologic agent, a human DNA papovavirus, JCV, establishes a lytic infection in the brain that targets the myelin-producing oligodendrocytes. The ensuing cytolytic destruction of these cells leads to multifocal areas of demyelination within the central nervous system. Neuroradiological signs of PML include white matter lesions which are typically concentrated at the gray-white matter junction of the cerebral hemispheres, however, these lesions can be found in other areas including the cerebellum and brain stem (Itoyama *et al*., 1982). While PML associated lesions correlate with a variety of clinical symptoms, the most common include motor, visual, and cognitive impairments (Berger and Major, 1994; Brooks and Walker, 1984).

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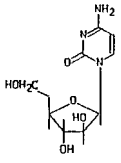
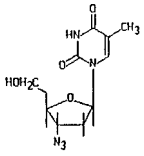
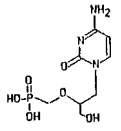
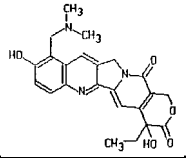
Although PML was once considered a rare disease, a higher incidence of HIV-1 infection in recent years has resulted in a large number of immunocompromised patients presenting with symptoms of PML. It is estimated that 85% of all reported PML cases occur with AIDS as the immunosuppressive background (Berger and Concha, 1995) and approximately 5% of all AIDS patients will eventually develop PML (Major and Curfman, 1997). Moreover, serological studies have demonstrated that greater than 80% of the general population has detectable levels of antibodies to JCV in the peripheral blood (Major *et al*., 1992; Padgett and Walker, 1983). As a result, there has been an increased interest in identifying those people that may be at risk to develop active infection. Despite the fact that the majority of the population has had initial exposure early in life and will remain asymptomatic thereafter, the increasing number of patients who develop immune disorders including AIDS may encourage future testing of individuals at risk for PML.

Although there have been sporadic reports of successful treatment with antiretroviral agents and even reports of spontaneous remission (Berger and

Mucke, 1988), as yet no effective therapy has been established to treat this disease. Trial therapies have investigated specific antiviral drug regimens targeting JCV as well as attempts to enhance a general immune response (Budka and Shah, 1983). The first relatively large-scale trials investigating drugs such as Adenine arabinoside (Ara-A) met with varied results. Although one such trial reported that patients suffering from PML showed no clinical improvement with Ara-A treatment (Marriot *et al*, 1975), isolated cases suggest its possible effectiveness (Portegies *et al*., 1991).

Nucleoside analogs such as Ara-A and Ara-C along with non-nucleoside analogs such as topotecan (Kerr *et al*., 1993) have been used for some time to treat various illnesses including PML. Table 1 illustrates the drugs and their proposed mechanism(s) of action which have been used in attempts to block JCV infection. The ability to terminate chain elongation and interfere with DNA synthesis confers antiviral activity and has been utilized in combating myeloproliferative diseases. Ara-C, for example, was first established as an anti-neoplastic drug and was only later recognized as a possible treatment for JCV infection. Physician reports of improved clinical symptoms, resolution of white matter lesions, and near-complete remission in PML patients following Ara-C therapy were encouraging (O'Riordan *et al*. 1990). Other base analogs, however, were not as successful. Since the majority of PML cases occur in AIDS patients treated with antiretrovirals, it was postulated that PML associated with HIV-1 would respond to antiretrovirals such as AZT as well (Conway *et al*, 1990). As yet there has been no correlation between clinical or neuroradiological improvement and AZT activity against JCV infection. As a result of preliminary experiments showing that it has significant activity against the herpes simplex virus (HSV), recent attention has been given to a non-cyclic nucleoside analog, cidofovir (Aduma *et al*., 1995). In addition, reports of the upregulation of JCV expression by cytomegalovirus (CMV), a member of the Herpesvirus family (Heilbronn *et al*, 1993) further fostered hopes that cidofovir would show some activity against JCV as well. A recent report of cidofovir activity against SV40 infection in monkey kidney cells suggested some effect (Andrei *et al*, 1997). However, the biology of JCV and particularly its host range in the human CNS is substantially different from SV40 to make useful comparisons. It was not possible for the reporting laboratory to culture human polyomaviruses, BKV or JCV, for their investigations, which resulted in the use of alternative

Table 1 Drugs used in treating viral diseases

Trade name	*Chemical name*	*Classification*	*Action*	*Uses*	*Structure*
Ara-C	1-β-D-arabinofurano-sylcytosine; cytosine arabinoside	Nucleoside analog	Terminates chain elongation, inhibits DNA polymerase	Antineoplastic agent, also used to treat PML	
AZT	3′-Azido-3′-deoxythymidine	Nucleoside analog, anti retroviral	Terminates chain elongation, inhibits reverse transcriptase	Treatment for HIV	
Cidofovir	(S)-1-[3-hydroxy-2-(phosphonylmethoxy)-propyl]cytosine	Acylic nucleoside phosphonate	Terminates chain elongation, inhibits viral polymerase function	Active against DNA viruses such as HSV	
Topotecan	[S]-9-dimethylamino-methyl-10-hydroxy-camptothecin	Topoisomerase inhibitor	Stabilizes DNA topoisomerase I, induces apoptosis	Effective antineoplastic agent	

mouse polyomavirus and simian virus 40 for study. Consequently, cidofovir has not been tested against JCV prior to this report nor has the drug been tested in human glial cell cultures which represent the main target of JCV infection.

To assess the efficacy of such drugs against JCV, a system needed to be developed through which a quantitative measurement of infection could be made. Of particular interest was the possibility of monitoring the effects of these drugs in cells which are normally involved in this disease rather than in cell types not normally targeted by JCV.

JCV has been shown to infect several cell types including hematopoietic progenitor cells, B lymphocytes (Monaco *et al*, 1996), and human fetal Schwann cells (Assouline *et al*, 1991), but substantial productive infection has only been reported in glial and stromal cells (Monaco *et al*, 1996). However, the establishment of a cell line particularly susceptible to JCV infection had been reported as early as 1985 (Major *et al*, 1985). These cells, then designated as SVG, were derived from primary human fetal brain cultures that had been immortalized with an origin defective mutant of simian virus 40 (SV 40). Once infected with JCV, the SVG cells supported multiplication of the virus at levels that were similar to those observed in primary cultures of human fetal brain cells. Figure 1 characterizes these persistently infected SVG cells and the detection of JCV in the nuclei by *in-situ* DNA hybridization. An example of one of the applications of this cell line is the use of the JCV infected SVG cells for the high level production of virus particles which are necessary for immune response studies (Frye *et al*, 1997).

For the purposes of this experiment, infection of SVG cells by JCV was initiated and cell cultures were passaged until a persistently infected population of cells was established in which approximately 40% of the cells were positive for JCV replication and multiplication. The cultures were evaluated for virus production by the ability of cell harvests to hemagglutinate human type O erythrocytes and by the number of replication positive cells determined by *in situ* DNA hybridization. Once a persistently infected cell line with a consistently high virus titer was achieved, the cells were used to test the ability of drugs to block viral DNA replication and multiplication. SVG-JC cells were grown in Minimal Essential Media supplemented with 10% fetal calf serum, 2 mM L-glutamine, and 50 μg/ml gentamycin. These cells were transferred onto glass coverslips in six well culture dishes at a concentration of 1×10^5 cells per well and allowed to grow to confluence. Day 0 was designated as the start of treatment with nucleoside analogs. Fresh stocks of Ara-C, AZT, and cidofovir were added to the cultures every 3 days at the specified concentrations when the cells were refed. At the end of days 7, 14, and 21, the coverslips were fixed in 5% paraformaldehyde and dehydrated in increasing ethanol concentrations. Cells were harvested from the culture dishes by trypsinization and split into two aliquots. One aliquot was stained with trypan blue and counted to assess viability and the other was reserved for hemagglutination assays (HA).

The reserved cells were treated with 0.25% deoxycholic acid (DOC) and incubated in a 37°C water bath for 30 min to release virion particles from nuclei. Fifty microliters of this virus suspension were serially diluted 1 : 2 in a 96 well U-bottom culture plate in Alsever's solution (20 mM Na nitrate, 72 mM NaCl, and 100 mM glucose, pH 6.5 adjusted with glacial acetic acid). Each well then received an equal volume of human type O erythrocytes. The virion particles form a lattice with the red blood cells keeping them in suspension, observed as hemagglutination, whereas the absence of virion particles results in erythrocytes aggregating at the bottom of the well. The virus titer was expressed as the reciprocal of the highest dilution which resulted in hemagglutination.

In situ DNA hybridization was performed on SVG-JC cells on coverslips that were attached to glass slides to measure the number of cells that are actively replicating viral DNA. Previous studies determined that high copy numbers of the JCV genome were necessary to generate a positive signal (Major *et al*, 1985). The complete probe mixture contained 50% formamide, 10% dextran sulfate, 0.4 μg/ml herring sperm DNA, and 2 μg/coverslip biotinylated probe (ENZO Biochem., New York) in $2 \times$SSC. The probe mixture for the negative control contained calf thymus DNA in place of the viral probe. Twenty-

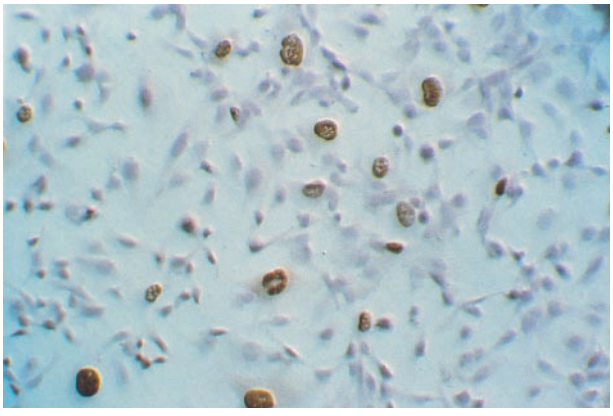


Figure 1 Characterization of SVG-JC cells. *In situ* DNA hybridization with the JC virus biotinylated DNA probe shows localization of JCV DNA to the nuclei of infected cells. Quantitative measurements demonstrate high levels of active DNA replication by *in situ* DNA hybridization and virion multiplication by hemagglutination assays. The average number of cells per coverslip positive for JCV DNA is 3280 and HA titers are consistently above 4096.

five microliters of the final mixture were applied to each coverslip and sealed under a larger coverslip with rubber cement. Incubating at 80°C for 10 min denatured the probe and cellular DNA after which hybridization at 37°C took place overnight. The larger coverslips were removed and the slides were washed in 2 × SSC, 0.1% Triton X-100 in PBS, and 1 × PBS.

A streptavidin-biotin-horseradish peroxidase signal generating system (ENZO Biochem., New York) was used to detect the biotinylated probe. The detection was done using the protocol suggested by the manufacturer. One hundred microliters of the avidin-biotin-hrp complex diluted 1 : 250 were added to each slide and incubated for 1 h at 37°C in a humidity chamber. The slides were washed in 0.1% Triton for 1 min and 1 × PBS for 3 min. Diaminobenzidine tetrahydrochloride (DAB) reagent was applied to the coverslips and allowed up to 45 min for the reaction product to precipitate. After washing, the slides were counterstained in hematoxylin, dehydrated in increasing concentrations of ethanol, and mounted using permount. Hybridization positives ranging in color from golden to dark brown were counted over the entire coverslip under phase contrast using a Zeiss ICM 405 inverted microscope.

The results from these experiments indicate that of the three nucleoside analogs tested, only Ara-C showed significant activity against JCV. *In situ* DNA hybridization was used to identify the percent of infected cells in culture and to identify cells in which JCV DNA was being replicated. The positive signals generated are not only a sign of infection but also of active viral DNA replication, making it useful to identify productively infected cells. The data in Table 2 show that there was a decrease in JCV replication and multiplication only in Ara-C treated cultures while AZT and cidofovir had minimal or no effect. Concentrations as high as 25 μg/ml of Ara-C were non-toxic and highly effective in decreasing DNA replication. Virus titers in the treated cell cultures measured by HA showed a corresponding drop which indicated that the drug was also affecting the rate of JC virion particle formation. At the end of 21 days of treatment, the number of cells actively replicating the virus had decreased by 93%.

The other nucleoside analogs tested showed no activity against JCV *in vitro*. Figure 2 is a photographic representation of the *in situ* hybridization results, showing a decrease in JCV replication in Ara-C, Figure 2a, treated cells as opposed to no change in AZT or cidofovir treated cultures (not shown). AZT was not toxic to the cells at concentrations twice as high as those used during Ara-C treatment. Even at 50 μg/ml, the drug did not significantly reduce the number of positive cells as seen by *in situ* hybridization. Although AZT is a nucleotide chain terminator, it also has been shown to impair the function of reverse transcriptase (Melton *et al*, 1997), which did not suggest it would be effective against a papovavirus such as JCV. However, reports of clinical improvement of PML in conjunction with AZT administration (Conway *et al*, 1990) promoted the use of AZT in these experiments to test its efficacy against JCV *in vitro*.

Both Ara-C and AZT treated cultures were able to retain mitotic ability and survive prolonged drug administration with no significant signs of toxicity. Cidofovir, on the other hand, proved to be more toxic to cells. Even at concentrations in the micromolar range (as opposed to millimolar concentrations used for Ara-C and AZT), a significant loss in viability, as determined by trypan blue staining, was seen with treatment. The concentrations of cidofovir that were low enough to spare the cells may have been too low to have any significant effect on JCV replication. Figure 3, which shows the number of replication positive cells at the end of

Table 2 JC virus multiplication in drug treated persistently infected SVG cells assayed by hemagglutination and *in situ* DNA hybridization

		7 days		*14 days*		*21 days*	
Drug	*Concentration (μg/ml)*	*HA titer*	*Number of in situ positive cells*	*HA titer*	*Number of in situ positive cells*	*HA titer*	*Number of in situ positive cells*
Ara-C	0	>4096	2974	>4096	3132	>4096	3025
	10.0	>4096	2256	2048	1017	512	767
	25.0	1048	1004	128	620	64	204
AZT	0	>4096	2025	>4096	3261	>4096	2663
	10.0	>4096	2395	>4096	2696	>4096	3027
	25.0	>4096	1913	>4096	3164	>4096	2480
		5 days		10 days		15 days	
Cidofovir	0	>4096	4388	>4096	3986	>4096	4067
	0.5	>4096	3721	2048	3899	2048	3769
	1.0	2048	3239	1024	3569	1024	2546

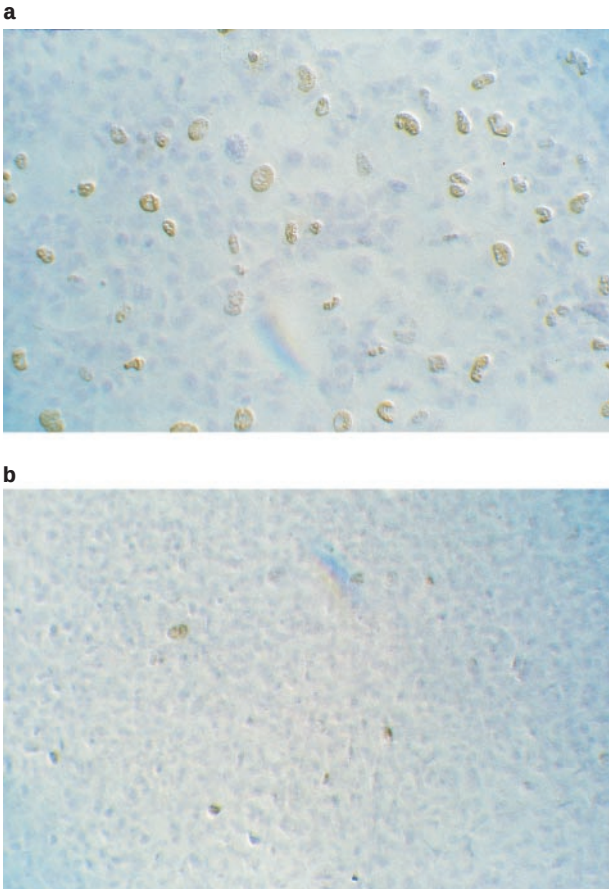


Figure 2 *In situ* DNA hybridization of untreated (**a**) and Ara-C treated (**b**) SVG-JC cells with a biotin labeled JCV DNA probe. Darkly staining nuclei result from biotin-avidin-peroxidase complex reacting with biotin labeled DNA in the presence of hydrogen peroxide and diaminobenzidene. Cells treated with Ara-C show a marked decrease in the number of JCV infected nuclei.

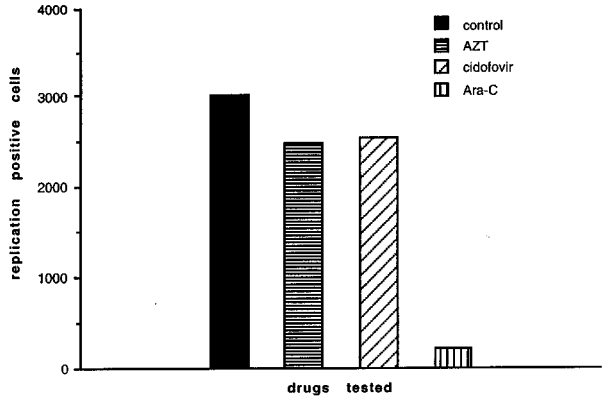


Figure 3 Number of cells actively replicating JCV DNA in drug treated SVG-JC cells measured by *in situ* DNA hybridization. Cidofovir concentration was 1 μg/ml; AZT at 25 μg/ml; and Ara-C at 25 μg/ml.

each treatment period at maximum effective dosage, shows a decrease only with Ara-C treatment.

The difficulties in evaluating neurological therapeutics *in vivo* are due in some part to the variability of cell types and differing susceptibilities to JCV infection found in primary human fetal brain cell cultures. To examine levels of JCV DNA replication and multiplication as a measure of a drug's efficacy, a cell culture system with a homogeneous population of cells is necessary. The cell population heterogeneity typically found in primary human fetal brain cultures makes it difficult to normalize results obtained using alternative tissue sources. Although Ara-C was similarly effective against JCV multiplication using primary cultures of human fetal brain (Major and Curfman, 1997), it was difficult to derive accurate quantitation in these cultures. The persistently infected SVG-JC cells of human neuroglial origin are rapidly proliferating (the cell cycle is approximately 18 h) and maintain a continuously high number of infected cells and virion particles formed through multiple passages. The cell cultures could be used to examine any synergistic effects that different drugs may have in combating viral infection in an attempt to mimic the combination therapies often prescribed for other viral infections.

The first controlled clinical trial of Ara-C was recently conducted in AIDS patients with biopsy proven PML (Hall *et al*, 1998). The conclusion of that trial was that Ara-C administration did not result in clinical improvement, the study end-point, in patients receiving drug through either intravenous or intraventricular administration. However, the trial design did not determine whether Ara-C was delivered to its site of action, the multifocal demyelinated lesions in the brain. Since Ara-C has been shown to be effective against JCV infection *in vitro*, an improved method of delivering Ara-C to the lesioned sites within the brain may have to be developed (Groothuis and Levy, 1997; Levy *et al*, 1997). A recent case report described the resolution of PML during the course of treatment with both Ara-C and cidofovir (Blick *et al*, 1998). It may be possible that cidofovir can effect the progression of PML in AIDS patients without directly effecting JCV multiplication. The combination of effective antivirals and a more efficient method of drug delivery that specifically targets the lesioned sites in the brain could greatly enhance the chances of finding an effective therapy for PML.

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