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Early events in the life cycle of JC virus as potential therapeutic targets for the treatment of progressive multifocal leukoencephalopathy

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The human polyomavirus, JC virus (JCV), is the etiological agent of progressive multifocal leukoencephalopathy (PML). PML occurs almost exclusively in the setting of severe and prolonged immunosuppression and it remains an important and life-threatening complication in the acquired immunodeficiency syndrome (AIDS) population. Several drugs that target DNA replication have shown efficacy at inhibiting JCV replication *in vitro* but none to date have shown *in vivo* efficacy. The authors' laboratory has been studying early events that contribute to infection of susceptible cells by JCV. They previously demonstrated that infection of glial cells by JCV requires clathrin-dependent endocytosis and that this early step in the viral life cycle can be blocked by the antipsychotic drug, chlorpromazine. As chlorpromazine is associated with the development of extrapyramidal symptoms that may be heightened in AIDS patients, the authors sought to test the atypical antipsychotic, clozapine, for antiviral activity against JCV. In this report, the authors show that clozapine is as effective as chlorpromazine at inhibiting infection. They further demonstrate that low-dose combinations of both drugs synergistically inhibit infection. *Journal of NeuroVirology* (2003) **9(suppl. 1),** 32–37.

Keywords: JC virus; polyomavirus; progressive multifocal leukoencephalopathy; thereutics

Introduction

Despite the introduction of highly active antiretroviral therapy (HAART), JC virus (JCV)-induced progressive multifocal leukoencephalopathy (PML) continues to be a life-threatening complication of acquired immunodeficiency syndrome (AIDS) (Antinori *et al*, 2001). Approximately 4% to 8% of human immunodeficiency virus (HIV)-infected individuals develop PML and the majority succumb to the disease within 1 year. Long-term survival beyond 1 year is rare but correlates with the restoration of anti-JCV–specific cellular immunity (Du Pasquier *et al*, 2001; Koralnik *et al*, 2002; Koralnik *et al*, 2001). To date, three antiviral compounds have been tested for activity against JCV. These include cytosine arabinoside (Ara-C), azidothymidine (AZT), and cidofovir. Of these, only Ara-C has been shown to inhibit JCV multiplication in tissue culture models (Hou and Major, 1998). Based on these data and anecdotal evidence that Ara-C and cidofovir might be effective against PML, both drugs were tested *in vivo*. Ara-C failed to show a significant benefit in a large-scale clinical trial and the trial was terminated (Hall *et al*, 1998). It was subsequently shown that Ara-C did not penetrate the blood-brain barrier in that trial and a subsequent trial involving convection-enhanced intraparenchymal delivery is currently underway (Levy *et al*, 2001). In a smaller pilot study, cidofovir has not shown beneficial effects against PML (Marra *et al*, 2002).

Our laboratory has been studying the early events that contribute to infection of glial cells by

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JCV. These early events include virus-host cell receptor interactions, mechanisms of virus entry, intracellular trafficking of virions towards the nucleus, and finally delivery of the viral genome across the nuclear membrane. In an earlier study, we used the drug chlorpromazine as a tool to specifically inhibit clathrin-dependent endocytosis, which is critical for JCV infectious entry into glial cells (Pho *et al*, 2000). As chlorpromazine was once a widely used antipsychotic drug, we proposed that it may be useful in the treatment of PML (Atwood, 2001). Like other "typical" antipsychotic drugs, chlorpromazine treatment is associated with elevated levels of serum prolactin, induction of extrapyramidal symptoms (EPSs), and with continued use, tardive dyskinesia (Seeman, 2002). As AIDS patients already show evidence of EPSs, attempts to treat PML with chlorpromazine may exacerbate these symptoms (Breitbart *et al*, 1996; Itoh *et al*, 2000; Kelly *et al*, 2002; Lopez *et al*, 1999; Ramachandran *et al*, 1997). The "atypical" antipsychotic drugs such as clozapine may be ideal for treating PML in AIDS patients as they maintain their antipsychotic effects without inducing significant EPSs. Both the typical and atypical drugs work by antagonizing the dopamine D2 receptor. The differences in side effects appear to be related to the relative affinities of chlorpromazine and clozapine for occupancy of the dopamine D2 receptor (Seeman, 2002).

In this report, we tested the ability of clozapine either alone or in combination with chlorpromazine for its ability to inhibit JCV infection of human glial cells. We found that clozapine when given alone inhibited JCV infection as well as chlorpromazine. Interestingly, we also found that lower doses of each drug when given simultaneously synergistically inhibited infection. These data suggest that the atypical antipsychotics may be effective therapeutically against JCV-induced neurological disease when given alone or in combination with lower doses of the typical antipsychotics.

Results

Chlorpromazine inhibits infection of SVG-A cells by JCV

As a first approach to evaluating the antiviral activity of chlorpromazine, we tested a range of drug concentrations for *in vitro* toxicity. The SV40-transformed human glial cell line, SVG-A, was incubated with increasing doses of chlorpromazine at 37°C for 48 h. Cells were then stained with Trypan blue and the percentage of dead cells calculated. Doses above 6.0 μM resulted in significant toxicity (Figure 1**A**). We then tested doses between 0.3 and 6.0 μM of drug for anti-JCV activity. SVG-A cells were pretreated with drug for 45 min at 37°C and then infected with JCV (Mad-1SVEΔ) in the presence of drug. The cells were washed 3○ in phosphate-buffered saline (PBS) and maintained for 48 h in the same concentration of drug. Cells were fixed in acetone and infection scored by indirect immunofluorescence analysis of JCV V antigen expression. Doses of 0.3 and 3.0 μM of drug inhibited infection 1.6-fold and the 6.0-μM dose inhibited by 2.2-fold (Figure 1**B**).

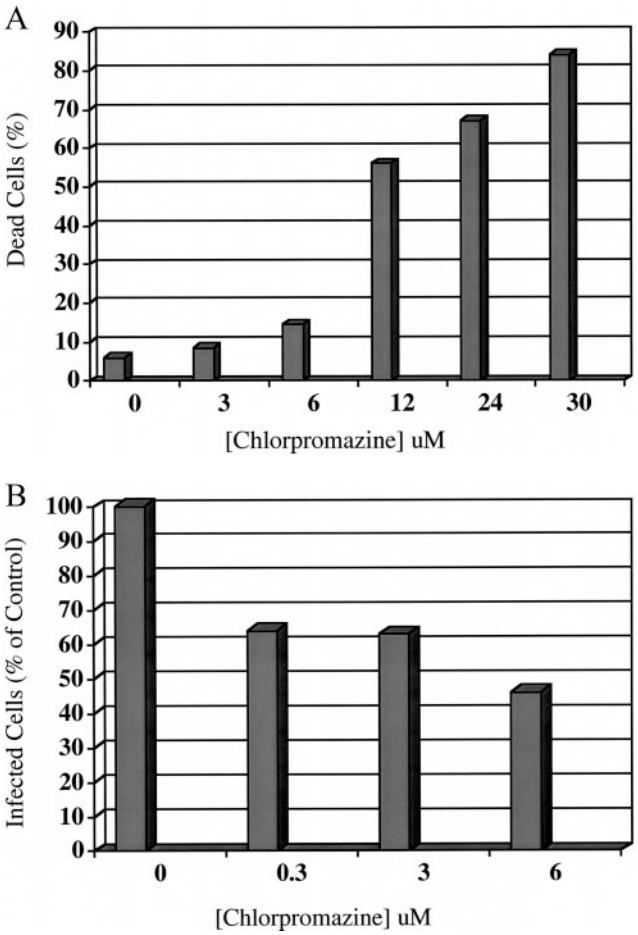

Figure 1 (**A**) Toxicity of chlorpromazine. SVG-A human glial cells were incubated with chlorpromazine for 48 h at 37°C. The cells were then stained with 0.1 ml of a 0.4% solution of trypan blue. The percentage of dead (blue) versus live cells were determined by counting on a hemacytometer. The graph is representative of three independent experiments. (**B**) Chlorpromazine inhibits infection of SVG-A cells by JCV. SVG-A cells were pretreated with the indicated concentrations of chlorpromazine for 45 min at 37°C. The cells were then incubated with 200 hemaglutination units/ml of JCV (Mad-1SVEΔ) for 1 h in the presence of drug. The cells were washed 3○ in PBS and then maintained in growth media containing drug. At 3 days post infection, the cells were fixed and stained for the late viral protein, V antigen. The percentage of infected cells in cultures not treated with drug was set at 100% and used to compare with the drug treated cultures.

Clozapine inhibits infection of SVG-A cells by JCV

We first tested the toxicity of clozapine in SVG-A cells as described above. Doses above 40.0 μM showed significant toxicity (Figure 2**A**). We then tested doses between 15 and 30 μM of drug for anti-JCV activity as described above. The 15- and 20 μM doses of clozapine had little effect on JCV infection (Figure 2**B**). The

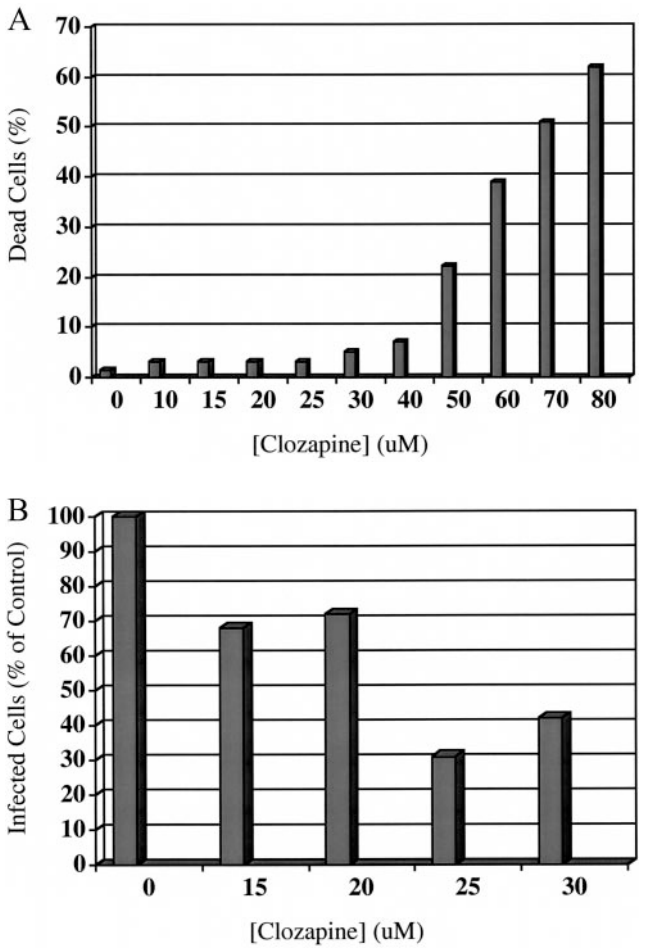

Figure 2 (**A**) Toxicity of clozapine. SVG-A cells were incubated with clozapine for 48 h at 37°C. The cells were then stained with 0.1 ml of a 0.4% solution of trypan blue. The percentage of dead (blue) versus live cells were determined by counting on a hemacytometer. The graph is representative of three independent experiments. (**B**) Clozapine inhibits infection of SVG-A cells by JCV. SVG-A cells were pretreated with the indicated concentrations of clozapine for 45 min at 37°C. The cells were then incubated with 200 hemaglutination units/ml of JCV (Mad-1SVEΔ) for 1 h in the presence of drug. The cells were washed 3○ in PBS and then maintained in growth media containing drug. At 3 days post infection, the cells were fixed and stained for the late viral protein, V antigen. The percentage of infected cells in cultures not treated with drug was set at 100% and used to compare with the drug treated cultures.

25- and 30-μM concentrations inhibited infection by approximately twofold (Figure 2**B**).

A combination of chlorpromazine and clozapine inhibits infection of SVG-A cells by JCV

As neither drug alone completely inhibited infection, we asked whether a combination of both drugs might have an effect. SVG-A cells were treated with either 30 or 25 μM chlorpromazine alone or in combination with either 6.0 or 3.0 μM of chlorpromazine. The combinations of both drugs did not result in increased toxicity in the cultures (not shown). At each of the tested concentrations, the combination of both drugs inhibited infection significantly better than either drug alone (Figure 3). This was most pronounced with the lower doses of chlorpromazine (Figure 3).

Discussion

JCV-induced disease continues to be a major complication in AIDS. Several drugs targeting viral replication have proven to be effective *in vitro* but have not been useful clinically. This is mainly due to poor penetrance of brain parencyma by many of these drugs. Our laboratory is focused on studying the early events in infection of cells by JCV (Figure 4). Several of these early steps in the viral life cycle could be targeted by antiviral therapy, including virus-receptor interactions and virus entry (Figure 4). We have successfully shown that the latter of these steps, virus entry, is mediated by clathrin-dependent endocytosis and that this step can be blocked by chlorpromazine. Interestingly, chlorpromazine was once a widely used antipsychotic drug and as such has good penetrance into brain parenchyma. A major concern with the use of chlorpromazine to treat PML in AIDS patients is that the drug is highly associated with the induction of EPSs. As AIDS patients already have basal ganglia deficits, the addition of chlorpromazine may exacerbate these effects and induce severe EPSs. For this reason, we have begun to analyze the "atypical" antipsychotic drugs for effectiveness at inhibiting JCV infection *in vitro*. Unlike the "typical" antipsychotics, the "atypicals" are associated with less severe side effects. We found that the "atypical" antipsychotic, clozapine, was as effective as chlorpromazine in inhibitng JCV infection of glial cells. More promising, however, was that a combination of lower doses of both chlorpromazine and clozapine showed synergistic anti-JCV activity. These data suggest that drug combinations could be found that are effective against JCV infection without induction of severe EPSs in the AIDS population. In support of this, one study found that low-dose chlorpromazine and haloperidol was effective at managing delirium in AIDS patients with only minimal induction of EPSs (Breitbart *et al*, 1996). We have shown that both typical and atypical antipsychotic drugs can inhibit initial infection of cells by JCV. We have not shown that this drug regimen can inhibit an already active infection. Further studies are required both *in vitro* and *in vivo* before definitive conclusions can be drawn regarding the efficacy of these drugs in the treatment of PML.

Materials and methods

Cells, virus, and antibody

The human glial cell line, SVG, was established by transformation of human fetal glial cells by an

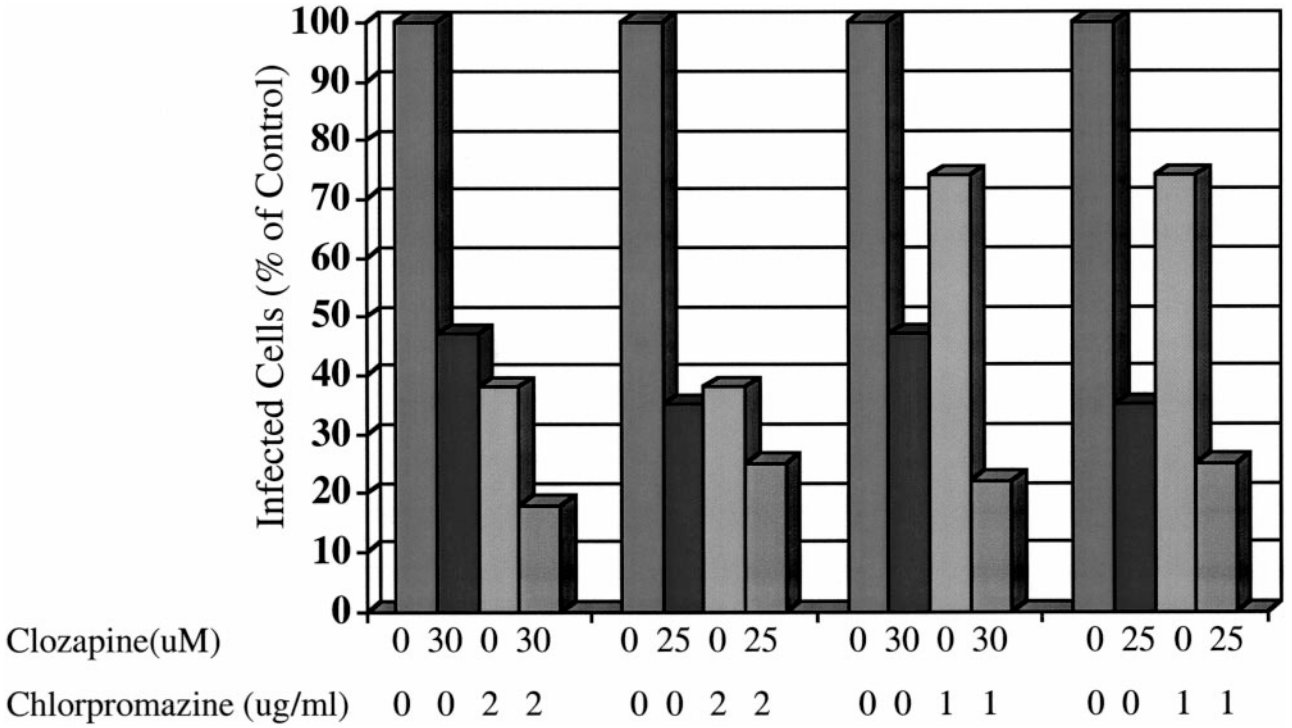

Figure 3 A combination of chlorpromazine and clozapine inhibits infection of SVG-A cells by JCV. SVG-A cells were treated with the indicated concentrations of chlorpromazine, clozapine, or a combination of both as indicated. The cells were then incubated with 200 hemaglutination units/ml of JCV (Mad-1SVEΔ) for 1 h in the presence of drug. The cells were washed 3○ in PBS and then maintained in growth media containing drug. At 3 days post infection, the cells were fixed and stained for the late viral protein, V antigen. The percentage of infected cells in cultures not treated with drug was set at 100% and used to compare with the drug treated cultures.

origin defective SV40 mutant and has been previously described (Major *et al*, 1985). The cell line was sublconed twice to generate SVG-A cells. SVG-A cells were maintained in a humidified 37°C CO_2 incubator in Eagles' minimum essential media (E-MEM; Mediatech, Herndon, VA) supplemented with 10% heat inactivated fetal bovine serum (Mediatech). The hybridoma, PAB597, which produces an antibody to SV40 V antigen, was obtained from E. Harlow and maintained in RPMI-1640 Hybrimax media (Sigma, St. Louis, MO) supplemented with 10% heat inactivated fetal bovine serum (Mediatech). The PAB597 monoclonal has previously been shown to cross-react with JCV VP1 (Atwood *et al*, 1995). The hybrid Mad-1/SVEΔ virus was constructed by insertion of the regulatory region of SV40 into the regulatory region of the Mad-1 strain of JCV (Mad-1/SVE) (Vacante *et al*, 1989). Propagation of Mad-1/SVE in human glial cells led to deletions and alterations exclusively in the regulatory region. The rearranged regulatory region contains the origin of replication, the TATA box, and 78 base pairs of the first 98–base pair repeat from JCV and one complete 72–base pair repeat from SV40. Most of one of the 72–base pair repeats and the 21–base pair repeats from SV40 were deleted. The virus is termed Mad-1/SVEΔ to indicate this fact. A comparison of the restriction patterns of Mad-1/SVEΔ DNA with the prototype Mad-1 DNA were identical except for the regulatory region changes just discussed (Vacante *et al*, 1989). No additional alterations were apparent following subsequent passage of Mad-1/SVEΔ in human fetal glial cells (Vacante *et al*, 1989). We sequenced the VP1 gene of the chimeric virus and it is identical to published sequence of VP1 from the prototype Mad-1 strain (Liu *et al*, 1998).

Toxicity assay

SVG-A cells were incubated with increasing doses of chlorpromazine or clozapine for 48 h at 37°C. The cells were then stained with a 0.1% solution of Trypan blue and counted on a hemocytometer. The results shown are from three independent experiments.

Inhibition of infection assay

SVG-A cells growing on coverslips were pretreated with chlorpromazine, clozapine, or various combinations of both drugs for 45 min at 37°C. The cells were then infected with 200 hemagglutination units (HAUs) of JCV for 1 h at 37°C. The cells were then washed and incubated for an additional 48 h at 37°C in the continued presence of drug. The cells were then fixed in acetone and the percentage of infected cells scored by staining with an anti–V antigen monoclonal antibody.

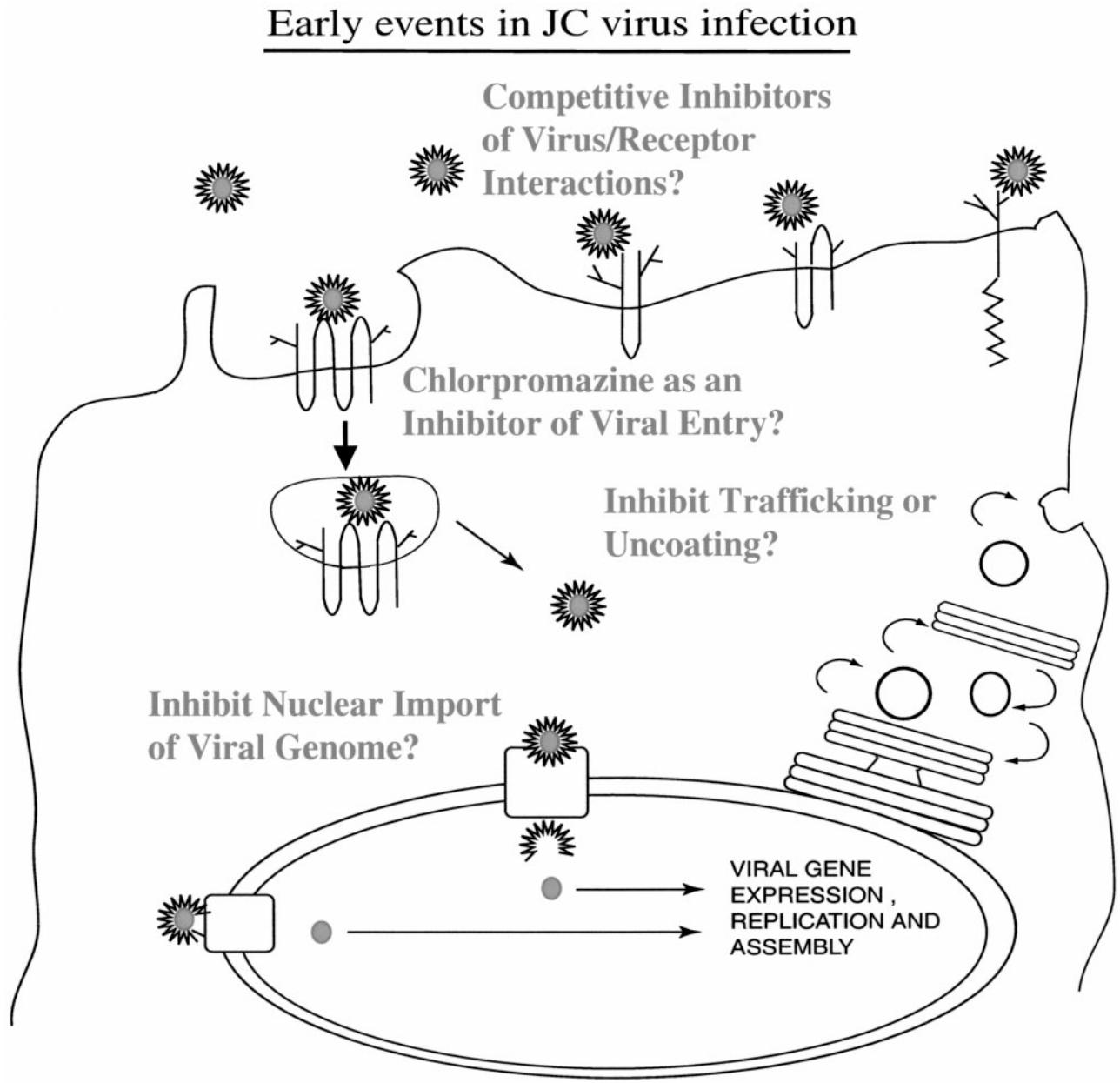

Figure 4 Early events as targets for anti-JCV–specific therapy. The model depicts the sequence of events that lead up to viral gene expression in the nucleus of the cell. As more information is learned about specific host cell receptors for JCV, it may be possible to design competitive inhibitors that would block this first step in the life cycle of the virus. Virus entry into cells represents the second critical step in the life cycle of JCV. Based on previous data and data presented in this paper, it may be possible to interfere at this stage of the viral life cycle using a combination of "typical" and "atypical" antipsychotic drugs such as chlorpromazine and clozapine. Little is known about the subsequent steps in the life cycle that lead to the delivery of the viral genome across the nuclear membrane. As we learn more about this process, specific inhibitors may be targeted to these steps in the life cycle as well. Several compounds that focus on disrupting events that occur in the nucleus (viral DNA replication) are either in the early phases of clinical trial or in the process of being developed.

References

Antinori A, Ammassari A, Giancola ML, Cingolani A, Grisetti S, Murri R, Alba L, Ciancio B, Soldani F, Larussa D, Ippolito G, De Luca A (2001). Epidemiology and prognosis of AIDS-associated progressive multifocal leukoencephalopathy in the HAART era. *J NeuroVirol* **7:** 323–328.

Atwood, WJ (2001). A combination of low-dose chlorpromazine and neutralizing antibodies inhibits the spread of JC virus (JCV) in a tissue culture model: implications for prophylactic and therapeutic treatment of progressive multifocal leukencephalopathy. *J NeuroVirol* **7:** 307–310.

Atwood WJ, Wang L, Durham LC, Amemiya K, Traub RG, Major, EO (1995). Evaluation of the role of cytokine activation in the multiplication of JC virus (JCV) in human fetal glial cells. *J NeuroVirol* **1:** 40–49.

Breitbart W, Marotta R, Platt MM, Weisman H, Derevenco M, Grau C, Corbera K, Raymond S, Lund S, Jacobson P (1996). A double-blind trial of haloperidol, chlorpromazine, and lorazepam in the treatment of delirium in hospitalized AIDS patients. *Am J Psychiatry* **153:** 231–237.

Du Pasquier RA, Clark KW, Smith PS, Joseph JT, Mazullo JM, De Girolami U, Letvin NL, Koralnik IJ (2001). JCV-specific cellular immune response correlates with a favorable clinical outcome in HIV-infected individuals with progressive multifocal leukoencephalopathy. *J NeuroVirol* **7:** 318–322.

Hall CD, Dafni U, Simpson D, Clifford D, Wetherill PE, Cohen B, McArthur J, Hollander H, Yainnoutsos C, Major E, Millar L, Timpone J (1998). Failure of cytarabine in progressive multifocal leukoencephalopathy associated with human immunodeficiency virus infection. AIDS Clinical Trials Group 243 Team. *N Engl J Med* **338:** 1345–1351.

Hou J, Major EO (1998). The efficacy of nucleoside analogs against JC virus multiplication in a persistently infected human fetal brain cell line. *J NeuroVirol* **4:** 451–456.

Itoh K, Mehraein P, Weis S (2000). Neuronal damage of the substantia nigra in HIV-1 infected brains. *Acta Neuropathol (Berl)* **99:** 376–384.

Kelly DV, Beique LC, Bowmer MI (2002). Extrapyramidal symptoms with ritonavir/indinavir plus risperidone. *Ann Pharmacother* **36:** 827–830.

Koralnik IJ, Du Pasquier RA, Kuroda MJ, Schmitz JE, Dang X, Zheng Y, Lifton M, Letvin NL (2002). Association of prolonged survival in HLA-A2+ progressive multifocal leukoencephalopathy patients with a CTL response specific for a commonly recognized JC virus epitope. *J Immunol* **168:** 499–504.

Koralnik IJ, Du Pasquier RA, Letvin NL (2001). JC virus-specific cytotoxic T lymphocytes in individuals with progressive multifocal leukoencephalopathy. *J Virol* **75:** 3483–3487.

Levy RM, Major E, Ali MJ, Cohen B, Groothius D (2001). Convection-enhanced intraparenchymal delivery (CEID) of cytosine arabinoside (AraC) for the treatment of HIV-related progressive multifocal leukoencephalopathy (PML). *J NeuroVirol* **7:** 382–385.

Liu CK, Wei G, Atwood WJ (1998). Infection of glial cells by the human polyomavirus JC is mediated by an *N*-linked glycoprotein containing terminal alpha 2-6 linked sialic acids. *J Virol* **72:** 4643–4649.

Lopez OL, Smith G, Meltzer CC, Becker JT (1999). Dopamine systems in human immunodeficiency virus-associated dementia. *Neuropsychiatry Neuropsychol Behav Neurol* **12:** 184–192.

Major EO, Miller AE, Mourrain P, Traub RG, de Widt E, Sever J (1985). Establishment of a line of human fetal glial cells that supports JC virus multiplication. *Proc Natl Acad Sci U S A* **82:** 1257–1261.

Marra CM, Rajicic N, Barker DE, Cohen BA, Clifford D, Donovan Post MJ, Ruiz A, Bowen BC, Huang ML, Queen-Baker J, Andersen J, Kelly S, Shriver S (2002). A pilot study of cidofovir for progressive multifocal leukoencephalopathy in AIDS. *AIDS* **16:** 1791–1797.

Pho MT, Ashok A, Atwood WJ (2000). JC virus enters human glial cells by clathrin dependent receptor mediated endocytosis. *J Virol* **74:** 2288–2292.

Ramachandran G, Glickman L, Levenson J, Rao C (1997). Incidence of extrapyramidal syndromes in AIDS patients and a comparison group of medically ill inpatients. *J Neuropsychiatry Clin Neurosci* **9:** 579–583.

Seeman P (2002). Atypical antipsychotics: mechanism of action. *Can J Psychiatry* **47:** 27–38.

Vacante DA, Traub R, Major EO (1989). Extension of JC virus host range to monkey cells by insertion of a simian virus 40 enhancer into the JC virus regulatory region. *Virology* **170:** 353–361.