CONCISE COMMUNICATION

Ribavirin Inhibits West Nile Virus Replication and Cytopathic Effect in Neural Cells

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West Nile virus (WNV) is an emerging mosquito-borne pathogen that was reported for the first time in the Western hemisphere in August 1999, when an encephalitis outbreak in New York City resulted in 62 clinical cases and 7 deaths. WNV, for which no antiviral therapy has been described, was recently recovered from a pool of mosquitoes collected in New York City. In anticipation of the recurrence of WNV during the summer of 2000, an analysis was made of the efficacy of the nucleoside analogue ribavirin, a broad-spectrum antiviral compound with activity against several RNA viruses, for treatment of WNV infection. High doses of ribavirin were found to inhibit WNV replication and cytopathogenicity in human neural cells in vitro.

The Flaviviridae, a family of enveloped positive-strand RNA viruses, include West Nile virus (WNV), as well as other significant human pathogens, such as hepatitis C, yellow fever, dengue, Japanese encephalitis, and St. Louis encephalitis viruses [1]. Whereas hepatitis C virus (genus *Hepacivirus*) may be transmitted by blood products or sexual activity, most members of the *Flavivirus* genus, including WNV, are transmitted by arthropods [1]. After an incubation period of 3–6 days, WNV infections typically result in subclinical or nonspecific, mild febrile illnesses lasting 3–5 days [2]. Host factors, including age and immune status, influence pathogenesis. Elderly individuals are at higher risk of progressing to fatal neurological disease, involving profound motor weakness and axonal neuropathy [3]. WNV is endemic in parts of Africa, Europe, the Middle East, and Asia [2], and it has potential to cause large epidemics in populations where background immunity is low. The largest African epidemic occurred in 1974 in the Cape province, South Africa, with 3000 clinical cases diagnosed after unusually heavy rainfalls; a large outbreak in Romania in 1996 resulted in 352 acute infections of the central nervous system, with a case-fatality rate of ~4% [2]. WNV was recognized for the first time in the Americas in August 1999, when an outbreak in New York City resulted in 62 cases of acute encephalitis. Seven patients died, and mortality among horses and birds was substantial [3]. Genetic analysis of the envelope protein sequence indicated that the viral strain most closely resembled an isolate from a goose in Israel in 1998, demonstrating the wide geographic distribution of this emerging pathogen [4, 5].

Antiviral therapy for hepatitis C has been described [6]; however, no specific treatment has been reported for WNV or other flavivirus infections. We recently reported that the presence of WNV RNA in cerebrospinal fluid may be associated with poor clinical outcome [7]. These findings, together with indications that WNV has persisted in mosquitoes in New York City during the winter [8], emphasize the importance of rapid diagnosis of WNV infections and of establishing antiviral treatment. We therefore tested a panel of nucleoside analogues for antiviral activity against WNV.

Materials and Methods

Cells and virus. Human oligodendroglial (OL) cells [9] were cultured in a humidified atmosphere at 37°C, 5% CO_2 in Dulbecco's modified Eagle medium/high glucose (DMEM; Irvine Scientific, Santa Ana, CA) supplemented with 10% heat-inactivated fetal calf serum (FCS), 2 m*M* glutamine, 100 U/mL penicillin, and 100 μg/mL streptomycin. *Aedes albopictus* C6/36 cells were cultured at 25°C in Leibovitz's L-15 medium (Gibco-BRL, Gaithersburg, MD) supplemented with 10% heat-inactivated FCS, 10% tryptose phosphate broth (Gibco-BRL), 100 U/mL penicillin, and 100 μg/mL streptomycin. Virus was recovered after DMRIE-C (Gibco-BRL) liposome-mediated transfection of WNV-NY1999 RNA into OL cells. Virus-maintenance medium was a 1:1 mixture of supplemented DMEM and OPTI-MEM I reduced-serum medium (Gibco-BRL) resulting in 5% final serum concentration. Virus was passaged only once in either OL or C6/36 cells after recovery by RNA transfection; virus stocks were prepared as clarified OL cul-

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ture supernatant when ~80% of the cells had cytopathic effect or as C6/36 culture supernatant 4 days after infection with the initial OL virus isolate. Plaque-forming units were determined on confluent OL cell layers by serial dilution of viral stock in duplicates after overlay with 1% low-melting agarose in DMEM.

Antiviral drug evaluation. Nucleoside analogues (3-deazaguanine; ICN 10169; ICN 10776; ICN 15100; 2′,3′-dideoxyinosine; ribavirin; ribavirin analogues ICN 17261 and ICN 17377; FTC [β-L-2′,3′-dideoxy-5-fluoro-3′-thiacytidine]; and pyrazofurin) were provided by ICN Pharmaceuticals (Costa Mesa, CA). The drugs were dissolved in dimethyl sulfoxide to 100 m*M* and in OPTI-MEM to working concentrations. Two hours before treatment with drug at various concentrations, OL cells were seeded in virus-maintenance medium to 80% confluence on microtiter plates and were infected at a multiplicity of 0.1. Half the wells of each plate were treated with drug but not infected. After 3 days, 50 μL of supernatant were transferred onto fresh microtiter plates for cytotoxicity assays (release of lactate dehydrogenase from lysed cells; CytoTox 96; Promega, Madison, WI); 50 μL of supernatant was extracted with 150 μL Tri-Reagent LS (Molecular Research, Cincinnati, OH) for real-time reverse transcriptase (RT)–polymerase chain reaction (PCR) analysis [7], using diagnostic primers directed against the NS5 region; the remaining 100 μL of supernatant and the underlying cell layer were supplemented with XTT labeling mix to assay cell proliferation (mitochondrial metabolism of XTT tetrazolium salt; Roche, Basel, Switzerland). The cytotoxicity and cell proliferation assays were measured with a v_{max} plate reader (Molecular Devices, Sunnyvale, CA) at 490 nm, with 650 nm as reference wavelength.

Results

A panel of 10 nucleoside analogues was screened for antiviral activity in WNV-NY1999–infected human OL cells at 20 μ*M* concentrations. This initial screening was performed by size fractionation and Northern blot analysis of total RNA followed by hybridization with probes against the NS5 polymerase gene and host-cell glyceraldehyde-3-phosphate-dehydrogenase (GAPDH) as reference (data not shown). Two compounds, pyrazofurin and ribavirin, had antiviral activity. Pyrazofurin affects activity of orotate monophosphate decarboxylase in pyrimidine biosynthesis. Because of its high cytotoxicity, pyrazofurin was not tested further. However, the drug's ability to reduce WNV genomic RNA to levels <1% of GAPDH-normalized infected untreated control values suggests that further drug screening using less toxic derivatives may be useful.

For a detailed evaluation of the activity of ribavirin, we measured 3 parameters (figure 1): (1) cell lysis due to cytopathic effect and drug-induced cytotoxicity was measured via the release of a stable cytoplasmic enzyme (lactate dehydrogenase) into the culture supernatant; (2) viral replication was measured with real-time PCR, using diagnostic primers that amplified a 69-bp fragment from the NS5 region, detected by a linear fluorochrome/quencher probe annealing within that region; and (3) inhibition of cellular proliferation and viability due to infection or drug side effects was measured at the end of treatment by mitochondrial metabolic activity.

Ribavirin reduced extracellular WNV RNA by ~50% at 60-μ*M* (ED_{50}) and by ~90% at 190-μ*M* (ED_{90}) concentrations (figure 1*A*); the reference value of 100% extracellular viral RNA was determined by real-time RT-PCR, using RNA isolated from the supernatant of infected untreated cells. At the ED_{50} and ED_{90} concentrations, cytopathogenicity decreased from 90% to 20% of the reference value obtained with infected untreated controls (figure 1*A*). Cell proliferation of infected untreated cells was ~55% of the noninfected untreated controls, and it recovered to 95% at 200 μ*M* ribavirin (figure 1*A*, 1*C*). The titer of WNV was reduced by 4 orders of magnitude (from 10^7 to 10^3 pfu/mL) in the presence of 200 μ*M* ribavirin (figure 1*D*). The greater reduction in plaque-forming units than in extracellular viral RNA at 200 μ*M* ribavirin suggests that not all RNA measured by RT-PCR is present as infectious units. In noninfected cells, ribavirin did not inhibit cell growth or produce cytotoxic effects at concentrations as high as 400 μ*M* (figure 1*B*). All studies were performed in duplicate. Results were similar for virus stocks generated on OL or C6/36 cells and for 2 batches of ribavirin. On the basis of these experiments, we estimate that ribavirin has an in vitro selectivity index of ~7.

Discussion

Ribavirin is a guanosine analogue with in vitro antiviral activity against a broad spectrum of RNA and DNA viruses, including members of the Arenaviridae, Bunyaviridae, Flaviviridae, Herpesviridae, Orthomyxoviridae, Paramoyxoviridae, and Bornaviridae families. Within the Flaviviridae, in vitro activity has been demonstrated for yellow fever, dengue types 1–4, banzi, Japanese encephalitis [10, 11], and hepatitis C viruses. Ribavirin has been used to treat human patients infected with respiratory syncytial [12], Lassa [13], Hantaan [14], La Crosse [15], and (in combination with interferon-α) hepatitis C [6] viruses. Ribavirin is phosphorylated by cellular enzymes and is postulated to exert antiviral effects by several mechanisms: (1) depletion of the intracellular GTP pool by interference with host-cell inosine monophosphate (IMP) dehydrogenase, the enzyme that converts IMP to xanthosine monophosphate, a precursor molecule in the biosynthesis of GTP and dGTP; (2) interference with mRNA capping guanylylation; (3) specific inhibition of some viral polymerases; and (4) enhancement of the Th1 antiviral immune response [11, 16].

After oral administration, ribavirin concentrations in cerebrospinal fluid are ~70% of those in serum [17]. We are not aware of published data concerning levels of ribavirin in the brain after intravenous administration; however, on the basis of results from the intravenous 7-day regimens employed for

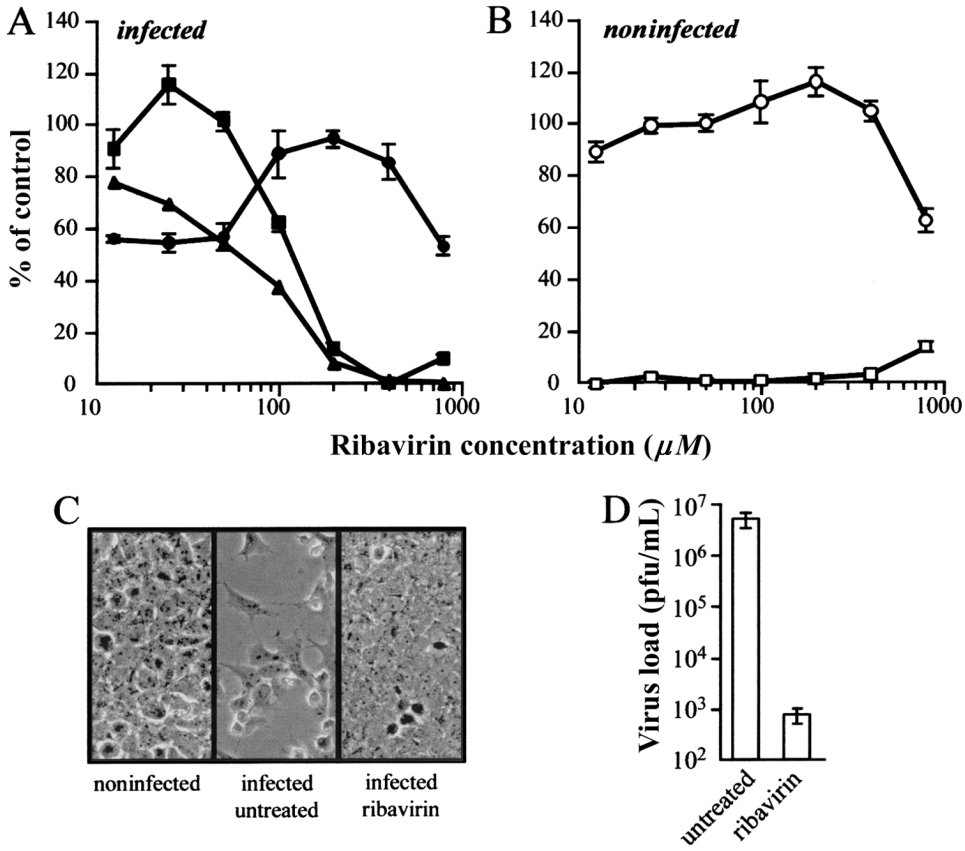

Figure 1. Concentration-dependent effect of ribavirin on viral replication, cell proliferation, and cytopathogenicity in oligodendroglial (OL) cells. Two hours before treatment with ribavirin at various concentrations, cells were infected with WNV-NY1999 at a multiplicity of 0.1. Cells were assayed 3 days later for host cell proliferation (●), cytopathogenicity (■), and amount of viral RNA in the culture supernatant (▲; *A*). Noninfected cells were assayed in parallel for cell proliferation (○) and drug-induced cytotoxic effect (□; *B*). Results are expressed as percentage of control values. One hundred percent cellular proliferation values were obtained from untreated uninfected cells. One hundred percent viral replication and cytopathogenicity values were obtained from untreated infected cells. Reduction of cytopathogenicity (magnification, ×200; *C*) and virus load (plaque-forming units per millimeter; *D*) after 3 days treatment with 200 μM ribavirin are also shown.

treatment of La Crosse encephalitis and Hantaan fever with a loading dose of 25–33 mg/kg of body weight followed by 24–64 mg/kg of body weight per day administered intravenously [14, 15], we extrapolate that intravenous administration of ribavirin will probably result in ~100 μM concentrations in the cerebrospinal fluid. This concentration exceeds our measured ED_{50} of 60 μM for reduction of extracellular viral RNA. Although a higher concentration of ribavirin in cerebrospinal fluid may be more desirable, the efficacy:benefit ratio will be revealed only through clinical studies. It is important to note that reversible hemolytic anemia can occur at these doses and may require transfusion [18].

One pool of mosquitoes collected in New York City in February 2000 yielded live WNV [8], indicating that this emerging pathogen has established its presence in the Western hemisphere. WNV is not yet endemic in the Americas, and the short period since its introduction, in August 1999, may aggravate outbreaks until background immunity has developed in the human and peridomestic animal population. It is clearly important to identify drugs for treatment of WNV that will have better selectivity and therapeutic indices than ribavirin. Furthermore, the efficacy of ribavirin in animal models of WNV encephalitis remains to be addressed. Nonetheless, our findings suggest that ribavirin at high doses may improve prognosis in individuals with WNV encephalitis.

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