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

Ingo Jordan,1 Thomas Briese,1 Nicole Fischer,1 Johnson Yiu-Nam Lau,2 and W. Ian Lipkin1

1Emerging Diseases Laboratory, Departments of Microbiology and Molecular Genetics, Neurology, and Anatomy and Neurobiology, University of California, Irvine, and 2ICN Pharmaceuticals, Costa Mesa, California

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.

Materials and Methods

Cells and virus. Human oligodendroglial (OL) cells [9] were cultured in a humidified atmosphere at 37°C, 5% CO2, in Dulbecco’s modified Eagle medium/high glucose (DMEM; Irvine Scientific, Santa Ana, CA) supplemented with 10% heat-inactivated fetal calf serum (FCS), 2 mM 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-
n culture 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-t-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 μM and in OPTIMEM 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 cytopathology 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 Vmax 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 μM 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-μM (ED50) and by ~90% at 190-μM (ED90) concentrations (figure 1A); 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 ED90 concentrations, cytopathogenicity decreased from 90% to 20% of the reference value obtained with infected untreated controls (figure 1A). Cell proliferation of infected untreated cells was ~55% of the noninfected untreated controls, and it recovered to 95% at 200 μM ribavirin (figure 1A, 1C). The titer of WNV was reduced by 4 orders of magnitude (from 10^7 to 10^3 pfu/mL) in the presence of 200 μM ribavirin (figure 1D). The greater reduction in plaque-forming units than in extracellular viral RNA at 200 μM 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 μM (figure 1B). 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, Paramyxoviridae, and Bornaviridae families. Within the Flaviviridae, in vitro activity has been demonstrated for yellow fever, dengue types 1–4, banz, 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 virus [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
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 \( \mu M \) concentrations in the cerebrospinal fluid. This concentration exceeds our measured ED\(_{50}\) of 60 \( \mu M \) 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.

Acknowledgments

We thank Charlie Calisher for helpful comments and Stanley Lipkin for research support.

References