

Inhibition of Borna Disease Virus Replication by Ribavirin

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The guanosine analogue ribavirin was tested for antiviral activity in two neural cell lines, human oligodendrocytes and rat glia, against Borna disease virus (BDV) strains V and He/80. Ribavirin treatment resulted in lower levels of virus and viral transcripts within 12 h. Addition of guanosine but not adenosine resulted in a profound reduction of the ribavirin effect. Ribavirin appears to be an effective antiviral agent for treatment of BDV infection in vitro. A likely mechanism for its activity is reduction of the intracellular GTP pool, resulting in inhibition of transcription and capping of BDV mRNAs.

Borna disease virus (BDV) is a nonsegmented negative-strand RNA virus that transcribes and replicates in the nucleus of infected cells (4, 7) and employs a number of strategies to direct expression of its genome including RNA splicing, overlap of transcription units and transcriptional signals, readthrough of transcription initiation sites, and differential use of translational initiation codons (reviewed in reference 39). In concert, these result in noncytolytic replication and persistence and may contribute to BDVs neurotropism. Although BDV is classically associated with meningoencephalitis of horses and sheep in central Europe, more recent data (3, 5, 26, 27, 29, 46) indicate that the host range of BDV probably extends to most warmblooded animals and suggest that the geographic distribution includes Asia and North America as well as greater Europe (14, 21, 26, 31, 32, 46).

The spectrum of clinical disease due to BDV infection ranges from subtle impairment of learning and memory to progressive, immune-mediated, frequently fatal meningoencephalitis (1, 9, 33, 38, 41). Asymptomatic infection has also been described (14, 21, 32, 37). Recognition of BDV's broad host range and predilection to target higher integrative circuits of the brain and the observation that disturbances in infected animals are reminiscent of some aspects of human neuropsychiatric diseases led to the proposal that BDV might be implicated in their pathogenesis. Although there is consensus that humans are likely to be susceptible to BDV infection, the epidemiology and clinical consequences of human infection remain controversial (reviewed in reference 18).

Amantadine has been reported to be effective in vivo and in vitro against one strain of BDV. Bode and coworkers (2) described clearance of strain Hu-HI from cultured oligodendrocytes and from peripheral blood mononuclear cells of a patient with bipolar disorder. However, an antiviral effect of amantadine was not confirmed with other BDV isolates in studies of infected cultured cells, rodents, or horses (6, 11, 13, 16, 42).

In an effort to identify antiviral agents for BDV, we tested an encoded panel of 10 nucleoside analogues (Table 1) for activity against the two best-characterized laboratory strains of BDV, strains V and He/80, in two neural cell lines, OL (human oligodendrocytes) and C6 (rat glia). Strain V was originally isolated from naturally infected horse brain in 1929 (48), and

strain He/80 was originally isolated from naturally infected horse brain in 1980 (19). Both viruses were later passaged in rabbits, rats, and a variety of cultured cells. Each of the compounds was initially tested with uninfected cells to establish a toxicity threshold defined as growth arrest (Table 1). Thereafter, efficacy against BDV was tested by using 10-fold dilutions of drug beginning at the toxicity threshold. One compound of the 10 examined, ribavirin, significantly reduced viral load at concentrations of 2 to 200 μ M. No effect was seen with the other compounds, including 2',3'-dideoxyinosine (ddI) (Table 1).

To further characterize the antiviral effect of ribavirin, OL cells persistently infected with strain V (Fig. 1A and C) and C6 cells persistently infected with strain He/80 (Fig. 1B and D) were treated for up to 13 days with 20 μ M ribavirin or ddI (negative control). At 3, 6, 9, and 12 days (OL cells) or 4, 7, 10, and 13 days (C6 cells) the numbers of host cells were determined and infectious virus and total cellular RNA were isolated from cells treated with ribavirin, ddI, or vehicle (dimethyl sulfoxide without antiviral drug). Virus was titered by focus immunoassay using fetal rat glial cells and antiserum against viral nucleocapsid and phosphoproteins (P) (35). mRNA originating from the second transcription unit encoding the BDV P was quantitated by Northern hybridization (24) and PhosphorIm-

TABLE 1. Toxic threshold and efficacy of nucleoside analogues tested for properties against BDV

Compound	Toxic threshold ^a (μ M) for:		Antiviral activity (fold) ^b
	OL	C6	
3-Deazaguanine	20	200	
ICN 10169	2	2	
ICN 10776	20	2	
ICN 15100	200	200	
2',3'-Dideoxyinosine (ddI)	200	200	
Ribavirin	200	200	>10
ICN 17261 ^c	20	200	
FTC ^d	200	200	
Pyrazofurin	2	2	
ICN 17377 ^c	200	20	5–10

^a Maximum concentration at which replication of treated cells was within 10% of that of the vehicle-treated cells after 1 week.

^b Reduction of virus load determined after 1 week of treatment and compared to vehicle-treated control at the highest nontoxic concentration.

^c Ribavirin analogue.

^d β -L-2',3'-Dideoxy-5-fluoro-3'-thiacytidine.

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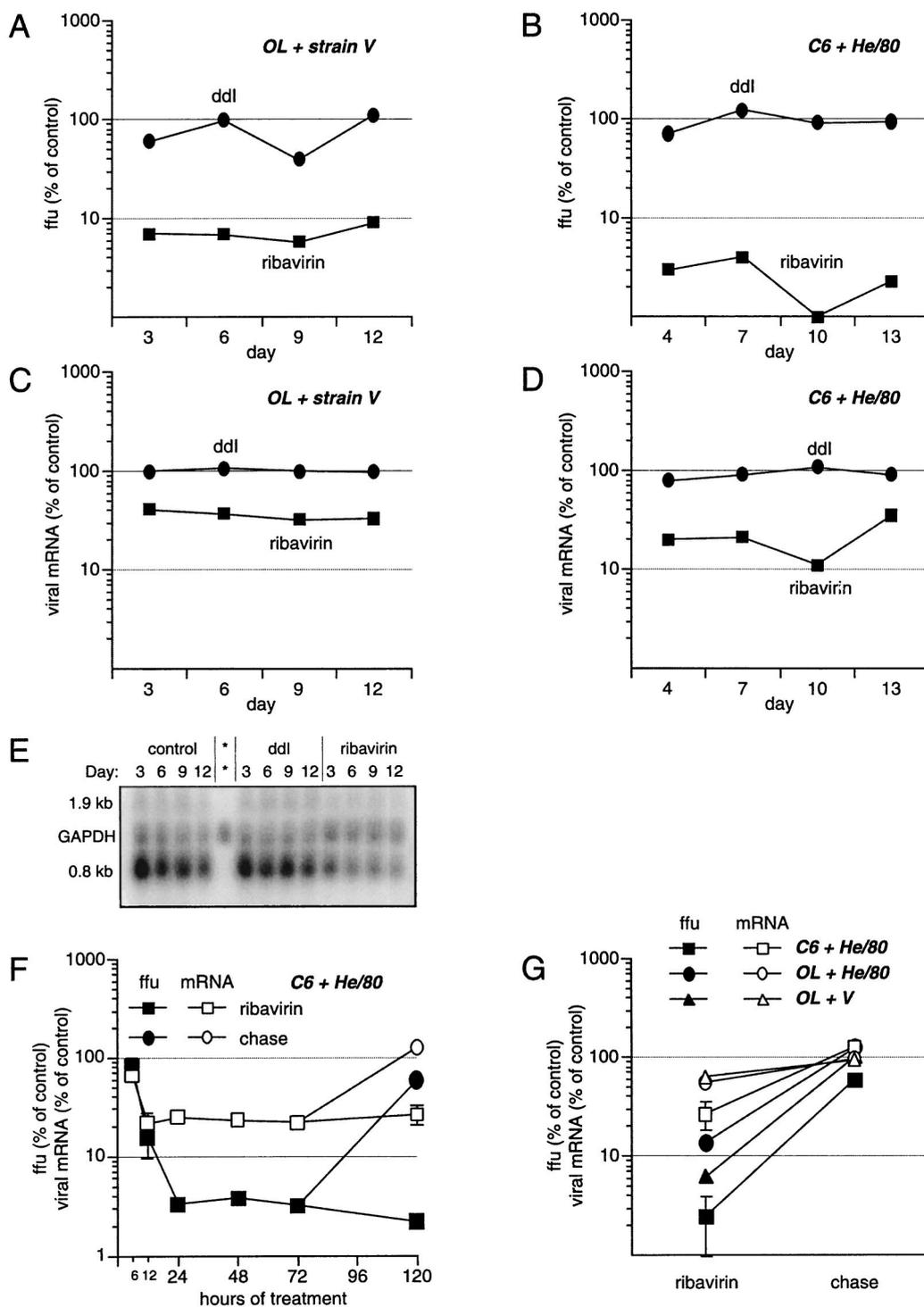


FIG. 1. Effect of ribavirin on viral titers and RNA levels. BDV strain V- (A and C) or He/80- (B and D) infected cells were treated with 20 μ M ribavirin (squares) or 20 μ M ddi (circles). Virus and RNA were isolated in parallel. All experiments were done in duplicate. (A and B) Viral titers in focus-forming units (ffu) normalized to the number of cells were determined in a focus immunoassay and are given as the percentages of the titers for vehicle-treated controls (with 100% corresponding to approximately 5×10^6 ffu/ml from 4×10^6 cells). (C and D) Amounts of viral 0.8-kb mRNA were determined by quantification of Northern blots using a PhosphorImager (Molecular Dynamics) and are given as the percentages of the amounts for vehicle-treated controls, normalized to the mRNA encoding GAPDH. (E) Representative Northern blot used for quantification of mRNA levels in strain V-infected OL cells. The asterisks indicate a negative control with RNA isolated from uninfected cells. The 1.9-kb RNA is a viral, non-mRNA also containing the P open reading frame. (F) Viral titers (filled squares) and amount of 0.8-kb mRNA (open squares) in the first 6, 12, 24, 48, and 72 h of ribavirin treatment, followed by a release from ribavirin for 2 days (filled circle for viral titer and open circle for viral mRNA). (G) Effect of the host cell on ribavirin susceptibility. Cells in the acute phase of infection with strain V or He/80 were treated for 2 weeks with ribavirin.

ager (Molecular Dynamics) analysis. GAPDH (glyceraldehyde-3-phosphate dehydrogenase) was used as host cell mRNA control.

Viral titers and viral transcripts rapidly declined in ribavirin-but not in ddI-treated cells. In OL cells, titers of strain V were reduced by more than 90% within 3 days and remained at this level through the end of the 12-day sampling period (Fig. 1A). The ribavirin effect was even more striking in C6 cells, where titers of He/80 were reduced by 97% within 4 days and 99% at day 10 (Fig. 1B). BDV transcripts were reduced by 60% in OL cells (Fig. 1C) and 80% in C6 cells (Fig. 1D).

To determine the kinetics of ribavirin's antiviral activity, BDV P gene mRNA expression and titers were studied at shorter time intervals in C6 cells infected with strain He/80. Six hours into treatment with ribavirin, levels of P gene mRNA were reduced to 65% of untreated control values; steady-state levels of approximately 20% of untreated control values were observed within 12 h of initiating ribavirin treatment (Fig. 1F, open squares). Viral titers also fell rapidly but did not reach nadirs until 24 h after the addition of ribavirin. The reduction in viral titer was 20% at 6 h, 85% at 12 h, and 97% at 24 h (Fig. 1F, filled squares). The response of BDV to ribavirin was also brisk following withdrawal of treatment, suggesting rapid turnover of viral RNA. Viral titers and mRNA expression recovered fully within 2 days after removal of ribavirin from cultures (Fig. 1F, filled and open circles; chase from 72 [day 3] to 120 h [day 5] posttreatment). The time course for the effect of ribavirin on BDV P gene expression and titer was similar in strain V-infected OL cells (data not shown).

The antiviral effect of ribavirin was less pronounced on strain V in OL cells (Fig. 1A) than on strain He/80 in C6 cells (Fig. 1B). Thus, to assess the relative importance of viral strain and host cell phenotype on ribavirin effect, OL cells were infected with He/80 and treated with ribavirin. Although strain He/80 was highly susceptible to ribavirin in C6 cells ($2.5\% \pm 1.5\%$ of control titers after 2 weeks of treatment; Fig. 1G), it was less inhibited in OL cells ($13.6\% \pm 1.7\%$ of control values; Fig. 1G). Interestingly, in OL cells, strain V was more sensitive to ribavirin ($6.2\% \pm 0.2\%$ of control titers) than He/80. Together, these findings suggest that the potency of ribavirin is determined in part by virus-host interactions.

In X-ray diffraction studies, ribavirin closely resembles guanosine (36) and is postulated to interfere with the activity of the inosine monophosphate (IMP) dehydrogenase (43), the enzyme that converts IMP to xanthosine monophosphate, a precursor molecule in the biosynthesis of GTP and dGTP. The resulting depletion of the intracellular GTP pool suggests two mechanisms by which ribavirin might exert antiviral activity: (i) interference with viral transcription and (ii) inhibition of mRNA capping guanylation after phosphorylation at the 5' position (10, 12, 47). There is evidence that both mechanisms are operative in BDV-infected cells treated with ribavirin. Ribavirin-treated cells had lower levels of viral mRNAs (Fig. 1B and D), a result consistent with inhibition of transcription. An even more profound ribavirin effect was observed with respect to viral titers (Fig. 1A and C). The latter finding is consistent with an additional functional deficit in BDV transcripts due to inhibition of capping and reduced efficiency of translation. Support for the hypothesis that ribavirin acts through interference with GTP/dGTP biosynthesis has been found in other viral systems, for example, measles virus, where the antiviral effect of ribavirin *in vitro* is abrogated by addition of guanosine but not adenosine (43). To test whether a related mechanism applies to the antiviral effect of ribavirin in BDV, we measured viral titers in strain V-infected OL cells after 3 days of treatment with ribavirin, adenosine, or guanosine, in-

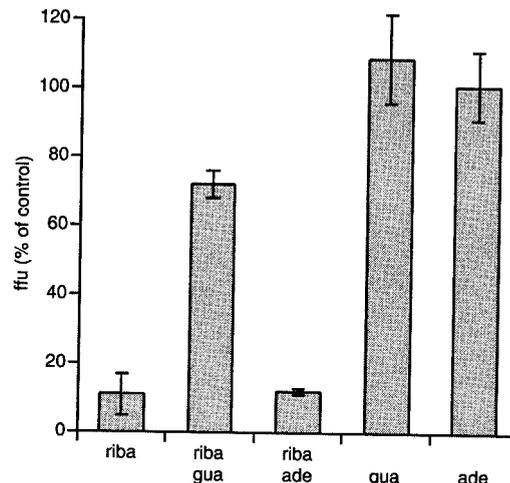


FIG. 2. BDV strain V-infected OL cells were treated for 3 days with 10 μ M ribavirin (riba), 10 μ M adenosine (ade), and 10 μ M guanosine (gua) individually or in the indicated combinations. Virus was quantified by focus-immunoassay titration and is given as the percentage of the vehicle-treated control.

dividually or in combination (Fig. 2). Viral titers were reduced to 10% in the presence of ribavirin or a combination of ribavirin and adenosine. In contrast, an equimolar amount of guanosine significantly inhibited the antiviral activity of ribavirin, allowing viral titers to reach 70% of the level obtained in the negative controls (Fig. 2). Guanosine or adenosine had no effect on viral replication in the absence of ribavirin.

While this article was in preparation, Mizutani and colleagues (30) showed through reverse transcription-PCR experiments that ribavirin treatment resulted in lower levels of BDV RNAs in a non-neural cell line (MDCK) infected with strain He/80 (30). Our experiments confirm and extend these findings by demonstrating that ribavirin has activity against two different strains of BDV (strain V and He/80) in two neural cell lines of different lineages (human oligodendrocytes and rat glia). The significance of our data is underscored by the controversy in the literature concerned with effects of amantadine on BDV replication where strains and cell lines are considered to be important variables. Furthermore, we have monitored the effect on virus titer as well as viral transcripts and provided data that suggest that ribavirin not only reduces the amount of viral RNA but also interferes with their function as messengers.

In vivo, ribavirin is effective in the treatment of respiratory syncytial virus infections (15, 44) and, in combination with interferon α , of chronic hepatitis C virus infections (8, 28). Although our *in vitro* studies indicate that ribavirin inhibits BDV at 2 μ M (data not shown), a concentration well below that associated with toxicity *in vitro* (200 μ M) and within the physiologic concentration in plasma of 0.5 to 10 μ M (22), it is conceivable that use of ribavirin in BDV-infected animals may enhance rather than inhibit disease. Ribavirin was recently shown to promote Th₁ responses (20, 25, 34, 45). It has been suggested that this Th₁ effect may contribute to antiviral activity in hepatitis C virus infections (20). However, severity of disease in BDV infections is correlated with the Th₁ immune response (17, 40). Indeed, although vaccination of rats with BDV nucleoprotein constructs results in enhanced Th₁ responses and viral clearance, the clinical outcome is adverse due to increased mortality and morbidity (23). Thus, it is difficult to predict whether the antiviral or Th₁ effect of ribavirin will prevail *in vivo*. Future work will be directed toward assessing

the therapeutic utility of ribavirin by treatment of experimental BDV infection and exploring ribavirin analogues that have selective antiviral or immunomodulatory activity.

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