

## Ribavirin Reduces Mortality in Enterovirus 71–Infected Mice by Decreasing Viral Replication

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**Enterovirus 71 (EV71) causes fatal encephalitis in young children. However, there is no effective antiviral drug available for infected patients. Ribavirin is currently used for the treatment of several RNA virus infections clinically, so its anti-EV71 efficacy was evaluated. In vitro results showed that ribavirin effectively reduced the viral yields (with an IC<sub>50</sub> of 65 µg/mL) and virus-induced cytopathic effect in human and mouse cell lines. In vivo results showed that ribavirin reduced the mortality, morbidity, and subsequent paralysis sequelae in infected mice by decreasing viral loads in tissues. Thus, ribavirin could be a potential anti-EV71 drug.**

Enterovirus 71 (EV71), a member of the family Picornaviridae, infects humans by the fecal-oral route before inducing herpangina, viremia, and hand-foot-and-mouth disease. The initial illness is self-limited, but it is sometimes followed by fatal neurological symptoms, such as aseptic meningitis, brain-stem encephalitis, encephalomyelitis, and acute flaccid paralysis similar to paralytic poliomyelitis, particularly in infants and young children [1, 2]. Subsequently, some survivors of severe cases are left with long-term neurological sequelae, delayed neurodevelopment, and reduced cognitive functioning [3]. EV71 outbreaks

have been reported periodically throughout the world. The largest and most severe outbreak occurred in Taiwan in 1998; 129,106 cases of herpangina and hand-foot-and-mouth disease, 405 cases of neurological and cardiopulmonary complications, and 78 deaths were reported [2]. Since then, EV71 infection has become endemic in Taiwan and caused >40 deaths per year in 2000 and 2001.

Currently, there is no effective antiviral drug available to treat patients with EV71 infection. Only supportive care and intravenous immunoglobulin are available for severe cases, but the therapeutic efficacy of intravenous immunoglobulin seems limited [1]. Among antiviral compounds, pleconaril has been shown to inhibit picornaviruses, including coxsackievirus and rhinovirus [4], but has failed to show a protective effect in EV71-infected patients [1] or to reduce the cytopathic effect (CPE) in cells induced by EV71 isolated from a patient during the outbreak in Taiwan in 1998 [4]. To date, pyridyl imidazolidinone derivatives are the only compounds with promising anti-EV71 activity in vitro [4]; however, it will take years for these new compounds to be approved for use in patients.

Ribavirin is a nucleoside analogue with broad-spectrum antiviral activity against a variety of viruses, such as coxsackievirus and echovirus among picornaviruses [5, 6]. It is currently used to treat patients infected with hepatitis C virus (HCV) or respiratory syncytial virus [7, 8]. It has also been shown to reduce the severity of disease in patients with encephalitis induced by Nipah virus, La Crosse virus, or measles virus and in patients with hemorrhagic fever induced by Lassa fever virus or hantavirus [9, 10]. In addition, it is recommended for clinical trials in patients with encephalitis induced by West Nile virus, Junin virus, or Rift Valley fever virus [10–12]. Thus, the anti-EV71 efficacy of ribavirin was evaluated in the present study. Our results showed that ribavirin reduces EV71 replication in vitro and in vivo.

**Methods.** Human muscular (rhabdomyosarcoma [RD]) and neuronal (SK-N-SH) and mouse neuronal (N18) cell lines were maintained in medium according to the instructions of the American Type Culture Collection. EV71 strain Tainan/4643/98 (GenBank accession number AF304458) [13] isolated from a patient with encephalomyelitis in Taiwan in 1998 was propagated and titrated in RD cells and used to infect human cell lines. Tainan/4643/98 was passed in mice 4 times to obtain mouse-adapted strain MP4 [13]. MP4 was passed in mice 2 more times in our laboratory and designated strain M2. M2 was propagated and titrated in RD cells and used to infect N18 cells or mice.

For the antiviral assay, RD and SK-N-SH cells ( $6 \times 10^5$ ) were infected with 5000 pfu of Tainan/4643/98, and N18 cells

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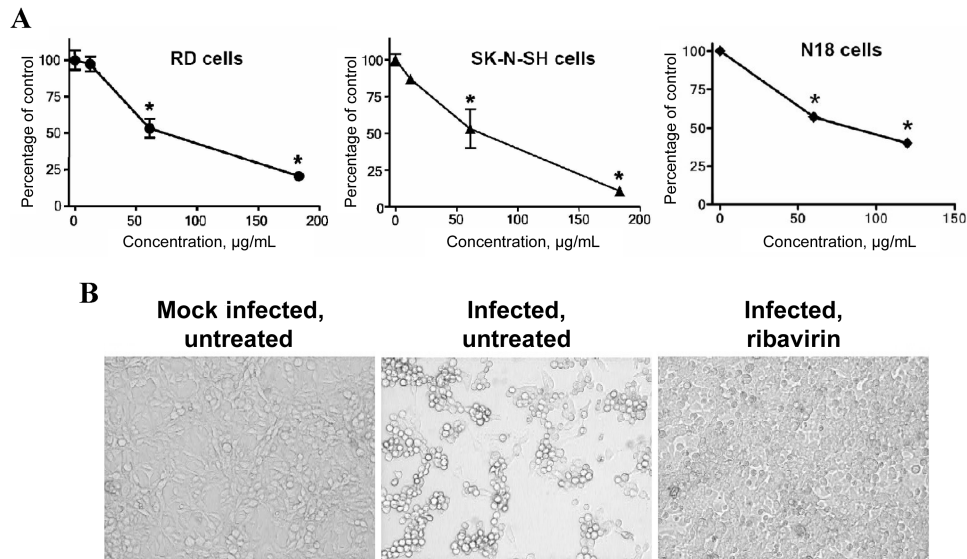
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**Figure 1.** A, Inhibition of EV71 replication by ribavirin in rhabdomyosarcoma (RD), SK-N-SH, and N18 cell lines. The viral titers in the ribavirin-treated cell sample were divided by those in the untreated sample and expressed as percentages of control values. The data shown are mean  $\pm$  SE values from 2 independent experiments, each done in duplicate. \* $P < .05$ , compared with untreated sample (Student's  $t$  test). B, Reduction of virus-induced cytopathic effect by ribavirin in human neuronal cells. SK-N-SH cells were mock-infected or infected with EV71, cultured in medium without or with ribavirin (60  $\mu\text{g}/\text{mL}$ ), and observed under a microscope. Photomicrographs were obtained 25 h after infection (original magnification,  $\times 100$ ). The data shown represent 2 experiments.

( $6 \times 10^5$ ) were infected with 5000 pfu of M2. The infected cells were cultured in medium with or without various concentrations of ribavirin (MP Biomedicals) 2 h after infection. Infected RD, SK-N-SH, and N18 cells were harvested at 48, 25, and 25 h after infection, respectively, to determine viral yields by plaque assay on RD cells.

To determine whether ribavirin reduces virus-induced CPE, SK-N-SH cells were mock-infected or infected with 5000 pfu of Tainan/4643/98, cultured in medium without or with ribavirin (60  $\mu\text{g}/\text{mL}$ ), and observed under a microscope. Photomicrographs were obtained at 25 h after infection.

All mouse experiment protocols for the mouse protection assay were approved by the Laboratory Animal Committee at National Cheng Kung University. To test the anti-EV71 efficacy of ribavirin in vivo, 12–14-day-old ICR mice (Charles River Laboratories) were infected with  $5 \times 10^4$  to  $1 \times 10^5$  pfu of M2 by intraperitoneal inoculation. Infected mice were given 1 intraperitoneal injection of PBS or ribavirin (100 mg/kg) daily for 9 days, starting 2 h after infection. Infected mice were monitored daily for 30 days for signs of disease and survival, and the clinical score was graded as follows: 0, healthy; 1, ruffled hair; 2, weakness in hind limbs; 3, paralysis in single hind limb; 4, paralysis in both hind limbs; and 5, death. In separate experiments, the brains and spinal cords of infected mice treated with ribavirin or PBS were collected on day 5 after infection. The brain stems (pons-medulla region) were dissected from the brains. Samples were frozen, homogenized, and frozen again. The frozen samples

were thawed and sonicated, and the viral titers of samples were determined by plaque assay on RD cells.

**Results.** The anti-EV71 efficacy of ribavirin was first tested in vitro using human muscular (RD) and neuronal (SK-N-SH) cell lines infected with a clinical isolate of EV71 (strain Tainan/4643/98) and a mouse neuronal (N18) cell line infected with a mouse-adapted EV71 strain (M2) derived from Tainan/4643/98. The infected cells were treated with or without various concentrations of ribavirin at 2 h after infection and then harvested to determine viral titers from 25 to 48 h after infection. The viral titers of RD, SK-N-SH, and N18 cell cultures without ribavirin treatment reached  $\sim 9, 9,$  and  $6 \times 10^4$  pfu/culture, respectively. Under these conditions, ribavirin was able to reduce the viral yields of RD, SK-N-SH, and N18 cells in a dose-dependent manner (figure 1A), demonstrating that the inhibition of EV71 replication by ribavirin is not a phenomenon specific to human or mouse cells or to a particular cell type. Additional experiments showed that ribavirin did not significantly affect the proliferation of SK-N-SH cells and had little toxicity to the cells (data not shown; figure 2).

Ribavirin effectively inhibited EV71 replication in infected cells, so we further investigated whether it could reduce virus-

The figure is available in its entirety in the online edition of the *Journal of Infectious Diseases*.

**Figure 2.** No significant effect of ribavirin on the proliferation of SK-N-SH cells, and little toxic effect on the cells.

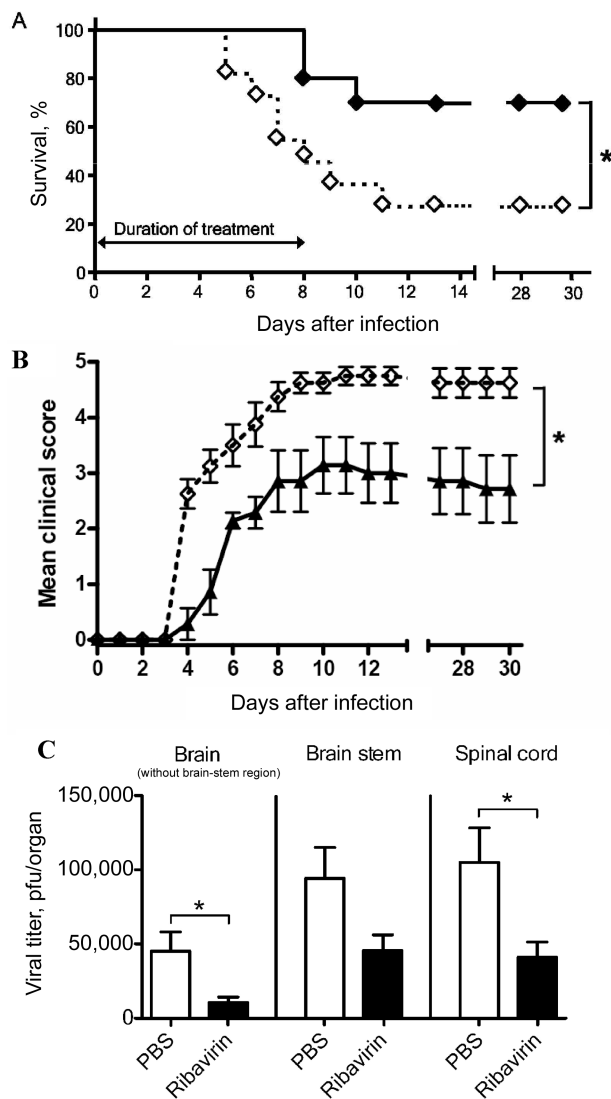
induced CPE. When infected SK-N-SH cells were not treated with ribavirin, >90% of cells developed CPE 25 h after infection (figure 1B). However, the monolayer of infected cells treated with 60  $\mu\text{g}/\text{mL}$  ribavirin remained as intact as that of mock-infected cells. These data indicate that ribavirin prevents virus-induced CPE.

The antiviral and protective effects of ribavirin were tested in vivo by assessing its ability to reduce mortality and morbidity in EV71-infected mice. Mice were intraperitoneally infected with M2 and then treated with ribavirin (100 mg/kg) once daily. All infected mice given PBS developed hind limb paralysis with signs of encephalitis manifested by hunched posture, lethargy, ataxia, and anorexia, and only 27% of mice (3/11) survived (figure 3A). The infected mice treated with ribavirin showed a marked reduction in mortality, with a survival rate of 70% (7/10), significantly higher than that in PBS-treated mice ( $P < .05$ , log-rank test). Ribavirin-treated mice did not show obvious symptoms on day 5 after infection, whereas PBS-treated mice began to develop paralysis in their hind limbs (figure 3B). The hind limbs of ribavirin-treated mice were slightly paralyzed, but the severity was significantly less than that in PBS-treated mice from day 5 to day 30 after infection ( $P < .05$ , Wilcoxon test). Thus, ribavirin treatment significantly reduced mortality, morbidity, and subsequent paralysis sequelae in infected mice.

It has been shown that the brain stem and spinal cord are the main targets of EV71 in severe cases of human infections [1]. Our previous study showed that high levels of virus were detected in the mouse central nervous system on day 5 after infection [13]. Accordingly, we next investigated the viral loads in the mouse central nervous system on day 5 after infection. Our data showed that ribavirin treatment reduced the viral loads in the brains (without brain-stem region), brain stems, and spinal cords of EV71-infected mice (figure 3C). Additional experiments showed that the virus harvested from the tissues of mice receiving ribavirin treatment was still sensitive to ribavirin treatment (data not shown).

**Discussion.** This is the first report that ribavirin, which is currently used to treat several RNA virus infections in patients, has antiviral activities against EV71 in vitro and particularly in vivo by reducing viral loads in tissues, mortality and morbidity during acute infection, and subsequent sequelae in infected mice. This finding is particularly important, because there is no effective antiviral drug currently available for the prevention, treatment, and control of fatal EV71 infection in humans.

Our in vitro experiments showed a direct anti-EV71 action by ribavirin in both human and mouse cell lines. The  $\text{IC}_{50}$  of ribavirin for EV71 is  $\sim 65 \mu\text{g}/\text{mL}$  (266  $\mu\text{mol}/\text{L}$ ) in both RD and SK-N-SH cells, which is lower than that found for other neurotropic viruses, such as Nipah virus, West Nile virus, and Japanese encephalitis virus ( $\text{IC}_{50}$  values of 100, 71, and 134  $\mu\text{g}/\text{mL}$ , respectively) [14, 15]. Previous studies showed that 80–400 mg/kg ribavirin daily reduces the mortality in rats



**Figure 3.** Reduction of the mortality and subsequent paralysis sequelae of EV71-infected mice by ribavirin, via reduction of viral loads in tissues. Infected mice were treated with PBS (white symbols;  $n = 11$ ) or ribavirin (black symbols;  $n = 10$ ). The survival rates (A) and clinical scores (B) of mice were recorded. C, In separate experiments, the brains (without brain-stem region), brain stems, and spinal cords of infected mice treated with PBS ( $n = 6$ ) or ribavirin ( $n = 6$ ) were collected on day 5 after infection to determine viral titers. Data are mean  $\pm$  SE values. \* $P < .05$ , by log-rank test (A), Wilcoxon test (B), and Mann-Whitney  $U$  test (C).

with encephalitis induced by Junin virus [11], in rhesus monkeys with encephalitis induced by Rift Valley fever virus [12], and in mice with myocarditis induced by coxsackievirus B3 [6]. On the basis of these studies, 100 mg/kg ribavirin was chosen to treat EV71-infected mice, and this dose showed a protective effect. In our study, ribavirin was given to infected mice during the viremic phase (data not shown). Further studies are needed to test the efficacy of ribavirin therapy beginning later in the disease course in mice.

For Nipah virus, despite the IC<sub>50</sub> of ribavirin being higher than that for EV71 and the fact that the drug fails to prevent the deaths of infected hamsters [14], ribavirin treatment significantly reduces mortality in patients with encephalitis without inducing the adverse effects (such as anemia, jaundice, and teratogenic effects) commonly associated with a 6–9-day regimen of oral ribavirin, starting with a loading dose of 2000 mg daily, or with a 7-day regimen of intravenous ribavirin, starting with a loading dose of 94 mg/kg daily [10]. Under these regimens, the plasma concentration of ribavirin is ~2.5 μg/mL, which is comparable to that found in HCV-infected patients treated with the drug [7]. The concentration of ribavirin in cerebrospinal fluid is ~70% of that in plasma in humans [16]. Interestingly, the IC<sub>50</sub> of ribavirin for Nipah virus is >40 times higher than the clinically achievable concentrations in plasma and cerebrospinal fluid. For HCV, the IC<sub>50</sub> of ribavirin is 230 μmol/L (56 μg/mL), >22 times higher than the plasma concentration [17]. In fact, the IC<sub>50</sub> of ribavirin for almost all viruses for which the drug shows therapeutic efficacy in patients—including measles virus, respiratory syncytial virus, La Crosse virus, hantavirus virus, and Lassa virus [5, 8, 9, 18–21]—are much higher than the plasma concentrations. Evidently, ribavirin inhibits viruses more efficiently in vivo than in vitro.

In the present study, we found that the IC<sub>50</sub> of ribavirin for EV71 is lower than that for Nipah virus [14]. Most importantly, unlike in Nipah virus [14], ribavirin protects mice from EV71 infection by reducing the viral loads in tissues. In addition, the dose of ribavirin that effectively protects mice from EV71 infection is close to the initial dose of the drug used intravenously to improve the conditions of patients with encephalitis induced by Nipah virus [10]. Collectively, these data demonstrate that ribavirin could be a potential anti-EV71 drug. Our previous study showed that type I interferons also reduce mortality in EV71-infected mice [22]. Alternatively, the combination of ribavirin and interferon, which has a synergistic effect and is used as a standard therapy for HCV-infected patients, could be considered for the treatment of potentially fatal EV71 infection.

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