In vivo ribavirin activity against severe pandemic H1N1 influenza A/Mexico/4108/2009

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The use of ribavirin in influenza treatment is a matter of debate. Due to adamantane- and oseltamivir-resistant strains of the current pandemic H1N1 (pdmH1N1) influenza viruses, the demand for alternative antiviral treatments has increased. This study demonstrated the potent antiviral effects of ribavirin in a mouse model of pdmH1N1 influenza infection (A/Mexico/4108/2009). It was found that treatment with 40 mg ribavirin kg⁻¹ day⁻¹ partially protected the animals if initiated immediately upon infection. Administration of similar concentrations on subsequent days or immediate therapy with lower doses efficiently delayed disease progression. Correlation studies showed a direct relationship between low viral titres in the lung during the early stages of infection with animal survival in ribavirin-treated animals. Reduced lung pathology in animals treated with ribavirin following infection also indicated the importance of immediate treatment. This study revealed the antiviral properties of ribavirin and these results justify comprehensive clinical studies for the use of ribavirin against influenza virus in future outbreaks.

INTRODUCTION

The H1N1 influenza pandemic, which began in 2009, is a matter of global concern. It has been the cause of an estimated 257,000 hospitalized cases with 11,000 mortalities in the USA alone, and more than 15,000 deaths worldwide (http://www.who.int/csr/disease/swineflu/en/). The epidemiology appears to be similar to regular seasonal influenza (Thompson et al., 2003), with the exception of higher transmission rates among the younger population. However, the actual number of infected cases is expected to be much higher due to changed priorities in public health monitoring systems, the unavailability of data from patients with mild sickness and insufficient surveillance in developing countries. Furthermore, patients with underlying co-morbidities are at higher risk of severe illness following pandemic H1N1 (pdmH1N1) influenza infection (Jain et al., 2009). Approximately 99% of novel pdmH1N1 virus isolates exhibit resistance to adamantanes (amantadine and rimantadine), leaving neuraminidase inhibitors (such as oseltamivir and zanamivir) as the only recommended options for treatment of hospitalized individuals.

This pandemic is among the four influenza pandemics over the past century (Belser et al., 2007; Uyeki et al., 2002) caused by a triple reassortment H1N1 influenza virus, carrying genes from porcine, avian and human origin influenza viruses (Garten et al., 2009). Current fears are concentrated on the potential of a second wave with a mutated virus causing increased morbidity, as was seen in the Spanish flu 1918 pandemic (Reid et al., 2004). An H275Y mutation in the neuraminidase gene of pdmH1N1, conferring oseltamivir resistance, has already been detected in some random cases (Centers for Disease Control and Prevention, 2009a, b; Speers et al., 2010). Despite the recent decline in the 2009 H1N1 pandemic, the identification of
oseltamivir-resistant isolates is still on the rise (World Health Organization, 2010). Therefore, there is a demand for a comprehensive treatment strategy, which includes the screening of available antiviral compounds.

The present study demonstrated the protective effects of ribavirin against pdmH1N1, strain A/Mexico/4108/2009, in a mouse model of infection.

Ribavirin is an antiviral pro-drug, created in 1972, that can be used against a broad spectrum of both DNA and RNA viruses including influenza A virus, influenza B virus, human respiratory syncytial virus (RSV), hepatitis C virus (HCV), Sendai virus and many others (Beigel & Bray, 2008). Following metabolization, ribavirin’s structure is similar to nucleosides and it is this conformation that gives the drug its antiviral activity (Gilbert & Knight, 1986). There are several hypothesized modes of action depending on the type of virus ribavirin is used against. These include decreasing intracellular GTP by inhibition of inosine monophosphate dehydrogenase, inhibition of mRNA 5‘-cap formation and increasing mutations in RNA replication by its resemblance to adenosine or guanosine. Although it has been reported to have teratogenic and mutagenic effects in some animal models, the compound has shown promising antiviral activity against HCV, RSV, herpes simplex virus and varicella-zoster virus (Eggleston, 1987; Saldana et al., 2009). Additionally, it is currently licensed for antiviral therapy of chronic HCV and RSV infections in oral and aerosolized formulations, respectively (Chan-Tack et al., 2009; Graci & Cameron, 2006; Knight et al., 1981).

Studies of the in vitro anti-influenza activity of ribavirin against different seasonal strains have been conducted previously (Graci & Cameron, 2006), but its potential teratogenic side effects have hampered clinical trial outcomes (Rodriguez et al., 1994). Considering the effect of severe pandemic influenza and the emergence of oseltamivir-resistant virus strains, ribavirin may yet represent an alternative therapeutic agent against future pandemic influenza outbreaks.

RESULTS

In vitro ribavirin antiviral activity against A/Mexico/4108/2009

Previously, ribavirin has been shown to be effective against influenza A viruses (Browne et al., 1983). Although ribavirin activity has been investigated in vitro against the pandemic A/California/07/2009 influenza strain (Selvam et al., 2010), its efficacy for the pdmH1N1 A/Mexico/4108/2009 strain has not been determined. We evaluated the antiviral activity of ribavirin against A/Mexico/4108/2009 in vitro using influenza infection in Madin–Darby canine kidney (MDCK) cells. MDCK cells were infected with 10³ or 10⁴ 50 % egg infectious doses (EID₅₀) A/Mexico/4108/2009 ml⁻¹ and 24 h later were administered ribavirin at various concentrations. At 7 days post-infection (p.i.), we observed that the highest dose of ribavirin (100 µg ml⁻¹) protected ~65 % of the infected cells (Fig. 1). Lower doses of ribavirin also reduced cell death following infection: 30 µg ribavirin ml⁻¹ conferred statistically significant protection in the infected cells (57 % of the cells infected with 10³ EID₅₀ ml⁻¹ and 28 % infected with 10⁴ EID₅₀ ml⁻¹). The lowest concentration of ribavirin (10 µg ml⁻¹) protected 22 and 37 % of cells infected with 10³ and 10⁴ EID₅₀ ml⁻¹, respectively. These results suggested that ribavirin had effective antiviral activity against the pdmH1N1 A/Mexico/4108/2009 strain.

![Fig. 1. In vitro antiviral effect of ribavirin against A/Mexico/4108/2009. MDCK cells were infected with 1.35×10⁴ (a) or 1.35×10³ (b) EID₅₀ A/Mexico/4108/2009 ml⁻¹, followed by treatment with ribavirin (40 mg kg⁻¹ day⁻¹) at day 1 p.i. At 6 days after ribavirin treatment, cell viability was evaluated by MTT assay and the results were expressed as percentage cell viability (infected cells/uninfected control cells). Values are shown as means±SD.](http://vir.sgmjournals.org)
BALB/c mice as an animal model of A/Mexico/4108/2009 infection and ribavirin treatment

Ribavirin was first discovered to be effective against influenza A and B viruses in the 1970s using *in vitro* and *in vivo* models (Huffman et al., 1973; Khare et al., 1973; Sidwell et al., 1972). Until recently (Kash et al., 2010; Triana-Baltzer et al., 2009), A/Mexico/4108/2009 influenza infection had not been investigated in mice. Here, we compared A/Mexico/4108/2009 influenza infection in BALB/c and C57BL/6 mouse strains (Fig. 2a and b, respectively). Mice were infected intranasally with A/Mexico/4108/2009 (10^4 EID_{50}), and animal survival and weight were recorded daily. C57BL/6 mice were more susceptible to pdmH1N1 influenza infection. When using 20% weight loss as an end point, 80% mortality was observed by day 8 p.i. with 10^4 EID_{50} A/Mexico/4108/2009 given intranasally for C57BL/6 mice. Interestingly, the same dose of virus administered to BALB/c mice did not result in death (P<0.005) (Fig. 2a). Mice receiving higher viral doses (10^5 and 10^6 EID_{50}) also responded in terms of weight loss kinetics, although no significant difference was noted in the number of survivors between the two mouse strains.

Our main objective was to determine the effect of ribavirin treatment for A/Mexico/4108/2009 infection. Therefore, we analysed the effectiveness of a single daily dose of ribavirin (40 mg kg^{-1}) for 10^5 and 10^6 EID_{50} A/Mexico/4108/2009 (non-mouse-adapted) infection in both C57BL/6 and BALB/c mice. This dose of ribavirin, administered once daily from the time of infection to 7 days p.i., protected at least 80% of BALB/c mice, irrespective of the infectious dose of virus given (Fig. 2a). Additionally, modest weight loss was observed in the ribavirin-treated mice p.i. In C57BL/6 mice, however, ribavirin only provided 50% protection in mice infected with the 10^5 EID_{50} viral dose and did not protect mice infected with 10^6 EID_{50} (Fig. 2b). These data suggested that C57BL/6 mice were more susceptible to A/Mexico/4108/2009 infection and thus less responsive to ribavirin treatment. The results also demonstrated a marked protective effect of daily ribavirin treatment against influenza infection. Therefore, BALB/c mice were more susceptible to pdmH1N1 influenza infection and less responsive to ribavirin treatment.

**Fig. 2.** *In vivo* effects of ribavirin against A/Mexico/4108/2009 virus in BALB/c and C57BL/6 mice. BALB/c (a) and C57BL/6 (b) mice were infected with A/Mexico/4108/2009 (10^5 EID_{50}). Survival curves (left panels) are shown for mice infected with varying concentrations of virus (10^3, 10^4, 10^5 and 10^6 EID_{50}) with or without ribavirin (Rv) treatment (40 mg kg^{-1} day^{-1}). Ribavirin treatment was initiated at the time of infection (day 0) and ended at day 7 p.i. Statistical evaluation of survival curves by log-rank test between various doses of virus as well as between ribavirin-treated and untreated groups was statistically significant (P<0.0005) for both animal strains. Differences in daily weights were plotted graphically as a percentage of weight lost (right panels). Significant differences were observed between ribavirin-treated and untreated groups (P<0.0005).
mice were chosen as an *in vivo* infection model for further analysis of the effects of ribavirin.

**Temporal variation of ribavirin treatment for influenza infection**

In 1981, it was shown that ribavirin could also be used to treat influenza in humans (Knight *et al.*, 1981). Here, we sought to determine the action of ribavirin treatment given at varying times after influenza infection. To investigate ribavirin treatment temporally, administration with 40 mg ribavirin kg\(^{-1}\) day\(^{-1}\) was initiated at the time of A/Mexico/4108/2009 infection or on subsequent days up to day 3 p.i. Mock-treated control animals infected with A/Mexico/4108/2009 virus suffered rapid weight loss (Fig. 3b), and 100% lethality occurred by day 11 p.i. (Fig. 3a). Ribavirin treatment initiated at day 0 p.i. conferred almost complete protection against a lethal dose of virus with significantly lower hazard ratios (see Supplementary Table S1, available in JGV Online). Similar trends were observed in terms of weight loss where most day 0 ribavirin-treated animals maintained their initial body weight throughout the infection period (*P* ≤ 0.0005). The protective effects of delayed therapeutic regimes, initiated at 1 and 2 days p.i., were less prominent throughout, but statistical analysis revealed significant differences in survival curves compared with mock-treated groups (*P* ≤ 0.05). Additionally, we found that administration of ribavirin after 1 day of infection shifted the median day of death from day 5 to 11 and had a lower risk of death (see Supplementary Table S2, available in JGV Online). However, neither the overall survivor count nor weight loss was affected by ribavirin treatments initiated after day 1 p.i. These results suggested that ribavirin therapy was most beneficial when started soon after infection. Furthermore, this protection diminished rapidly if given at later time points p.i., indicating the importance of early treatment.

As the mice had increased survival and health when given ribavirin early after infection, we went on to determine viral loads in the early-treated animals. To confirm that the protection by ribavirin in influenza infection was due to the inhibition of virus replication, we assessed viral titres in the lungs of BALB/c mice at 3 days p.i. As expected, viral loads were reduced in ribavirin-treated mice. Interestingly, a reduction in virus replication was also observed in groups

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**Fig. 3.** Effect of delayed ribavirin treatment (40 mg kg\(^{-1}\) day\(^{-1}\)) on severe pandemic influenza 2009 infection in BALB/c mice. Animals infected with 10\(^5\) EID\(_{50}\) A/Mexico/4108/2009 received daily ribavirin treatment beginning on the day of infection (Rv days 0–7) or on day 1 (Rv days 1–7), day 2 (Rv days 2–7) or day 3 (Rv days 3–7) p.i. (a) Significant antiviral effects were observed on the survival curve (*P* ≤ 0.005). A Gehan–Breslow–Wilcoxon test indicated that the survival curves Rv days 0–7 (*P* ≤ 0.0005) and Rv days 1–7 (*P* ≤ 0.05) were significantly different from the control. (b) Ribavirin treatment was effective in terms of animal body weight throughout the infection study. The results were expressed as the percentage of original weight at the time of experimental infection (*P* ≤ 0.0005). (c) MDCK cells were used to determine viral titres in serially diluted lung homogenates collected at 3 days p.i. Results are expressed as log\(_{10}\) values of the mean of viral load ± sem in each group of mice (*n* = 3).
receiving delayed ribavirin treatment p.i., despite the treatments resulting in less than optimal protection (Fig. 3c). Statistical analysis indicated that the difference in lung viral load at 3 days p.i. correlated with shifts in the median day of death (correlation coefficient 0.89 with \( P \leq 0.05 \)). In conclusion, the lung viral loads were inversely proportional to the time of initiation of ribavirin treatment in influenza-infected mice.

**Concentration-dependent protection of ribavirin therapy**

As we had determined the optimal time to begin ribavirin treatment, we next sought to determine whether the ribavirin protection against A/Mexico/4108/2009 was dose-dependent. While maintaining the same infection conditions in BALB/c mice, different concentrations of ribavirin (40, 20, 10 and 5 mg kg\(^{-1}\) day\(^{-1}\)) were administered to separate mouse groups from days 0 to 7 p.i. (Fig. 4). We found that different concentrations of ribavirin affected the survival of the animals (\( P \leq 0.0005 \)). Similar to the highly effective ribavirin dose of 40 mg ribavirin kg\(^{-1}\) day\(^{-1}\), 20 and 10 mg ribavirin kg\(^{-1}\) day\(^{-1}\) also resulted in markedly improved animal survival (\( P \leq 0.0005 \) and \( P \leq 0.05 \), respectively). Furthermore, these ribavirin treatments resulted in a shift in the median day of death from day 5 to 11 (see Supplementary Table S3, available in JGV Online). Full protection against a lethal challenge of A/Mexico/4108/2009 was not induced by lower concentrations of ribavirin, but it was sufficient to decrease the risk of death by more than 90\% (hazard ratios of 0.04–0.13 with \( P \leq 0.05 \)). Mock-treated control animals suffered massive weight loss after 6 days, which was significantly lower than that observed in the ribavirin-treated groups (Fig. 4b). Weight loss and animal survival were not improved by the lowest dose of ribavirin tested (5 mg kg\(^{-1}\) day\(^{-1}\)). These results suggested that ribavirin was not clinically effective at a 5 mg kg\(^{-1}\) day\(^{-1}\) dose but was effective to varying degrees at the higher doses. Thus, the protective clinical effects of ribavirin in influenza-infected mice against A/Mexico/4108/2009 were dose-dependent.

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**Fig. 4.** Ribavirin protection against A/Mexico/4108/2009 (10\(^5\) EID\(_{50}\)) is dose dependent in BALB/c mice. Daily ribavirin treatment was initiated on the day of infection at varying concentrations and ended on day 7 p.i. (a) Survival curves were found to be significantly different (\( P \leq 0.0005 \)) with differences between the number of survivors in each group performed by \( \chi^2 \) test (\( P \leq 0.0005 \)). Pairwise comparisons of survival curves were performed by a Gehan–Breslow–Wilcoxon test showing statistically significant differences in the survival curves at low doses, i.e. Rv 20 mg kg\(^{-1}\) day\(^{-1}\) (\( P \leq 0.0005 \)) and Rv 10 mg kg\(^{-1}\) day\(^{-1}\) (\( P \leq 0.05 \)), compared with the control. (b) Maintenance of animal body weights was observed in ribavirin-treated cases. The results are expressed as the percentage of original weight at the time of experiment infection (\( P \leq 0.0005 \)). (c) Viral load was determined by MDCK cell culture with diluted lung homogenates. Ribavirin concentration varied from 20 to 5 mg kg\(^{-1}\) day\(^{-1}\). Results are expressed as log\(_{10}\) values of the mean of viral loads ± SEM in each group of mice (\( n = 3 \)).
After monitoring the clinical signs of A/Mexico/4108/2009-infected mice treated with varying concentrations of ribavirin, we went on to determine the viral load following these ribavirin treatments. Although we established that treatment with lower doses of ribavirin was not as protective against A/Mexico/4108/2009, a significant reduction in virus replication was observed in groups that received immediate ribavirin therapy of doses from 20 to 5 mg kg\(^{-1}\) day\(^{-1}\) (Fig. 4c). These results showed that low concentrations of ribavirin reduced viral loads in A/Mexico/4108/2009-infected BALB/c mice.

Ribavirin treatment reduces lung pathology

We examined lung histology to determine whether ribavirin treatment improved lung pathology following influenza infection in BALB/c mice. Using haematoxylin and eosin (H&E) staining, the lung tissues of infected animals were found to have considerable inflammation with a pronounced cuff of cell exudates in the lung parenchyma and small airways. Following ribavirin treatment, however, significant eradication of perivascular inflammation and fewer cell exudates were observed. The response of inflammatory cell accumulation was subtle at 3 days p.i., but low levels of inflammation were clearly observed at day 6 p.i. (Fig. 5a, b) compared with mock-treated infected groups (Fig. 5c, d). Taken together with our previous results, which showed reduced clinical disease and viral loads following ribavirin treatment, these results suggested that ribavirin treatment improved the lung pathology of influenza-infected animals.

**DISCUSSION**

This study demonstrated the *in vitro* and *in vivo* antiviral efficacy of ribavirin against the 2009 severe pdmH1N1 influenza virus strain A/Mexico/4108/2009. Experiments conducted in BALB/c mice showed almost complete survival if a 40 mg kg\(^{-1}\) day\(^{-1}\) treatment regime of ribavirin was initiated immediately after infection. Delayed therapy or lower-dose regimes also prolonged disease progression to an extent but not as effectively. Outcomes were dependent on the dosage and timing of ribavirin treatment. We observed a correlation between lung viral loads measured on day 3 p.i. and survival of the mice, as mice with low viral loads had increased survival. For instance, the administration of 40 mg ribavirin kg\(^{-1}\) day\(^{-1}\) beginning at 24 h p.i., and 20 or 10 mg kg\(^{-1}\) day\(^{-1}\) beginning on the same day of infection, resulted in a pronounced reduction in viral loads at 3 days p.i. Consequently, we observed improved morbidity and mortality in the infected mice that received these treatments. However, these ribavirin dose regimes were not sufficient to provide 100 % protection from infection. Our data indicate that ribavirin could be an effective antiviral in the treatment of the pdmH1N1 A/Mexico/4108/2009 strain.

Typically, ferrets are used to investigate human influenza isolates and influenza treatments where there is significant virus replication and abundant viral shedding (Munster et al., 2009). Although ferrets are a good influenza model due to similar symptomology to humans (Martina et al., 2003), it is not practical to use ferrets for high-throughput screening. Mice are the gold standard for *in vivo* studies as they are relatively inexpensive and easy to manipulate. Despite these attributes, mice are not always applicable to human studies, as they are not always susceptible to human disease or relative treatments. Here, we infected mice with a direct human isolate of pdmH1N1 influenza, A/Mexico/4108/2009, and showed that the mice became ill. We observed 100 % lethality in mice at day 11 p.i. with A/Mexico/4108/2009, and consistent weight loss that indicated sufficient viral infection to perform antiviral

Fig. 5. Tissue inflammation is reduced in ribavirin-treated animals. BALB/c mice were infected with A/Mexico/4108/2009 and the lungs were removed for H&E histology on day 6 p.i. Observed perivascular inflammation (arrows) on day 6 p.i. was reduced in ribavirin-treated mice (a, b) compared with infected animals who received mock treatment (c, d).
screening. Consistent with our results, other groups have also reported efficient virus replication in the lungs of mice (Maines et al., 2009). Moreover, we found that infected mice responded well to ribavirin antiviral treatment. Our findings suggest that mice are a robust model for the investigation of influenza infection and potential therapeutics, as well as for antiviral screening.

Currently, the treatment of choice for pandemic H1N1 viruses consists of oseltamivir and zanamivir. A 75 mg kg⁻¹ dose of oseltamivir twice daily is able to lessen symptoms and delay virus replication by 5 days (Smith et al., 2010). In hospitalized cases, however, oral oseltamivir treatment is not able to decrease the case fatality ratio below 50% (Hayden, 2009). In addition to the drastically high ratio of amantadine resistance in circulating influenza virus strains, there has also been an increasing number of oseltamivir-resistant pdmH1N1 viruses (Centers for Disease Control and Prevention, 2009a, b; Speers et al., 2010). To this end, there has been discussion regarding multiple drug treatment strategies for influenza (Smeet al., 2006, 2009, 2010). Due to these emerging issues, coupled with the unavailability of other antivirals in developing countries, it is critical to investigate the use of ribavirin alone and in combination with other antiviral drugs.

Ribavirin consistently shows influenza antiviral activity in vitro (Bernstein et al., 1988; Chan-Tack et al., 2009; Gilbert et al., 1985; Knight et al., 1981; McClung et al., 1983; Wilson et al., 1984). In a series of studies published on human influenza outbreaks in Texas in the 1980s, aerosolized ribavirin was investigated to treat A/England/333/80 (H1N1), influenza B, A/Victoria/7/83 (H1N1) and A/England/333/80-like viruses (Bernstein et al., 1988; Gilbert et al., 1985; Knight et al., 1981; McClung et al., 1983; Wilson et al., 1984). In all these reports, aerosolized ribavirin was shown to be effective against influenza viruses determined by a reduction in fever, inhibition of virus shedding, increased polymorphonuclear leukocyte counts and a faster recovery rate in treated patients compared with controls. There were no reported adverse effects of the ribavirin aerosol therapy with respect to respiratory, liver and haematological functions in these studies. Taken together with our data showing ribavirin as a treatment for a pdmH1N1 virus, ribavirin has potential as an alternative treatment paradigm for seasonal and emerging influenza viruses.

Ribavirin has also been shown to be effective towards H2N2 and H3N2 strains of influenza A virus (Browne et al., 1983). Currently, there are many reports investigating the use of ribavirin in combination with other drugs such as oseltamivir and the cyclopentane neuraminidase inhibitor RWJ-270201 against highly pathogenic H5N1 and a highly pathogenic strain of H1N1 (A/NWS/33) (Ilyushina et al., 2008; Smeet al., 2002). Ilyushina et al. (2008) found that, by combining oseltamivir and ribavirin, there was a significant reduction in H5N1 replication and H5N1 spreading beyond the respiratory tract and an inhibition of cytokine production in mice. Ribavirin was also found by Smeet al. (2002) to have significant inhibition against the lethal A/NWS/33 (H1N1) strain in mice when used in combination with RWJ-270201. Some minimal effects were observed when ribavirin was used alone, but the synergistic combination of ribavirin and RWJ-270201 was more effective. It is imperative to investigate the effects of ribavirin in combination with other antiviral drugs such as oseltamivir or cyclopentane in our mouse model for A/Mexico/4108/2009.

Although, ribavirin consistently shows antiviral activity in vitro (Chan-Tack et al., 2009), its clinical use is controversial due to inconsistent clinical results and the potential risk of side effects, such as haemolytic anaemia (Rodriguez et al., 1994). It is possible that changing the route of ribavirin administration or the treatment dosage might lessen these side effects. In addition, ribavirin cannot be administered to pregnant women and patients with severe co-morbidities (Jain et al., 2009), but, from our data, it is still a possible treatment to be considered for the rest of the population. Published data based on clinical records have indicated that the success of ribavirin in treating critically ill influenza patients has been uncertain, but that ribavirin should be considered as a valuable alternative to oseltamivir (Chan-Tack et al., 2009; Hayden, 2009). Furthermore, inconsistencies with regard to ribavirin treatment regimes in vivo may be due to the viral strain used and the therapeutic routes and schedules of ribavirin administration, as well as antiviral dosing.

In conclusion, this study has provided the first direct evidence for the antiviral effect of ribavirin against pdmH1N1 A/Mexico/4108/2009 strain. These findings demonstrate the potential for the use of ribavirin, where necessary, as an alternative antiviral drug to use against future pandemic influenza virus outbreaks.

METHODS

Virus, cells and antiviral drugs. Experiments were conducted using pdmH1N1 influenza strain A/Mexico/4108/2009, obtained from the Centers for Disease Control and Prevention (Atlanta, GA, USA). The virus was propagated and titrated in embryonated eggs prior to animal challenge. Viral stocks were stored in liquid nitrogen and thawed prior to use. The 50% mouse lethal dose (MLD₅₀) was determined. Ribavirin (Sigma-Aldrich) was stored at 4 °C. The powder was reconstituted in sterile MilliQ water and working aliquots of ribavirin were stored at −20 °C.

Cell culture. All cell culture reagents were obtained from Invitrogen. MDCK cells (ATCC) used for tissue viral load experiments were maintained in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% heat-inactivated FBS, 10 mM l-glutamine and antibiotics (100 U penicillin ml⁻¹, 100 μg streptomycin ml⁻¹). During and following infections, the cells were cultured in vDMEM (DMEM with 1% BSA, 10 mM l-glutamine, antibiotics as above and 1 μg TPCK-treated trypsin ml⁻¹). Cultures were maintained at 37 °C in a 5% CO₂-enriched environment.

Cell-based antiviral assay. In order to determine the antiviral activity of ribavirin in vitro, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazo- lium bromide (MTT; Sigma-Aldrich) assays were performed. Briefly,
MDCK cells were seeded in a 96-well plate (Nunc) at a concentration of 2.5 × 10^4 cells per well prior to infection. The cells were infected with A/Mexico/4108/2009 virus at concentrations of 1.35 × 10^4 (m.o.i.=0.05) and 1.35 × 10^5 (m.o.i.=0.005) EID_{50} ml^{-1} in vDMEM. At 2 h p.i., the cell culture supernatant was replaced with fresh vDMEM. Ribavirin was added at 24 h p.i. at concentrations of 10, 30 or 100 μg ml^{-1} followed by 6 days of incubation. At day 7 p.i., MTI (0.5 mg ml^{-1}) was added to each well and incubated for 4 h at 37 °C with 5% CO₂. Aciddent SDS (10%, w/v) was then added to each well and the absorbance of the solution was determined at 560 and 670 nm using a μQuant spectrophotometer (BioTek Instruments). The cytotoxic effects of influenza infection were compared with 100% cell death induced by hydrogen peroxide treatment and 100% cell survival in uninfected cells incubated in medium containing the same concentrations of ribavirin.

**Animals.** Female BALB/c and C57BL/6 mice (8–10 weeks of age) were purchased from Harlan Laboratories and used in influenza infection studies. Mice were maintained on standard animal feed and water *ad libitum* in clean environmental conditions and controlled temperature and humidity with a 12 h light/dark cycle. For infection studies, animals were housed in cage racks, which were HEPA filtered under ABSL2 + conditions (Toronto General Hospital Animal Resource Centre, Toronto, Canada). All animal procedures were performed in a certified Class II biosafety cabinet (Baker Co.). The animal use protocol was approved by the Animal Care Committee of the University Health Network, Toronto, Ontario. All experiments were conducted in accordance with committee recommendations.

**Viral infection and treatment experiments.** BALB/c and C57BL/6 mice were used to measure the efficacy of the infection model as well as to evaluate the *in vivo* ribavirin activity. To determine the MLD_{50}, animals were divided into several groups (*n*=10) and infected intranasally with tenfold dilutions of A/Mexico/4108/2009 influenza virus strain ranging from 10^0 to 10^6 EID_{50} in a final volume of 50 μl. Separate groups infected with 10^6 and 10^3 EID_{50} were also treated with daily intraperitoneal injections of 40 mg ribavirin kg^{-1} day^{-1} from day 0 to 7 p.i.

Further studies were conducted in BALB/c mice (grouped; *n*=16) and infected intranasally with A/Mexico/4108/2009 (10^6 EID per mouse, ~4 MLD_{50}). Ribavirin treatment was initiated as described above. Test groups received drug concentrations ranging from 40 to 5 mg kg^{-1} day^{-1} for 7 days. Mock treatment consisting of MilliQ water was given to control animals.

For temporal infection, infected BALB/c mice were treated with 40 mg ribavirin kg^{-1} day^{-1} initiated at different time points ranging from day 0 to 3 p.i. Mock treatment of MilliQ water was given to control groups. Animals were observed daily for weight loss, morbidity and mortality up to day 14 p.i.

The humane end point for mouse mortality was 20% weight loss. Healthy controls were included in each experiment for comparison.

**Determination of viral load.** Animals were euthanized at day 3 p.i. and lung tissues were homogenized by sonication and counted in a 96-well plate and incubated for 2 h. Homogenates were removed from the cells and replaced with fresh vDMEM. The cells were cultured for 6 days, at which time the cell culture supernatants were tested for virus titre by a haemagglutination assay using 0.5% (v/v) turkey red blood cells (LAMPIRE Biological Laboratories).

**Histology.** Animals were euthanized at day 3 or 6 p.i. and lung tissues were perfused with 10% buffered formalin, harvested and processed as paraffin wax-embedded thin sections for histology studies. Slides were stained with H&E and observed under a light microscope (AccuScope). Images were captured using a digital camera and SEM Premium software (Micrometrics).

**Statistical analyses.** Survival analysis was performed using GraphPad Prism software (GraphPad Software Inc.). Survival differences among treatment types were measured by a log-rank test, whereas concentration or dose-dependent effects were confirmed by a log-rank trend test. Contingency analysis using a χ² test was applied to measure statistical significance between the survivors of each group. The significance of the median day of death and the hazard ratio in each group compared with the mock-treated control group was determined by Student’s t-test. Similar analyses were performed to determine differences in viral load between ribavirin-treated and untreated groups. Significant differences in weight loss between groups were confirmed by two-way analysis of variance. Spearman’s rank one-tailed and Gaussian Pearson correlation analyses were applied to correlate direct association between viral load measurements and animal mortality and morbidity.

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