Combined chloroquine and ribavirin treatment does not prevent death in a hamster model of Nipah and Hendra virus infection

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Hendra virus (HeV) and Nipah virus (NiV) are recently emerged, closely related and highly pathogenic paramyxoviruses that cause severe disease such as encephalitis in animals and humans with fatality rates of up to 75%. Due to their high case fatality rate following human infection and because of the lack of effective vaccines or therapy, they are classified as Biosafety Level 4 pathogens. A recent study reported that chloroquine, an anti-malarial drug, was effective in preventing NiV and HeV infection in cell culture experiments. In the present study, the antiviral efficacy of chloroquine was analysed, individually and in combination with ribavirin, in the treatment of NiV and HeV infection in in vivo experiments, using a golden hamster model. Although the results confirmed the strong antiviral activity of both drugs in inhibiting viral spread in vitro, they did not prove to be protective in the in vivo model. Ribavirin delayed death from viral disease in NiV-infected hamsters by approximately 5 days, but no significant effect in HeV-infected hamsters was observed. Chloroquine did not protect hamsters when administered either individually or in combination with ribavirin, the latter indicating the lack of a favourable drug–drug interaction.

INTRODUCTION

Hendra virus and Nipah virus form a separate genus, Henipavirus, within the family Paramyxoviridae (Harcourt et al., 2000; Wang et al., 2000). Both viruses are capable of causing severe disease in humans, horses and swine, and of infecting a number of other mammalian species. The natural reservoir of both viruses appears to be pteropid fruit bats, which eliminate the virus in their urine (Chua et al., 2002; Halpin et al., 1999, 2000; Reynes et al., 2005).

Hendra virus (HeV) was identified as the causative agent of an outbreak of acute respiratory disease in humans and horses in September 1994 in Hendra, a suburb of Brisbane, Australia, that resulted in the deaths of 13 horses (Murray et al., 1995a, b). Since its first appearance, other outbreaks have been reported in Queensland between 1994 and 2008, which caused only one or two horse fatalities in each instance (Hanna et al., 2006; ProMED-mail, 2008a). The most recent outbreak is ongoing (September 2009) in Queensland and has been associated with a total of one human and four horse fatalities (ProMED-mail, 2009b, c). Of the few reported clinical cases of human HeV infection, which were caused by transmission from infected horses to veterinarians and horse-handlers (Murray et al., 1995b), all were associated with direct exposure to secretions and tissues from very ill, moribund or dead horses during their treatment, nursing or necropsy (Hanna et al., 2006; O’Sullivan et al., 1997; Paterson et al., 1998; ProMED-mail, 2009b; Selvey et al., 1995).

Nipah virus (NiV) was detected for the first time in 1998 in Malaysia, where it caused disease in pigs and humans (Chua et al., 2000a) and was first isolated from infected cerebrospinal fluid taken from a patient from the village of...
Nipah close to Ipoh (Chua et al., 1999). In March 1999, the outbreak spread to neighbouring Singapore as a result of the trading and movement of pigs from Malaysia. During this outbreak, human infections were observed primarily in pig farmers, and the main source of human infections was considered to be the close contact with infected pigs during parturition and carcass handling. From a total of 265 human cases in both countries, 105 resulted in fatal parturition and carcass handling. From a total of 265 human cases in both countries, 105 resulted in fatal parturition and carcass handling. From a total of 265 human cases in both countries, 105 resulted in fatal parturition and carcass handling. From a total of 265 human cases in both countries, 105 resulted in fatal parturition and carcass handling.

Human infections have also been identified in several outbreaks in India and Bangladesh between 2001 and 2008 (Chadha et al., 2006; Harcourt et al., 2005; Hossain et al., 2008; Hsu et al., 2004; Luby et al., 2006; ProMED-mail, 2008b; World Health Organization, 2004). The reported human death rates varied from 40 % in Malaysia to 75 % in Bangladesh and India, and some outbreaks were associated with human-to-human transmission (Gurley et al., 2007). Similar to HeV, NiV virus can persist over a long period in the infected patient before causing fatal disease (Tan et al., 2002; Wong et al., 2001).

NiV and HeV are classified as Biosafety Level 4 pathogens due to their high case fatality rate following human infection and because of the lack of effective vaccines or therapeutics for treatment of the disease. During the 1998 outbreak in Malaysia, a limited non-randomized trial of ribavirin was conducted with 140 NiV-infected patients and showed ribavirin therapy to be able to reduce the mortality of acute NiV encephalitis (Chong et al., 2001). The effect of ribavirin on HeV was shown in an in vitro study by Wright et al. (2005) which resulted in a reduction in virus yield of more than 50-fold. A study performed by Georges-Courbot et al. (2006) using a hamster model for NiV infection showed that treatment with ribavirin delayed death from viral disease by 2 days but could not prevent death. A more recent study performed by Porotto et al. (2009) identified chloroquine, an anti-malarial drug, as a potent antiviral in in vitro experiments, where it inhibited live HeV and NiV infection. These studies apparently supported the use of a combination of intravenous ribavirin and oral chloroquine to treat four people for 5 days during the latest HeV outbreak in Queensland (ProMED-mail, 2009a). These people had extensive exposure to blood and nasal secretions of confirmed or highly suspect horse cases. All four survived, but the combination treatment was only poorly tolerated and individuals were quite ill following treatment. The reports did not provide confirmation that these individuals tested positive for HeV prior to receiving prophylactic treatment.

In the present study, we wanted to determine whether the antiviral effects found for chloroquine in in vitro experiments would also be observed in the treatment of henipavirus-infected hamsters. We also wanted to discover whether combining monotherapy treatments with ribavirin and chloroquine would result in any protection indicative of favourable drug–drug interactions when treatment was initiated 6 h after infection with a lethal inoculum of NiV or HeV. Our studies found that, whilst both drugs were highly potent in the reduction and inhibition of viral spread in vitro, they did not prove to be protective in an in vivo animal model. Whilst ribavirin delayed death from viral disease in NiV-infected hamsters by 5 days, it did not have a significant effect on HeV-infected hamsters. Chloroquine did not protect hamsters when administered either individually or in combination with ribavirin.

RESULTS

Chloroquine and ribavirin are effective in preventing henipavirus spread in HeLa cells after infection

Previous studies have shown the antiviral activity of ribavirin and recently also of chloroquine on henipavirus-infected cells (Aljofan et al., 2008, 2009; Porotto et al., 2009; Wright et al., 2005). In order to investigate the antiviral efficacy of ribavirin and chloroquine in permissive HeLa cells and to confirm that both drugs were working in our hands, we carried out dose–response assays. HeLa cells were infected with HeV (Fig. 1) or NiV (data not shown), and fresh medium supplemented with various concentrations of chloroquine and ribavirin were added at 1 h post-infection (p.i.). Infected cells were incubated for 24 h. The effect of the drugs was assessed by determining viral titres in the supernatants of infected cells (Fig. 1). The IC50 was calculated to be 0.71 and 0.62 μM chloroquine for HeV and NiV, respectively. For ribavirin, we determined an IC50 of 4.96 and 4.18 μM for HeV and NiV, respectively. These data clearly showed that chloroquine and chloroquine can effectively reduce the spread and infection of NiV and HeV in HeLa cells if the drugs are added after infection.

In vitro prophylactic and therapeutic advantages of chloroquine for henipavirus infection

The experiments described above were carried out with drugs added at 1 h p.i. In the next step, we wanted to examine the optimal time point of addition at which chloroquine and ribavirin exerted their antiviral activity. We used the highest chloroquine (20 μM) and ribavirin (100 μM) concentrations that were shown to strongly reduce or abolish the release of henipavirus particles from infected cells in previous assays. HeLa cells were infected under three conditions: (i) cells were pre-treated with chloroquine or ribavirin for 12 h prior to virus infection; (ii) cells were infected in the presence of the drugs, and the drugs remained present for the duration of the experiment; and (iii) untreated cells were infected and the drugs were added at 6 or 12 h p.i. Cell culture supernatants were harvested at 24 h p.i. and viral titres were determined by plaque assay. The results shown in Fig. 2 for HeV infection indicate that treatment of HeLa cells with 20 μM chloroquine or 100 μM ribavirin prior to infection reduced the release of virions by...
approximately 100 and 75%, respectively. Similar results were achieved with NiV-infected cells (data not shown). With ribavirin, an increased level of inhibition was observed when the drug was present during or after infection (Fig. 2b). This most likely reflects the mechanism of ribavirin activity acting at the step of RNA replication. Both drugs proved equally active in their inhibitory effect when added during infection or up to 12 h p.i. (Fig. 2).

The observed strong antiviral effect of chloroquine during pre- and post-infection conditions suggested that it might have both prophylactic and therapeutic advantages in the treatment of henipavirus infections.

**Combined chloroquine and ribavirin treatment does not prevent death in a hamster model of NiV and HeV infection**

As ribavirin and chloroquine proved to be active in inhibiting henipavirus release from infected cells, we asked whether both drugs would show an increased antiviral effect when given in combination, indicative of a favourable drug–drug interaction. To investigate this, we challenged 8–10-week-old hamsters with a lethal dose of $10^6$ TCID$_{50}$ NiV or HeV, and treated them with chloroquine and ribavirin post-challenge. Cell culture experiments showed the effectiveness of both drugs in inhibiting virus spread when added during infection or up to 12 h p.i. (Fig. 2).

Fig. 1. Dose–response curves for chloroquine (a) and ribavirin (b). HeLa cells were infected with $10^3$ TCID$_{50}$ HeV and fresh medium containing serial twofold dilutions of ribavirin or chloroquine were added at 1 h pi. Cells were incubated for 24 h prior to harvesting of the cell supernatants for titration in Vero cells. Non-linear regression analysis was performed using SigmaPlot software to determine IC$_{50}$. Values are expressed as the mean log drug concentration and plotted against infectious HeV titres ± SEM ($n=3$). The continuous line represents a non-linear sigmoidal fit.

Fig. 2. Prophylactic effect of chloroquine. HeLa cells were pre-treated with 20 μM chloroquine (a) or 100 μM ribavirin (b) for 12 h prior to virus infection, or the drugs were added at the time of infection (0 h) or at the times indicated. Cells were infected with $10^3$ TCID$_{50}$ HeV. At 1 h p.i., cells were washed with DMEM and fresh medium was added. Pre-treated cells were maintained in the absence of drugs, whilst for the other time points, the medium contained 20 μM chloroquine or 100 μM ribavirin. Cells were incubated for 24 h prior to harvesting of the cell supernatants for titration in Vero cells. Results are shown as means ± SEM ($n=3$).
control animals received drugs only. Hamsters were observed daily, and body weight, temperature and clinical signs were recorded. The dose–survival graphs are shown in Fig. 3 and the mean day of death for NiV- and HeV-infected hamsters is summarized in Table 1. Animals that lost \( \geq 10\% \) of their body weight were euthanized.

Animals in the untreated groups succumbed between day 5 and 8 (NiV) and on day 4 (HeV) after infection (Fig. 3a, b). One animal in the NiV-infected group survived until day 21 (Fig. 3a), the end of the experiment, and one animal in the HeV-infected group survived until day 14 (Fig. 3b). The survivor in the NiV-infected control group (animal \#9) did not show any clinical signs of illness except for ulcers in the mouth that developed on day 10 p.i., and no virus could be detected in serum or brain samples by cell culture titration. The survivor in the HeV-infected control group (animal \#40) became sick on day 6 p.i. and developed neurological signs (head tilt) on day 12 p.i.; the hamster was euthanized on day 14 due to a loss of

Table 1. Mean days to death for hamsters infected with NiV or HeV in different drug treatment groups (n=5 per group)

<table>
<thead>
<tr>
<th>Group</th>
<th>Nipah Malaysia</th>
<th>Hendra</th>
<th>Control*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Untreated</td>
<td>9.2 ± 3.0</td>
<td>6.0 ± 2.0</td>
<td>–</td>
</tr>
<tr>
<td>Ribavirin†</td>
<td>14.0 ± 2.9</td>
<td>5.2 ± 0.4</td>
<td>21.0 ± 0.0</td>
</tr>
<tr>
<td>Chloroquine‡</td>
<td>6.2 ± 0.7</td>
<td>4.2 ± 0.2</td>
<td>21.0 ± 0.0</td>
</tr>
<tr>
<td>Ribavirin+/chloroquine‡</td>
<td>7.0 ± 0.0</td>
<td>6.6 ± 2.1</td>
<td>21.0 ± 0.0</td>
</tr>
</tbody>
</table>

*Two animals per control group.
†30 mg kg\(^{-1}\) twice daily.
‡50 mg kg\(^{-1}\) once every other day.

>10 % body weight. A possible explanation for the delayed death or survival could be the higher body weight, and consequently age, of these animals compared with the remaining four animals in the group (Fig. 4a, b). Guillaume et al. (2009) demonstrated that 11-week-old hamsters are less sensitive to HeV infection than 5-week-old hamsters.

Fig. 3. Effects of ribavirin and chloroquine on the survival of NiV- and HeV-infected hamsters. Animals were infected by the i.p. route with \( 10^4 \) TCID\(_{50} \) NiV (a) or HeV (b) in 100 \( \mu L \). At 6 h p.i., treatment was initiated by dosing with 30 mg ribavirin kg\(^{-1}\) twice daily (○), 50 mg chloroquine kg\(^{-1}\) every other day (▼) or a combination of both drugs (△). Infected control animals received vehicle solution only (●).

Fig. 4. Effect of body weight on the sensitivity to NiV or HeV infection in hamsters. Change in body weight (g) of NiV-infected control animals (a) and HeV-infected animals (b) over the course of the experiment. The numbers on the graph indicate the animal number referred to in Results.
Under our experimental conditions, ribavirin delayed death by around 5 days in NiV-infected hamsters but could not prevent NiV-induced mortality. However, in this group, two hamsters survived until day 21, the end of the experiment (Fig. 3a and Table 1). Similar to the survivor of the virus control group, no infectious virus was detected in the sera or brains of these animals by cell culture titration. For the HeV-infected hamsters, we did not observe a positive effect of ribavirin on the survival rate (Fig. 3b and Table 1). Treatment of infected animals with chloroquine alone was found neither to delay nor to prevent virus-induced mortality (Fig. 3a, b). Rather, it appeared that the administration of chloroquine decreased survival by 3 (NiV) or 2 (HeV) days (Table 1). Animals treated with ribavirin in combination with chloroquine had a mean survival time that was approximately comparable to the untreated virus-infected group (Table 1). One of the HeV-infected animals survived until day 15; however, this animal became sick on day 5 p.i. and hypothermic on day 6 p.i.

**Optimization of the efficacy of chloroquine and ribavirin in vivo**

Having observed no clear curative effect of ribavirin or chloroquine, individually and in combined treatment, we chose to focus our efforts on examining whether higher doses of the drugs might have a positive outcome on the survival rates of infected hamsters. As ribavirin proved to be able to delay death caused by NiV, we opted to perform the concentration optimization experiments on NiV-infected animals only. We ensured that the body weight of animals included in this experiment were within ±10% of 100 g body weight (6–7-week-old hamsters). Hamsters were inoculated with $10^4$ TCID$_{50}$ NiV and received three different doses of ribavirin (100, 150 or 200 mg kg$^{-1}$ day$^{-1}$) or chloroquine (50, 100 or 150 mg kg$^{-1}$ day$^{-1}$). Animals were observed daily and body weight, temperature and clinical signs were recorded. The animal survival data are summarized in Table 2.

As demonstrated in Table 2, the 100 mg kg$^{-1}$ day$^{-1}$ dose of ribavirin was the most effective at increasing animal survival following infection. At this dose, death was delayed by ~3 days, which is in good agreement with our first experiment, where ribavirin was administered at 60 mg kg$^{-1}$ day$^{-1}$ (Fig. 3a). The 150 and 200 mg kg$^{-1}$ day$^{-1}$ doses were less effective, as the mean survival time was extended by only approximately 1 day (Table 2). In the uninfected control group receiving only ribavirin at a dose of 200 mg kg$^{-1}$ day$^{-1}$, all three animals started losing weight on day 3 or 4. Two of the three control animals became sick on day 6 and were euthanized on day 7, indicating that the maximal tolerable ribavirin concentration was between 150 and 200 mg kg$^{-1}$ day$^{-1}$ using a twice-daily dosing schedule (Table 2). A similar finding has been described by Ishii et al. (1996).

The mean day of death of hamsters receiving chloroquine at a daily dose of 50 mg kg$^{-1}$ was approximately 5 days, similar to untreated infected animals (Table 2). Chloroquine administered at doses of 100 or 150 mg kg$^{-1}$ day$^{-1}$ were not well tolerated and animals died on day 2 or 1 p.i., respectively. Numerous animals in the infected groups had blood in their nasal secretions. Uninfected control animals receiving 100 or 150 mg chloroquine kg$^{-1}$ day$^{-1}$ died at day 2 after initiation of treatment, indicating highly toxic effects at these doses. Initially, chloroquine was given subcutaneously, but almost all of the animals receiving the drug subcutaneously developed a skin reaction close to the injection site. Therefore, after the first drug treatment, chloroquine was injected via the intraperitoneal (i.p.) route.

**DISCUSSION**

HeV and NiV are newly emerging zoonotic viruses that cause severe disease such as encephalitis in animals and humans with fatality rates of up to 75% (Chua et al., 2000a, b; Harcourt et al., 2000; Hoasain et al., 2008; Murray et al., 1995b; O’Sullivan et al., 1997; Wang et al., 2000). Both viruses may cause asymptomatic infection in up to 60% of exposed people and can lead to late-onset disease or the relapse of encephalitis years after initial infection (Sejvar et al., 2007), as well as persistent or delayed neurological sequelae (Lee, 2007). Both viruses have been associated with the fruit bat (Pteropus spp.) mammalian reservoir. Interestingly, a recent study described the detection of putative henipavirus RNA in Eidolon helvum, a fruit-eating bat that occurs in Africa but not in Asia (Drexler et al., 2009), suggesting that the distribution of henipaviruses might be more global than initially suspected. These findings are particularly concerning because there is no effective treatment or vaccine for the human disease caused by these viruses.

The aim of this study was to determine whether the antiviral activity of individually administered chloroquine and ribavirin against NiV and HeV observed in *in vitro* experiments could also protect against fatal disease caused by NiV and HeV infection in a hamster model. Using HeLa
cells in our in vitro assays, we confirmed that chloroquine and ribavirin were effective in reducing viral spread, with IC50 values of ~0.7 and ~4.5 μM, respectively. These results are in good agreement with previously published in vitro experiments (Aljofan et al., 2008, 2009; Porotto et al., 2009; Wright et al., 2005). Furthermore, we detected inhibition of virus spread when chloroquine was added at different time points before or after infection. Chloroquine was significantly effective at inhibiting viral infectivity (almost 100%), even when the drug was added at up to 12 h p.i. The mechanism of action for chloroquine has been proposed as preventing the proteolytic cleavage of NiV and HeV fusion protein by cathepsin L (Porotto et al., 2009). Diederich et al. (2008) found that proteolytic activation of NiV fusion protein by endosomal cathepsin L is required before incorporation into virions. Taken together with the findings of Porotto et al. (2009), our study provides further evidence that chloroquine is effective against NiV and HeV infection under cell culture conditions, as evidenced by its inhibitory effect when the drug was added prior to, during or after infection. These results suggest that chloroquine might have both prophylactic and therapeutic advantages in the treatment of henipavirus infections. However, ribavirin had a slightly higher inhibitory effect on virus spread when the drug was present during or after infection, probably reflecting the mechanism of ribavirin acting at the step of viral RNA replication (Crotty et al., 2002).

In our initial hamster experiment, ribavirin (60 mg kg\(^{-1}\) day\(^{-1}\)) delayed death by viral disease by only 3 days in NiV-infected hamsters, and did not effect a cure. These results are similar to findings by Georges-Courbot et al. (2006) on the use of ribavirin (50 mg kg\(^{-1}\) day\(^{-1}\)) for the treatment of NiV infection in hamsters. In our study, animals received 30 mg ribavirin kg\(^{-1}\) twice daily, and death from viral disease was delayed by approximately 5 days. Although ribavirin was subcutive in NiV-infected hamsters, it did not have any beneficial effect on HeV-infected animals in our experiments. Chloroquine, given independently at 50 mg kg\(^{-1}\) every other day, or in combination with ribavirin, did not extend the mean survival times of animals at the doses we used, and henipavirus-induced illness was unaltered with control animals. One possibility explaining the lack of protective activity of both drugs might be the concentrations used. It is possible that an antiviral effect could be achieved with a higher dosage and a daily administration of both drugs. Although we could achieve a delay of death by 3 days with ribavirin, we were unable to extend the delay of death or to achieve complete protection with increased ribavirin doses. Interestingly, treatment with chloroquine did not have a favourable effect in in vivo experiments compared with its strong antiviral activity found in cell culture experiments. It should be noted that our findings were restricted to post-challenge treatment of henipavirus-infected hamsters. Other possibilities explaining the failure of the drugs’ antiviral effects could be the route of administration. In humans, ribavirin is given intravenously and chloroquine orally; consequently, the oral route of chloroquine treatment could also be considered in future hamster studies. For treatment against malaria, the daily prescribed adult dosage of chloroquine is 300 mg of the base. The drug is normally well tolerated at the prophylactic dose regimen; however, side effects are common when curing clinical attacks. During a recent outbreak of HeV in Queensland, Australia, four individuals with extensive exposure to HeV-infected material received prophylactic treatment using a combination of intravenous ribavirin and oral chloroquine for 5 days (ProMED-mail, 2009a). All four survived, but the combination was only poorly tolerated, as the treatment regimen made individuals feel quite ill. Infected hamsters receiving chloroquine in our study also appeared ill, indicating the presence of possible side effects. Ribavirin has been reported to be active in hamster models of subacute sclerosing panencephalitis, where it was not active when administered via the i.p. route, but improved survival when administered intracranially (Honda et al., 1994). As NiV and HeV cause encephalitis and in up to 60% of exposed people relapsed encephalitis may occur years after initial infection (Sejvar et al., 2007), it would be interesting to investigate whether intracranial administration of ribavirin would improve the survival of henipavirus-infected hamsters; however, treatment by this route will damage the blood–brain barrier and is just as likely to exacerbate disease. Furthermore, the infective dose used in our study may be higher than that occurring in natural infections of humans, possibly limiting the effectiveness of both drugs.

In summary, the results of our studies indicated that ribavirin was moderately effective at increasing survival time as a post-henipavirus-challenge treatment in a hamster model. Treatment with chloroquine was found to be ineffective in the treatment of NiV- and HeV-infected hamsters, and daily doses of more than 100 mg (kg body weight\(^{-1}\)) were found to be highly toxic. Furthermore, combining monotherapeutic treatments of ribavirin and chloroquine did not result in protection indicative of additive drug–drug effects against henipavirus infection in vivo.

During the preparation of this manuscript, a study was published investigating the effect of chloroquine in a ferret model of lethal NiV infection (Pallister et al., 2009). Ferrets received 25 mg chloroquine kg\(^{-1}\) day\(^{-1}\) either 24 h before or 10 h after viral challenge, but this was found to be ineffective against NiV infection in vivo.

**METHODS**

**Cells and viruses.** VeroE6, Vero and permissive HeLa cells were propagated in Dulbecco’s modified Eagle’s medium (DMEM; Gibco) supplemented with 10% fetal bovine serum (FBS; Sigma-Aldrich), 100 U penicillin ml\(^{-1}\) (Sigma-Aldrich), 100 μg streptomycin ml\(^{-1}\) (Sigma-Aldrich) and 1% sodium pyruvate (Cellgro). Virus-infected cells were maintained at 37°C in 5% CO\(_2\) in DMEM supplemented...
with 2% FBS, 100 U penicillin ml$^{-1}$ and 100 μg streptomycin ml$^{-1}$. NIV Malaysia and HeV were kindly provided by the Special Pathogens Branch (Centers for Disease Control and Prevention, GA, USA) and Dr Heinz Feldman (Special Pathogens Program, National Microbiology Laboratory, Public Health Agency of Canada, Manitoba, Canada), respectively. NIV and HeV stocks were prepared by infecting VeroE6 cells, and the virus was titrated by determination of TCID$_{50}$ using the method of Reed & Muench (1938). All work with live viruses was performed in the Robert E. Shope Biosafety Level 4 (BSL-4) laboratory at the University of Texas Medical Branch, TX, USA.

**Compounds.** Stock solutions (100 mM) of chloroquine diphosphate salt (Sigma) and ribavirin (VIRAZOLE; Valeant) were prepared in DMEM supplemented with 20 mM HEPES (pH 7; Cellgro) (vehicle solution).

**Antiviral assay.** HeLa cells were infected with $10^3$ TCID$_{50}$ NIV or HeV (approximate m.o.i. of 0.03). After 1 h of adsorption at 37 °C, cell monolayers were washed with PBS and fresh medium containing various concentrations of chloroquine or ribavirin was added. At 24 h p.i., virus-containing supernatants were collected and viral titres were determined by plaque assay. For titrations, confluent monolayers of Vero cells were infected with 100 μl of serial 10-fold dilutions of virus-containing cell supernatant. After 1 h incubation at 37 °C and 5% CO$_2$, the inocula were removed and the wells overlaid with a mixture of one part 1% methylcellulose (Fisher Scientific) and one part 2 x MEM (Gibco) supplemented with 4% FBS, 200 U penicillin ml$^{-1}$ and 200 μg streptomycin ml$^{-1}$. The plates were incubated at 37 °C and 5% CO$_2$ for 3 days and then stained with 0.25% crystal violet in 10% buffered formalin. The plates were washed and the plaques enumerated.

To calculate IC$_{50}$, the resulting data were fitted to the symmetrical sigmoidal log drug dose–response equation (SigmaPlot software version 11.0): % inhibition = maximal inhibition/1 + exp[−[(log drug concentration−log IC$_{50}$)/minimal inhibition]]).

**Time-of-addition assay.** Subconfluent monolayers of HeLa cells were pre-treated with 20 μM chloroquine or 100 μM ribavirin for 12 h prior to virus infection. Alternatively, the drugs were added at the time of infection or at specified times thereafter. Cells were then infected with $10^3$ TCID$_{50}$ NIV or HeV. At 1 h p.i., the cells were washed with DMEM and fresh medium was added. Pre-treated cells were maintained in the absence of drugs, whilst for the other time points, the medium contained 20 μM chloroquine or 100 μM ribavirin, respectively.

**Hamster infection experiments.** Two sets of animal studies were carried out. In the first study, the antiviral activity of ribavirin and chloroquine (treatment with either drug or in combination) was tested on NIV- and HeV-infected 8–10-week-old female golden Syrian hamsters (Mesocricetus auratus; Harlan Laboratories). Animals were inoculated by the i.p. route with $10^3$ TCID$_{50}$ (100 μl) of NIV or HeV. Five animals were used for each treatment group; control animals (two per group) received the drugs only. Doses and treatment schedules for ribavirin and chloroquine were deduced from paediatric recommendations using up to 200 mg kg$^{-1}$ twice daily (considering the short half-life of ribavirin of 12 h) for children with body weight less than 36 kg and <300 mg base [Rebetol (Schering) and Aralan (Sanofi-Aventis) product inserts]. For these studies, one group received ribavirin (30 mg kg$^{-1}$) twice daily, another group received chloroquine (50 mg kg$^{-1}$) every other day and the third group received a combination of both drugs under the above-mentioned conditions. The infected control group received vehicle solution only.

Drugs were administered via the i.p. route and treatment was initiated at 6 h p.i. and continued throughout the course of the experiment (21 days).

Based on the results of the first study, a second study was then performed to determine the optimal and maximal ribavirin and chloroquine concentration administrable. Hamsters were infected with $10^4$ TCID$_{50}$ (100 μl) NIV only using the i.p. inoculation route. Groups of three hamsters received three individual ribavirin concentrations (50, 75 or 150 mg kg$^{-1}$) twice daily or three individual chloroquine concentrations (50, 100 or 150 mg kg$^{-1}$) daily. Treatment was initiated at 6 h p.i. and continued throughout the course of the experiment (21 days).

The physical conditions of the animals were observed and the body weight and temperature of hamsters with implanted subdermal transponders were measured daily. The animals were housed in microisolator caging equipped with HEPA filters in the BSL-4 laboratory according to Institutional Animal Care and Use Committee guidelines, and in accordance with the Principles of Laboratory Animal Care.

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