Antiviral Activity of Chloroquine against Human Coronavirus OC43 Infection in Newborn Mice

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Until recently, human coronaviruses (HCoVs), such as HCoV strain OC43 (HCoV-OC43), were mainly known to cause 15 to 30% of mild upper respiratory tract infections. In recent years, the identification of new HCoVs, including severe acute respiratory syndrome coronavirus, revealed that HCoVs can be highly pathogenic and can cause more severe upper and lower respiratory tract infections, including bronchiolitis and pneumonia. To date, no specific antiviral drugs to prevent or treat HCoV infections are available. We demonstrate that chloroquine, a widely used drug with well-known antimalarial effects, inhibits HCoV-OC43 replication in HRT-18 cells, with a 50% effective concentration (± standard deviation) of 0.306 ± 0.0091 μM and a 50% cytotoxic concentration (± standard deviation) of 419 ± 192.5 μM, resulting in a selectivity index of 1,369. Further, we investigated whether chloroquine could prevent HCoV-OC43-induced death in newborn mice. Our results show that a lethal HCoV-OC43 infection in newborn C57BL/6 mice can be treated with chloroquine acquired transplacentally or via maternal milk. The highest survival rate (98.6%) of the pups was found when mother mice were treated daily with a concentration of 15 mg of chloroquine per kg of body weight. Survival rates declined in a dose-dependent manner, with 88% survival when treated with 5 mg/kg chloroquine and 13% survival when treated with 1 mg/kg chloroquine. Our results show that chloroquine can be highly effective against HCoV-OC43 infection in newborn mice and may be considered as a future drug against HCoVs.

Coronaviruses are large, enveloped, single-stranded, positive-sense RNA viruses with a genome of approximately 30 kb in length, the largest found in any of the RNA viruses. The genus Coronavirus belongs to the family Coronaviridae in the order Nidovirales. The coronaviruses are classified into three groups based on genetic and serological relationships. Group 1 contains porcine epidemic diarrhea virus, porcine transmissible gastroenteritis virus, canine coronavirus, feline infectious peritonitis virus, human coronavirus 229E (HCoV-229E), and HCoV-NL63. Group 2 contains murine hepatitis virus, bovine coronavirus (BCoV), HCoV-OC43, rat sialodacryoadenitis virus, porcine hemagglutinating encephalomyelitis virus (PHEV), canine respiratory coronavirus, and equine coronavirus. The severe acute respiratory syndrome coronavirus (SARS-CoV) is considered a distant member of group 2 and is therefore placed in a subgroup, 2b (13). Group 3 thus far contains only avian coronaviruses, such as infectious bronchitis virus and turkey coronavirus. Within group 2, HCoV-OC43 is most closely genetic related to BCoV (33). A comparative analysis of the complete genomes of HCoV-OC43, BCoV, and PHEV demonstrated a high genetic similarity among these three coronaviruses in more than two-thirds of their genomes, except in the hemagglutinin-esterase and spike gene region, in which PHEV was remarkably divergent from HCoV-OC43 and BCoV (30, 31, 33).

HCoVs cause respiratory infections, but gastroenteritis and neurological disorders can also occur (3, 20). Until now, five HCoVs have been described. HCoV-OC43 and HCoV-229E are responsible for 10 to 30% of all common colds, and infections occur mainly during the winter and early spring (21). In 2003, a novel HCoV displaying only distant antigenic and genetic similarities to the two previously known HCoVs was identified as the causal agent of SARS, and this causes severe lung disorder, leading in some cases to systemic infection and eventually death in about 10% of cases (19). During the two years after the SARS outbreak, two additional previously unrecognized coronaviruses affecting humans, HCoV-NL63 and HCoV-HKU1, were identified (29, 35). HCoV-NL63 infection is related to acute respiratory dysfunction in infected individuals. Furthermore, HCoV-NL63 was identified as the major pathogen responsible for croup in young children (9, 10, 22). The clinical features of HCoV-NL63 infections appear to be more severe than those commonly attributed to infections by HCoV-OC43 and HCoV-229E (4, 12, 29). Infection with HCoV-HKU1 is mostly associated with bronchiolitis and pneumonia (35, 36).

Although coronaviruses have been recognized as human pathogens for about 50 years, no effective treatment strategy has been approved. This shortcoming became evident during the SARS-CoV outbreak and was the start of numerous studies. Nevertheless, 5 years after the outbreak, we are still lacking an effective commercially available drug. Chloroquine is a clinically approved drug effective against malaria, and it is known as an effective commercially available drug.

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to elicit antiviral effects against several viruses, including human immunodeficiency virus type 1 (23, 26, 28), hepatitis B virus (18), and herpes simplex virus type 1 (27). Savarino and colleagues hypothesized that chloroquine might be of some use for the clinical management of SARS (25). Moreover, chloroquine was reported to inhibit the replication of HCoV-229E (7) and SARS-CoV (16) in vitro. In a previous study, we showed that the 50% effective concentration (EC\textsubscript{50}) of chloroquine (8.8 ± 1.2 μM) for the inhibition of SARS-CoV in vitro was significantly lower than its cytostatic activity (261.3 ± 145.5 μM), yielding a selectivity index of 30, and that this EC\textsubscript{50} approximates the plasma concentrations of chloroquine reached during treatment of acute malaria. The addition of chloroquine to infected cultures could be delayed for up to 5 h postinfection without a significant drop in antiviral activity (16). The antiviral effects of chloroquine against human immunodeficiency virus type 1 replication are currently being tested in clinical trials (25). Chloroquine is a weak base that increases the pH of acidic vesicles. When added extracellularly, the nonprotonated portion of chloroquine enters the cell, where it becomes protonated and concentrated in acidic, low-pH organelles, such as endosomes, Golgi vesicles, and lysosomes. Chloroquine can affect virus infection in many ways, and the antiviral effect depends in part on the extent to which the virus utilizes endosomes for entry. Besides having a direct antiviral effect, chloroquine is endowed with an immunomodulatory activity, suppressing the production and release of tumor necrosis factor alpha and interleukin 6, which mediate the inflammatory complications of several viral diseases (25).

In the present study, we further investigated the anticornaviral properties of chloroquine by testing the in vitro antiviral activity of chloroquine against HCoV-OC43. In addition, we developed a lethal in vivo challenge model to test the antiviral effect of chloroquine against HCoV-OC43.

**MATERIALS AND METHODS**

**Test compounds.** We tested chloroquine phosphate (7-chloro-4-[4-(diethylamino)-1-methylbutyl]amino) quinoline phosphate; Alpha Pharma, Braines- l’Alleud, Belgium).

**Virus and cells.** The OC43 strain was originally obtained from the American Type Culture Collection (ATCC, Rockville, MD). HRT-18 cells (ATCC, Rockville, MD) were propagated in minimal essential medium (MEM; Gibco, Life Technologies, Rockville, MD) supplemented with 10% fetal calf serum (Integro, Zaandam, The Netherlands), 1% l-glutamine (Gibco), and 1.4% sodium bicarbonate (Gibco). Virus-infected cells were maintained in MEM supplemented with 2% fetal calf serum. For antiviral activity and cytotoxicity measurements, supernatant was harvested after 4 days of incubation at 37°C in the presence of 5% CO\textsubscript{2}.

**Real-time RT-PCR.** The methodology of the real-time reverse transcriptase PCR (RT-PCR) assay has been described previously (32). Briefly, the quantitative RT-PCR (qRT-PCR) was performed with a 25-μl reaction mixture with 5 μl extracted RNA, 12.5 μl of Eurogentec one-step qRT-PCR master mix containing Rox as a passive reference dye, 0.125 μl Euroscript reverse transcriptase/RNase inhibitor (Eurogentec, Seraing, Belgium), 300 nM forward and reverse primers, and 200 nM MGB (3′-minor groove binder) probe. Amplification and detection were performed in an ABI Prism 7700 sequence detection system (Applied Biosystems, Foster City, CA). In order to allow absolute HCoV-OC43 quantitation, cRNA standards were constructed and used for the generation of a standard curve as described previously (32).

**qRT-PCR-based antiviral activity assay.** Antiviral measurements were based on the reduction in viral titer of coronavirus-infected cells. HRT-18 cells were seeded at a density of 6 × 10\textsuperscript{4} cells per well into a 24-well culture plate. After 4 days of growth, cells were infected with 1 × 10\textsuperscript{6} HCoV-OC43 copies per ml in the presence of various concentrations of chloroquine ranging from 0.032 to 500 μM.

After 4 days of incubation at 37°C in the presence of 5% CO\textsubscript{2}, cell supernatants were collected. Viral RNA was extracted using the QiAamp viral RNA kit (Qiagen, Venlo, The Netherlands) to determine the viral RNA load in the cell supernatant by using the qRT-PCR described above.

**Cytotoxicity assay.** Cytotoxicity measurements were based on the viability of HRT-18 cells in the presence of various concentrations of the test compounds. Four days after the addition of the compounds, viable cells were quantified by a tetrazolium-based colorimetric method in which the reduction of the MTS-[3-(4,5-dimethylthiazol-2-y1)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)]-2H-tetrazolium dye (CellTiter 96 AQueous One Solution kit; Promega, The Netherlands) by mitochondrial dehydrogenases to a soluble colored formazan was measured with a spectrophotometer (Multiskan EX, Thermo Labsystems, Belgium) at 492 nm. The 50% cytotonic concentration (CC\textsubscript{50}) was defined as the concentration of the compound that reduced cell viability by 50%.

**Time-of-addition assay.** The methodology of the real-time reverse transcriptase PCR of HRT-18 cells in 24-well plates were infected with 4 × 10\textsuperscript{4} HCoV-OC43 genome equivalents per ml MEM. After 30 min of adsorption, cell monolayers were washed five times with MEM. Chloroquine was added in triplicate at a concentration 30-fold higher than the EC\textsubscript{50} at the time of infection or at different time points thereafter. Twenty-eight hours after infection, cell supernatant was collected. Viral RNA was extracted using the QiAamp viral RNA kit (Qiagen) to determine the viral RNA load in the cell supernatant by using the qRT-PCR described above.

**Mouse infection studies.** Six-week-old male and female C57BL/6 mice (El- evage Janvier, Le Genest Saint Isle, France) were coupled. Female pregnant mice were not treated or were injected subcutaneously with 200 μl of different dilutions of chloroquine (corresponding to 1, 5 or 15 mg/kg of body weight) daily starting from 2 days before labor. Subsequently, five-day-old C57BL/6 pups were inoculated intracerebrally with 10 μl of virus dilution (i.e., 1 × 10\textsuperscript{5} HCoV-OC43 viral copies/μl) using a sterile 30G Micro-Fine syringe (BD Consumer Health care, Franklin Lakes, NJ). The suckling mice were monitored daily for mortality. The surviving mice were followed during 60 days after the infection. In the switching experiments, three groups of pregnant C57BL/6 mice were used. One group of mice were treated once with 15 mg/kg chloroquine 1 day prepartum, one group of mice were treated daily with 15 mg/kg postpartum, and one group did not received any chloroquine treatment and served as the infection control. At the day of birth, the litter of group 1 and the litter of group 2 were switched. In the third group, the newborns in group 1 were transferred to the maternal milk and the newborns in group 2 received chloroquine only transplacentally. After 5 days, the 5-day-old suckling mice were inoculated intracerebrally with 1 × 10\textsuperscript{5} genome copies of HCoV-OC43. All mice were treated according to the laboratory animal control guidelines of our institute, which conform to those of the European Commission. All animal experiments were carried out in a bio- safety level 2 facility.

**Statistical analysis.** All statistical tests were carried out using GraphPad Prism version 4.00. The significance level was set at a P value of <0.05.

**RESULTS**

In vitro antiviral activity of chloroquine. qRT-PCR represents a useful tool in determining the antiviral activity of a compound against HCoV-OC43. In this antiviral assay, the inhibitory effect of the compound on viral replication is calculated by quantitation of the viral growth (i.e., RNA genome equivalents) in the presence of the compound versus the viral growth without the compound (i.e., the positive control). The EC\textsubscript{50} of the compound was defined as the concentration that inhibited the viral RNA increase by 50%. To allow absolute HCoV-OC43 RNA quantitation, cRNA standards were constructed and used for the generation of a standard curve. A chloroquine concentration higher than 0.16 μM was calculated to be 0.306 ± 0.091 μM. The CC\textsubscript{50} (± standard deviation) of chloroquine was calculated to be 419.0 ± 192.5 μM. Chloroquine inhibits the in vitro replication of HCoV-OC43 with a selectivity index of 1,369. The selectivity index was determined as the ratio of the CC\textsubscript{50} to the EC\textsubscript{50}.
Antiviral activity of chloroquine administered at different time points after coronavirus infection. A time-of-addition assay was performed to determine the in vitro antiviral activity of chloroquine when added at various time points after virus infection. For this purpose, HRT-18 cells were infected with HCoV-OC43. Chloroquine was added at a concentration of 10 μM (i.e., 32.7 times the EC50) at different time points after infection. Viral RNA levels in the cell supernatants were determined 28 h postinfection. The measurement of the viral load shows that chloroquine is required at the moment of infection to block the HCoV-OC43 replication (Fig. 1). When chloroquine was added at the time of infection, viral RNA could still be detected, but the viral load was reduced 100-fold in comparison to the positive control viral load. At later time points, a loss of the antiviral activity of chloroquine was noted.

Antiviral activity of chloroquine against a lethal HCoV-OC43 infection in newborn C57BL/6 mice. We investigated whether chloroquine could prevent death in newborn mice as a result of infection with HCoV-OC43. C57BL/6 mice were selected in view of their susceptibility to HCoV-OC43 (14, 15). Pregnant mice were left untreated or treated subcutaneously 1 day prepartum or 1 day postpartum with chloroquine (1, 5, or 15 mg/kg). Subsequently, 5-day-old suckling mice were inoculated intracerebrally with 1 × 108 genome copies of HCoV-OC43. In litters of untreated mothers (group 3), all the pups died within 6 days after HCoV-OC43 challenge (Fig. 3). The survival rate of newborn mice that received the chloroquine only transplacentally (group 2) was 0% (0/27) (Table 1). The survival rate of newborn mice that received the chloroquine via maternal milk (group 1) was 69.0% (20/29). A log rank test indicated that there was a significant difference between the survival curve for the pups that received chloroquine transplacentally and the survival curve for the pups that received the chloroquine only via maternal milk (P < 0.0001).

DISCUSSION

As HCoVs are no longer considered to be as harmless as previously assumed and as they can be associated with severe respiratory tract infections, the identification of compounds effective against these viruses is an important issue. Chloroquine is a clinically approved drug effective against malaria, and it is known to elicit antiviral effects against several viruses, including SARS-CoV and HCoV-229E (7, 16, 17). However, in vivo studies were unable to show the antiviral effectiveness of chloroquine against SARS-CoV (6). In the present study, we investigated the antiviral properties of chloroquine by testing its in vitro and in vivo antiviral activities against the group 2 HCoV-OC43 virus.

In the in vitro antiviral experiments, chloroquine showed in vitro antiviral properties against HCoV-OC43 replication in HRT-18 cells, with an EC50 of 0.306 μM. The 50% CC50 was 419 μM, resulting in a selectivity index of 1,369. In comparison...
to the anti-SARS-CoV activity of chloroquine, with an EC50 of 8.8 μM and a selectivity index of 30 (16), the inhibition of HCoV-OC43 replication by chloroquine is more potent. The exact mechanism of antiviral intervention by chloroquine is not yet elucidated but is possibly a multitarget mechanism, depending on the time point at which the drug is added. When added during and shortly after infection, chloroquine probably affects the endosome-mediated fusion, and when the drug is given after this first target, it can still act on later stages of the viral life cycle, as reported for other viruses (25). For the anti-SARS-CoV activity, chloroquine was proven equally active when added during virus adsorption or 1 h after infection. Later, a gradual loss of antiviral activity was seen (16). Vincent and colleagues found that chloroquine is effective in preventing SARS-CoV infection in cell culture. When the drug is added 24 h prior to the infection at a concentration of 10 μM, the infectivity was completely eliminated. Moreover, they found that chloroquine was significantly effective even when the drug was added 3 to 5 h after infection, suggesting an antiviral effect even after the establishment of infection. Since these researchers obtained similar results by NH4Cl treatment of Vero cells, the underlying mechanisms of action of chloroquine and NH4Cl might be similar and might be attributed to the alkaline properties of both compounds (34). Interestingly, our time-of-addition experiments with HCoV-OC43 pointed out that chloroquine was required at the moment of infection to block HCoV-OC43 replication. When added at the time of infection, chloroquine reduced the viral load by 2 logs in comparison to the positive control value. At later time points, a loss of antiviral activity of chloroquine was noted. Thus, chloroquine appears to act only on the entry of HCoV-OC43 and not on the later stages of the replication cycle.

Because of this markedly broad activity spectrum against coronaviruses, we developed an in vivo model to test the antiviral properties of chloroquine against HCoV-OC43. Our

![Graph of survival curves for newborn mice after HCoV-OC43 infection.](image)

**FIG. 2.** Survival curves for newborn mice after HCoV-OC43 infection. Newborn mice were infected intracerebrally with 10^3 HCoV-OC43 copies, and mother mice were treated subcutaneously with 1, 5, or 15 mg/kg chloroquine (CQ) prepartum (A) or postpartum (B). N represents the number of mother mice per group, and n represents the number of newborn mice per group.

<table>
<thead>
<tr>
<th>Chloroquine administration method</th>
<th>No. of surviving mice/total (% survival) given indicated amt of chloroquine (mg/kg)</th>
</tr>
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<tbody>
<tr>
<td>Transplacental and via maternal milk</td>
<td>70/70 (100) 39/42 (92.9) 7/21 (33.3) 0/132 (0)</td>
</tr>
<tr>
<td>Via maternal milk</td>
<td>74/76 (97.4) 35/42 (83.3) 0/31 (0) ND</td>
</tr>
<tr>
<td>Switch ext</td>
<td>0/27 (0) ND ND ND</td>
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<tr>
<td>Transplacental</td>
<td>20/29 (69.0) ND ND ND</td>
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* C57BL/6 mice were treated with 15, 5, 1 or 0 mg/kg chloroquine before challenge. ND, not done.

**TABLE 1.** Absolute numbers of animals surviving 60 days after HCoV-OC43 challenge.
results show that a lethal HCoV-OC43 infection in newborn C57BL/6 mice can be treated with an effective antiviral concentration of chloroquine acquired transplacentally or through maternal milk. Pregnant mice were treated once with several doses of chloroquine prepartum or postpartum. Since the survival rate was higher for newborn mice when the mother was treated prepartum (Fig. 2A), we investigated the roles of transplacentally and orally (i.e., via maternal milk) acquired chloroquine in a second experiment. In litters of untreated mothers, all the pups died within 6 days after HCoV-OC43 challenge. All newborn mice that received chloroquine exclusively transplacentally died (Fig. 3). A possible reason for the 100% mortality can be that chloroquine is not transferred through the placenta. This is highly unlikely, because almost all xenobiotics that are given during pregnancy can enter the fetal blood circulation through passive diffusion. Moreover, transplacental distribution of chloroquine in humans and rabbits has already been documented (1, 2, 11). In sheep, on the other hand, the transfer rate of chloroquine from the mother to the fetus was low (5). No data are available for transplacental transfer in mice, although placental transfer of chloroquine in mice seems likely, given the survival rate of newborn mice treated both before and after birth being higher than the survival rate of newborn mice treated solely after birth.

The 100% mortality of the pups that received chloroquine only transplacentally is most likely attributable to an ineffectively low concentration of chloroquine reached in the newborn mice. Chloroquine accumulates in tissue and organs of the mother and the fetus, and shortly after administration, a balance between the mother and fetus is reached (2, 24). The concentration of chloroquine reached after equilibrium in the fetus, in the case of treatment with 15 mg/kg chloroquine prepartum, is possibly not sufficient to protect against a lethal HCoV-OC43 infection. A higher dose of chloroquine in this animal model is not an option, since previous experiments with 30 mg/kg chloroquine administered prepartum or postpartum revealed that this dose was toxic for the mother mice and lethal for the pups (data not shown).

In humans, chloroquine accumulates in the milk glands, resulting in high chloroquine concentrations in maternal milk (1, 8). The chloroquine concentration in the blood of the suckling mice increases with the amount of maternal milk they drink. In this way, an effective antiviral chloroquine concentration can be reached.

The survival rate of newborn mice that were switched after birth and received chloroquine only via maternal milk (the mothers were treated with 15 mg/kg chloroquine) was 69.0% (20/29). This rate is lower than the survival rate of newborn mice whose mothers received chloroquine postpartum (97.4%). One reason for this lower survival rate in the switched litters can be attributed to the fact that the mother mice, treated prepartum with chloroquine in the second experiment, received the chloroquine 2 days earlier than the mother mice from the first experiment that were treated postpartum. In this way, the chloroquine was already distributed to their biological pups. Thus, part of the chloroquine was already metabolized and excreted, leading to lower chloroquine concentrations in the switched litters. Additionally, the stress for the mother mice, caused by the switch of the pups and possibly resulting in poor nursing of the pups, can also explain the higher death rate of the switched pups.

In an in vivo study by Barnard and colleagues, BALB/c mice were infected with SARS-CoV and treated with chloroquine (6). In that study, no significant reduction in the replication of SARS-CoV in vitro and in vivo was seen, and this was in contrast with the findings of other published in vitro studies (16, 34) and our in vivo study. This difference in findings is possibly due to a difference in animal model, an infection (nonlethal) model in BALB/c mice versus a lethal model in newborn C57BL/6 mice, and a different antiviral treatment scheme. Moreover, the in vitro antiviral effect of chloroquine against SARS-CoV is less potent than the antiviral effect against HCoV-OC43 in vitro. For SARS-CoV, the plasma concentrations reached in mice are possibly not high enough to protect against a SARS-CoV infection.

In conclusion, we demonstrate here that chloroquine shows strong in vitro and in vivo antiviral activities against HCoV-OC43. Moreover, treatment with daily doses of chloroquine has a long-lasting protective effect against lethal coronavirus OC43 infection in newborn mice.
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