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Hydroxychloroquine and azithromycin used alone or combined are not effective against SARS-CoV-2 ex vivo and in a hamster model

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A B S T R A C T

Drug repositioning has been used extensively since the beginning of the COVID-19 pandemic in an attempt to identify antiviral molecules for use in human therapeutics. Hydroxychloroquine and azithromycin have shown inhibitory activity against SARS-CoV-2 replication in different cell lines. Based on such in vitro data and despite the weakness of preclinical assessment, many clinical trials were set up using these molecules. In the present study, we show that hydroxychloroquine and azithromycin alone or combined does not block SARS-CoV-2 replication in human bronchial airway epithelia. When tested in a Syrian hamster model, hydroxychloroquine and azithromycin administrated alone or combined displayed no significant effect on viral replication, clinical course of the disease and lung impairments, even at high doses. Hydroxychloroquine quantification in lung tissues confirmed strong exposure to the drug, above in vitro inhibitory concentrations. Overall, this study does not support the use of hydroxychloroquine and azithromycin as antiviral drugs for the treatment of SARS-CoV-2 infections.

1. Introduction

A novel member of the Betacoronavirus genus, named severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), emerged in late 2019 in Wuhan, China. As other emerging betacoronaviruses such as severe acute respiratory syndrome coronavirus (SARS-CoV) and Middle East respiratory syndrome coronavirus (MERS-CoV), this new virus causes severe pneumonia and acute respiratory distress, particularly in the elderly population. In March 2020, the World Health Organization (WHO) declared the coronavirus disease 2019 (COVID-19) as pandemic. As of November 2nd, 2021, the numbers of SARS-CoV-2-related infections and deaths listed by the WHO were over 246 million and near 5 million, respectively (WHO, 2021). During the first months of the pandemic, repositioning of drugs has been proposed to offer the possibility of rapid availability of molecules with antiviral activity (Mercerelli et al., 2018). In this regard, significant antiviral activity was reported for chloroquine (CQ) on VeroE6 cells at a clinically relevant concentration (Wang et al., 2020). This article was quickly followed by a press release claiming efficacy in humans (Gao et al., 2020). After these two reports, CQ and derivatives have been viewed by many as serious candidates for therapeutic use.

Chloroquine and its hydroxylated form, hydroxychloroquine (HCQ), have been developed as antimarial drugs. HCQ is now also used against autoimmune diseases. Both drugs have already been reported to be effective in inhibiting the replication of many viruses in vitro, including emerging coronaviruses (Barnard et al., 2006; Dyall et al., 2014). Their antiviral activity against several RNA viruses has been assessed in rodent models with positive (Li et al., 2017; Yan et al., 2013)}
and negative results (Falzarano et al., 2015; Vigerust and McCullers, 2007). However, some clinical trials conducted before the current pandemic with several RNA viruses indicated that CQ had no effect on acute virus infection in humans (De Lamballerie et al., 2008; Paton et al., 2007). At the beginning of the pandemic, the pandemic with several RNA viruses indicated that CQ had no effect on antiviral activity of HCQ against SARS-CoV-2 was widely investigated. In VeroE6 cells, 50% effective concentrations (EC50) were ranging from 4.2 to 4.5 μM (Liu et al., 2020; Touret et al., 2020), and in CaCo2 cells EC50 were below 5.0 μM (Touret et al., 2020). However, the inconsistency of the antiviral activity of HCQ and CQ in other models such as in Calu-3 cells, A549 cells and in a reconstituted human airspace epithelium (HAE) has raised doubts about their repurposing potential for clinical use (Grosse et al., 2021; Hofmann et al., 2020; Maisonnasse et al., 2020; Ou et al., 2021). Currently, several hypotheses on the mechanism of antiviral action of HCQ in vitro have been proposed, such as inhibition of virus entry through the ACE2 receptor and GM1 ganglioside pathways (Panini et al., 2020a, 2020b), and blockage of virus fusion with endosomal membranes by inhibiting endosomal maturation (Liu et al., 2020).

Azithromycin (AZM) is an antibiotic of the macrolide class commonly used to treat respiratory infections and having a strong pulmonary diffusion (Ishida et al., 1994). AZM has a broad-spectrum antiviral activity in cell culture and some studies reported its efficacy against severe RNA viruses infections using ex vivo and preclinical models (Madrid et al., 2015; Menzel et al., 2016; Schögler et al., 2015; Wu et al., 2018; Zeng et al., 2019). Notably, AZM demonstrated in vitro antiviral activity against SARS-CoV-2 with an EC50 value of 2.1 μM and less than 5.0 μM in VeroE6 and CaCo2 cell cultures respectively (Touret et al., 2020). Its ability to prevent bacterial superinfection and its immunomodulatory properties have been proposed as an additional argument for therapeutic use in SARS-CoV-2 infection (Beigelman et al., 2010; Menzel et al., 2017; Schögler et al., 2015) and 132 clinical trials assessing HCQ and AZM on COVID-19 patients have been launched (www.clinicaltrials.gov) before the release of first preclinical data on non-human primates (Maisonnasse et al., 2020).

Here, we completed the ex vivo assessment of AZM and the combination of HCQ and AZM by testing their antiviral activity against SARS-CoV-2 in a HAE model and we assessed the antiviral activity of high doses of HCQ and AZM, alone or combined, against SARS-CoV-2 using a Syrian hamster model (Mesocricetus auratus).

2. Material and methods

2.1. Cell line

VeroE6 cells (ATCC CRL-1586) were grown in minimal essential medium (Life Technologies) supplemented with 7.5% heat-inactivated fetal calf serum (FCS; Life Technologies), incubated at 37 °C and 5% CO2 with 1% penicillin/streptomycin (PS, 5000 U/mL) and 5000 μg/mL respectively; Life Technologies) and supplemented with 1% non-essential amino acids (Life Technologies).

2.2. Human airway epithelia (HAE)

Mucilair™ HAE recovered from human primary cells of bronchial biopsies were purchased from Epithelix SARS (Geneva, Switzerland). MucilAir™ HAE is composed of approximately 400,000 cells (Essaidi-Laziosi et al., 2018). Specific Epithelix media had been used to maintain bronchial epithelium in air-liquid interface. Epithelium was derived from a 56-year-old Caucasian female donor with no known pathology. All samples purchased from Epithelix SARI have been obtained with informed consent. These studies received approval from local ethic committees, and were conducted according to the declaration of Helsinki on biomedical research (Hong Kong amendment, 1989).

2.3. Virus

SARS-CoV-2 strain BavPat1 was provided by Christian Drosten (Berlin, Germany) through the European Virus Archive GLOBAL (www.european-virus-archive.com). Virus stock was prepared by inoculating at multiplicity of infection (MOI) of 0.001 a 25 cm² culture flask of confluent VeroE6 cells with medium supplemented with 2.5% FCS. At the peak of replication, cell supernatant medium was harvested, supplemented with 25 mM HEPES (Sigma) and then stored at −80 °C. Whole-genome sequencing of virus stock was performed as previously described (Driouich et al., 2021) to check the absence of furin-cleavage-site adaptation mutations (Klimstra et al., 2020).

2.4. Antiviral assay using HAE

Before inoculation, epithelia were gently washed with pre-warmed OPTI-MEM medium (Life technologies). Infection with SARS-CoV-2 was performed on the apical side at a multiplicity of infection of 0.1 as previously described (Pizzorno et al., 2020; Touret et al., 2021). Epithelial cells were cultivated in basolateral media with different concentrations of azithromycin (Sigma) (from 0 to 80 μM), with hydroxychloroquine (Sigma) (5 and 10 μM) and with remdesivir (BLD pharm) used as a positive compound control (10 μM). For the combination experiment, two concentrations of both hydroxychloroquine and azithromycin were evaluated: 5 and 10 μM. Media were replaced every day during the experiment. At the apical side, samples were collected by washing with 200 μL of pre-warmed OptiMEM medium. A volume of 100 μL of this washing medium was used for RNA extraction (see below) and the remaining volume was used to perform a TCID50 assay. For the cytotoxicity evaluation, LDH (lactate dehydrogenase) release was quantified in the basal medium using the Cytotoxicity Detection KitPLUS (LDH) (Roche) according to the manufacturer instructions at day 4 post infection using a Tecxan Infinite 200Pro plate reader (TECAN). Assessing cytotoxicity in HAE model, based on LDH detection has been previously described (Essaidi-Laziosi et al., 2018). The media from each insert was analyzed in duplicate.

2.5. In vivo experiments

In vivo experiments were approved by the local ethical committee (C2EA—14) and the French ‘Ministère de l’Enseignement Supérieur, de la Recherche et de l’Innovation’ (APAFIS#23975) and performed in accordance with the French national guidelines and the European legislation covering the use of animals for scientific purposes. All experiments were conducted in biosafety level 3 laboratory.

2.5.1. Animal handling

Three-week-old female golden Syrian hamsters were provided by Janvier Labs. Animals were maintained in ISOcage P - Bioexclusion System (Techniplast) with unlimited access to water/food and 14 h/10 h light/dark cycle. Animals were weighed and monitored daily for the duration of the study to detect the appearance of any clinical signs of illness/suffering.

2.5.2. Infection

Four-week-old anesthetized (isoflurane) animals were intranasally infected with 10⁴ TCID50 of virus diluted in a final volume of 50 μL with 0.9% sodium chloride solution. The mock-infected group was intranasally inoculated with 50 μL of 0.9% sodium chloride solution.

2.5.3. Drug administration

AZM for oral suspension (Zithromax, Pfizer) was dissolved in ultrapure distilled water to obtain a 20 mg/mL suspension. Two tablets of 200 mg of HCQ (Plaquenil, Sanofi) were crushed and then dissolved in aqueous vehicle solution supplemented with 0.5% of hydroxypropylcellulose and 0.1% of Tween80 to obtain a 10 mg/mL...
solution. AZM and HCQ were orally administered under general anesthesia (isoflurane). Untreated animals received the vehicle of HCQ only. As a positive drug control, a distinct group of hamsters was treated with intraperitoneal favipiravir twice a day (25 mg/mL; solubilized in 0.9% sodium chloride solution; Courtesy of Toyama-Chemical) as previously described (Driouich et al., 2021).

2.5.4. Lung and plasma collection

Lung and blood samples were collected directly after the time of sacrifice. The left lung lobe was washed in 10 mL of 0.9% sodium chloride solution, blotted with filter paper, weighed and transferred into a 2 mL tube that contain 1 mL of 0.9% sodium chloride solution and 3 mm glass beads. It was crushed using a Tissue Lyser machine (Retsch MM400) at 30 cycles/s for 20 min and centrifuged at 16,200 g for 10 min. The supernatant medium was transferred into a 1.5 mL tube, centrifuged at 16,200 g for 10 min. For plasma collection, 1 mL of blood was harvested into a 2 mL tube containing 100 μL of 0.5 M EDTA (Thermo-Fischer Scientific), centrifuged at 16,200 g for 10 min and plasma was transferred into a 1.5 mL tube. Lung clarified homogenates and plasma were stored at –80 °C.

2.6. Quantitative real-time RT-PCR (RT-qPCR) assays

To prevent contaminations, all experiments were conducted in a molecular biology laboratory specifically designed for clinical diagnosis which includes separate laboratories to respect walking forward principle. All animal samples were spiked with 10 μL of internal control (bacteriophage MS2) before acid nucleic extraction as previously described (Ninove et al., 2011). Nucleic acid from HAE apical side washes, lung clarified homogenates and plasma were extracted using the Qiacube HT robot and the QIAamp 96 DNA kit (both from Qiagen) as previously described (Ninove et al., 2011). Nucleic acid from HAE apical side washes, lung clarified homogenates and plasma were extracted using the Qiacube HT robot and the QIAamp 96 DNA kit (both from Qiagen) following manufacturer’s instructions. Before extraction, 100 μL of each sample was transferred in a 5-Block (Qiagen) previously loaded with VXl lysis buffer containing RNA carrier and proteinase K.

Real-time RT-qPCR assays (GoTaq 1-step qRT-PCR, Promega) were performed using 3.8 μL of extracted RNA, 6.2 μL of RT-qPCR mix and standard fast cycling parameters (i.e. 10 min at 50 °C, 2 min at 95 °C, and 40 amplification cycles at 95 °C for 3 sec followed by 30 sec at 60 °C). RNA quantities were determined using four serial dilutions of appropriate quantified T7-generated synthetic RNA standards. RT-qPCR reactions were performed on QuintStudio 12 K Flex Real-Time PCR System (Applied Biosystems) and analyzed with the QuintStudio 12 K Flex Applied Biosystems software v1.2.3. For antiviral assay using HAE, primers and probe sequences, which target SARS-CoV-2 N gene, were: Forward: 5′-GGCGGCAAATTGCAACAAT-3′; Reverse: 5′-CCAAATGGCGC-GACATTCC-3′; Probe: FAM-CCCCCAGCGCTTCAGCGTTCT-BHQ1. For animal experiments, primers and probe sequences, which target SARS-CoV-2 RdRp gene, were: Forward: 5′-GTGARATGTGTCATGTGGTGCGG-3′; Reverse 5′-CARATGTTAASACACATTTAGCATA-3′; Probe: 5′-FAM-CAGGTGGAACCTCATCAGGAGATGC-TAMRA-3′ (Corman et al., 2020).

2.7. Tissue-culture infectious dose 50 (TCID50) assays

To determine infectious titers, 96-well culture plates of confluent VeroE6 cells were inoculated with serial dilutions of samples (ten-fold or four-fold dilutions for HAE apical side washes and animal samples respectively). Each sample dilution was performed in sextuplicate. Plates were read for presence or absence of cytopathic effect after an incubation of 4–6 days at 37 °C and 5% CO2. Infectious titers were estimated using the method described by Reed & Muench and expressed as TCID50/mL or TCID50/g of lung (Reed, 1938).

2.8. Histology

Animal handling, hamster infections and drug administration were performed as described above in the ‘in vivo experiments’ section. Lungs were collected following intratracheal instillation of 4% (w/v) formaldehyde solution and fixed 72 h at room temperature with a 4% (w/v) formaldehyde solution and then embedded in paraffin. Histological analysis was performed as previously described (Driouich et al., 2021). Briefly, tissue sections of 3.5 μm were stained with hematoxylin-eosin (H&E) and blindly analyzed by a certified veterinary pathologist. Different lung compartments were examined: (1) regarding bronchial and alveolar walls, a score of 0–4 was assigned based on inflammation severity; (2) for alveoli, a score of 0–2 was assigned based on presence and severity of hemorrhagic necrosis; (3) for vessel changes (leukocyte accumulation in sub-endothelial space and tunica media), absence or presence was scored 0 or 1 respectively. A cumulative score (see Supplemental Table 1) was then calculated and assigned to a grade of severity.

2.9. Determination of HCQ concentrations in plasma and lung of hamsters

Quantification of hydroxychloroquine in plasma and lung tissues of infected hamsters was performed by a sensitive and selective validated high-performance liquid chromatography method with tandem mass spectrometry method (Quattro Premier XE LC-MS/MS, Waters), with lower limits of quantification of 0.015 μg/mL for plasma and 0.05 μg/mL for lung tissue (Doudika et al., 2020). Plasma samples and lung tissue homogenates were collected at euthanasia, 14 h after last drug intake of 3 days of treatment. Hydroxychloroquine was extracted by a simple protein precipitation method, using methanol and ice-cold acetonitrile for plasma and lung tissue homogenates respectively, as previously described (Maisonnasse et al., 2020). Briefly, 100 μL of sample matrix spiked with 10 μL of internal standard working solution (HCQ-d5, Alsachim) was vortexed for 2 min and then centrifuged at 4 °C for 10 min. Tissue-homogenate supernatants were evaporated. Dry residues or plasma supernatants were then transferred to 96-well plates and 5 μL was injected. To assess the selectivity and the specificity of the method and matrix effect, blank plasma and lung tissue homogenates from untreated hamsters were processed and compared with HCQ and internal standard-spiked samples from the same untreated controls animals.

2.10. Statistical analysis

Statistical analyses and graphical representations were realized using Graphpad Prism 7 (Graphpad software). When relevant, two-sided statistical tests were always used. P-values lower than 0.05 were considered statistically significant.

3. Results

3.1. AZM exhibit no antiviral activity in an ex vivo model of HAE

As previously described, the ex vivo model of reconstituted HAE is a relevant tool to assess the antiviral activity of compounds against SARS-CoV-2 (Pizzorno et al., 2020; Touret et al., 2021). In this section, we focused on AZM and HCQ-AZM combination because we previously demonstrated that HCQ alone does not exhibit antiviral activity with that model (Maisonnasse et al., 2020) (Fig. 1). Remdesivir was used as antiviral positive control at 10 μM. In a first experiment, several AZM concentrations (0; 10; 20; 40; 80 μM) were tested. This experiment was repeated once, concomitantly with two HCQ-AZM combinations (5 and 10 μM) (Fig. 1 and Supplemental Fig. 1). Epithelia were exposed to drugs through their basolateral side from the day of infection. Viral excretion was monitored by performing apical side washes from 2 to 4 days post-infection (dpi) by measuring infectious titres (Fig. 1a,c) and viral RNA yields (Fig. 1b,d) using TCID50 and quantitative real time RT-qPCR assays respectively. The cytotoxic effect of each condition (measure of lactate dehydrogenase activity) was also assessed to strengthen the interpretation of the results as previously
described (Essaidi-Laziosi et al., 2018) (Supplemental Fig. 2).

Taken together, results from all experiments showed that AZM alone or combined with HCQ did not significantly reduce (i) infectious titres and RNA yields in apical washes from 2 to 4 dpi (p ≥ 0.8926 and p ≥ 0.4614 respectively) (Fig. 1). Results also confirmed that HCQ alone was not effective in this model (Maisonnasse et al., 2020) (Supplemental Fig. 1). By contrast, remdesivir significantly reduced infectious titers at 3 and 4 dpi (p ≤ 0.0001) and RNA yields in apical washes from 2 to 4 dpi (p ≤ 0.0482). HCQ at 10 μM when used alone or in combination with AZM induced cytotoxicity (p ≤ 0.0397) (Supplemental Fig. 2) but this did not impact viral replication. The overall results indicate a lack of antiviral activity of AZM, HCQ and combination of both drugs using this ex vivo model of HAE.

3.2. HCQ and AZM alone or combined have no antiviral effect in a hamster model

To assess in vivo efficacy of HCQ and AZM alone or combined, groups of Syrian hamsters were intranasally infected with 1 × 10^4 TCID_{50} of SARS-CoV-2 and orally treated following a preemptive strategy (infection and first dose of treatment at the same time; Fig. 2a). Several dosing regimens were tested: high dose of HCQ (176.9 ± 16.3 mg/kg/day BID); low dose of HCQ (135.6 ± 11.6 mg/kg/day BID); AZM (90.6 ± 6.9 mg/kg/day QD); high dose of HCQ combined with AZM; low dose of HCQ combined with AZM.

All treatments were initiated at the time of infection (afternoon of day 0). HCQ treatments began with a single drug administration of 176.9 or 135.6 mg/kg for high and low dose treatments respectively at day 0. Then, similar daily drug amounts were administrated but following a BID scheme from day 1 post-infection (ie. 176.9 or 135.6 mg/kg/day BID for high and low dose treatments respectively). In all animal experiments, an untreated group of hamsters was infected and orally received the HCQ vehicle twice a day (called “vehicle” in graphs and “vehicle group” below). A positive control group was used in some experiments and received intraperitoneal favipiravir (863.9 ± 79.5 mg/kg/day BID) following a preemptive strategy as previously described (Driouich et al., 2021).

In a first experiment (Fig. 2), groups of 6 animals treated with high dose of HCQ, low dose of HCQ, AZM, high dose of HCQ combined with AZM or low dose of HCQ combined with AZM were sacrificed at 3 dpi (Fig. 2a). Several control groups of 8 and 6 animals received the vehicle only or the favipiravir respectively. Compared to the vehicle group, no treatment regimen induced a significant reduction of infectious titers and viral RNA yields in lungs, while significant reductions were observed in the group treated with favipiravir (p = 0.0002) (Fig. 2b-c). When assessing the viral replication in plasma, a slight significant
reduction of viral RNA yields was observed in the group treated with AZM alone \((p = 0.0483)\) (Fig. 2d). To confirm these results, we replicated this experiment in two steps, with only three dosing regimens (groups of 6 animals) (Supplemental Fig. 4). Firstly, with high dose of HCQ, high dose of HCQ combined with AZM and the vehicle only as control (Supplemental Fig. 4a,c,e,g). Secondly, with AZM and the vehicle only as control (Supplemental Fig. 4b,d,f,h). For high dose of HCQ, alone or combined with AZM, no significant reduction was found. For AZM, the results did not confirm the significant reduction observed during the first experiment in plasma, but another non-reproducible slightly significant reduction of viral RNA yields in lungs was detected \((p = 0.0290)\).

During this first set of experiments, a lack of weight gain was observed from 2 dpi with all infected animals as previously described \((p \leq 0.0208)\) (Driouich et al., 2021). Moreover, immediately after the first administration, all groups treated with AZM alone or combined with HCQ exhibited weight losses (~9.8% to ~2.9% of initial weight at 1 dpi), while the others infected groups did not (Supplemental Figs. 3 and 4g-h).

To further investigate the effect of three treatment regimens (high dose of HCQ, AZM alone and high dose of HCQ combined with AZM) on the viral clearance and on the clinical course of the disease, groups of 6 animals were treated from 0 to 3 dpi and sacrificed at 5 dpi (Fig. 3a). Two control groups of 6 animals received the vehicle only or the favipiravir. The favipiravir group was used only for clinical follow-up because animals were sacrificed at 7 dpi since we combined different experiments. One animal in the group treated with high dose of HCQ combined with AZM and one animal in the favipiravir group died at 1 dpi (unexplained death). Compared to the vehicle group, no treatment regimen induced a significant reduction of lung infectious titers (Fig. 3b) \((p \geq 0.0519)\), lung viral RNA yields (Fig. 3c) \((p \geq 0.1371)\) and plasma viral loads (Fig. 3d) \((p \geq 0.4165)\). Only animals treated with favipiravir gained weight until 4 dpi \((p \leq 0.0143\) at 4 and 5 dpi when compared to vehicle group), in accordance with previous experiments with this drug (Driouich et al., 2021) (Fig. 4e). As described above, animals treated with AZM, alone or combined with HCQ, were subjected to early weight losses (Fig. 4e).

In a last experiment (Fig. 4), we investigated the impact of high dose of HCQ, AZM alone and high dose of HCQ combined with AZM on lung pathological changes induced by SARS-CoV-2 infection. Groups of 4 animals were infected and treated from 0 to 3 dpi and sacrificed at 5 dpi (Fig. 4a). As a control, a group of 4 animals received the vehicle only. For each animal, a cumulative score from 0 to 10 was calculated and assigned to a grade of severity (normal; I = mild; II = moderate; III = marked and IV = severe; details in Supplemental Table 5), based on the severity of broncho-interstitial inflammation, alveolar haemorrhagic necrosis and vessel lesions as previously described (Driouich et al., 2021). Almost all animals exhibited marked or severe broncho-interstitial pneumonia, except one in the group treated with AZM which displayed moderate pulmonary pathological changes (Fig. 4b). Compared to the vehicle group, no treatment regimen induced a significant reduction of cumulative scores \((p \geq 0.2110)\).
Finally, the exposure to HCQ in plasma and lungs was assessed from animals sacrificed during the first experiment (i.e., determination of trough concentrations after multiple administration; Table 1). With both high and low doses of HCQ, alone or combined with AZM, a high accumulation of HCQ was observed with lung/plasma ratios ranging from 57.5 to 362. At both dosing regimens, lung concentrations of HCQ were above the EC\textsubscript{50}. The inhibitory quotient (IQ: ratio between the concentration and EC\textsubscript{50} of 5 μM) in plasma and lungs was 0.07 and 9.14 for treatment with high dose of HCQ, 0.04 and 8.40 for treatment with low dose of HCQ, 0.13 and 10.3 for treatment with high dose of HCQ combined with AZM and 0.09 and 11.01 for treatment with low dose of HCQ combined with AZM, respectively.

4. Discussion

Early in the pandemic, HCQ and AZM were found to inhibit SARS-CoV-2 replication \textit{in vitro} (Touret et al., 2020; Wang et al., 2020), leading to numerous clinical trials assessing the efficacy of HCQ alone or in combination with AZM to treat COVID-19. In the present study, we investigate the efficacy of HCQ, AZM and combination of both drugs \textit{ex vivo} and \textit{in vivo}, in a hamster model. The doses used in the present study were chosen in order to induce high lung exposition with both molecules and consequently avoid ambiguity regarding their antiviral activity \textit{in vivo}.

Regarding azithromycin, we completed previous \textit{in vitro} investigations by using an \textit{ex vivo} model of human airway epithelium with...
drug concentrations up to 16 times the EC$_{50}$ identified in VeroE6 and CaCo2 cells (i.e. 2.1 μM and 5.0 μM, respectively) (Touret et al., 2020). We found that AZM does not inhibit SARS-CoV-2 replication in this model which is relevant for the evaluation of antiviral molecules in SARS-CoV-2 infection (Pizzorno et al., 2020; Touret et al., 2021). In vivo, AZM has previously been evaluated only in combination with HCQ, with doses of 10 mg/kg/day in Syrian hamsters (Kaptein et al., 2020) and 18 mg/kg/day after a loading dose of 36 mg/kg in cynomolgus macaques (Maisonnasse et al., 2020; Rosenke et al., 2020). In the current study, we used a higher dose monotherapy of AZM (91 mg/kg/day) and found no
reproducible antiviral effect, with unmodified lung infectious titres and viral RNA yields, and no improvement of pulmonary histopathological impairments. The good pulmonary diffusion of the molecule has been well documented in several animal models (Davila et al., 1991; Resendez et al., 2020; Rivulgo et al., 2013). In particular, a study assessed the lung diffusion of the drug after a single oral intake of 50 mg/kg in Syrian hamsters. Lung maximal concentrations reached 28.5 μg/g 1 h after the administration of the drug (corresponding to 38.1 μM) and a long half-life of AZM in lungs was reported (22.5 h). These data suggest that the AZM dosing regimen used in the present study, around 2 times higher than the dose used in the work previously cited (Ishida et al., 1994), can induce a lung concentration at least 3.8 times higher than EC50 values between the first drug intake and the sacrifice of the animals used in our investigation.

Altogether, our results suggest that despite favourable pharmacokinetics and lung exposure, AZM has no antiviral activity against SARS-CoV-2 in the hamster model, even at concentrations far above in vitro inhibitory concentrations. Of note, AZM treatment was associated with weight losses, especially at 1 dpi, in accordance with previous studies that showed the toxicity of this class of antibiotic in small rodents when administered orally (DeSalva et al., 1969; Faine and Kaipainen, 1955).

Regarding the HCQ, previous studies have reported antiviral activity in different cell lines (Liu et al., 2020; Touret et al., 2020; Weston et al., 2020) but also reduced or lacking antiviral efficacy of the molecule in cells expressing ACE2 and TMPRSS2 (Dittmar et al., 2021; Grosse et al., 2021; Hoffmann et al., 2020; Ou et al., 2021) and the absence of antiviral activity in the HAE model (Maisonasse et al., 2020). Here we confirmed that HCQ does not inhibit SARS-CoV-2 replication in the HAE model with concentrations near or at least 2 times above the EC50 values identified in VeroE6 and CaCo2 cells (i.e. from 4.2 to 4.5 μM and below 5.0 μM, respectively). Furthermore, a few preclinical studies investigated the efficacy of HCQ against SARS-CoV-2 in hamster and macaque models (Kaptein et al., 2020; Maisonasse et al., 2020; Rosenke et al., 2020). Syrian hamsters were treated with doses ranging between 6.5 and 50 mg/kg/day (Kaptein et al., 2020; Rosenke et al., 2020). Rhesus and cynomolgus macaques were treated with maintenance doses ranging between 6.5 and 45 mg/kg/day (Maisonasse et al., 2020; Rosenke et al., 2020). In these models, preemptive and preventive treatments were ineffective in reducing respiratory viral loads and lung impairments. To ensure a good lung exposure of the drug, we used higher dosing regimens than previously investigated: the high dose of HCQ in our work was 3.5 times higher than the highest dose administrated in the hamster model of SARS-CoV-2 infection (Kaptein et al., 2020; Rosenke et al., 2020).

As expected, the mean lung trough concentration for all treatment was 16.3 μg/g (48.6 μM), well above the EC50 values determined in vitro with a mean IQ of 9.72. In fact, 4-aminoquinolines such as HCQ have a large volume of distribution, leading to a high lung accumulation thanks to their physicochemical properties (Browning, 2014; Mackenzie, 1983; Rowland Yeo et al., 2020). Recent results of bronchoalveolar lavages on COVID-19 patients have shown that HCQ is found in the lung epithelial lining fluid at concentrations above EC50 values, once again suggesting a good exposition of the tissue of concern (Ruiz et al., 2021).

Despite such high lung concentrations, our results were in accordance with previous studies: HCQ treatment had no impact on (i) viral load at peak of replication, (ii) viral clearance at 5 dpi, (iii) clinical disease and (iv) lung pathological changes. However, the ability of HCQ and AZM to prevent transmission between animals has not been studied since no nasal washes were performed during in vivo experiments. This could be a limit of the current study.

After providing experimental evidence that both HCQ and AZM have no antiviral activity ex vivo and in vivo against SARS-CoV-2 when used in monotherapy, we observed no antiviral activity with the combination of both drugs in the same models, despite a synergistic effect predicted in silico (Pantini et al., 2020a). Of note, the only report of synergistic effect of these two drugs in vitro failed to demonstrate it in a robust way (Andreani et al., 2020). Indeed, no combination tested with concentrations below the EC50 values showed a significant antiviral effect and no robust synergy evaluation experiments were performed (Meyer et al., 2019). Finally, other preclinical studies have also shown no synergistic effect with these two drugs (Kaptein et al., 2020; Maisonasse et al., 2020).

Interestingly, recent investigations shed a new light on the link between phospholipidosis and the in vitro activity of antiviral candidates against SARS-CoV-2 (Tummino et al., 2021). A correlation between the induction of phospholipidosis and antiviral activity has been described for several drugs and demonstrated an in vitro antiviral effect solely due to physicochemical properties and not to a specific targeting of SARS-CoV-2. Hence, the in vitro antiviral activity of HCQ and AZM must be rediscussed since these molecules are known or strongly suspected to induce this biological disorder in various models (Baronas et al., 2007; Tsukimura et al., 2021; Tummino et al., 2021; Van Bambekte et al., 1996) and clinical cases (Costa et al., 2013; Khubchandani and Bichle, 2013; Manabe et al., 2021; Obeidat et al., 2020).

In conclusion, our results showed a lack of antiviral activity of HCQ, AZM and combination of both drugs against SARS-CoV-2 ex vivo using a reconstituted HAE model. Moreover, the assessment of efficacy of high doses of HCQ and AZM, used alone or combined, in the hamster model showed no effect on viral replication, viral clearance, clinical disease and pulmonary histopathological impairments. This lack of efficacy in vivo cannot be correlated with an unfavourable pharmacokinetics, since both molecules showed good lung diffusion and lung concentrations above EC50 values determined in vitro. Altogether, these results do not support the use of HCQ and AZM for the treatment of COVID-19.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.antiviral.2021.105212.

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