In vitro testing of Hydroxychloroquine and Azithromycin on SARS-CoV-2 shows synergistic effect

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Abstract

Human coronaviruses SARS-CoV-2 appeared at the end of 2019 and led to a pandemic with high morbidity and mortality. As there are currently no effective drugs targeting this virus, drug repurposing represents a short-term strategy to treat millions of infected patients at low costs. Hydroxychloroquine showed an antiviral effect *in vitro. In vivo* it also showed efficacy, especially when combined with azithromycin in a preliminary clinical trial. Here we demonstrate that the combination of hydroxychloroquine and azithromycin has a synergistic effect *in vitro* on SARS-CoV-2 at concentrations compatible with that obtained in Human lung.

Background

Since the end of 2019, the world has encountered pandemic conditions attributable to a novel Coronavirus SARS-CoV 2 (*1-3*). This is the 7th Coronavirus identified to infect the human population (*1;4;5*) and the first one that had pandemic potential in non-immune populations in the 21st century (*6)*. Finding therapeutics is thus crucial, and it is proposed to do so by repurposing existing drugs (*7-9*). This strategy presents the advantages that safety profiles of such drugs are known and that they could be easily produced at relatively low cost, thus being quicker to deploy than new drugs or a vaccine. Chloroquine, a decades-old antimalarial agent, an analog of quinine, was known to inhibit the acidification of intracellular compartments (*10*) and has shown *in vitro* and *in vivo* (mice models) activity against different subtypes of Coronaviruses: SARS-CoV-1, MERS-CoV, HCoV-229E and HCoV-OC43 (*11-16*). In 2004 it was tested *in vitro* against SARS-CoV 1 (*17*) and caused a 99% reduction of viral replication after 3 days at 16 μM. Moreover, tests *in vitro* have shown inhibition of viral replication on SARS-CoV 2 detected by PCR and by CCK-8 assay (*18*). Hydroxychloroquine (hydroxychloroquine sulfate; 7-Chloro-4-[4-(N-ethyl-N-b-hydroxyethylamino)-1-methylbutylamino]quinoline sulfate) has shown activity against SARS-CoV2 *in vitro* and exhibited a less toxic profile (*19*). This drug is well known and currently used mostly to treat autoimmune diseases and also by our team to treat Q fever disease (*20;21*) and Whipple's disease (*22;23*). In those clinical contexts, concentrations obtained in serum are close to 0.4-1 μg/mL at the dose of 600 mg per day over several months (*24*). Clinical tests of chloroquine and hydroxychloroquine to treat COVID-19 are underway in China (*25*), with such trials using hydroxychloroquine in progress in the US (ClinicalTrials.gov Identifier: NCT04307693) and in Europe with the Discovery Trial. In this drug repurposing effort, antibacterial components have also been tested. Teicoplanin, a glycopeptide, was demonstrated *in vitro* to inhibit cellular penetration of Ebola virus (26) and SARS-CoV 2

(*27*). Azithromycin (azithromycin dihydrate), a macrolide, N-Methyl-11-aza-10-deoxo-10-dihydroerythromycin A, has shown antiviral activity against Zika (*28-30*) . Azithromycin is a well-known and safe drug, widely prescribed in the US, for example, with 12 million treatment courses in children under 19 years of age alone. (*31*). A recent study has identified these two compounds (azithromycin and hydroxychloroquine) among 97 total potentially active agents as possible treatments for this disease (*32*).

In a preliminary clinical study, hydroxychloroquine and, with even greater potency, the combination of hydroxychloroquine and azithromycin were found effective in reducing the SARS-CoV-2 viral load in COVID-19 patients (*33*). Since the beginning of the epidemic in the Marseille region we isolated numerous strains and we tested one of them, the SARS-CoV-2 IHUMI-3, using different concentrations of hydroxychloroquine and azithromycin, alone and in combination, with Vero E6 cells.

Materials and Methods

Viral isolation procedure and viral stock

The procedure of viral isolation of our SARS-Cov 2 strain IHUMI-3 was detailed elsewhere (33). The viral production was done in 75 cm^2 cell culture flask containing Vero E6 cells (American type culture collection ATCC® CRL-1586™) in Minimum Essential Media (Gibco, ThermoFischer) (MEM) with 4% of fetal bovine serum and 1% glutamine. Cytopathic effect was monitored daily under an inverted microscope (Figure 1). After nearly complete cell lysis (approximately 96 hours), viral supernatant was used for inoculation on 96-well plate.

Testing procedure for drugs

Briefly, we prepared 96-well plates with 5.10^5 cells/mL of Vero E6 (200µL per well), using MEM with 4% of fetal bovine serum and 1% L-glutamine. Plates were incubated overnight at 37°C in a CO_2 atmosphere. Drug concentrations tested were 1, 2 and 5 µM for

hydroxychloroquine and 2, 5 and 10 μM for azithromycin. We also tested combinations of these agents at these concentrations, each test done at least in triplicate. Four hours before infection, cell culture supernatant was removed and replaced by drugs diluted in the culture medium. At t=0, virus suspension in culture medium was added to all wells except in negative controls where 50μL of the medium was added. We tested two multiplicities of infection (MOI) at 2.5 and at 0.25. Then RT-PCR was done 30 minutes post-infection in one plate and again at 60 hours post-infection on a second plate. For this, 100 μL from each well was collected and added to 100 μL of the ready-use VXL buffer from QIAcube kit (Qiagen, Germany). The extraction was done using the manual High Pure RNA Isolation Kit (Roche Life Science), following the recommended procedures. The RT-PCR was done using the Roche RealTime PCR Ready RNA Virus Master Kit. The primers were designed against the E gene using the protocol of Amrane et al. (*34*) in the Roche LightCycler® 480 Instrument II.

Transmission electron microscopy and scanning electron microscopy procedures.

Well supernatants samples (50μL) were fixed with 2.5% glutaraldehyde in 0.1 M cacodylate buffer for at least 1hour. For transmission electron microscopy negative staining, a drop of sample solution was adsorbed for 5 minutes onto formvar carbon films on 400 mesh nickel grids (FCF400-Ni, EMS). Grids were stained for 10 seconds with 0.2% Oolong Tea Extract (OTE) in 0.1 M cacodylate buffer. All steps were performed at room temperature. Electron micrographs were acquired on a Tecnai G2 transmission electron microscope (Thermo-Fischer/FEI) operated at 200 keV and equipped with a 4096 × 4096 pixels resolution Eagle camera (FEI). The same grids were observed on scanning electron microscope SEM SU5000 microscope

Results

No cytotoxicity was associated with drugs alone or in combination in control wells (without viruses). We detected RNA viral production from 25 to 16 cycle-thresholds (Ct,

inversely correlated with RNA copy numbers) for the positive control that was associated with cell lysis. In all cases, cell lysis at 60 hours was correlated with viral production as compared to control (Figure 2). At low MOI, azithromycin or hydroxychloroquine alone had no or low impact on the viral production compared to the positive control. We observed only a moderate effect for hydroxychloroquine at 5 μM in 2 of the 3 replicates (Figure 2a). For the combination of azithromycin and hydroxychloroquine, we observed inhibition of viral replication for wells containing hydroxychloroquine at 5 μM in combination with azithromycin at 10 and 5 μM (Figure 2b). Moreover, one cytopathic effect was observed at 60 hours post infection in these ten wells (Figure 3). Lack of multiplication of the virus in wells with azithromycin and hydroxychloroquine combination was confirmed by TEM and SEM observations (Figure 4). At high MOI, neither drug showed any effect on the cell lysis. The only condition where an effect was observed was the combination of hydroxychloroquine at 2 μM and azithromycin at 10 μM, leading to inhibition of viral replication measured by RT-PCR.

Discussion

In this present work, we could confirm a moderate effect of hydroxychloroquine alone on SARS-CoV2 at low MOI as previously observed with the lowest concentrations used in a prior study (*19*). The most striking observation was the synergistic effect of the combination of hydroxychloroquine and azithromycin. As compared to other studies testing hydroxychloroquine for which viral growth was evaluated at 48h, our conditions with prolonged incubation time of 60 hours showed that this effect remained observable. As for MOI, even at the higher MOI of 2.5, as compared to the data of Liu et al. where the highest MOI was of 0.8, the effect of the combination to inhibit viral growth was quantified by RT-PCR. Hydroxychloroquine has been demonstrated *in vitro* to inhibit replication of SARS-CoVs 1 and 2 (*17;19*). Concentrations of drugs for our study were based on the known

cytotoxicity of the drugs (50% of cytotoxicity, EC 50) and their effect on microorganisms (50% inhibitory concentration, IC50). With Zika virus, azithromycin showed activity with an IC 50 range from 2.1 to 5.1 μM depending on MOI (28) without notable effect on EC 50 at high concentration (*29)*. On Vero E6 it was shown that for hydroxychloroquine, EC 50 is close to 250 μM (249.50 μM), which is significantly above the concentrations we tested herein (*19*). Against SARS-CoV 2, the IC 50 of hydroxychloroquine was determined to be 4.51, 4.06, 17.31, and 12.96 μM with various MOI of 0.01, 0.02, 0.2, and 0.8, respectively. One of the main criticisms of previously published data was that drug concentrations for viral inhibition used *in vitro* are difficult to translate clinically due to side effects that would occur at those concentrations. The synergy between azithromycin and hydroxychloroquine that we observed herein is at concentrations achieved *in vivo* and detected in pulmonary tissues (*35-37*). Our data are thus in agreement with the clinical efficacy of the combination of hydroxychloroquine and azithromycin demonstrated by Gautret et al. (*33*). They support the clinical use of this drug combination, especially at the early stage of the COVID-19 infection before the patients develop respiratory distress syndrome with associated cytokine storm and become less treatable by any antiviral treatment.

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Conflicts of Interest:

Authors would like to declare that Didier Raoult is a consultant in microbiology for Hitachi High-Tech Corporation. Funding sources had no role in the design and conduct of the study, collection, management, analysis, and interpretation of the data; and preparation, review, or approval of the manuscript. The others authors declare no conflict of interest.

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Figure 1: Observations of infected Vero E6 Monolayer.

Observation was done 48 hours post infection by the SARS-CoV 2 strain IHUMI-3.

Magnitude X400. The picture was captured on ZEISS AxioCam ERC 5s.

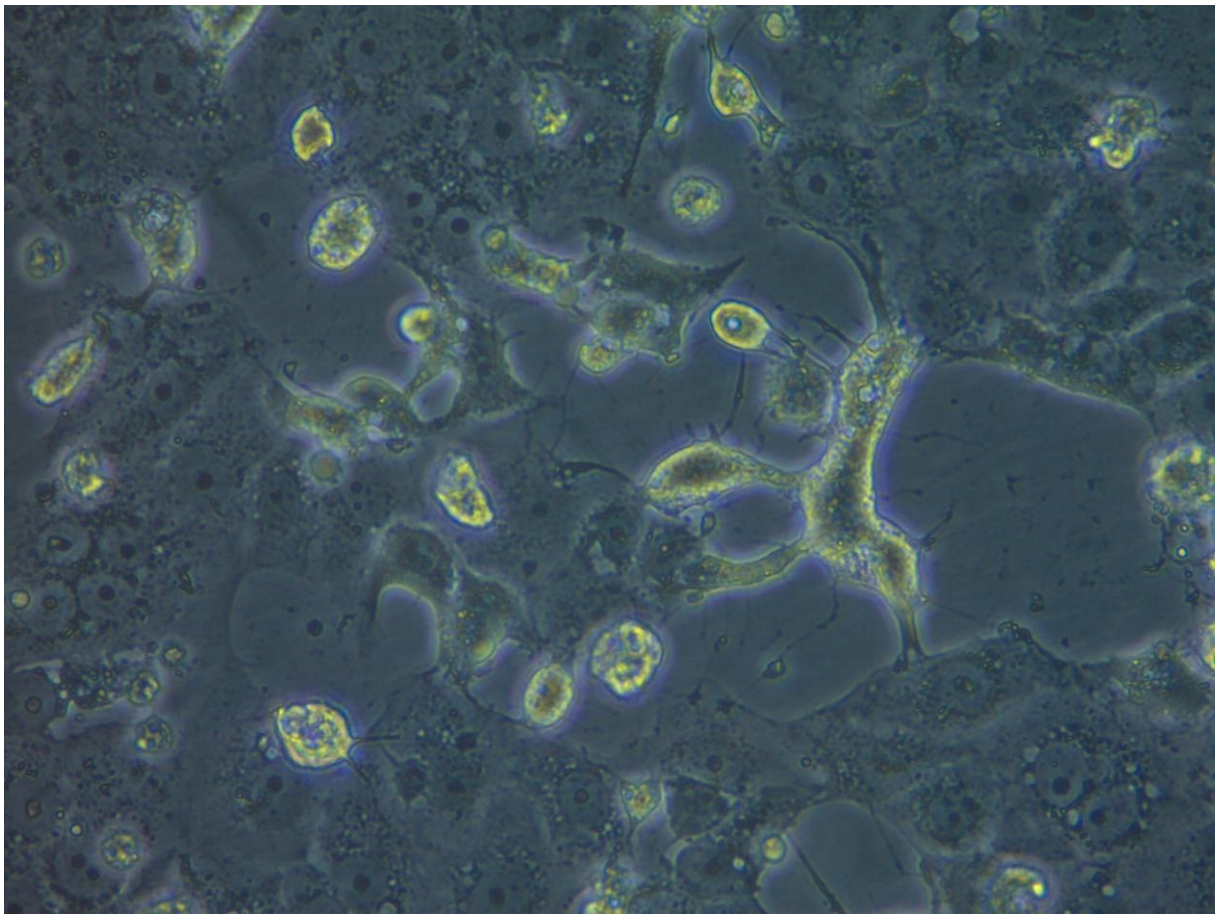

Figure 2: RNA viral quantification between 0 and 60 hours post infection.

Ordered axis represents the number of cycle-thresholds obtained by RT-PCR. For each condition, the first histogram, in blue, represents average RNA cycle-thresholds quantification at H0, and the second histogram, in green, represents average RNA viral quantification 60 hours post-infection. Standard deviation scales are present for each condition (number of replicates was indicated for all conditions as n=Y and n=7 for the positive control).

2A. represents molecules tested alone, A10 is for azithromycin at 10 µM, A5 at 5 µM, A2 is at 2 µM, H5 is for hydroxychloroquine at 5 µM, H2 for 2 µM, H1 for 1 µM. **2B**. represents the combination of molecules tested.

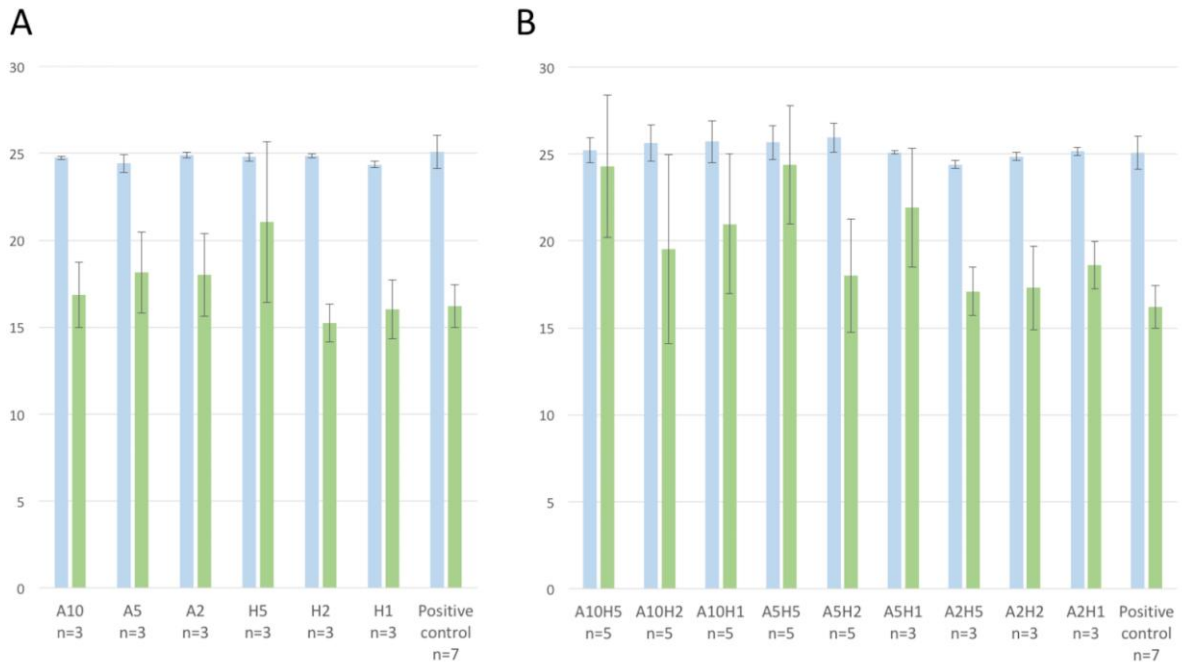

Figure 3: Observations of infected cells resistant or not to viral replication.

Pictures were captured on ZEISS AxioCam ERC 5s, 58 hours post infection by the SARS-CoV 2 strain IHUMI-3. Magnitude X200. **3A-B-C.** overview of the monolayer in each well for the condition of azithromycin 5 µM associated with hydroxychloroquine at 5 µM, **3D.** shows a cytopathic effect observed in one well in the condition azithromycin 10 µM combined with hydroxychloroquine at 2 µM **3E.** negative control well and **3F.** positive control well.

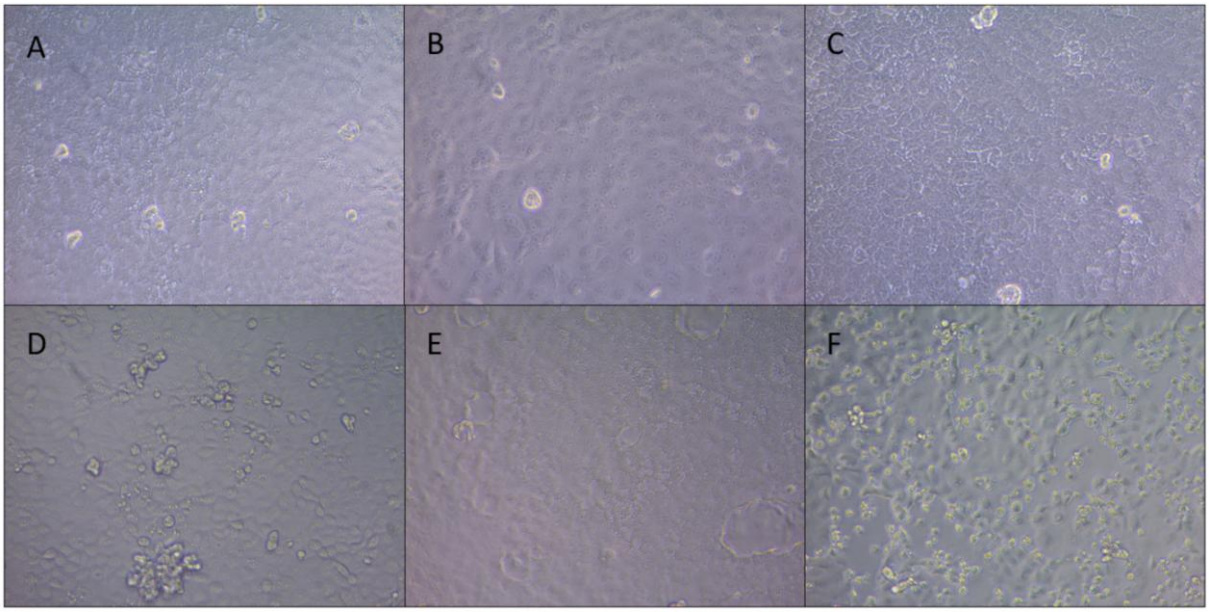

Figure 4: Electron microscopy observations.

4 A-B-C pictures were captured on Tecnai **4 D-E-F** pictures were captured on SU 5000. **4A -D** correspond to the condition at H0 on the well with azithromycin and hydroxychloroquine both at 5 µM . **4 B-E** correspond to the condition H60 on the well with azithromycin and hydroxychloroquine both at 5 µM. **4 C-F** correspond to the positive control at H60 allowing to observe viral particles. Scales bars are indicated below each panel.