

**Short communication**

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

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E6

**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 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.

## 1.Introduction

Since the end of 2019, the world has encountered pandemic conditions attributable to a novel Coronavirus SARS-CoV 2 (1-3). This is the 7<sup>th</sup> Coronavirus identified to infect the human population (1;4;5) and the first one that had pandemic potential in non-immune populations in the 21<sup>st</sup> 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 (17) and caused a 99% reduction of viral replication after 3 days at 16  $\mu$ M. 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  $\mu$ g/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

(26;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 in combination, with Vero E6 cells.

## **2. Materials and Methods**

### **2.1. 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<sup>2</sup> 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. We determined the TCID<sub>50</sub> of the strain at 5.10<sup>5</sup> infectious particles per mL.

### **2.2. Testing procedure for drugs**

Briefly, we prepared 96-well plates with 5.10<sup>5</sup> 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<sub>2</sub> atmosphere. Drug concentrations tested, expressed in micromoles per liter

( $\mu\text{M}$ ), were 1, 2 or 5  $\mu\text{M}$  for hydroxychloroquine associated with 5 or 10  $\mu\text{M}$  for azithromycin. Each test was done at least in triplicate and repeated two times except conditions with 5  $\mu\text{M}$  for hydroxychloroquine associated with 5 or 10  $\mu\text{M}$  for azithromycin that were repeated a third time. 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 50 $\mu\text{L}$  of the medium was added. Multiplicity of infection (MOI) was of 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  $\mu\text{L}$  from each well was collected and added to 100  $\mu\text{L}$  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. Relative viral quantification was done compare to the positive control (viruses without drugs) by the  $2^{(-\Delta\Delta\text{CT})}$  method (35). We performed a statistical analysis using GraphPad Prism v9.0.0 (GraphPad Software, La Jolla California USA). Distribution of the data not followed a normal law. So, non parametric Kruskal-Wallis test was used to compare each combinations against positive controls using  $\Delta\text{Ct}$  between H0 and H60. Then, Dunn's test was used to correct the multiple comparison. All test was used at  $p=0,05$  parameter and were bilateral (two-sides) and significant P-value was indicated on the figure 2. All others conditions was not significant.

### **3. Results**

No cytotoxicity was associated with drugs in combination in all 13 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 lyses. In all cases, cell lyses at 60 hours was correlated with viral production as compared to control (Figure 1). Combination of azithromycin and hydroxychloroquine led to significant inhibition of viral replication for wells containing hydroxychloroquine at 5  $\mu$ M in combination with azithromycin at 10 and 5  $\mu$ M (P-values at 0,0003 for A10H5 and at 0,0004 for A5H5) (Figure 2A) with relative viral inhibition of 97.5% and 99.1% respectively (Figure 2B). Others conditions were not significant. In agreement with the relative viral RNA load reduction, a cytopathic effect could be observed in 5/31 wells at 60 hours post infection as compared to 13/13 in positive controls.

#### **4. Discussion**

In the work we identified a strong synergistic effect of the combination of hydroxychloroquine and azithromycin. 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, CC 50) and their effect on microorganisms (50% inhibitory concentration, IC<sub>50</sub>). With Zika virus, azithromycin showed activity with an IC<sub>50</sub> range from 2.1 to 5.1  $\mu$ M depending on MOI (28) without notable effect on EC<sub>50</sub> at high concentration (29). The observation of efficacy of azithromycin on RNA viruses is probably shared by some other macrolides. Clarithromycin or the non antibiotic macrolide EM900 were observed as effective on rhinovirus in vitro (35;36). In vivo sulfate of hydroxychloroquine could be imply in the modulation of the immune response by reducing pro-inflammatory cytokines and by modification of the lysosome acidification procedure (37). Those aspects may play a keystone role in severe cases of SARS-coronaviruses. Indeed, in mouse models from SARS-CoV pneumonia and lung affections was associated with cytokines storm (38). In parallel azithromycin was known as inhibit the viral replication of Zika virus in vitro (29). And in enlarge viral infection context, azithromycin was associated to up-regulate interferons I and III (30). Concerning the

respiratory syncytial virus, it was also shown that Macrolides reduce the acidity of the lysosome and by the down-regulation of the ICAM-1 protein (36). So, in the SARS-CoV 2 context, azithromycin could potentialize the effect of hydroxychloroquine by similar mechanism.

On Vero E6 it was shown that for hydroxychloroquine, CC 50 is close to 250  $\mu$ M (249.50  $\mu$ M), 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  $\mu$ M 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 hydroxychloroquine and azithromycin that we observed herein is at concentrations achieved *in vivo* and detected in serum (35) and pulmonary tissues (36-37) respectively. Our data are thus in agreement with the clinical efficacy of the combination of hydroxychloroquine and azithromycin observed 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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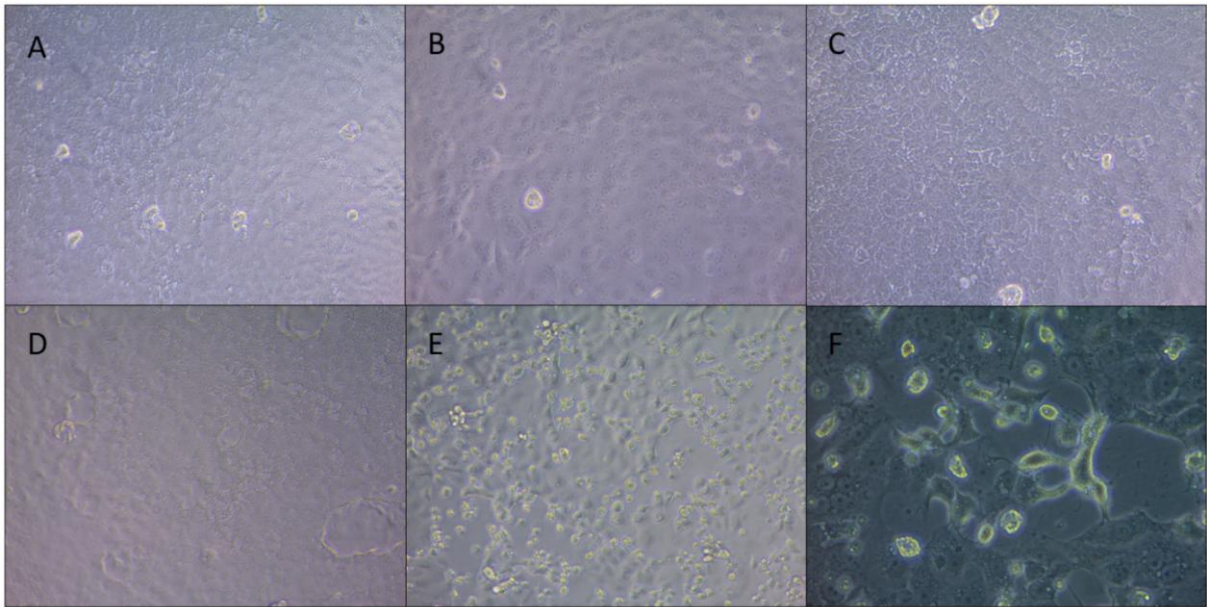
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**Figure legends:**

**Figure 1: Observations of infected cells resistant or not to viral replication after inoculation of SARS-CoV 2 strain IHUMI-3 at MOI 0.25.**

Pictures were captured on ZEISS AxioCam ERC 5s, 58 hours post infection. Magnitude X200. **3A-B-C.** overview of the monolayer in each well for the condition of azithromycin 5  $\mu$ M associated with hydroxychloroquine at 5  $\mu$ M, **3D.** Negative control well and **3E.** Positive control well. **1F.** Observation was done 48 hours post infection by the SARS-CoV 2 strain IHUMI-3 for the viral stock production. Magnitude X400.



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**Figure 2: Effect of hydroxychloroquine and azithromycin association on SARS-CoV 2 replication.**

**2A. Delta Ct between 0 and 60 hours post infection.** Ordered axis represents the variation of delta cycle-thresholds obtained by RT-PCR between H0 and H60 for each condition. Each point represents data obtained for one well. Number of replicates was indicated for each conditions are A10H5 n=16, A10H2 n= 5, A10H1 n= 5, A5H5 n=15, A5H2 n=5, A5H1 n=3, A2H5 n=3, A2H2 n=3, A2H1 n=3 and n=13 for the positive control. Median and interquartile range were indicated for each condition. \*\*\* represent significant results under  $p < 0,0005$ . Others are not significant compared to the control.

**2B. Percentage of inhibition as compared to control by the combinations of 5  $\mu$ M of hydroxychloroquine associated with 5 or 10  $\mu$ M for azithromycin.** Data represent the mean  $\pm$  SD, representing three independent experiments conducted at least in triplicate.

