Heterogeneity in susceptibility to hydroxychloroquine of SARS-CoV-2 isolates

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Abstract

Background

Hydroxychloroquine has been demonstrated *in vitro* to control SARS-Cov2 multiplication on Vero E6 cells. We herein tested the possibility that some patients with prolonged excretion of virus could be infected by less susceptible viral strains.

Methods

Using high-content screening method, we screened 30 different selected isolates of SARS-CoV-2 from different patients. All these patients received azithromycin and/or hydroxychloroquine. We focused our work on patients with viral persistence defined as patient having a positive detection of virus in nasopharyngeal sample for at least 10 days and tested during the two episodes of French epidemic, late winter-spring then summer. Dose-response curves in single-molecule assays with hydroxychloroquine were done for isolates with suspected reduced susceptibility. Genome clustering was done for all isolates.

Results

Among 30 tested strains, 3 were detected as replicating in presence of azithromycin and hydroxychloroquine at 5µM both. Dose-response model showed a decrease of susceptibility of these 3 strains to hydroxychloroquine. Whole genome sequencing showed that these three strains are all from the second epidemic episode and 2 clusters with isolates from Africa.

Conclusions

Reduced susceptibility to hydroxychloroquine was not associated with persistence of the virus in naso-pharyngeal sample. It was rather associated with occurrence during the second epidemic episode starting during summer and with strains clustering with those having a genotype which is common in Africa. This continent is the continent where hydroxychloroquine was the most used.

Keywords: SARS-CoV 2; Hydroxychloroquine; azithromycin; in vitro model; Vero E6; WGS; Africa.

Abbreviations : IHU Mediterranean infection, Hospitalo-Universitaire Institute Mediterranean infection ; µM, micromolar; NSB3 laboratory, Biosafety Level 3 laboratory; azithromycin, AZT; hydroxychloroquine, HCQ ; A5H5, azithromycin 5 µM and hydroxychloroquine 5 µM; RT-PCR, real-time, Polymerase Chain Reaction; MOI, multiplicity of infection; HCS, High-content screening

Introduction

In December 2019, a novel coronavirus named SARS-CoV-2 emerged in Wuhan, China, in Hubei province [1–3]. Quickly, SARS-CoV-2 was spread around the world and the number of cases and deaths has increased rapidly (https://www.nature.com/articles/d41586-020-00758-2). Since then, finding effective treatments and vaccines remains a major issue in the world. Several molecules with an antiviral effect have been tested *in vitro* and *in vivo*, drug repurposing being one of the strategies applied. In this objective many drugs have shown an inhibition *in vitro* such as remdesivir, chloroquine, hydroxychloroquine, ivermectin, azithromycin, spiramycin, several protease inhibitors and some antimalarial drugs [4–16]. The combination of azithromycin and hydroxychloroquine showed synergistic effects *in vitro* at concentrations of 5 μM for each molecule and the combination was massively used in our institute to treat infected patients [17], as well as in several countries in particular in Africa (Zambia, Uganda, Egypt, Algeria, Morocco, Tunisia, Senegal, Cameroon) [18,19]. In literature, and in our experience, some patients, especially those immunocompromised or with other comorbidities (as hypertension, diabetes) have presented a positive viral load with a late clearance [20–23]. In our institute, we could isolate at least one SARS-CoV-2 strain for several patients defined as “persistent” with a positive RT-PCR test for more than 10 days after admission in spite of the fact that they received the combination of azithromycin and hydroxychloroquine [24]. In those cases the question of susceptibility to the antiviral drugs of the responsible strain was raised. Indeed, most *in vitro* studies evaluating susceptibility to antiviral drugs used a unique strain or clone of SARS-Cov 2, considering that this clone is representative of all, in spite of the fact that the variability of antiviral activities on an enlarged panel of strains is unknown. Herein, we decided to screen in an automated model of Vero E6 a single association of azithromycin-hydroxychloroquine on multiple strains issued of persistent and non-persistent patients randomly chosen to detect reduced susceptibility to

this combination [25]. After this preliminary screening, a dose-response study to hydroxychloroquine was done on suspect isolates and controls.

Materials and Methods

Ethic concerns and sample collection

Nasopharyngeal samples were done at the IHU Mediterranean infection as part of Covid-19 diagnosis and follow-up of patients. The study was approved by the ethical committee of the University Hospital Institute Méditerranée Infection (N°: 2020-029). Regarding both the French and the local situations, we defined 2 periods of time in this pandemic situation: a first one consisting in the arrival of the virus then the lockdown from February to May 2020, and a second one since June 2020, still ongoing. Information on the sample collection, name of the strains, and treatments are summarized in Table 1. All patients received treatment by Azithromycin that was in most cases associated to hydroxychloroquine [24]. Persistent patients are defined as presenting 2 viral swabs positive by RT-PCR during a period up to 10 days [25]. For these patients, when it was possible, we evaluated the susceptibility of 2 strains, the first isolated at admission and the second isolated during evolution under treatment. IHUMI-3 isolate was among the first strains isolated in the laboratory and used as control, as in all our previous experiments [17]. Viral isolation was done following the procedure described [26]. After isolation, the viruses were harvested and frozen at -80°C. TCID50 were performed for each strain and MOI for inoculation was adjusted in function of the RT-PCR values in order to inoculate the same virus concentration for each virus. Before inoculations for antiviral assays the viral stock was diluted into the M4 medium.

Screening for reduced susceptibility and dose-response to hydroxychloroquine of selected isolates

All 30 strains from 20 patients (Table 1) and IHUMI-3 strain control were screened using the high-content screening procedure to the combination of hydroxychloroquine and azithromycin (Sigma-Aldrich) at 5 µM each to evaluate the possible reduction of susceptibility. First, 200 µL of 5.10^5 cells /mL of Vero-E6 were incubated overnight at 37°C with 5% CO_2 in 96 well plates. Supernatant was removed four hours before the infection by SARS-CoV 2 and drug dilutions were incubated in the M4 medium 4 hours before. Viral infection of each strain was achieved with a MOI 0,001 (50uL per well) except in negative controls. Imaging and cell analyses were performed by high-content-screening using the CX7 automated cell-insight optical microscope (Thermofisher Scientific, USA). The proof of concept was used by Francis *et al.* and developed to automatically detect infections in cells [25]. Briefly at time points (H0 and 72 hours post-infection) wells were stained by NucBlue™ Live ReadyProbes™ reagent (Molecular Probes, Life Technologies, USA) at a final concentration of 2 ng/mL (5 µL per well directly from stock solution). Image acquisition and analyses were performed using the automated CellInsight™ CX7 High-Content Analysis Platform coupled with an automation system including an Orbitor™ RS Microplate mover and a Cytomat™ 2C-LIN (Thermo Scientific) incubator. We evaluated the protective effect of A5H5 by comparison to the positive control without addition of drugs and measured the difference in total cell count and % on infected cells according to the following formula [total cell counts (A5H5 -positive control)] * [% injured cells (A5H5 – positive control) / 10)]. As a consequence of this initial screening, 3 strains suspected to have possible reduced susceptibility to the combination (IHUMI-2123, IHUMI-2137 and IHUMI-2178) were first tested against hydroxychloroquine and azithromycin at 5 µM each then in a serial dilution range from 25 µM to 0,39 µM of hydroxychloroquine to determine dose-response assays. In order to confirm that the effect was not a genotype-selection effect, we tested IHUMI-2122, IHUMI-2177 and IHUMI-3 as control. Dose-effect curve was determined using a range of

hydroxychloroquine doses (from 25 µM to 0,39 µM) at MOI of 0,001. Hydroxychloroquine dilutions were done from a stock solution in M4 and then concentrations were adjusted. Each test was done at least in sixplicates and repeated twice independently and the potential effect was monitored by RT-PCR after 48H of incubation under previously described conditions [27], except concerning the polymerase replaced by the SuperScript™ III Platinum™ SYBR with ROX (Sigma-Aldrich, catalog number: 11736051). Relative viral quantification was done compared to the positive control (viruses without drugs) by the $2^{-\Delta\Delta Ct}$ (–delta delta CT) method [28]. Statistical analyses were performed using GraphPad Prism v8.0.0 (GraphPad Software, La Jolla California USA).

Viral preparation and genomic sequencing, genomic assembly and bioinformatic analyses

In parallel of the antiviral assays, 500 µL of the viral supernatant obtained from co-culture were centrifuged through UFC-filter (see previous section). Then viral RNA was extracted from 200 µL of the filtrate supernatant using the QIAcube kit. Then, it was reverse transcribed using SuperScript IV (ThermoFisher Scientific, Waltham, MA, USA) prior to cDNA second strand synthesis with Klenow Fragment DNA polymerase (New England Biolabs, Beverly, MA, USA). The next step concerned the DNA purification done by using Agencourt AMPure XP beads (Beckman Coulter, Villepinte, France) and finally sequenced on Illumina technology with the Illumina Nextera XT Paired-end strategy on a MiSeq instrument (Illumina Inc., San Diego, CA, USA). The Wuhan-Hu-1 isolate genome served as reference (consensus sequences GenBank Accession no. MN908947) and mapping was done by CLC Genomics workbench v.7. Sequences were compared to the GISAID database and a phylogenetic tree was done by using next train ncov (https://github.com/nextstrain/ncov).

Results

High-content screening for reduced susceptibility detection

Among the 30 strains (plus IHUMI-3 strain control) screened 72 hours after the viral infection by SARS-COV-2 on the high-content screening with or without treatment by the combination of hydroxychloroquine and azithromycin both at 5 µM, IHUMI-2123, IHUMI-2137 and IHUMI-2178 had a low threshold obtained on the HCS software (1099, -1021 and -257 respectively), suggesting possible reduced susceptibility to A5H5 (Figure 1a and supplementary Table 1). This result was confirmed by SARS-CoV-2 replication analysis (Figure 1b).

Dose-effect curves of hydroxychloroquine assays

Concerning the IHUMI-3, IHUMI-2122 and IHUMI-2177 strains used as controls, we observed a consistent viral inhibition compatible with the results observed previously in SARS-CoV-2 isolates. On the contrary, concerning IHUMI-2123, IHUMI-2137 and IHUMI-2178 we observed a displacement of susceptibility to hydroxychloroquine (Figure 2) confirming a specific pattern of reduced susceptibility for these isolates.

Genome analyzis

We first conduced a global genome-to-genome comparison on the couple of strains isolated in persistent patients and could not detect any modification (Table 1). We also analyzed 20 genomes to place them in a phylogenetic tree. Regarding the quality score on the next clade, all strains received a good quality score (Supplementary file S1). We could detect that all the strains of the second period have 10 or more amino-acid changes in their genome compared to strains of the first period. On the contrary all the strains of the first period have less than 10 amino-acid mutations except one (patient 12). All viruses in those studies have the D614G mutation in the spike, described elsewhere as potentially increasing the infectious effects [29]. The phylogenetic tree was reconstructed by integrating all IHUMI strains and evolutionary relationships were evidenced (Figure 3). All 5 strains from the second period

belonged to separate clades provisionally named Marseille 1 and Marseille 5 [30]. Specifically, the strains with reduced susceptibility to hydroxychloroquine were from Marseille clades 1 (IHUMI-2123 and IHUMI-2178) and 5 (IHUMI-2137). All strains belonging to the Marseille 1 clade were positioned in proximity of genomes originating from Africa i.e; Senegal and Gambia. The phenotype with reduced hydroxychloroquine in Marseille 1 genotype was shared by IHUMI-2123 and IHUMI-2178 isolates but not by IHUMI-2122 and IHUMI-2177 isolates. IHUMI-2137 grouped within Marseille 5 clade. Meanwhile, regarding IHUMI-2123 and IHUMI-2178 isolates with a reduced susceptibility to hydroxychloroquine, they did not present mutations compared to close to IHUMI-2122 and IHUMI-2177 isolates without reduced susceptibility.

4. Discussion

To the best of our knowledge, it was the first time that three SARS-CoV2 strains showed a profile of reduced susceptibility to hydroxychloroquine *in vitro*. Susceptibility to azythromycin was not tested independently as its effect alone *in vitro* is limited. The high-content screening technology, first applied to the high-throughput culture of giant viruses of amoeba then to SARS-CoV-2 [25], was used for the first time herein to quickly screen susceptibility to drugs of a large panel of viruses. If other work will be needed to confirm clearly that the procedure can be standardized enough to provide efficient large screening of strains, it was proven as efficient to detect isolates with reduced susceptibility. However, even if highly time-consuming and susceptible to many confounding factors as presented below, for fine determination of susceptibility, dose-effect determination by molecular biology remains necessary. Indeed, *in vitro* sensitivity assays carried out on the same virus can provide divergent results according to the great discrepancies due to several essential determinants in the experiments. First, cell lines used, although all the ones used in these assays need to be permissive, may harbor different permissivity levels resulting in differences

in viral titers. For SARS-CoV2, the entry step involves the ACE2 receptor and two independent host protease pathways TMPRSS2 or the cathepsins B/L that activate the Spike viral protein. The virus may not use these two ways similarly, and the expression level of these receptors mediating virus entry are differentially expressed according to the cell lines [31]. For example, VeroE6 engineered for expressing greater amounts of TMPRSS2 were used elsewhere, resulting in 100-fold higher titers of SARS-CoV-2 [32]. Inversely, viral titers provided by SARS-CoV2 infected calu-3 cells (Continuous human lung epithelial cell line) are lower than in Vero cells. [32]. It could make sense for the sensitivity assays to use the cells physiologically closest from those of the replication site in vivo. In this view, primary cells derived from organ explants were used for sensitivity assays and it seemed to be a relevant approach. However, variable effects which are donor-dependent on the sensitivity for some tested drugs should be expected due to differences in viral replication and gene expression [33]. Thus this approach could be a false good idea and testing molecules in a coarse model such as in Vero E6 that have genetic defect in interferon production could help in evidencing an effect. Secondly, the multiplicity of infection reported the drug concentration is not standardized. It seems obvious that the higher the MOI, the lower is the relative drug concentration, and the more likely the virus can replicate. This MOI is not even mentioned in some studies. Finally, time for end point evaluation and the method used for assessment of viral replication can also vary according to the studies from 1 hour to 120 hours [34,35]. Besides, assessment of viral replication by PCR or fluorescent assay or visual inspection to monitor cell viability may also not have the same sensitivity. In example for the latter, some permissive cells such as human intestinal epithelial Caco-2 do not produce cytopathic effects after SARS-CoV2 infection and thus cannot be evaluated with such method [36]. As a result, it is risky to draw conclusions on a single sensitivity test, especially when testing a virus with a high genomic variability.

One of the most interesting perspectives should be also to test multiple viral strains to check concordance of the results. Currently, *in vitro* assays use essentially 1 or 2 SARS-CoV2 strains. Our work suggests it is risky to draw conclusions on a single sensitivity test when testing a virus with a high genomic variability. Indeed we observe an heterogeneity in the hydroxychloroquine antiviral activity screening of 30 strains and could detect three strains with a lower susceptibility profile. For isolates from patients of the first period of the epidemic, persistence was clearly not associated to a lowered susceptibility profile to hydroxychloroquine *in vitro.* This confirmed the observation that persistence and severity are rather associated with host factors as suggested by recent genetic research on Covid 19 severity-associated factors [37,38] or immunocompromised status [39,40]. Moreover, genomic analyses did not reveal any modification in these isolates that could explain persistence, neither in the sequence of the strain isolated at admission nor in that of the strain isolated during the course of the disease. The less susceptible hydroxychloroquine strain, IHUMI-2123, that belongs to Marseille 1 genotype was isolated in early summer at the beginning of the second episode from a patient returning from Tunisia [30], which is a country where hydroxychloroquine was massively used [19]. We evidenced a close phylogenetic proximity between all strains of the Marseille 1 clade (IHUMI-2123, IHUMI-2122, IHUMI-2178, and IHUMI-2177) with strains isolated in Senegal and Gambia, both countries using hydroxychloroquine to treat patients with COVID-19 [41,42]. We believe that it is possible that a large use of hydroxychloroquine in these countries selected strains with reduced susceptibility that were latter transmitted to Marseille population. Paradoxically, the patients tested in Marseille hospitals during the period of early summer and infected by isolates of this genotype presented milder infections and lower mortality than observed during the first part of the epidemic although the viral loads in their respiratory secretions were higher [43]. This observation raises several questions that will be difficult to resolve soon

such as does the lowered susceptibility to hydroxychloroquine reduces severity of infection or is it useful to use hydroxychloroquine in such case or only in patients with severity markers or risk factors such as anticoagulant lupus for which hydroxychloroquine is the treatment and thus, efficiency likely, not due to an antiviral effect [44–46]. But finally, the hydroxychloroquine concentration to achieve 50% of viral inhibition was around 3.125 µM for the three strains with high hydroxychloroquine susceptibility and > 12.5 µM for the three strains with reduced susceptibility, which require at least four times more hydroxychloroquine for the same effect (Figure 2). Moreover, a 90% viral inhibition required around 12.5 µM for susceptible strains and > 25 µM for less susceptible strains. However, these concentrations remain consistent with concentrations observed in human plasma and lungs. An oral uptake of 400 mg of hydroxychloroquine led to a maximum blood concentration (C_{max}) of 1.22 µM [47]. But, hydroxychloroquine accumulated 30 times more in lungs than in blood [48], allowing a potential efficiency of hydroxychloroquine even against strains with reduced susceptibility. An oral uptake of 400 mg of hydroxychloroquine would still be effective *in vivo* in humans infected with the current strains with *in vitro* reduced susceptibility to hydroxychloroquine.

However, these genotypic and phenotypic variations could be frequent in the viral populations in the future and could apply to more drugs and need to be considered in the global repurposing strategy. By the description of Korber et al we know that the spike population evolved during February - April 2020 and constituted a fast replacing situation by the G614 [29]. Recently a major situation was noticed by Denmark where minks were infected with a strain presenting a few mutations notably in the spike protein and associated with a selection pressure in a potential zoonotic transfer. Those aspects need to be carefully considered, for testing and using antiviral compounds but also for epidemiology and vaccination strategy.

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Table 1. Strains and patients information

Patients number	Strains	Month of sampling	Persistence	Days of the lastest isolate after onset	RT-PCR Ct values	Treatment	Clade	GISAID access number
Patient 1	IHUMI-11	March 2020	No	NA	29	AZT HCQ	20A/15324T	IHUCOVID-0760
Patient 2	IHUMI-15	March 2020	No	NA	23	HCQ	20B	IHUCOVID-0649
Patient 3	IHUMI-240	March 2020	No	NA	22	AZT HCQ	20C-5	IHUCOVID-0152
Patient 4	IHUMI-243	March 2020	No	NA	29	AZT HCQ	20A/15324T	IHUCOVID-0762
Patient 5	IHUMI-597	March 2020	No	NA	20	/	20A/25563T	IHUCOVID-0142
Patient 6	IHUMI-215	March 2020	Yes	8	23	AZT HCQ	20A/25563T-1B	IHUCOVID-756
	IHUMI-611				32,2		20A/25563T-1B	
Patient 7	IHUMI-364	March 2020	Yes	4	21	AZT HCQ	20A/15324T	IHUCOVID-143
	IHUMI-599				29,1			
Patient 8	IHUMI-284	March 2020	Yes	4	30		20A/A0268G-2	IHU-0144
	IHUMI-538				20,5	AZT	20A/A0268G-2	
Patient 9	IHUMI-713	March 2020	Yes	3	31	AZT HCQ	20A/25563T-1	IHUCOVID-0272
	IHUMI-800				23,1		20A/25563T-1	

Patient 10	IHUMI-684	March 2020	Yes	4	21,6	AZT HCQ	20A/25563T	IHUCOVID-0147
	IHUMI-743				20,4		20A/25563T	
Patient 11	IHUMI-598	March 2020	Yes	4	20,5	AZT HCQ	20C-5	IHUCOVID-0151
	IHUMI-801				20,7		20C-5	
Patient 12	IHUMI-717	March 2020	Yes	2	21,2	AZT HCQ	20B-1a	IHUCOVID-0312
	IHUMI-742				19,1		20B-1a	
Patient 13	IHUMI-624	March 2020	Yes	2	16,1	AZT HCQ	20A/25563T-1b	IHUCOVID-0749
	IHUMI-719				17,7			
Patient 14	IHUMI-288	March 2020	Yes	5	23	AZT HCQ	20C-4	IHUCOVID-0752
	IHUMI-614				26			
Patient 15	IHUMI-880	April 2020	Yes	3	19	HCQ	20B	IHUCOVID-°0641
	IHUMI-990				21,4		20B	
Patient 16	IHUMI-2122	July 2020	Unknown	NA	17,8	AZT	Marseille 1	IHUCOVID0976
Patient 17	IHUMI-2123	July 2020	Yes	NA	17,7	AZT HCQ	Marseille 1	IHUCOVID0982
Patient 18	IHUMI-2137	August 2020	Yes	NA	14,7	AZT HCQ	Marseille 5b	IHUCOVID1329
Patient 19	IHUMI-2177	August 2020	No	NA	25,1	AZT	Marseille 1A	

Patient 20	IHUMI-2178	August 2020	Uknown	NA	21,6	AZT HCQ	Marseille 1A	IHUCOVID1212

NA : not applicable because only one strain was obtained

Figure legends

Figure 1. Initial screening of the 31 selected SARS-CoV-2 isolates to a combination of hydroxychloroquine and azithromycin at 5 µM each. (1a) Difference observed between cells treated or not treated calculated by high-content screening for each strain. **(1b)** Effect of hydroxychloroquine and azithromycin association on SARS-CoV 2 replication on selected isolates. Delta Ct between 0 and 48 h post infection. Ordered axis represents the variation of delta cycle-thresholds obtained by RT-PCR between H0 and H48 for each condition. Each point represents data obtained for one well. Median and interquartile range were indicated for each condition. *** represent significant results under $p < 0,0005$. Others are not significant compared to the control

Figure 2: Exploration of effect-dose curves of hydroxychloroquine. The range used from 25 µM to 0,39 µM tested on IHUMI-3, IHUMI-2122, IHUMI-2123, IHUMI-2137, IHUMI-2177 and IHUMI-2178 strains.

Abbreviations : p.i, post-infection; HCQ, hydroxychloroquine ; µM, micromolar

Figure 3: Phylogenetic tree of whole genomes from IHUMI strains including closely related genomes available from GISAID. Mutation scales are compared to the Wuhan reference genome.