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Title: Enantiomers of Chloroquine and Hydroxychloroquine Exhibit Different Activities Against SARS-CoV-2 *in vitro*, Evidencing *S*-Hydroxychloroquine as a Potentially Superior Drug for COVID-19

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Keywords: chloroquine, hydroxychloroquine, enantiomer, enantioselectivity, chiral switch, antiviral activity, SARS-CoV-2, COVID-19

ABSTRACT

In all of the clinical trials for COVID-19 conducted thus far and among those ongoing involving chloroquine or hydroxychloroquine, the drug substance used has invariably been chloroquine (CQ) diphosphate or hydroxychloroquine (HCQ) sulfate, i.e., the phosphoric or sulfuric acid salt of a racemic mixture of *R*- and *S*-enantiomer (50/50), respectively. As a result, the clinical outcome from previous CQ or HCQ trials were, in fact, the collective manifestation of both *R* and *S*-enantiomers with inherent different pharmacodynamic and pharmacokinetic properties, and toxicity liabilities. Our data for the first time demonstrated the stereoselective difference of CQ and HCQ against live SARS-CoV-2 virus in a Biosafety Level 3 laboratory. *S*-chloroquine (*S*-CQ) and *S*-hydroxychloroquine (*S*-HCQ) were found to be 31% and 60% more active against SARS-CoV-2, as compared to *R*-CQ and *R*-HCQ, respectively. With these data and previous work on stereoselective metabolism of CQ and HCQ, we recommend that future clinical studies should employ *S*-HCQ as a potentially superior drug substance for the treatment of COVID-19 for improved therapeutic index.

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INTRODUCTION

In the last few months, the virulence and lethality of coronavirus breakout have presented an unprecedented challenge to the medical community and world governments. The coronavirus disease 2019 (COVID-19), caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) [1-2] has become a global pandemic. It has now affected more than 184 countries and regions, [1, 3-6] becoming a global pandemic as declared by the World Health Organization (WHO). By July 13th, 2020, the cumulative number of confirmed cases of COVID-19 infection has exceeded 12.9 million worldwide, with more than 569,128 recorded deaths (a fatality rate of 4.4 % approximately), according to real-time data released by Johns Hopkins University.[7]

With the prospect of effective vaccines months away from being developed and launched, scientists and physicians alike have turned to existing drugs with known toxicity and human pharmacokinetic data to deal with this global healthcare crisis. Numerous treatment regimens for COVID-19 have been proposed and tested in clinical trials with varying degrees of controls and sizes of subject enrollment. With limited access to compounds and live virus for testing, research institutions around the world have also resorted to tools like virtual screening and modeling to compile lists of potential drugs that may be efficacious for COVID-19.[8-10] Many of these have been evaluated against SARS-CoV-2 in vitro. These include some medications commonly used for decades, such as chloroquine,[11-13] arbidol,[14] ribavirin,[15-17] lopinavir, etc.,[18-20] The list also contains some drugs in development, for instance, remdesivir,[11, 17] a previously studied drug candidate for treating Ebola infection.[21-22]

Among the most notable and also controversial repurposed drugs are chloroquine (CQ) and hydroxychloroquine (HCQ) as shown in Figure 1A, with several initial promising results especially when combined with azithromycin or zinc supplement being reported,[23] only to be followed by contradicting reports on lack of efficacy and presence of severe side effects, especially among the higher dosed patients. Based on the preclinical results, both CQ and HCQ have displayed inhibition of viral replication.[11-13] The 70-year-old CQ was first used in clinical practice as an anti-malarial drug, and its indications were extended to treat systemic lupus erythematosus, rheumatoid arthritis, and several infections.[24] Originally developed as a safer alternative to CQ for treating malaria, the analogous compound hydroxychloroquine sulfate[25] has become a popular drug for treating autoimmune diseases including rheumatoid arthritis (RA) and system lupus emphysema (SLE).[26-27] In fact, it is the 2nd most prescribed medicine for rheumatoid arthritis in the United States, with nearly half a billion tablets dispensed in 2019. Both drugs have a long history as inexpensive and well-received treatment options for malaria, autoimmune, as well as infectious diseases, even for women patients during pregnancy.[24] We believe the verdict is still out on their ultimate safety and efficacy. By the last account, there are currently 41 and 133 ongoing or planned clinical trials (clinicaltrials.gov) against COVID-19 involving the use of CQ and HCQ, respectively.

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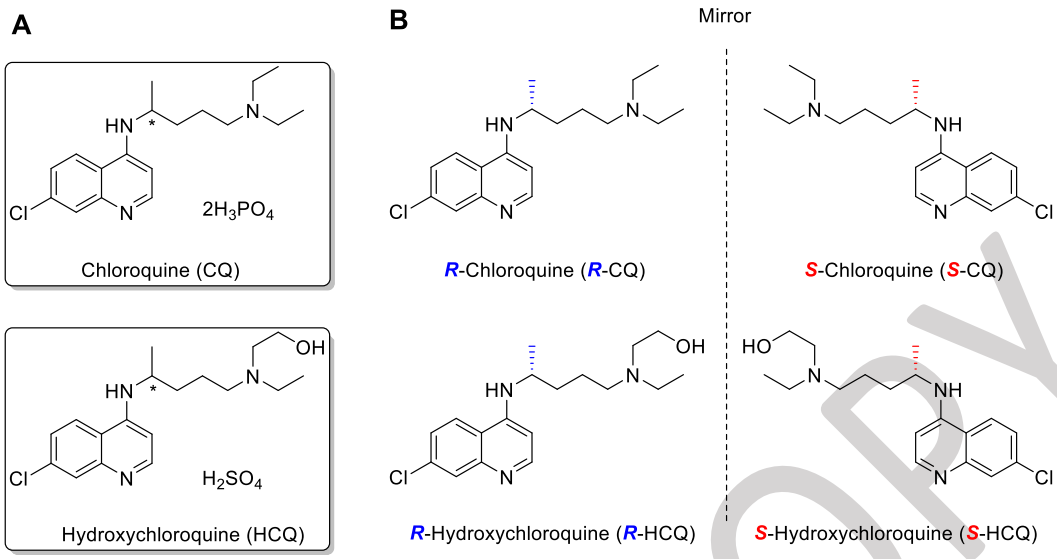

Figure 1. Structures of chloroquine and hydroxychloroquine (A), as well as their enantiomers as mirror images to each other (B).

While remdesivir, another repurposed antiviral that has also generated enormous excitement in the global communities, has also been approved by FDA under an EUA for treating COVID-19 and is emerging as the standard of care, its demonstrated efficacy is far from ideal. The development of less expensive, safer, more effective, and more accessible treatment options is still an urgent unmet medical need.

We noticed certain caveats about CQ and HCQ that are worthy of being taken into accounts by stakeholders. First, CQ and HCQ have been in use for decades by millions of patients with diverse health conditions since their approval in the mid-1900s; hence, a wealth of information about the adverse events is available.[28-30] Secondly, unlike i.v. administered drugs including remdesivir, CQ and HCQ can be taken conveniently as oral tablets. Thirdly, CQ and HCQ are among the least expensive drugs being considered for COVID-19, with an excellent prospect of accessibility for the global population should its safety and efficacy be proven by further and more definitive, randomized, and controlled clinical trials with more appropriate tailoring according to patient conditions, time of intervention, and dosing regimen. Last but not least, all preclinical and clinical trials of CQ and HCQ for COVID-19 thus far have been carried out using the common CQ and HCQ formulations,[31] *i.e.*, the phosphoric or sulfuric acid salt of a ***racemic*** mixture of CQ or HCQ, respectively.

Commercial chloroquine and hydroxychloroquine drug products are administrated as their respective racemates of a 50:50 mixture of two enantiomers: *S*- and *R*-isomers. The *R*- and *S*-isomers are in an enantiomeric relationship, meaning that these two compounds have the same chemical compositions but are a mirror image relationship to each in the spatial arrangement, as shown in Figure 1B. Compounds that cannot superimpose with their own mirror images are defined as chiral compounds. Life, the biological system, is chiral in nature, as proteins are composed of amino acids of predominately that of the *L*-configuration, and sugars of mostly *D*-configurations. This predisposes different enantiomers of a chiral drug to interact with proteins differently, and

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hence the different pharmacological effects would be expected. As chiral drugs, the two enantiomers of CQ or HCQ, might exhibit distinct efficacy and safety profiles in patients. These differences, big or small, can translate into performance variances that could potentially influence the decision process on whether either enantiomer of CQ or HCQ can ultimately become a viable therapeutic agent for COVID-19 through further testing, including clinical trials. The clinical trials for COVID-19 conducted so far have all been using a racemic mixture of either CQ or HCQ; hence the observations were actually the collective manifestation of the two different albeit very similar optical isomers of these drugs. Although CQ, as racemic mixtures, exhibited some antiviral activity against SARS-CoV-2 *in vitro* (EC_{50}= 1.13 μM in Vero E6 cells), and HCQ was also active with less toxicity,[11-13, 31] the efficacy and possible toxicity of each individual isomer against SARS-CoV-2 have not been reported in any preclinical or clinical studies yet. In this paper, we wish to present the preliminary results on such a difference exhibited by the enantiomers of CQ and HCQ, respectively, when subjected to *in vitro* test against SARS-CoV-2.

METHODS

Material: Virus and cells

African green monkey kidney Vero E6 cell line was obtained from American Type Culture Collection (ATCC, cat no. 1586) and maintained in Dulbecco's Modified Eagle's medium (DMEM, Gibco) supplemented with 10% fetal bovine serum (FBS) at 37 °C incubator with 5% CO_2. A clinical isolate SARS-CoV-2 virus (Genebank accession no. MT123290.1) was propagated in Vero E6 cells and viral titer was determined by plaque assay. All the infection experiments were performed in a Biosafety Level 3 (BLS 3) laboratory.

Material: antivirals

The racemic chloroquine diphosphate (CAS NO. 50-63-5) and hydroxychloroquine sulfate (CAS NO. 747-36-4), purchased from Bidepharm, were converted into free racemic chloroquine and hydroxychloroquine, respectively, under basic conditions. The enantiomers of the racemic mixtures were separated by preparative chiral chromatography as free-bases with a Shimadzu LC-20AD HPLC equipment: chloroquine or hydroxychloroquine was dissolved in isocratic *n*-hexane/*iso*propanol/diethylamine with a ratio of 85:15:0.1 (v/v/v), and the loading needed for CQ and HCQ was 24.0 mg/mL and 23.5 mg/mL, respectively. The resulting solution was loaded onto a CHIRALPAK AY-H (AYH0CE-VC001) chiral column, which was then eluted with the same solvent system. The preparation conditions were as follows: the flow rate was 1.0 mL/min, the detection wavelength was UV 254 nm, and the temperature was 35 ℃. The fractions of each enantiomer were collected and combined. The pure optical isomer was obtained by removing the solvent under reduced pressure with a rotary evaporator. The ee value of each enantiomer of both chloroquine and hydroxychloroquine was higher than 98%. The spectral data were identical to those reported in the literature. [32-33] These free-base enantiomers were converted to the enantiomerically pure diphosphate salt and sulfate salt, respectively, as described in the experimental section.

SARS-CoV-2 antiviral assay and Immunofluorescence microscopy

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Antiviral assays against SARS-CoV-2 *in vitro* were performed in a biosafety level-3 laboratory (BSL-3) at the State Key Laboratory of Respiratory Disease, Guangzhou Medical University, Guangzhou Customs District Technology Center.

Log phase Vero E6 cells were plated overnight in 96-well cell culture plate (Greiner, Cat no.655090) and then pretreated with the indicated concentration of compounds for 1 h (each concentration had three replicates). SARS-CoV-2, with a multiplicity of infection (MOI), of 0.05, was subsequently added. After 1 h, the medium was replaced with a fresh drug-containing one. 24 hours later, the cells were fixed with 4% paraformaldehyde and permeabilized with 0.2% Triton X 100. After blocking with 5% bovine serum albumin (BSA) at room temperature for 1 h, the rabbit polyclonal antibody against SARS Coronavirus Nucleoprotein (NP) (1:1000) as the primary antibody was used to incubate the cells, and then Alexa 488 labeled secondary antibody (Donkey Anti-Rabbit IgG;1:500; Jackson) was added. The nuclei were stained with DAPI (Sigma). The plate was scanned using Celigo Image Cytometer and fluorescence microscopy images were taken. The inhibition rate was calculated using the mean intensity of a green channel versus a DAPI channel. The IC_{50} value for each compound was calculated using GraphPad Prism software (version 7.0) using a non-linear regression equation.

RESULTS

Chiral HPLC separation of both chloroquine and hydroxychloroquine

From the chiral HPLC separation of the racemic chloroquine, the first eluting compound at 4.86 minutes was found to be dextrorotary (+) and thus assigned as the *S*-CQ and the second compound at 5.33 minutes as *R*-CQ, with a specific rotation of 79.7 and -74.4, respectively, as depicted in Figure 2D-F. For the case of hydroxychloroquine in Figure 2A-C, the first eluting compound at 10.17 minutes was determined as *S*-HCQ, and the second compound at 11.85 minutes was *R*-HCQ, with a specific rotation of 95.6 and -107.8, respectively. The enantiomeric excess value of each enantiomer for both HCQ and CQ was found to be higher than 98%, as shown in Figure 2B, C, E, and F. In all cases, baseline separation was achieved. Material recovery was 8.32 g for loaded 9.64 g of HCQ case, and 5.04 g for loaded 5.04 g of CQ.

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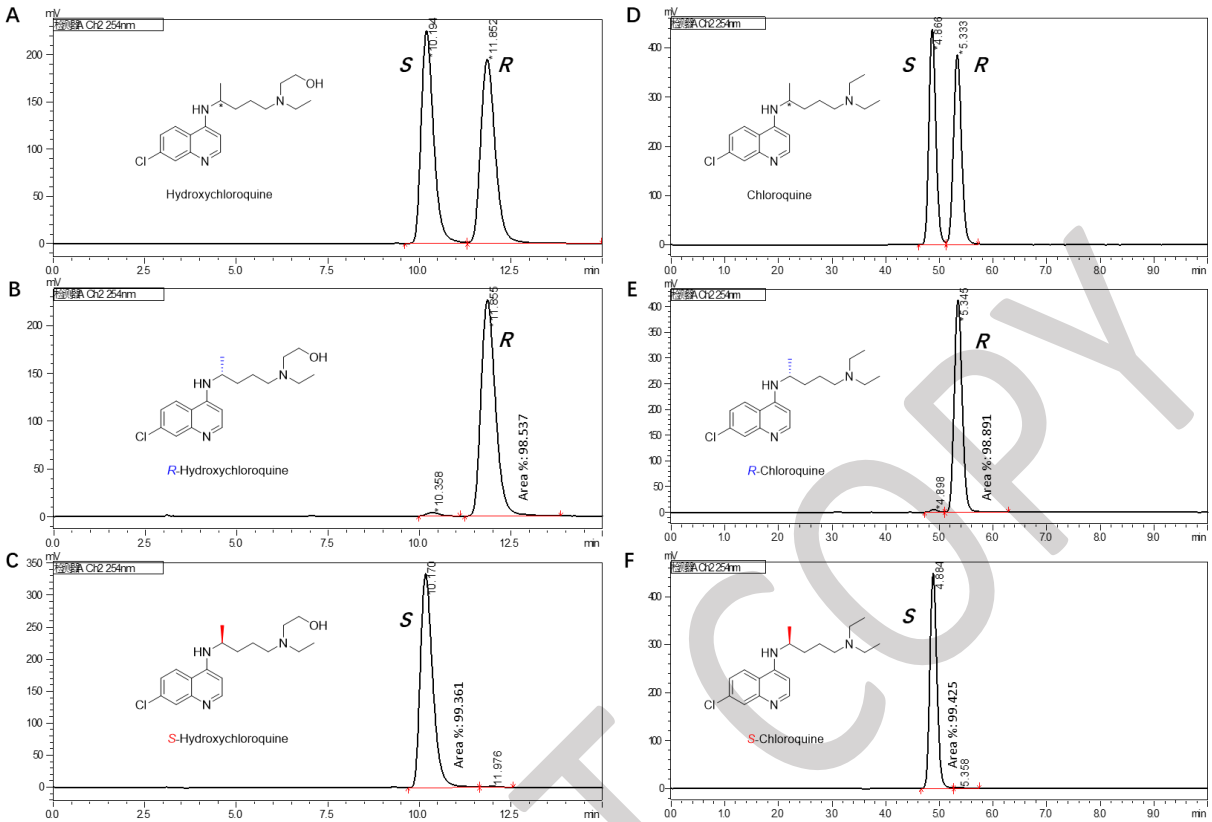

Figure 2. Chromatograms for the chiral HPLC separation from the racemic hydroxychloroquine (A-C) and chloroquine (D-F). Optimal chromatographic conditions for the two racemic mixtures: mobile phase, isocratic *n*-hexane/*iso*propanol/diethylamine with a ratio of 85:15:0.1 (v/v/v); eluent flow rate, 1.0 mL/min; column temperature, 35 °C; detection wavelength, 254 nm.

Antiviral activity in vitro

Results from the *in vitro* antiviral activity against SARS-CoV-2 in Vero E6 cells showed that both CQ and HCQ and their enantiomers exhibited respectable antiviral effect in a concentration-dependent manner. The IC_{50} values for the racemic chloroquine diphosphate (Rac-CQ) and its *R* and *S* enantiomer against SARS-CoV-2 *in vitro* were 1.801 μM, 1.975 μM, and 1.761 μM, respectively, as displayed in Figure 3A. Shown in Figure 3B, the IC_{50} values for the racemic hydroxychloroquine sulfate (Rac-HCQ) and its *R* and *S* enantiomer were 1.752 μM, 2.445 μM, and 1.444 μM, respectively. In addition, we tested the antiviral activity of azithromycin that was used as a combination drug with HCQ for treating COVID-19 in patients.[34] The single-dose of azithromycin afforded an IC_{50} value of 14.57 μM, shown in Figure 3C. The results from immunofluorescence microscopy of SARS-CoV-2 infection displayed visually a pronounced antiviral effect of the S-enantiomers of both CQ and HCQ, as compared to their *R*-enantiomer starting at the dose of 1 μM, respectively. The data showed that the antiviral activity of *S*-CQ was were relatively similar to the racemic CQ (Figure 4A), whereas the efficacy of the *S*-HCQ was much higher than its racemates (Figure 4B). On the other hand, azithromycin alone started to exhibit an antiviral effect at a relatively high concentration, which is over 10 μM (Figure 4C).

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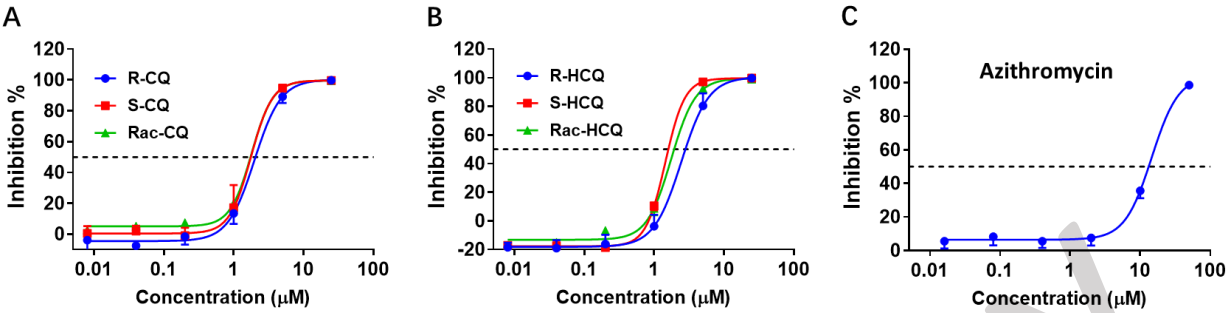

	R-CQ	S-CQ	Rac-CQ
IC_{50} (μM)	1.975	1.761	1.801

	R-HCQ	S-HCQ	Rac-HCQ
IC_{50} (μM)	2.445	1.444	1.752

	Azithromycin
IC_{50} (μM)	14.57

Figure 3. The antiviral activities of racemic and enantiomeric chloroquine diphosphate (A) and hydroxychloroquine sulfate (B), as well as azithromycin (C) against SARS-CoV-2 *in vitro*. Vero E6 cells were infected with SARS-CoV-2 (MOI = 0.05) at different concentrations: 0.008, 0.04, 0.2, 1, 5, and 25 μM for CQ and HCQ for 24 h (concentrations at 0.008, 0.04, 2, 10 and 50 μM for AZM). Data represented are the mean value of % inhibition of SARS-CoV-2 on Vero E6 cells.

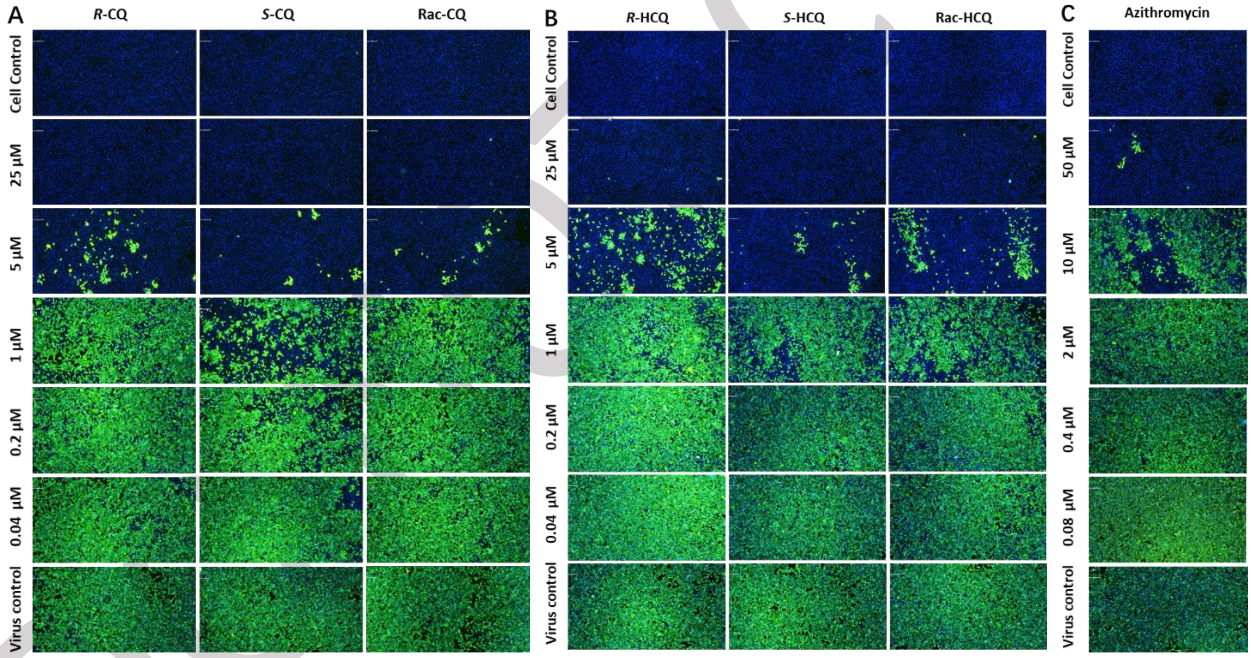

Figure 4. Immunofluorescence microscopy of virus infection with the treatment of chloroquine diphosphate (A) and hydroxychloroquine sulfate (B), as well as their enantiomers, and azithromycin (C) against SARS-CoV-2 *in vitro*. Experiments of virus infection and drug administration were conducted, as described in Figure 3. At 24 h p.i., the infected cells were fixed and then incubated with the primary antibody (rabbit polyclonal antibody against SARS Coronavirus Nucleoprotein), followed by Alexa 488 labeled secondary antibody. The nuclei were stained with DAPI. Bars, at 100 μm.

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DISCUSSION

Chiral drugs and performance difference of enantiomers

R- and *S*- enantiomers are different substances and by definition should not be expected to have the same biological effect in a living system, which is inherently chiral. Enantiomers often have different pharmacodynamics and pharmacokinetics behaviors and hence different therapeutic efficacy and safety profiles. However, the significance of this principle in drug development has not been fully appreciated at the time when CQ and HCQ were developed 70 some years ago.

One of the most notorious examples of drug chirality is thalidomide, a chiral drug first marketed as a racemic form in 1957 in Europe as an antiemetic for treating morning sickness and other ailments. The drug was withdrawn from the market in 1961 amid the emergence of birth defects among newborn children of woman patients. It was later determined that the two enantiomers caused distinctly different effects from one another, and the *S*-isomer was attributed to being the culprit for the teratogenic properties.[35] Since the enantiomers of thalidomide interconverts *in vivo*, efforts to develop a stable, single-enantiomer thalidomide was thwarted. Eventually, the racemic form was repurposed and launched for oncology applications under the brand Thalomid, strictly contraindicated for pregnancy. The thalidomide tragedy brought up a lot of attention and concerns about the importance of chirality in the drug development process. This eventually culminated in more specific guidance and regulations regarding chiral drug development by the FDA in the 1990s. CQ or HCQ would unlikely be approved as they had been submitted to a current regulatory authority, who will likely demand the delineation of each enantiomer's properties and demonstration of the non-inferiority of the racemate in the context of safety and efficacy before it is allowed for marketing approval.

Unlike thalidomide, CQ and HCQ are stereochemically stable and the interconversion between the two enantiomers has not been observed *in vivo* and is unlikely from a mechanistic point of view. The separation of the *R*- and *S*-enantiomers of CQ and HCQ has not been reported until the early 1990s,[36-37] as part of the metabolite profiling studies for HCQ.[36] With the enantio-discriminating analytical methods available, for CQ, HCQ and their respective metabolites, several studies demonstrated varying degrees of differences of pharmacological properties between the two enantiomers of CQ and HCQ.

Pharmacokinetics, pharmacology, and toxicology aspect of CQ and HCQ enantiomers

Both CQ and HCQ have extremely long half-lives approximating 50 days, predominantly due to their huge volume of distribution.[38] With such a low metabolic rate, the drugs accumulate excessively in tissues over time, especially eyes, skin, brain, heart, and liver. Long term and higher doses other than the recommended maximal dose for chloroquine and hydroxychloroquine (300 mg/kg and 400 mg/kg, respectively, according to the FDA) put the patients at risk of adverse effects such as irreversible retinopathy, neuropathic, and cardiac toxicities.[24, 26-27, 39] One of the major adverse effects associated with the clinical applications of CQ and HCQ is ocular toxicity manifested as pigmentary retinopathy, especially at higher doses, while the mechanism remains unelucidated.

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The pharmacokinetics differences between the two enantiomers of CQ and HCQ have been documented both in animals and human.[28, 38, 40-41] In a study of hydroxychloroquine distribution in rabbits, it was found that the *R*-enantiomer was stereoselectively accumulated in the ocular tissue with an *R/S*-HCQ ratio of 60/40 after the administration of the racemates. however, with an opposite ratio of 40:60 for the total plasma concentration, implying a possibility for a more significant contribution of the *R*-isomer for the ocular toxicity.[42] The results were consistent with the data of clinical PK study in humans, where the whole blood concentration of the *R*-enantiomer was much higher than its antipode, whereas the ratio of R/S concentrations of the chiral metabolites was less than 1. Besides, the renal clearance for the *S*-enantiomer was twice greater than the *R*-enantiomer, and a shorter elimination half-life was observed with the *S*-enantiomer.[28, 38, 40-41] *In vitro* assays using human plasma indicated that *R*-and *S*-HCQ enantiomers were 37% and 64% protein-bound, respectively, implying *R*-HCQ would have a higher volume of distribution than *S*-HCQ. These results indicated a faster renal excretion and/or more accentuated liver metabolism, and lower volume of distribution of the *S*-enantiomer, as compared to those of the *R*-enantiomer.[43] Thus, a stereoselective elimination for the S-isomer and a stereoselective protein for the R-isomer were considered as attribution to the differences in disposition and distribution into tissues such as ocular compartment.

The pharmacological effects were also proven to be different between the two enantiomers of CQ and HCQ for various applications. The difference in their antimalarial activities was reported in 1994.[30] It was noted that by scientists at Sterling Winthrop Inc. that *S*-HCQ was approximately 70% more active than *R*-HCQ in the Rat Pleurisy Macrophage Model.[44] Later on, *S*- and *R*-HCQ were found to suppress HIV-1 replication to a similar extent and dose proportionally, and were no more toxic than racemic HCQ.[45] Since HCQ's antimalarial and antiviral effects had been purported to originate from its ability to increase endosomal pH, the authors demonstrated that both enantiomers of HCQ increased endosomal pH to similar levels in a dose proportionally manner.

Unlike the much-explored pharmacokinetics and pharmacological effects of the racemates, limited toxicity information has shown that *S*-chloroquine seems to be less toxic than the *R*-chloroquine.[29] No further animal studies or clinical evidence was presented about the pharmacological activities of each enantiomer in the studies of malaria or autoimmune diseases.

The efficacy and safety of CQ and/or HCQ for treating COVD-19 need to be validated by larger and more definitive clinical trials. The complicated effects of ages, doses,[46] disease states, prior treatments, underlying physiological conditions of enrolled patients should be taking into consideration, for all of which can cause bias in the trial outcomes. However, running trials with a racemic drug represents one of the most significant variances, i.e. with a drug substance of only 50% potency of the preferred isomer, and will unnecessarily complicate the delineation of clinical outcomes contributed by each enantiomer. Fortunately, racemic HCQ is readily available with a worldwide production volume exceeding 300 metric tons and growing. Separation of a racemic mixture into pure enantiomer forms is industrially feasible, and asymmetric synthesis is also very promising as long-term solutions. There seems to be no excuse to continue running a trial using racemic CQ or HCQ other than ready availability.

The anti-SARS-CoV-2 activity of CQ, HCQ, as well as their enantiomers

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With this backdrop, we separated both racemic mixtures of CQ and HCQ by chiral HPLC, and tested the antiviral effects of *R*- and *S*-CQ and HCQ, as well as the racemic CQ, HCQ, and azithromycin in parallel, and the result is illustrated in Figure 3 and 4.

The most significant finding from this study is that both *S*-CQ and *S*-HCQ exhibited more pronounced activities than their respective *R*-enantiomer in the assay against SARS-CoV-2 *in vitro.* Specifically, the result with *S*-HCQ demonstrated significant enantioselectivity with a 60 % more efficiency than *R*-HCQ. The IC_{50} of *S*-enantiomer of CQ and HCQ were 1.761 µM and 1.444 µM, respectively, compared with their *R*-enantiomers with a respective IC_{50} value of 1.975 µM and 2.445 µM. The IC_{50} value of the racemic mixtures of CQ and HCQ was 1.801 µM and 1.752 µM, respectively, which fell between the two enantiomers, as expected. The IC_{50} of the racemic mixture of both CQ and HCQ indicated that at the testing conditions the *S*-isomers were the major contributors to the antiviral activities, implying that the *R*-isomers were either coming for the ride, being minor contributors to the antiviral activities, providing hitherto unknown benefits such as cytokine storm management, or contributing to untoward side effects.

With our preliminary data, the antiviral potency against SARS-CoV-2 *in vitro* of both enantiomers from the racemates was differentiated in the presented condition. This suggested that COVID-19 may be treated with the more effective and safer *S*-enantiomer substantially free of the *R*-enantiomer that might introduce adverse effects and dilute the therapeutic efficacy. The single *S*-enantiomer can be possibly administrated at a lower dose weight than that of the racemate for the same or improved therapeutic efficacy and fewer side effects, as a potentially superior treatment for COVID-19 with a broader therapeutic index. Since the mechanism of HCQ's antiviral effect remains to be elucidated, we remain cautious at explaining the synergies between the antiviral and anti-inflammatory activities without enantioselectivity data for the latter.

Conclusion and recommendations for future work

Our data clearly indicated that *R*- and *S*-enantiomer of CQ and HCQ exhibited significantly different properties against SARS-CoV-2. This is the first time that the enantioselective antiviral activities of CQ and HCQ against SARS-CoV-2 have been demonstrated *in vitro*. The *S*-enantiomers of both CQ and HCQ, proved to be 31% and 60% more active than their corresponding *R*-enantiomers.

Since we have demonstrated the *in vitro* antiviral activities mainly derived from the *S*-isomer, it is reasonable to expect the drug dose can be decreased to half of that or even less than the racemates for a similar antiviral effect, while eliminating the untoward side effects brought upon by the *R*-isomer. Moreover, our data showed a more significant difference in antiviral activity between two enantiomers of HCQ as compared to CQ. Taken all these together with previous studies on malaria, HIV, and human pharmacokinetic data implicating greater ocular accumulation of *R*-isomers, we opine a clear preference for the further development of *S*-HCQ as an enantiomerically pure drug for treating COVID-9 within this compound class.

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In summary, we have demonstrated unequivocally a pronounced difference in antiviral activities of enantiomers of CQ and HCQ. Racemate CQ or HCQ would be less preferred for COVID-19 applications unless shown to be so by further pharmacological testing. With the prospect of reducing or eliminating adverse effects attributed to the presence of *R*-HCQ, we strongly recommend further studies especially human clinical trials be conducted with pure *S*-HCQ, constituting the most expedient and highly probable way to double the therapeutic index of HCQ.

Experimental

Preparation of racemic chloroquine as a free-base (CAS NO. 54-05-7)

The purchased chloroquine diphosphate (13 g, 25.2 mmol) was dissolved in water (100 mL), followed by the addition of a 12% NaOH solution (50 mL) dropwise at 0 ℃. After stirring for 0.5 h, EtOAc (25 mL) was added and the mixture kept stirring for another 0.5 h at rt. The reaction mixture was extracted with EtOAc (3 x 100 mL). The organic phase was combined and washed with brine, then dried with anhydrous Na_2SO_4. The organic solvent was removed by a rotary evaporator under reduced pressure to obtain a yellow residue as the free chloroquine (7.6 g, 23.8 mmol, 94.3 % yield). The spectral data were identical to the literature. [47-48]

Preparation of racemic hydroxychloroquine as a free-base (CAS NO. 118-42-3)

The purchased hydroxychloroquine sulfate (10.9 g, 27.4 mmol) was dissolved in water (75 mL), followed by the addition of a 12% NaOH solution (50 mL) dropwise at 0 ℃. After stirring for 0.5 h, EtOAc (25 mL) was added and the mixture kept stirring for another 0.5 h at rt. The reaction mixture was extracted with EtOAc (3 x 100 mL). The organic phase was combined and washed with brine, then dried with anhydrous Na_2SO_4. The organic solvent was removed by a rotary evaporator under reduced pressure to obtain a yellow residue as the free hydroxychloroquine (7.7 g, 22.9 mmol, 83.7 % yield). The spectral data were identical to the literature.[49]

Preparation of the enantiomers of chloroquine and hydroxychloroquine by chiral HPLC

The chiral high-pressure liquid chromatography (HPLC) method was used to separate the enantiomers of the racemic mixture as free-bases by with a Shimadzu LC-20AD HPLC equipment. Chloroquine or hydroxychloroquine was dissolved in isocratic *n*-hexane/*iso*propanol/diethylamine with a ratio of 85:15:0.1 (v/v/v), and the loading needed for CQ and HCQ was 24.0 mg/mL and 23.5 mg/mL, respectively. The resulting solution was loaded onto a CHIRALPAK AY-H (AYH0CE-VC001) chiral column, which was then eluted with the same solvent system. The preparation conditions were as follows: the flow rate was 1.0 mL/min, the detection wavelength was UV 254 nm, and the temperature was 35 ℃. The fractions of each enantiomer were collected and combined. The pure optical isomer was obtained by removing the solvent under reduced pressure with a rotary evaporator. The ee of each enantiomer of both chloroquine and hydroxychloroquine was higher than 98 %. The spectral data were identical to the literature. [32-33]

General conversion from enantiomerically pure chloroquine as a free-base to its corresponding salt

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R-chloroquine or *S*-chloroquine (640 mg, 2.0 mmol) was dissolved in EtOH (4 mL) and heated to reflux, followed by the addition of 85% phosphoric acid (0.25 mL) for 2 h. A large amount of white solid precipitated out. The reaction mixture was cooled to rt and filtered. The resulting solid was washed with EtOH (3 x 1 mL) to obtain the product as (*R*)-chloroquine diphosphate or (*S*)-chloroquine diphosphate.

(4R)-4-N-(7-chloroquinolin-4-yl)-1-N,1-N-diethylpentane-1,4-diamine; phosphoric acid (R-chloroquine diphosphate salt)

The compound obtained was a white solid,868 mg, 1.68 mmol, 84.1 % yield, $[\alpha]_D^{26.8}$ = -74.4 (*c* = 0.5, H_2O). ^{1}H NMR (400 MHz, D_2O) δ 8.22 (d, *J* = 7.3 Hz, 1H), 8.06 (d, *J* = 9.1 Hz, 1H), 7.62 (d, *J* = 2.1 Hz, 1H), 7.46 (dd, *J* = 9.1, 2.1 Hz, 1H), 6.79 (d, *J* = 7.3 Hz, 1H), 4.09 (d, *J* = 5.9 Hz, 1H), 3.24 – 3.00 (m, 6H), 1.93 – 1.68 (m, 4H), 1.40 (d, *J* = 6.5 Hz, 3H), 1.22 (td, *J* = 7.3, 1.9 Hz, 6H); ^{13}C NMR (101 MHz, D_2O) δ 155.17, 142.10, 139.11, 137.88, 127.13, 123.92, 118.81, 114.95, 98.52, 51.13, 49.45, 47.22, 31.85, 20.21, 18.71, 8.09.

(4S)-4-N-(7-chloroquinolin-4-yl)-1-N,1-N-diethylpentane-1,4-diamine; phosphoric acid (S-chloroquine diphosphate salt)

The compound obtained was a white solid, 887 mg, 1.72 mmol, 86.0 % yield, $[\alpha]_D^{27.8}$ = 79.7 (*c* = 0.5, H_2O). ^{1}H NMR (400 MHz, D_2O) δ 8.27 (d, *J* = 7.3 Hz, 1H), 8.16 (d, *J* = 9.1 Hz, 1H), 7.76 (d, *J* = 2.0 Hz, 1H), 7.57 (dd, *J* = 9.1, 2.1 Hz, 1H), 6.84 (d, *J* = 7.3 Hz, 1H), 4.23 – 3.97 (m, 1H), 3.29 – 3.10 (m, 6H), 1.90 – 1.73 (m, 4H), 1.41 (d, *J* = 6.5 Hz, 3H), 1.24 (td, *J* = 7.3, 1.9 Hz, 6H); ^{13}C NMR (101 MHz, D_2O) δ 155.39, 142.13, 139.23, 138.10, 127.23, 124.02, 119.00, 115.17, 98.48, 51.13, 49.43, 47.30, 31.88, 20.21, 18.73, 8.10.

General conversion from enantiomerically pure hydroxychloroquine as a free-base to its corresponding salt

R-hydroxychloroquine or *S*-hydroxychloroquine, 700 mg, 2.1 mmol) was dissolved in EtOH (2 mL) and heated to 60 ℃, followed by addition of 80% phosphoric acid (188 mg) for 1 h. The reaction mixture was cooled to -20 ℃, a large amount of white solid precipitated out and the solid was filtered. The resulting solid was washed with cold EtOH (3 x 1 mL) to obtain the product as (*R*)-hydroxychloroquine sulfate or (*S*)-hydroxychloroquine sulfate.

2-[[(4R)-4-[(7-chloroquinolin-4-yl)amino]pentyl]-ethylamino]ethanol; sulfuric acid (R-hydroxychloroquine sulfate salt)

The compound obtained was a white solid, 745 mg, 1.71 mmol, 81.7 % yield, $[\alpha]_D^{26.1}$ = -107.75 (*c* = 0.32, H_2O). ^{1}H NMR (400 MHz, D_2O) δ 8.28 (d, *J* = 7.3 Hz, 1H), 8.16 (d, *J* = 9.1 Hz, 1H), 7.75 (d, *J* = 2.1 Hz, 1H), 7.56 (dd, *J* = 9.1, 2.1 Hz, 1H), 6.84 (d, *J* = 7.3 Hz, 1H), 4.14 (d, *J* = 6.5 Hz, 1H), 3.88 (t, *J* = 4.8 Hz, 2H), 3.34 – 3.16 (m, 6H), 1.96 – 1.71 (m, 4H), 1.42 (d, *J* = 6.5 Hz, 3H), 1.28 (td, *J* = 7.3, 1.8 Hz, 3H); ^{13}C NMR (101 MHz, D_2O) δ 155.34, 142.15, 139.17, 138.05, 127.20, 124.06, 118.98, 115.14, 98.53, 55.26, 53.77, 52.02, 49.46, 48.34, 31.86, 20.00, 18.74, 7.85.

2-[[(4S)-4-[(7-chloroquinolin-4-yl)amino]pentyl]-ethylamino]ethanol; sulfuric acid (S-hydroxychloroquine sulfate salt)

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The compound obtained was a white solid (805 mg, 1.85 mmol, 88.3 % yield, $[\alpha]_D^{26.8}$ = 95.6 (*c* = 0.32, H_2O). ^{1}H NMR (400 MHz, D_2O) δ 8.26 (d, *J* = 7.3 Hz, 1H), 8.13 (d, *J* = 9.1 Hz, 1H), 7.69 (d, *J* = 2.1 Hz, 1H), 7.52 (dd, *J* = 9.1, 2.1 Hz, 1H), 6.83 (d, *J* = 7.3 Hz, 1H), 4.12 (dd, *J* = 12.6, 6.3 Hz, 1H), 3.88 (t, *J* = 5.0 Hz, 2H), 3.37 – 3.21 (m, 6H), 1.92 – 1.78 (m, 4H), 1.42 (d, *J* = 6.5 Hz, 3H), 1.28 (td, *J* = 7.3, 2.0 Hz, 3H); ^{13}C NMR (101 MHz, D_2O) δ 155.26, 142.15, 139.12, 137.96, 127.15, 124.03, 118.90, 115.05, 98.54, 55.27, 53.77, 52.03, 49.47, 48.26, 31.84, 20.01, 18.73, 7.85.

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Conflict of Interest

All other authors have no potential conflict of interest.

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