OFFICE OF CLINICAL PHARMACOLOGY CONSULT REVIEW

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REQUESTOR: OID/DAV – John Farley, MD, MPH; Mary Singer MD, Clinical Team Lead; Karen Winestock, RPM (301-796-0834)

NAME OF DRUG/FORMULATION:	**ROUTE OF ADMINISTRATION:**
Chloroquine bis (phosphate) and hydroxychloroquine sulfate: Chloroquine phosphate 250 mg (RESOCHIN/ARALEN) Hydroxychloroquine sulfate 200 mg (PLAQUENIL)	Oral
INDICATION: to treat adult and adolescent patients who weigh 50 kg or more hospitalized with COVID-19 for whom a clinical trial is not available, or participation is not feasible	**DOSE/DOSING REGIMEN:** Chloroquine phosphate: 1g orally once on day one followed by 500 mg orally once a day for four to seven days of total treatment based on clinical evaluation Hydroxychloroquine sulfate: 800 mg orally once on day one followed by 400 mg orally once a day for four to seven days of total treatment based on clinical evaluation

CONSULT REQUEST

On March 28, 2020, FDA authorized the emergency use of chloroquine phosphate and hydroxychloroquine sulfate supplied from the Strategic National Stockpile to treat adults and adolescents who weigh 50 kg or more and are hospitalized with COVID-19 for whom a clinical trial is not available, or participation is not feasible. The EUA was based upon limited in-vitro data on antiviral activity of chloroquine phosphate and hydroxychloroquine sulfate and anecdotal clinical data in case series of chloroquine phosphate and hydroxychloroquine sulfate administered in the setting of COVID-19. The suggested dose for chloroquine was 1 gram of chloroquine phosphate on day one, followed by 500 milligrams daily for four to seven days of total treatment based on clinical evaluation. The suggested dose for hydroxychloroquine sulfate was 800 milligrams of hydroxychloroquine sulfate on the first day of treatment and then 400 milligrams daily for four to seven days of total treatment based on clinical evaluation. The suggested dosing relied in part upon the simulations published by Yao X, Ye F, Zhang M et al. which predicted adequate antiviral activity in lung.[1] Please review subsequent relevant publications and analyses and comment on the predicted antiviral activity of the suggested dosing in lung and other relevant tissues. Please summarize and assess any additional information that Clin Pharm believes is relevant to benefit risk considerations for ongoing authorization of the EUA.

EXECUTIVE SUMMARY

Selection of the suggested dosing regimen of hydroxychloroquine sulfate in EUA 39 (800 mg on the first day followed by 400 mg daily for four to seven days) relied in part upon the simulations published by Yao X, et al. which predicted adequate antiviral activity in lung.[1] Based on subsequent OCP analyses and review of relevant publications, coupled with the assumption that in vivo cellular accumulation is similar to that from in vitro studies, the calculated free lung concentrations resulting from the proposed hydroxychloroquine sulfate dosing regimen are well below in vitro EC50/EC90 values for SARS-CoV-2.

Much is unknown regarding both the pathogenesis of SARS-CoV-2-induced COVID-19 as well as the relevant mechanism of action for treatments that may ultimately prove to be safe and effective for COVID-19 prophylaxis and treatment. It has been hypothesized that the immunomodulatory effect of hydroxychloroquine (HCQ) may be beneficial during the moderate/late stage of COVID-19 disease progression. However, it has also been hypothesized that the immunosuppressive effect of CQ/HCQ could potentially be harmful to some patients with COVID-19, as it was for some other viral infections. Well-designed clinical trials that leverage an understanding of drug pharmacology and disposition, as well as disease pathogenesis, including interventions at various stages of infection and disease, will be necessary to definitively determine the risk/benefit balance for HCQ.

In vivo antiviral efficacy requires sufficiently high drug concentrations at the site of action, presumably the lung for COVID-19. This review describes analyses that indicate much of the current literature regarding doses of HCQ that will provide antiviral activity in COVID-19 patients underestimates the required dose. However, HCQ has potential to prolong QT, especially when used in combination

with other QT prolonging drugs, such as azithromycin. Thus, adequate concentrations for antiviral activity are not likely achievable with a safe oral dosing regimen of HCQ. Therefore, a strategy to increase the drug exposure at the site of action (e.g., through targeted delivery) while minimizing the systemic exposure may be desirable.

CLINICAL PHARMACOLOGY ASSESSMENT OF CONSULT

This Office of Clinical Pharmacology (OCP) review addresses an interoffice consult from CDER Office of Infectious Diseases Division of Antivirals (OID/DAV) on EUA 39 and the questions posed, as follows:

1. ***Please review subsequent relevant publications and analyses and comment on the predicted antiviral activity of the suggested dosing in lung and other relevant tissues.***

The dosing regimens for chloroquine (CQ) and HCQ proposed for emergency use relied in part upon the recently published investigation by Yao X, et al. that aimed to derive optimized dosing regimens of HCQ for the treatment of SARS-CoV-2 based on in vitro antiviral pharmacology experiments and physiologically-based pharmacokinetic (PBPK) modeling and simulation (M&S). The in vivo antiviral activity of a drug is typically estimated by calculating the ratio of free extracellular drug concentration in tissue in vivo to the in vitro EC50 or EC90 value. The higher this ratio, the greater the confidence in achieving in vivo antiviral efficacy. In the Yao, et al. study, PBPK models were used to predict the lung tissue concentrations of CQ and HCQ under different dosing regimens. The ratio of the free lung trough concentration (adjusted for protein binding) to the in vitro EC50 values obtained in the study using a VERO cell antiviral potency assay (RLTEC) was calculated for comparisons. The PBPK model-simulated lung trough concentrations were based on the lung-to-plasma partition coefficient obtained from rats and assumed to be the same in both rats and humans, as no human data are available. The authors indicated that the free lung trough HCQ concentrations would be approximately 21- to 169-fold of the EC50 value under different dosing regimens, resulting in high HCQ RLTEC values. They indicated that these results provide a rationale to support HCQ as a potentially efficacious antiviral drug against SARS-CoV-2.

Subsequent to the publication of the Yao et al. findings, multiple articles used the modeling approach and conclusions by the authors to inform HCQ dosing in clinical studies. Given the reliance of investigators and clinicians on the Yao findings, OCP evaluated the PBPK model and assumptions in this study and conducted a thorough literature review. OCP's review focused on the general range of HCQ dosing regimens being studied in clinical trials (including the regimen proposed in EUA 39), corresponding exposure, and predicted antiviral activity at relevant sites, as summarized below.[2]

- **HCQ/CQ potential mechanism of action and in vitro antiviral activity against SARS-CoV-2**
  HCQ and CQ are known to accumulate highly in acidic organelles, such as endosomes, the Golgi apparatus, and lysosomes, resulting in intracellular concentrations up to 1000-fold higher than extracellular drug concentrations (e.g., the concentrations in the cell culture media in the reported in vitro studies).[3, 4] The proposed mechanism of CQ's antiviral activity against coronavirus is related to its intracellular pH modulation effect. The increased endosomal pH is believed to block virus/cell fusion. The impairment of terminal glycosylation of angiotensin converting enzyme 2 (ACE2) caused by pH elevated Golgi apparatus may result in reduced binding affinities between ACE2 and SARS-CoV spike protein.[5] A more recent study confirmed the endosomal pH-related mechanism for CQ and explored the antiviral mechanism for HCQ.[6] Both CQ and HCQ affected the number and/or size/morphology of early endosomes and endolysosomes, and the authors hypothesized that this could result in failure of further transport of virions to the ultimate release site.

  In two papers, including the paper by Yao, the in vitro antiviral activity of CQ and HCQ against SARS-CoV-2 for both treatment and prophylaxis was reported using EC50 values that represent the drug concentrations initially added to the cell culture media instead of the intracellular drug concentration.[1, 6] It was reported that the initial drug concentration could decrease significantly due to intracellular accumulation during the incubation.[7] This could lead to a much lower estimated EC50 value if the measured steady state drug concentration had been used to estimate EC50. However, after examining the experimental conditions reported by both studies,[1, 6] we consider the impact of extracellular drug concentration drop during the in vitro study on EC50 estimate is insignificant.

- **Considerations when estimating in vivo drug exposure**
  CQ and HCQ have significantly higher tissue concentrations compared to those in plasma. The CQ product label reports tissue concentrations 200- to 700-fold higher than plasma in animals[8] while MacIntyre et al.[7] suggests HCQ may have similarly high tissue/plasma ratio in the rat. The mechanism for the high tissue/plasma ratio is due to the accumulation of CQ/HCQ in acidic organelles such as endosome, Golgi apparatus, and lysosomes inside tissue cells.[7] Despite the high tissue intracellular concentrations, the free tissue extracellular concentration should be similar to the free plasma concentration.[9] Given free drug is active drug to exert antiviral effect, linking in vitro antiviral activity and in vivo exposure necessitates that HCQ concentrations in different matrices (whole blood, serum or plasma) be converted to the unbound concentrations in plasma.

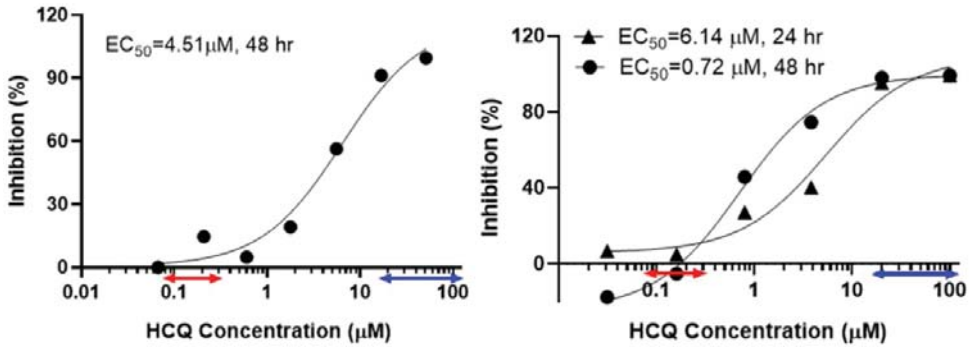
  A study investigated the distribution of CQ in blood and showed an average blood-to-plasma concentration ratio of 7.6 and serum-to-plasma concentration ratio of 2.[10] The higher concentrations of CQ in serum might be due to the release of CQ from leukocytes and thrombocytes during the clotting process. Therefore, at least 1000 g centrifugal force was recommended to obtain reliable plasma concentration of CQ due to the extensive accumulation of CQ in various blood cells. HCQ showed a similar mean blood-to-plasma concentration ratio of 7.2.[11] Given the similar intracellular accumulation between CQ and HCQ, the serum-to-plasma concentration ratio for HCQ is expected to be approximately 2 as well for the same reason.

In addition to distribution properties of HCQ/CQ, another critical consideration in linking in vitro antiviral activity to in vivo HCQ concentrations is that plasma concentrations of HCQ were shown to be altered by improper separation technique, specifically variables such as the time between blood sample collection and plasma separation by centrifugation, and centrifugation speed.[11] Tett SE et al. highlighted the importance of high centrifugal force in their study of HCQ PK and applied 1200 g for 20 minutes within 30 minutes of collection. However, as indicated by Lim et al.,[12] the plasma samples were obtained after the blood samples were centrifugated at only 250 g for 10 min. Centrifuging at such a low speed cannot efficiently remove platelets,[13] cells in which HCQ was concentrated.[10] Thus, much higher HCQ "plasma" concentrations were used for model development in the Lim et al. study (summarized in Appendix A), and the developed model significantly over-predicted HCQ plasma concentrations with different dosing regimens. The predicted "plasma" concentrations based on this model are as high as the observed whole blood HCQ concentrations from other reliable HCQ PK studies (based on data from the reference arm of multiple HCQ generic drug submissions). The over-predicted HCQ plasma concentrations using Lim's model thereafter were used in multiple investigations to support different HCQ dosing regimens. These results should be considered unreliable.

- **Linking in vitro antiviral activity and in vivo HCQ concentrations**

Based on the above considerations, we re-calculated the RLTEC values using free lung extracellular trough concentrations which should be similar to the free plasma concentrations (Cplasma*fu,plasma) extracted from Figure 3 in Yao et al.[1] instead of the predicted "free lung trough concentrations" (Cplasma*Kp*fu,plasma) reported by Yao et al. that included the highly accumulated intracellular concentration as discussed previously. Our results show lower RLTEC values (0.11-0.34) compared to those reported by Yao et al. (21-169). When a higher EC50 value as reported by Liu et al.[6] was used, even lower RLTEC values (0.017-0.054) were obtained, suggesting the in vivo concentrations of HCQ that would be achieved with the proposed dosing regimen in EUA 39 (800 mg orally once on day one followed by 400 mg orally once a day for four to seven days) may not result in adequate clinical antiviral activity against SARS-CoV-2, as the RLTEC values ranged from 0.005 to 0.34, depending on the values of EC50/EC90. Concentration-inhibition (%) plots from both Yao et al. and Liu et al. with the expected in vivo HCQ concentration range are displayed in Figure 1 below. It should be noted that our calculation assumed similar in vivo cellular accumulation as those seen in in vitro studies. Even though we used model-predicted HCQ plasma concentration from Yao et al. for comparison purposes, observed concentrations from various clinical trials can be used for similar calculations (see Appendix A). When using reported PK parameters, blood and serum concentrations should be properly converted to free plasma concentration before comparison with EC50/EC90 values.

Figure 1. Predicted HCQ free lung extracellular concentration (equal to free plasma concentration) range (0.077-0.305 μM, red double-end arrows) with different dosing regimens and HCQ SARS-CoV-2 inhibition concentration-response curves at multiplicity of infection (MOI, the ratio between the number of viruses and the number of host cells) of 0.01. The blue double-end arrows (15.1-121.7 μM) represent the "free lung trough concentration" obtained from Yao et al.[1]; HCQ SARS-CoV-2 inhibition concentration-response curves were adapted from Liu et al. (left)[6] and Yao et al. (right)[1].



Footnote: Multiple other reports[6, 14-18] also cited the significantly higher lung concentrations relative to the in vitro EC50 as the rationale to support CQ/HCQ as a potentially efficacious regimen against SARS-CoV-2. However, as stated earlier, the in vitro EC50 values used in these reports were based on the drug concentrations in cell culture media (i.e., extracellular concentration). In order to consider the significantly higher lung (intracellular) concentration to predict the potential in vivo antiviral efficacy, the corresponding in vitro antiviral potency parameter, e.g. EC50_intracellular, should be calculated based on the intracellular concentrations in the antiviral experiments. EC50_intracellular will be significantly greater than the currently reported EC50 values. As a result, the ratio between in vivo intracellular concentration and EC50_intracellular would still be low, suggesting low potential for in vivo antiviral activity at doses that would not be rate-limiting from the standpoint of toxicity.

Summary of assessment of predicted antiviral activity in lung and other relevant tissues of the suggested dosing regimen

The translation of in vitro antiviral activity to appropriate clinical dosing regimens is complex and multifactorial. For the case of CQ/HCQ, the in vitro antiviral EC50 values reported in the literature[1, 6] were extracellular drug concentrations present in cell culture media and should be compared with in vivo free drug concentration in the plasma (likely to be equal to free extracellular tissue concentration). Under the assumption that in vivo cellular accumulation is similar to that from the in vitro studies, the calculated free lung concentrations that would result from proposed dosing regimens studied, including the HCQ sulfate regimen proposed in EUA 39 (800 mg on the first day of treatment and then 400 mg daily for four to seven days of total treatment), are well below the in vitro EC50/EC90 values, making the antiviral effect against SARS-CoV-2 not likely achievable with a safe oral dosing regimen.

Multiple other reports also cited the significantly higher lung concentration relative to the in vitro EC50 as the rationale to support CQ/HCQ as a potentially efficacious regimen against SARS-CoV-2.[6, 14-18] A summary of additional relevant publications and analysis published as of May 12, 2020 and comments on the predicted in vivo antiviral activity is provided in Appendix A. The same limitations discussed above are shared by all these publications except one by Maharaj et al.[19] This study used PBPK modeling and allometric scaling to extrapolate currently investigated adult dosages to children for HCQ and remdesivir, respectively. Consistent with our findings, Maharaj et al. raised similar concerns regarding HCQ use for COVID-19 treatment since unbound plasma concentrations of HCQ were notably below thresholds for antiviral effect. Given viruses are intracellular pathogens, the authors postulated intracellular lung concentrations would likely be the more relevant concentrations exerting antiviral effect.

Well-designed clinical trials that leverage full understanding of drug pharmacology and disposition, particularly in the lung, as well as disease pathogenesis at various stages of infection and disease, will be necessary to definitively determine whether the risk/benefit balance is favorable for HCQ.

2. ***Please summarize and assess any additional information that OCP believes is relevant to benefit risk considerations for ongoing authorization of the EUA.***

The analysis above only considered viral inhibition activity in the calculation, while HCQ may have additional relevant pharmacological properties (e.g., anti-inflammatory/immunomodulatory effects). Our assessment should be put in proper context, as much is unknown regarding both the pathogenesis of SARS-CoV-2-induced COVID-19 as well as the relevant mechanism of action for treatments that ultimately prove to be safe and effective for COVID-19 prophylaxis and treatment.

It has been hypothesized that the immunomodulatory effect of HCQ may be beneficial during the moderate/late stage of COVID-19 disease progression.[20] Conversely, it has also been hypothesized that the immunosuppressive effect of CQ/HCQ could potentially be harmful to some patients with COVID-19, as it has been demonstrated with other viral infections.[20] Regarding drug-vaccine interactions, data from a randomized, controlled trial with CQ at prophylactic doses for malaria indicates the drug may influence vaccine take. The product labeling for CQ states, "The blood concentrations of chloroquine and desethylchloroquine (the major metabolite of chloroquine, which also has antimalarial properties) were negatively associated with log antibody titers. Chloroquine taken in the dose recommended for malaria prophylaxis can reduce the antibody response to primary immunization with intradermal human diploid-cell rabies vaccine."[8] The data raises a potential concern that the use of CQ/HCQ might also have a detrimental effect for the formation of neutralizing antibodies against SARS-CoV-2 during the infection and future vaccination efficacy.

Adequate and well-controlled clinical trials will ultimately be critical in determining which treatment modalities will be safe and effective, at what stages of infection and disease, and at what dose regimens. As in vitro studies showed antiviral activities for CQ and HCQ, in vivo antiviral efficacy may be possible only if the in vivo concentration is sufficiently high. However, CQ and HCQ have potential QT prolongation risk, especially when used in combination with other QT prolonging drugs, such as azithromycin.[21, 22] Therefore, a strategy to increase the drug exposure at the site of action (e.g., through targeted delivery) while minimizing the systemic exposure may be desirable.

References:

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2. Fan J, Zhang X, Liu J, et al. Connecting hydroxychloroquine in vitro antiviral activity to in vivo concentration for prediction of antiviral effect: a critical step in treating COVID-19 patients [submitted to CTS, peer review in progress; see APPENDIX B]
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COVID-19. J Crit Care 2020.
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APPENDIX A Summary of additional relevant publications and analysis published as of May 12, 2020 and comments on the predicted in vivo antiviral activity

Publication	Approach	Data Source	Key Findings	Reviewer's Comments
Optimizing hydroxychloroquine dosing for patients with COVID-19: An integrative modeling approach for effective drug repurposing. *Garcia-Cremades et al.2020* [1]	1. A published population PK model [2] was used to predict HCQ plasma concentration with different dosing regiments. 2. Plasma and serum HCQ concentrations were assumed to be comparable. 3. In vitro viral growth, death, and saturable growth were simulated using data from SARS-CoV-1. 4. Drug effect over time on viral replication rate was established.	1. Clinical data: non-randomized single arm open label study of HCQ 200 mg TID, with or without azithromycin for treatment of COVID-19 in France [3]. 2. In vitro viral replication data: Growth of SARS-CoV-1 in Vero cells over 11 days [4]. 3. In vitro anti-SARS-Cov-2 activity in Vero E6 cells [5-7]. 4. Model simulated HCQ plasma concentrations.	HCQ doses > 400 mg BID for ≥ 5 days were predicted to rapidly decrease viral loads, reduce the proportion of patients with detectable SARS-CoV-2 infection, and shorten treatment courses, compared to lower dose (≤ 400 mg daily) regimens.	1. For population PK model issues, please refer to comments on Lim et al [2]. 2. The assumption that plasma and serum HCQ concentrations were comparable is not valid. 3. In vitro SARS-CoV-1 replication data were used to describe the viral dynamic with time. However, the SARS-CoV-2 infection efficiency and replication kinetics were indicated to be different from those of SARS-CoV-1 [8]. 4. Drug effect vs virologic data were obtained from a clinical study for which concerns have been raised regarding the study design, patients recruited, and study result evaluation [9-12].
Hydroxychloroquine for treatment of SARS-CoV-2 infection? Improving our confidence in a model-based approach to dose selection. *Arnold et al. 2020* [13].	1. A published HCQ PBPK model [5] was used to predict HCQ plasma, blood and lung concentrations with different dosing regimens. 2. Impact of different HCQ lung Kp values on predicted lung concentration was investigated. 3. Simulated unbound lung tissue concentration was compared with HCQ in vitro efficacy data. 4. Unbound heart tissue concentration was predicted. 5. The fraction unbound in lung tissue was assumed to be the same as in the plasma (0.5).	1. In vitro anti-SARS-Cov-2 activity in VeroE6 cells [5]. 2. Model simulated HCQ plasma, blood, lung and heart concentrations.	Unbound HCQ lung trough concentrations were predicted to be well above the in vitro EC50 value using a Kp value of 220 which was assumed to be the same in both rats and humans[5].	1. The in vitro antiviral EC50 values should be compared with in vivo free drug concentration in the plasma (likely to be equal to free extracellular tissue concentration). 2. The assumption that the fraction unbound in lung tissue was the same as in the plasma is not valid, given the extensive accumulation of HCQ in the tissues. 3. The unbound lung and heart tissue concentrations were over-predicted. 4. The anti-SARS-CoV-2 activity was significantly over-predicted.
Finding the dose for hydroxychloroquine prophylaxis for COVID-19: the desperate search for effectiveness. *Al-Kofahi et al. 2020* [14]	1. A published population PK model [2] was used to predict HCQ plasma concentrations with different dosing regiments. 2. Various HCQ dosing strategies was examined and predicted plasma exposures were compared to in vitro efficacy data.	1. In vitro anti-SARS-Cov-2 activity in VeroE6 cells [5]. 2. Model simulated HCQ plasma concentrations.	Model-based dosing regimens were recommended for both pre-exposure prophylaxis and post-exposure prophylaxis settings.	1. For population PK model issues, please refer to comments on Lim et al [2]. 2. The predicted "plasma" HCQ concentration is similar to the HCQ whole blood concentration. 3. The anti-SARS-CoV-2 activity was significantly over-predicted.
Towards Optimization of Hydroxychloroquine Dosing in Intensive Care Unit COVID-19 Patients. *Perinel et al. 2020 [15]*	1. A published population PK model [16] was used to predict HCQ blood concentration with different dosing regiments. 2. Various HCQ dosing strategies was	1. Small prospective PK study to characterize HCQ PK in patients with SARS-CoV-2 infection (n=13) in critical care unit. 2. Model simulated HCQ blood	Based on the simulation, a loading dose of 800 mg once daily on day 1, followed by 200 mg BID for 7 days was proposed.	1. For Yao et al. PBPK modeling and simulation and data analysis issues, please refer to this consult and Fan et al. (Appendix A). 2. For population PK model issues, please refer to comments on

	examined. 3. HCQ blood trough levels >1 mg/L and <2 mg/L were considered to be therapeutic based on Yao et al. reported PBPK modeling and simulation results and HCQ in vitro anti-SARS-CoV-2 activity [5].	concentrations.		Carmichael et al[16]. 3. The in vitro antiviral EC50 values should be compared with in vivo free drug concentration in the plasma (likely to be equal to free extracellular tissue concentration) instead of blood concentration. 4. The anti-SARS-CoV-2 activity was significantly over-predicted.
Pharmacokinetic bases of the hydroxychloroquine response in COVID-19: implications for therapy and prevention. *Tarek et al. 2020 [17]*	1. A mathematical model was used to simulate the possible scenarios of response to HCQ in COVID-19 patients. 2. HCQ trough blood concentrations provided by Morita et al. [18] were used to estimate the response of SARS-CoV-2 to HCQ.	1. Model simulated HCQ blood concentrations. 2. Serum PK data in Gautret et al., were converted to blood concentration using a plasma/blood concentration ratio of 4. 3. It appears that the author assumed that plasma and serum HCQ concentrations were comparable.	Computer-aided simulations show that HCQ may have an impact on the amplitude of the viral load peak but that viral clearance is not significantly accelerated if the drug is not administered early enough	1. For population PK model issues, please refer to comments on Mortia et al [18]. 2. The in vitro antiviral EC50 values should be compared with in vivo free drug concentration in the plasma (likely to be equal to free extracellular tissue concentration) instead of blood concentration. 3. The anti-SARS-CoV-2 activity was significantly over-predicted.
Timing of antiviral treatment initiation is critical to reduce SARS-Cov-2 viral load. *Gonçalves, et al. 2020[19]*	1. Modeling of viral dynamics 2. Morita et al. model [18] was used to simulate HCQ PK	1. Viral dynamics data were from 13 untreated patients infected with SARS-CoV-2 in 4 Singapore hospitals. 2. Model simulated HCQ concentrations.	Using the total drug concentration and in vitro EC50 of 0.72 uM, a 33% efficacy was estimated for HCQ.	1. For population PK model issues, please refer to comments on Mortia et al [18]. 2. The in vitro antiviral EC50 values should be compared with in vivo free drug concentration in the plasma (likely to be equal to free extracellular tissue concentration) instead of total plasma concentration. 3. The anti-SARS-CoV-2 activity was significantly over-predicted.
Simulated Assessment of Pharmacokinetically-Guided Dosing for Investigational Treatments of Pediatric Patients with COVID-19. *Maharaj et al. 2020 Submitted to JAMA Pediatrics*	1. Adult and pediatric PBPK M&S 2. HCQ dosing regimen (D1:400mg BID, D2-D5: 200mg BID) was used based on the previously published PBPK analysis [5]. 3. Predicted HCQ unbound plasma and unbound lung interstitial fluid concentration were compared with in vitro efficacy data.	1. In vitro anti-SARS-Cov-2 activity in VeroE6 cells [5]. 2. Model simulated HCQ blood, plasma and unbound interstitial fluid concentrations in adults and pediatric subjects.	The analysis raised concerns regarding HCQ use for COVID-19 treatment since unbound plasma concentrations were notably below in vitro concentrations needed to mediate antiviral activity.	**This article's data analysis and conclusion are consistent with reviewers' independent data analysis and findings.**
Pharmacokinetics of hydroxychloroquine and its clinical implications in chemoprophylaxis against malaria caused by Plasmodium vivax. *Lim et al. 2009* [2].	Population PK	Plasma concentration from a total of 91 healthy subjects and patients receiving HCQ monotherapy. Dense PK was collected from 6 healthy subjects after a single dose 400 mg (a total of 77 samples), and sparse PK was collected 16 healthy subjects taking 400 mg (a total of 64 samples, collected up to 7 days post-dose) and 69 patients receiving a total of 2 mg HCQ in 48 hours (a total of 290 samples, collected up to 9 days post-dose).	A two-compartment model with first-order absorption and linear kinetics was developed to describe the plasma concentrations of HCQ.	The reported plasma concentration after a single dose of 400 mg HCQ plasma is substantially higher than expected and is comparable to the estimated blood HCQ level based on bioequivalence studies submitted to the FDA. The model predicted, steady state, plasma concentration (reached in about 1 month) is comparable to or higher than historical data for blood concentrations reported in literature. The centrifugal force (250 g) used to process blood is too low to remove

				platelets which contain accumulated HCQ.
Population Pharmacokinetics of Hydroxychloroquine in Patients with Rheumatoid Arthritis. *Carmichael et al.2003* [16].	Population PK	Blood concentration from a total of 123 patients receiving HCQ: 1) single dose of HCQ at 155 mg base as a 30-infusion; or 2) oral doses of HCQ at 155 mg or 310 mg, with or without methotrex (MTX). 9 patients who received both IV and oral doses provided dense PK data in the first 8 hours post-dose (followed by 24 and 32 hr post-dose only). The remaining patients on oral treatment provided through concentrations only.	A one-compartment model was supported for HCQ. MTX does not affect HCQ clearance.	The data do not support a characterization of the distribution phases of HCQ PK. The 1-compartment model predicted a half-life less than 2 days, which results in rapid accumulation after short term use and is not consistent with historical data that steady state is expected after months.
Population Pharmacokinetics of Hydroxychloroquine in Japanese Patients with Cutaneous or Systemic Lupus Erythematosus. *Morita et al. 2016* [18].	Population PK	3 samples from each patient were drawn at 3-5 hours, 7-11 hours, and 20-28 hours after or just before dosing during 36–48 weeks after initiation of HCQ therapy (200-400 mg daily dose). Both blood and plasma concentrations were measured.	A one-compartment model was developed for blood and plasma data. The authors reported a B/P ratio of 4. The authors recommended body weight-based dosing for Japanese patients.	The data do not support a characterization of the distribution phases of HCQ PK. The 1-compartment model predicted a half-life less than 2 days (shorter than Carmichael paper).

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APPENDIX B **Fan J, Zhang X, Liu J, et al. Connecting hydroxychloroquine in vitro antiviral activity to in vivo concentration for prediction of antiviral effect: a critical step in treating COVID-19 patients**

Connecting hydroxychloroquine in vitro antiviral activity to in vivo concentration for prediction of antiviral effect: a critical step in treating COVID-19 patients

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Abstract:

Translation of in vitro antiviral activity to the in vivo setting is crucial to identify potentially effective dosing regimens of hydroxychloroquine. In vitro EC50/EC90 values for hydroxychloroquine should be compared to the in vivo free extracellular tissue concentration, which is similar to the free plasma hydroxychloroquine concentration.

Keywords: Hydroxychloroquine, SARS-CoV-2, COVID-19, antiviral activity

Topic: anti-SARS-CoV-2 activity, in vitro-in vivo extrapolation

Section/Category: Brief reports

Introduction:

The recently published article by Yao et al. aimed to derive optimized dosing regimens of hydroxychloroquine (HCQ) for the treatment of SARS-CoV-2 based on in vitro antiviral pharmacology experiments and physiologically-based pharmacokinetic (PBPK) modeling and simulation (M&S) [1]. The unprecedented global COVID pandemic necessitates expeditious, pharmacologically-anchored development of therapeutic agents to both treat and prevent the adverse clinical sequelae of SARS-CoV-2. Mechanistically-informed approaches including, but not limited to, PBPK and other M&S strategies may be helpful in 1) deriving dosing regimens of therapeutic agents likely to have acceptable risk/benefit profiles; 2) identifying where in the course of disease treatment should be initiated; and 3) providing mechanistic insights into data derived from clinical trials. To the extent that translational research similar to that conducted by Yao et al. may be used to inform future drug development programs and clinical management strategies, herein, we wish to share our perspectives on how to link in vitro antiviral activity and drug exposure at the putative target site of action in predicting the in vivo antiviral effect of HCQ.

A key goal in PBPK modeling, as illustrated by Yao et al., is to derive appropriate dosing regimens by integrating in vitro experimental pharmacological data with understanding of physiological process and drug properties in order to simulate which regimens would achieve adequate concentrations in target tissues of relevance. To estimate in vivo antiviral activity, the ratio of free extracellular drug concentration in tissue in vivo to the in vitro EC50 or EC90 value is generally calculated. The higher this ratio, the greater the confidence in achieving in vivo antiviral efficacy.

Yao et al. recommended dosing regimens based on the ratios of free lung trough concentration to the in vitro EC50 value (R_{LTEC}=Ctrough,lung/EC50), where the free lung trough concentration was calculated as the PBPK model-simulated lung trough concentration adjusted with the chloroquine (CQ)/HCQ unbound fraction (fu,plasma) in plasma, and the EC50 values were the initial CQ/HCQ concentrations in the cell culture media that led to 50% of the maximum antiviral activity. The PBPK model-simulated lung trough concentration was based on the lung-to-plasma partition coefficient obtained from rats and assumed to be the same in both rats and humans as no human data are available. The authors indicated that the free lung trough HCQ concentrations would be approximately 21- to 169-fold of the EC50 value under different dosing regimens resulting in high HCQ R_{LTEC} values; this would suggest high likelihood for in vivo antiviral activity and thus provide a rationale to support HCQ as a potentially efficacious regimen inhibiting SARS-CoV-2 assuming an antiviral mechanism of action/benefit.

In this brief report, we summarize HCQ's potential mechanism of action against SARS-CoV-2, in vitro anti-SARS-CoV-2 studies, pharmacokinetic (PK) properties for HCQ, and provide a high-level assessment regarding how to link in vitro antiviral activity and in vivo drug concentration to assess the antiviral effect of HCQ for SARS-CoV-2.

HCQ/CQ's potential mechanism of action against SARS-CoV-2

HCQ and CQ are known to accumulate highly in acidic organelles, such as endosomes, the Golgi apparatus, and lysosomes. The intracellular concentrations can be up to 1000-fold higher than the extracellular drug concentrations (e.g., the concentrations in the cell culture media in the reported in vitro studies) [2, 3] (Figure 1). The proposed mechanism of CQ's anti-coronavirus activity is related to its intracellular pH modulation effect. The increased endosomal pH was believed to block virus/cell fusion. The impairment of terminal glycosylation of angiotensin converting enzyme 2 (ACE2) caused by pH elevated Golgi apparatus may result in reduced binding affinities between ACE2 and SARS-CoV spike protein [4]. A more recent study confirmed the endosomal pH-related mechanism for CQ and explored the antiviral mechanism for HCQ [5]. Both CQ and HCQ affected the number and/or size/morphology of early endosomes and endolysosomes, and the authors hypothesized that this could result in failure of further transport of virions to the ultimate release site.

In vitro antiviral activity against SARS-CoV-2

In two papers, the in vitro antiviral activity of CQ and HCQ against SARS-CoV-2 for both treatment and prophylaxis was reported using EC50 values that represent the drug concentrations initially added to the cell culture media instead of the intracellular drug concentration [1, 5]. It was reported that the initial drug concentration could decrease significantly due to intracellular accumulation during the incubation [6]. This could lead to a much lower estimated EC50 value if the measured steady state drug concentration had been used to estimate EC50. However, after examining the experimental conditions reported by both studies [1, 5], we consider the impact of extracellular drug concentration drop during the in vitro study on EC50 estimate is insignificant.

In vivo drug exposure

CQ and HCQ are known to have significantly higher tissue concentrations compared to those in plasma. The CQ product label reports tissue concentrations 200-700 fold higher than plasma in animals [7] while MacIntyre et al. [6] suggests HCQ may have similarly high tissue/plasma ratio in the rat (Figure 1). The mechanism for the high tissue/plasma ratio is due to the accumulation of CQ/HCQ in acidic organelles such as endosome, Golgi apparatus, and lysosomes inside tissue cells [6]. Therefore, despite the high tissue intracellular concentrations, the free tissue extracellular concentration should be similar to the free plasma concentration [8] (Figure 1). It should be noted that various types of concentrations have been reported, such as blood, serum, and plasma concentrations with different units. A study investigated the distribution of CQ in blood and showed an average blood-to-plasma concentration ratio of 7.6 and serum-to-plasma concentration ratio of 2 [9]. The higher concentrations of CQ in serum might be due to the release of CQ from leucocytes and thrombocytes during the clotting process. Therefore, at least 1000 g centrifugal force was recommended to process the blood samples and obtain reliable plasma concentration of CQ. HCQ showed a similar mean blood-to-plasma concentration ratio of 7.2 [10]. A similarly high centrifugal force (1200 g) had to be applied to obtain reliable plasma concentration of HCQ[10, 11]. Given the similar intracellular accumulation between CQ and HCQ, the serum-to-plasma concentration ratio for HCQ is expected to be approximately 2 as well for the same reason. When linking in vitro antiviral activity and in vivo exposure, the HCQ concentrations in different matrices (whole blood, serum or plasma) need to be converted to the unbound concentration in plasma. PK models developed from improperly processed plasma samples can lead to a "plasma" concentration prediction as high as the whole blood concentration [12]. Any application of such PK models to support HCQ dosing regimen is questionable [13, 14].

Results:

Linking in vitro antiviral activity and in vivo hydroxychloroquine concentrations

Based on the above considerations, we re-calculated the R_{LTEC} values using free lung extracellular trough concentrations which should be similar to the free plasma concentrations (Cplasma*fu,plasma) extracted from figure 3 in Yao et al.[1] instead of the predicted "free lung trough concentrations" (Cplasma*Kp*fu,plasma) reported by Yao et al which included the highly accumulated intracellular concentration as discussed previously. These results are listed in Table 1B and showed lower R_{LTEC} values (0.11-0.34) compared to those reported by Yao et al (21-169). When a higher EC50 value as reported by Liu et al. [5] was used, even lower R_{LTEC} values (0.017-0.054) were obtained, suggesting the possibility that in vivo concentrations of HCQ that would be achieved with the highest proposed dosing regimen (D1 800 mg + 400 mg, D2-D10 400 mg QD) may not result in adequate clinical antiviral activity against SARS-CoV-2, as the R_{LTEC} values ranged from

0.005 to 0.34, depending on the values of EC50/EC90 . Similar R_{LTEC} range (0.03-0.89 when compared to EC50, and 0.007 to 0.064 when compared to EC90) was obtained for CQ (D1-D10 500 mg BID) (data not shown). The in vivo HCQ concentration range is added to the concentration-inhibition (%) plots from both Yao et al. and Liu et al. to visualize the magnitude of in vivo concentration range relative to the in vitro concentration ranges (Figure 2).
It should be noted that our calculation assumed similar in vivo cellular accumulation as those seen in in vitro studies. Even though we used model-predicted HCQ plasma concentration from Yao et al. for comparison purposes, observed concentrations from various clinical trials can be used for similar calculations. When using reported PK parameters, blood and serum concentrations should be properly converted to free plasma concentration before comparison with EC50/EC90 values.

Discussion:

Multiple other reports [5, 15-19] also cited the significantly higher lung concentration relative to the in vitro EC50 as the rationale to support CQ/HCQ as a potentially efficacious regimen against SARS-CoV-2. However, as stated earlier, the in vitro EC50 values used in these reports were based on the drug concentrations in the cell culture media (extracellular concentration). In order to use the significantly higher lung (intracellular) concentration to predict the potential in vivo antiviral efficacy, we believe the in vitro corresponding antiviral potency parameter, e.g. EC50_intracellular, should be calculated based on the intracellular concentrations in the antiviral experiments. EC50_intracellular will be significantly greater than the currently reported EC50 values. As a result, the ratio between in vivo intracellular concentration and EC50_intracellular would still be low, suggesting low potential for in vivo antiviral activity at doses that would not be rate-limiting from the standpoint of toxicity.

Our assessment should be put in proper context. It should be noted that much is unknown about both the pathogenesis of SARS-CoV-2-induced COVID-19 as well as the relevant mechanism of action for treatments that ultimately prove to be safe and effective for COVID-19 prophylaxis and treatment. We only considered viral inhibition activity in the calculation, while HCQ may have additional relevant pharmacological properties (e.g., anti-inflammatory/immunomodulatory effects). It has been hypothesized that the immunomodulatory effect of HCQ may be beneficial during the moderate/late stage of COVID-19 disease progression [20]. Adequate and well-controlled clinical trials will ultimately be critical in determining which treatment modalities will be safe and effective, at what stages of infection and disease, and at what dose regimens.

As in vitro studies showed antiviral activities for CQ and HCQ, in vivo antiviral efficacy may be possible only if the in vivo concentration is sufficiently high. However, CQ and HCQ have potential QT prolongation risk, especially when being used in combination with another QT prolonger, such as azithromycin [21, 22]. Therefore, a strategy to increase the drug exposure at the site of action (e.g., through targeted delivery) while minimizing the systemic exposure may be desirable.

In conclusion, the translation of in vitro antiviral activity to appropriate clinical dosing regimens is complex and multifactorial. For the case of CQ/HCQ, the in vitro antiviral EC50 values reported in the literature [1, 5] were extracellular drug concentrations present in cell culture media, and should be compared with in vivo free drug concentration in the plasma (likely to be equal to free extracellular tissue concentration). Under the assumption that in vivo cellular accumulation is similar to that from the in vitro studies, the calculated free lung concentrations that would result from proposed dosing regimens are well below the in vitro EC50/EC90 values, making the antiviral effect against SARS-CoV-2 not likely achievable with a safe oral dosing regimen. Well-designed clinical trials that leverage full understanding of drug pharmacology and disposition, as well as disease pathogenesis, will be necessary to definitively determine whether the risk/benefit balance is favorable for a given treatment.

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Conflict of interest: None

Table 1. **A:** Model predicted HCQ free trough concentration (Ctrough,u) and free maximal concentration (Cmax,u) in plasma on days 1, 3, 5 and 10 following different proposed dosing regimens in healthy subjects (data were digitized from Figure 3 in Yao et al. article[1].). **B**: Re-calculated ratios of free lung extracellular (or free plasma) trough concentration to in vitro extracellular EC50 or EC90 (R_{LTEC}) with different dosing regimens of HCQ.

A

Dosing Regimen[a]	Ctrough,u (μM)				Cmax,u (μM)			
	Day 1	Day 3	Day 5	Day 10	Day 1	Day 3	Day 5	Day 10
D1 800 mg + 400 mg D2-D10 400 mg QD	0.108	0.118	0.155	0.228	0.165	0.186	0.223	0.305
D1 600 mg BID D2-D10 400 mg QD	0.115	0.118	0.155	0.228	0.185	0.185	0.221	0.302
D1 600 mg BID D2-D10 200 mg BID	0.115	0.131	0.164	0.244	0.182	0.159	0.192	0.271
D1 400 mg BID D2-D10 200 mg BID	0.077	0.109	0.145	0.225	0.122	0.134	0.174	0.259
D1 400 mg BID D2-D5 200 mg BID	0.077	0.109	0.145	0.101	0.122	0.134	0.173	0.102

B

Dosing Regimen[a]	R_{LTEC} (Ctrough,u/EC50)							
	EC50 = 0.72 μM[1][b]				EC50 = 4.51 μM[5] [b]			
	Day 1	Day 3	Day 5	Day 10	Day 1	Day 3	Day 5	Day 10
D1 800 mg + 400 mg D2-D10 400 mg QD	0.15	0.16	0.22	0.32	0.024	0.026	0.034	0.051
D1 600 mg BID D2-D10 400 mg QD	0.16	0.16	0.22	0.32	0.026	0.026	0.034	0.051
D1 600 mg BID D2-D10 200 mg BID	0.16	0.18	0.23	0.34	0.026	0.029	0.036	0.054
D1 400 mg BID D2-D10 200 mg BID	0.11	0.15	0.20	0.31	0.017	0.024	0.032	0.050
D1 400 mg BID D2-D5 200 mg BID	0.11	0.15	0.20	0.14	0.017	0.024	0.032	0.022
Dosing Regimen [a]	R_{LTEC} (Ctrough,u/EC90)							
	EC90 = 10 μM[1] [b]				EC90 = 16.9 μM[5] [b]			
	Day 1	Day 3	Day 5	Day 10	Day 1	Day 3	Day 5	Day 10
D1 800 mg + 400 mg D2-D10 400 mg QD	0.011	0.012	0.015	0.023	0.006	0.007	0.009	0.013
D1 600 mg BID D2-D10 400 mg QD	0.012	0.012	0.015	0.023	0.007	0.007	0.009	0.013
D1 600 mg BID D2-D10 200 mg BID	0.012	0.013	0.016	0.024	0.007	0.008	0.010	0.014
D1 400 mg BID D2-D10 200 mg BID	0.008	0.011	0.014	0.023	0.005	0.006	0.009	0.013
D1 400 mg BID D2-D5 200 mg BID	0.008	0.011	0.014	0.010	0.005	0.006	0.009	0.006

Notes:

[a] Dosing regimen information was obtained from Table 1 in Yao et al. article[1]. The Ctrough concentrations were digitized from Figure 3 in Yao et al. article[1]. The fraction unbound in plasma (fu,plasma) is 0.5. The model performance regarding the HCQ plasma concentration prediction has been independently verified by the authors of this report.

[b] The EC50 values for HCQ against SARS-CoV-2 infection in vitro at different multiplicity of infection (MOI, the ratio between the number of viruses and the number of host cells) reported by Liu et al were 4.51 μM (MOI=0.01), 4.06 μM (MOI = 0.02), 17.31 μM (MOI = 0.2), and 12.96 μM (MOI = 0.8) at 48 hours post-infection[5]. The EC50 values reported by Yao et al. were 6.14 μM (MOI = 0.01 for at 24 hours post-infection), and 0.72 μM (MOI = 0.01 at 48 hours post-infection)[1]. The EC90 values for HCQ reported by Liu et al were 16.9 μM (MOI=0.01), 22.3 μM (MOI = 0.02), and >50 μM (MOI = 0.2 and 0.8)[5]. The EC90 values reported by Yao et al. were 16.5 μM (MOI = 0.01 at 24 hours post-infection), and 10 μM (MOI = 0.01 at 48 hours post-infection)[1]. Only EC50 and EC90 values representing MOI of 0.01 were used in the calculation above.

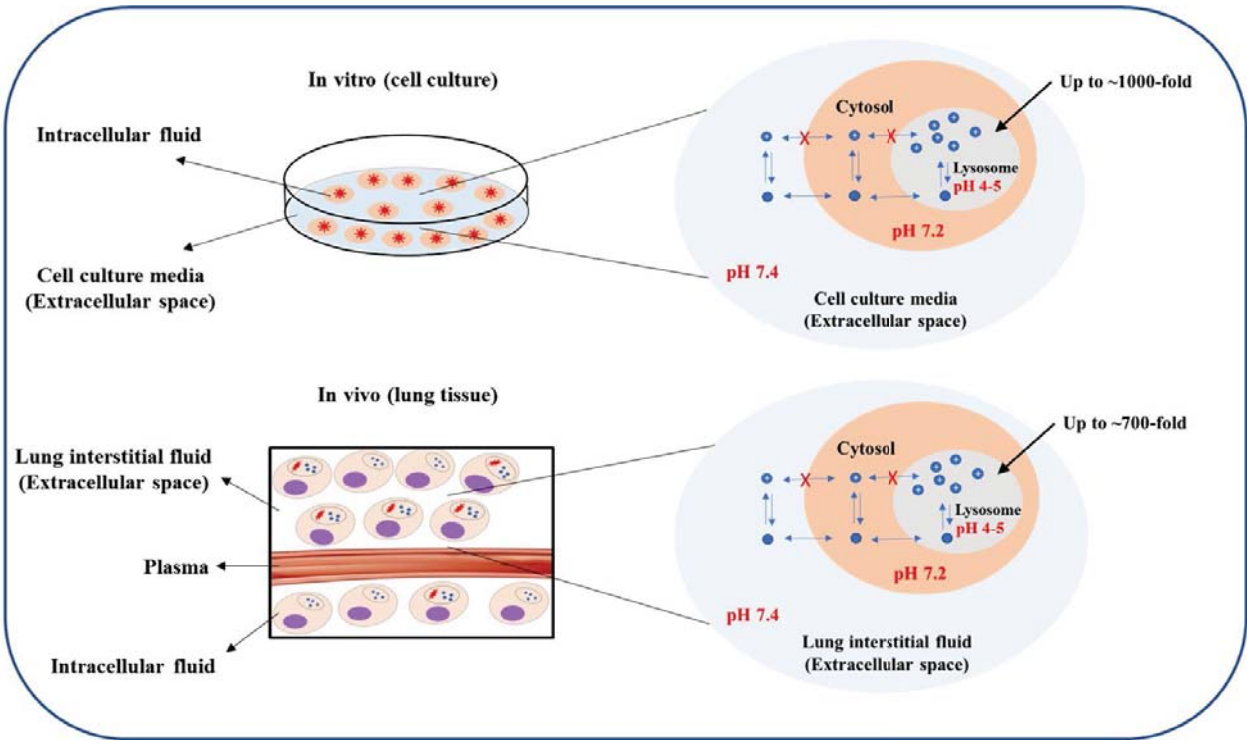

Figure 1 Mechanism of pH-driven intracellular accumulation of HCQ in the in vitro cell culture system and in vivo lung tissue. HCQ has a high logP (3.84) and pKa (9.67, 8.27) and can freely diffuse across the cell membrane in its unionized from to enter the cell and the lysosome. Once inside the lysosome, HCQ becomes protonated in the acidic environment, preventing it from crossing the lysosomal membrane back to the cytoplasm.

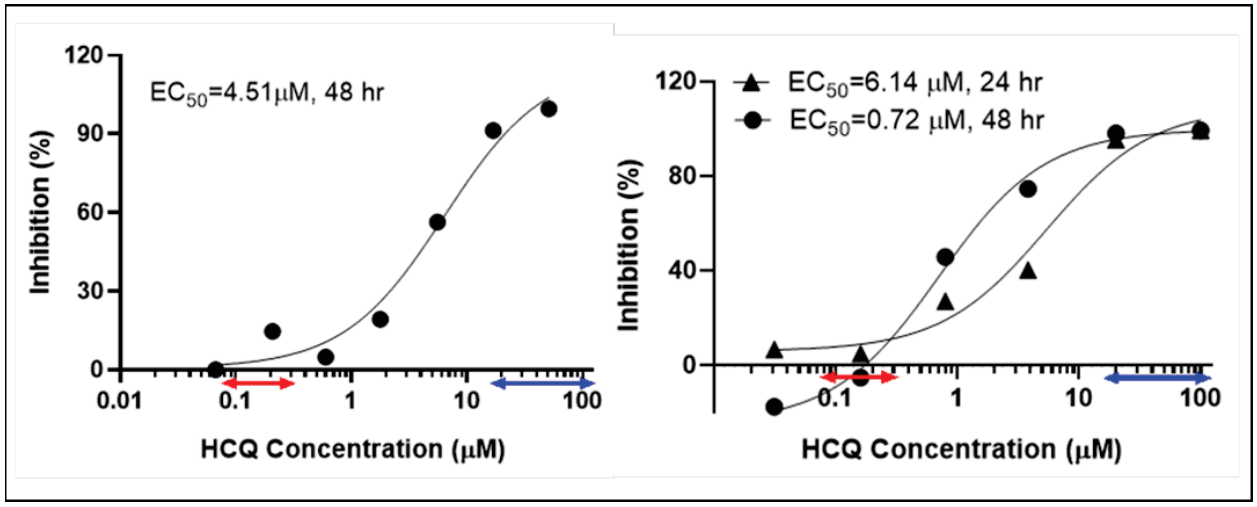

Figure 2 Predicted HCQ free lung extracellular concentration (equal to free plasma concentration) range (0.077-0.305 μM, red double-end arrows) with different dosing regimens (Table 1) and HCQ SARS-CoV-2 inhibition concentration-response curves at MOI of 0.01. The blue double-end arrows (15.1-121.7 μM) represent the "free lung trough concentration" obtained from Yao et al.[1]. The HCQ SARS-CoV-2 inhibition concentration-response curves were adapted from Liu et al.(left) [5], and Yao et al.(right) [1].

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