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

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.

Acknowledgements

Grateful acknowledgement is made to Dr. Mary Singer, Dr. Patrick Harrington, and Dr. Issam Zineh for their critical evaluation of this report.

Disclaimer:
This article reflects the views of the author and should not be construed to represent FDA's views or policies.

Conflict of interest: None

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Figure Legends:

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].

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

	R_{LTEC} (Ctrough,u/EC50)							
Dosing Regimen[a]	**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
	R_{LTEC} (Ctrough,u/EC90)							
Dosing Regimen [a]	**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

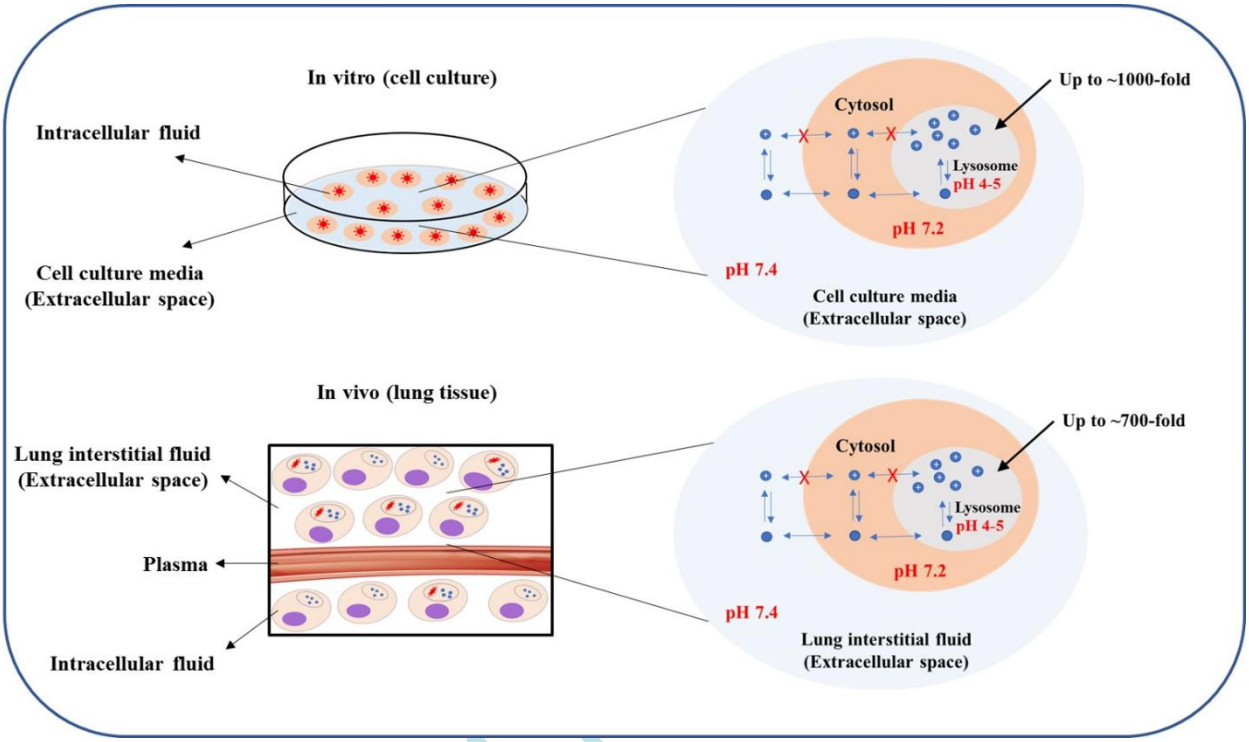

Figure 2