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A Strategy to Treat COVID-19 Disease with Targeted Delivery of Inhalable Liposomal Hydroxychloroquine: A Non-clinical Pharmacokinetic Study

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2 **ABSTRACT**

3 Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) is a newly identified
4 pathogen causing coronavirus disease 2019 (COVID-19) pandemic. Hydroxychloroquine
5 (HCQ), an antimalarial and anti-inflammatory drug, has been shown to inhibit SARS-CoV-2
6 infection *in vitro* and tested in clinical studies. However, lung concentration (6.7 µg/mL) to
7 predict the *in vivo* antiviral efficacy might not be achievable with the currently proposed oral
8 dosing regimen. Further, a high cumulative doses of HCQ may raise concerns of systemic
9 toxicity, including cardiotoxicity. Here, we described a non-clinical study to investigate the
10 pharmacokinetics of a novel formulation of liposomal HCQ administrated by intratracheal
11 (IT) instillation in Sprague-Dawley (SD) rats which achieved 129.4 µg/g (C_{max}) in the lung.
12 Compared to unformulated HCQ administered intravenous (IV), liposomal HCQ with
13 normalized dose showed higher (~30-fold) lung exposure, longer (~2.5-fold) half-life in lung,
14 but lower blood exposure with ~20% of C_{max} and 74% of AUC and lower heart exposure
15 with 24% of C_{max} and 58% of AUC. In conclusion, the pharmacokinetics results in an animal
16 model demonstrate the proof of concept that inhalable liposomal HCQ may provide clinical
17 benefit and serve as a potential treatment for COVID-19.

18 INTRODUCTION

19 Hydroxychloroquine (HCQ), an antimalarial and anti-inflammatory drug, is inexpensive,
20 safe, and well tolerated by most patient populations, including those with chronic diseases or
21 immunocompromised status. HCQ is a weak diprotic base that can pass through the lipid cell
22 membrane and preferentially concentrate in acidic cytoplasmic vesicles. HCQ is being
23 studied to prevent and treat coronavirus disease 2019 (COVID-19), possibly via blocking the
24 interactions between virus and angiotensin-converting enzyme-2 (ACE-2) receptor as well as
25 sialic acids receptor and has shown potential *in vitro* (1, 2) and preliminary clinical results (3,
26 4). As of Jul 8, 2020, a total of 232 studies involves HCQ use among 2478 clinical trials for
27 COVID-19 registered with the clinicaltrials.gov.

28 However, the effective *in vivo* levels as well as the optimal dosing regimen of HCQ for
29 treating COVID-19 remains unclear. The *in vitro* EC₅₀ values (0.72 to 17.31 μ M) proposed
30 for optimized dosing regimens are based on extracellular drug concentration (1, 2). A higher
31 lung (intracellular) concentration to predict the *in vivo* antiviral efficacy was suggested (5).
32 The HCQ concentration (6.7 μ g/mL) required to clear 100% of SARS-CoV-2 *in vitro* might
33 not be achievable with the currently proposed oral dosing regimen of 800 mg HCQ sulfate
34 orally daily, followed by a maintenance dose of 400 mg given daily for 4 days (2, 6). Further,
35 a high cumulative doses of HCQ may raise concerns of systemic toxicity, including

36 cardiotoxicity (7).

37 An alternative strategy is needed to bridge the gap between *in vitro* and clinical use of a
38 potentially effective agent in the context of COVID-19 pandemic. A drug delivery directly to
39 the respiratory tracts while minimizing the systemic exposure is a desirable alternative (5). In
40 this report, a proof-of-concept study was conducted to evaluate the systemic
41 pharmacokinetics (PK) profiles and tissue distribution following a single dose of inhalable
42 liposomal HCQ in a rat model and compared to that of unformulated HCQ through
43 intravenous (IV) delivery.

44 **RESULTS**

45 **HCQ pharmacokinetics in lung.** Upon IT administration of liposomal HCQ,
46 significantly more HCQ disposition (Fig. 1A) and longer half-life in lung was observed
47 compared to that those of HCQ by either IV or IT administration (37.5 hours vs. 15.2 hours
48 and 17.7 hours for HCQ-IV and HCQ-IT, respectively) (Table 1). Notably, even at 72 hours
49 post-dose, liposomal HCQ-IT had 77-fold (to HCQ-IV) and 43-fold (to HCQ-IT) higher
50 HCQ concentrations in lung. A single dose of 0.284 mg liposomal HCQ-IT achieved 129.4
51 $\mu\text{g/g}$ in C_{max} and 4193.2 $\text{h} \cdot \mu\text{g/g}$ in AUC_{0-72} and showed overall greater lung exposure with
52 29-fold in C_{max} and 35-fold in AUC_{0-72} compared to HCQ-IV with normalized dose (Table
53 2).

54 **HCQ pharmacokinetics in blood and heart.** With IV administration, HCQ showed
55 similar PK profile and systemic exposures as HCQ-IT in overall, including C_{max} and AUC
56 (Fig. 1B and Table 1). On the contrary, liposomal HCQ-IT showed significantly lower
57 systemic exposure in blood with only around 20% of C_{max} and 74% of AUC_{0-72} after
58 normalizing dose compared to HCQ-IV (Table 2). As observed in blood, liposomal HCQ-IT
59 showed significantly lower exposure in the heart from 0.25 hours to 24 hours (Fig. 1C and
60 Table 1). With normalized dose, liposomal HCQ-IT yielded only 24% of C_{max} and 58% of
61 AUC_{0-24} compared to HCQ-IV (Table 2).

62 **DISCUSSION**

63 In this rat pharmacokinetics study, a significantly higher exposure of HCQ with
64 sustained release in the lung was observed by targeted delivery of inhalable liposomal HCQ,
65 suggesting a potential treatment strategy for COVID-19 pulmonary disease. Fan et al. and
66 others have suggested that significantly higher lung (intracellular) concentrations relative to
67 the *in vitro* EC₅₀ would be required to achieve *in vivo* antiviral efficacy SARS-CoV-2 (5, 6).
68 Also, it has been proposed that the prediction of *in vivo* efficacy should be driven primarily
69 by high lung HCQ concentrations for treatment of viral pneumonia instead of HCQ blood
70 exposure (6). Given the current dosing regimen for oral HCQ is unlikely to produce an
71 antiviral effect, FDA recently revoked the emergency use authorization (EUA) to use HCQ to
72 treat COVID-19 in certain hospitalized patients and WHO also stopped the HCQ arm of the
73 COVID-19 Solidarity Trial. Here our findings supported the feasibility of alternative targeted
74 delivery of HCQ to the lungs with potentially effective antiviral levels while minimizing the
75 systemic exposure.

76 The aerosolized delivery of therapeutic drugs to the lower respiratory tract has been
77 applied for the treatment of various lung infectious and inflammatory disorders. For example,
78 aerosolized HCQ (AHCQ) has been applied in clinical trials for asthma and showed well
79 tolerance without significant toxicity after 21 days of dosing (8). In the current study, it is

80 found that HCQ lung exposure remains low with short half-life even with dosing of
81 unformulated HCQ directly to trachea, indicating unformulated HCQ is possibly to be
82 retained in lung only transiently and distributed rapidly from lung to systemic circulation.
83 The results suggest a sustained release formulation of HCQ, like inhalable liposomal HCQ
84 demonstrated here might be able to provide preferentially higher concentrations in the lung
85 than an aerosolized, non-liposomal HCQ formulation.

86 It was demonstrated that the inhalable liposomal formulation could provide efficient
87 aerosolized delivery of liposomal drugs to lung and increase drug exposure in airways and
88 lung with lower doses than used for systemic administration (9). For instance, ARIKAYCE[®],
89 an inhalable liposomal amikacin has been developed and accelerated approved by FDA in
90 2018 for treating *Mycobacterium avium* complex lung disease (10). As shown in this
91 proof-of-concept study, the inhalable liposomal HCQ did increase exposure in the lung with
92 extended residence time compared to systemically administered HCQ. Notably, the
93 significantly increased lung exposure at normalized dose suggested the lung HCQ
94 concentration with *in vivo* antiviral efficacy could be achieved at a relatively lower dose of
95 inhalable liposomal HCQ than that of oral regimens with loading dose initiation (11).

96 In addition to prolonging the residence time and increasing the exposure in lung,
97 significantly lower effective dose with less dosing frequency of inhalable liposomal HCQ

98 might also reduce systemic exposure and potential adverse events. One of the well-known
99 side effects of HCQ in the treatment of rheumatological disorders is cardiotoxicity, including
100 abnormal heart rhythms such as QTc interval prolongation and ventricular tachycardia, a
101 dangerously rapid heart rate (12). In recent study, among 84 COVID-19 patients who
102 received HCQ and azithromycin at NYU's Langone Medical Center, 11% of them had
103 prolonged QT intervals to be considered at high risk of arrhythmia (13). The present study
104 showed liposomal HCQ-IT not only reduced systemic exposure but also decreased heart
105 tissue distribution of HCQ compared to HCQ-IV. It suggested inhalable liposomal HCQ at a
106 significantly lower effective dose should possess less cardiotoxicity concerns than
107 conventional oral HCQ tablets.

108 While there is no commercially available aerosolized formulation of HCQ, a recent
109 empirical study of inhaled HCQ aerosols at 4 mg per day over one week found it was well
110 tolerated without significant adverse events (14). This further supports the rationale of
111 applying inhalable liposomal HCQ with direct lung targeting in COVID-19 infection/disease
112 prevention and treatment.

113 A limitation of the current study is that the pharmacokinetics of liposomal HCQ were
114 evaluated in a rat model using IT instillation to mimic the intended inhalation administration.
115 The typical lung deposition efficiency with inhaled aerosols is very likely lower than that of

116 the IT instilled microsprayed-droplets. Furthermore, it is suggested the aerosol-generating
117 procedure (nebulization) on patients with known or suspected COVID-19 should be
118 performed cautiously given that SARS-CoV-2 is highly contagious through the respiratory
119 route

120 (<https://www.cdc.gov/coronavirus/2019-ncov/hcp/infection-control-recommendations.html>)

121 (15). Therefore, we have developed a disposable closed-loop system connected to the
122 nebulizer to maximize the targeted delivery of inhaled liposomal HCQ while minimize the
123 spreading and contaminating the air and environment (data not shown).

124 In conclusion, this study in a rat model demonstrate the desirable pharmacokinetics of
125 inhalable liposomal HCQ *in vivo*. It supports the working hypothesis that inhalable liposomal
126 HCQ might serve as a potential treatment option for the delivery of HCQ for COVID-19
127 pulmonary disease by achieving targeted antiviral levels with less frequent dosing and at a
128 relatively lower dose.

129 **MATERIALS AND METHODS**

130 **Drugs and reagents.** The study drug (liposomal HCQ) was prepared by Taiwan Liposome
131 Company, Ltd., Taiwan. It is composed of HCQ encapsulated in liposomes with mean
132 particle size around 200 nm. The liposomes are composed of
133 dipalmitoylphosphatidylcholine (Lipoid GMBH, Germany) and cholesterol (Carbogen
134 Amcis B.V., The Netherlands), both of which are natural components of lung surfactant (16,
135 17). The formulation was manufactured in the following processes. Briefly, appropriate
136 amounts of lipid mixture were dissolved in ethanol (J. T. Baker, USA) and injected to a
137 hydroxychloroquine sulfate (SCI Pharmtech, Inc., Taiwan) solution at 50°C. The size of the
138 liposomes was adjusted to 200 nm by an extruder with 0.2 µm polycarbonate membrane to
139 standardize the sizes of the liposomes. A tangential flow filtration (TFF, VIVAFLOW 200,
140 MWCO 100,000 PES, Sartorius Stedim Biotech GmbH, Germany) was used to remove
141 non-incorporated HCQ and ethanol to obtain the liposomal HCQ in 0.9% sodium chloride
142 (Merck KGaA, Germany).

143 **Study Design.** A total of 52 female SD rats (BioLASCOTaiwan Co., Ltd.) were assigned
144 to one of three treatment groups: (1) HCQ-IV: 12 rats received a single dose of 0.590 mg
145 HCQ sulfate per animal via IV injection; (2) HCQ-IT: 20 rats received a single dose of
146 0.590 mg HCQ sulfate per animal via intratracheal (IT) administration; and (3) liposomal

147 HCQ-IT: 20 rats received a single dose of 0.284 mg liposomal HCQ sulfate per animal via
148 IT administration. The sampling time points for blood samples were 0.25, 1, 4, 24 and 72
149 hours post-dose and for tissue/organ samples were 0.25, 4, 24 and 72 hours post-dose. In
150 this study, inhalable liposomal HCQ was administered through IT instillation to mimic the
151 intended inhaled administration in a clinical setting. All procedures involving animals were
152 performed in TLC animal facility and in accordance with the ethical guidelines of
153 Institutional Animal Care and Use Committee (IACUC) at TLC, Taiwan
154 (#TLC20IACUC012).

155 Blood was collected from jugular veins at scheduled sampling time points into
156 collection tubes with K₂EDTA as the anticoagulant and stored at -80°C until analysis. After
157 blood draw, each animal was perfused with 2 mM K₂EDTA/saline solution before lungs and
158 hearts were removed, weighed and stored at -80°C.

159 **Bioanalysis and PK calculation.** Blood samples were mixed well with acetonitrile with
160 0.1% formic acid for protein precipitation. The supernatant was dried by nitrogen and
161 reconstituted with 30% acetonitrile with 0.1% formic acid containing internal standard (IS)
162 prior to injection into a liquid chromatography-tandem mass spectrometer (LC-MS/MS).
163 For lung and heart samples, tissue/organ was homogenized with 50% methanol with 0.1%
164 formic acid. The tissue/organ homogenate was added with IS and mixed well with

165 acetonitrile with 0.1% formic acid for protein precipitation. The resulting sample solution
166 was mixed well with 0.1% formic acid to final 30% acetonitrile with 0.1% formic acid prior
167 to injection into a LC-MS/MS.

168 Concentrations of HCQ in blood and tissue/organ samples were determined by
169 LC-MS/MS on a Waters ACQUITY UPLC-I CALSS coupled with Water Xevo TQS or AB
170 Sciex Triple Quad 5500. The ACQUITY BEH C18 column (2.1×50 mm) was selected for
171 sample separation with gradient elution consist of 0.1% formic acid in water (mobile phase
172 A) and acetonitrile containing 0.1% formic acid (mobile phase B) at 0.4 mL/min flow rate.
173 Total run time was 4.5 minutes and the column oven was set at 40°C. The mass
174 spectrometer was set up in MRM mode to monitor the transition 336 → 247 for HCQ. The
175 linear range of the assay is 0.5-500 ng/mL, 0.5-500 ng/mL and 20-10,000 ng/mL for blood,
176 heart and lung assay, respectively. PK parameters of HCQ were calculated by a
177 non-compartmental method using Phoenix[®] WinNonlin[®] (version 8.0). Sparse sampling
178 computation was applied for the calculation of PK parameters in tissue/organ.

179 **Statistical Analysis.** Two-sample *t* tests were used to assess differences in concentration
180 between liposomal HCQ-IT and HCQ-IV. *P* < 0.05 is considered significant.

181

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251

TABLE 1 Pharmacokinetic parameters of HCQ in rat lung, blood and heart after a single administration of HCQ-IV, HCQ-IT or liposomal HCQ-IT

PK parameters	Lung			Blood ^f			Heart		
	HCQ-IV	HCQ-IT	Liposomal HCQ-IT	HCQ-IV	HCQ-IT	Liposomal HCQ-IT	HCQ-IV	HCQ-IT	Liposomal HCQ-IT
T_{max} (h) ^a	0.25	0.25	0.25	0.25	0.25	0.25	0.25	0.25	24
C_{max} ($\mu\text{g/g}$)/(ng/mL) ^b	9.4	47.8	129.4	433.4 \pm 63.6	476.7 \pm 63.2	42.5 \pm 18.5	4.5	3.8	0.5
AUC_{0-t} ^c ($\text{h}\cdot\mu\text{g/g}$)/($\text{h}\cdot\text{ng/mL}$) ^d	251.6	328.6	4193.2	2333.2 \pm 247.6	2257 \pm 420.7	827.6 \pm 286.0	67.2	64.2	32.1
AUC_{∞} ($\text{h}\cdot\mu\text{g/g}$)/($\text{h}\cdot\text{ng/mL}$) ^e	259.9	345.8	5760.7	2434.8 \pm 291.4	2538.6 \pm 509.6	1234.1 \pm 323.6	-	-	-
$t_{1/2}$ (h)	15.2	17.7	37.5	15.3 \pm 2.1	22.1 \pm 5.7	55.2 \pm 13.0	-	-	-

^a T_{max} was presented as median for blood

^b C_{max} unit: $\mu\text{g/g}$ for lung and heart; ng/mL for blood

^c AUC_{0-72} for blood and lung; AUC_{0-24} for heart

^d AUC_{0-t} unit: $\text{h}\cdot\mu\text{g/g}$ for lung and heart; $\text{h}\cdot\text{ng/mL}$ for blood

^e AUC_{∞} unit: $\text{h}\cdot\mu\text{g/g}$ for lung and heart; $\text{h}\cdot\text{ng/mL}$ for blood

^fPharmacokinetic parameters was presented as mean \pm SD for blood

TABLE 2 Ratios of dose-normalized maximum concentration and area under the concentration-time curve for liposomal HCQ-IT to HCQ-IV or to HCQ-IT

Specimen	Liposomal HCQ-IT to HCQ-IV		Liposomal HCQ-IT to HCQ-IT	
	C _{max} Ratio	AUC Ratio ^a	C _{max} Ratio	AUC Ratio ^a
Lung	29	35	6.0	27
Blood	0.20	0.74	0.19	0.76
Heart	0.24	0.58	0.29	0.60

^aAUC₀₋₇₂ for lung and blood; AUC₀₋₂₄ for heart

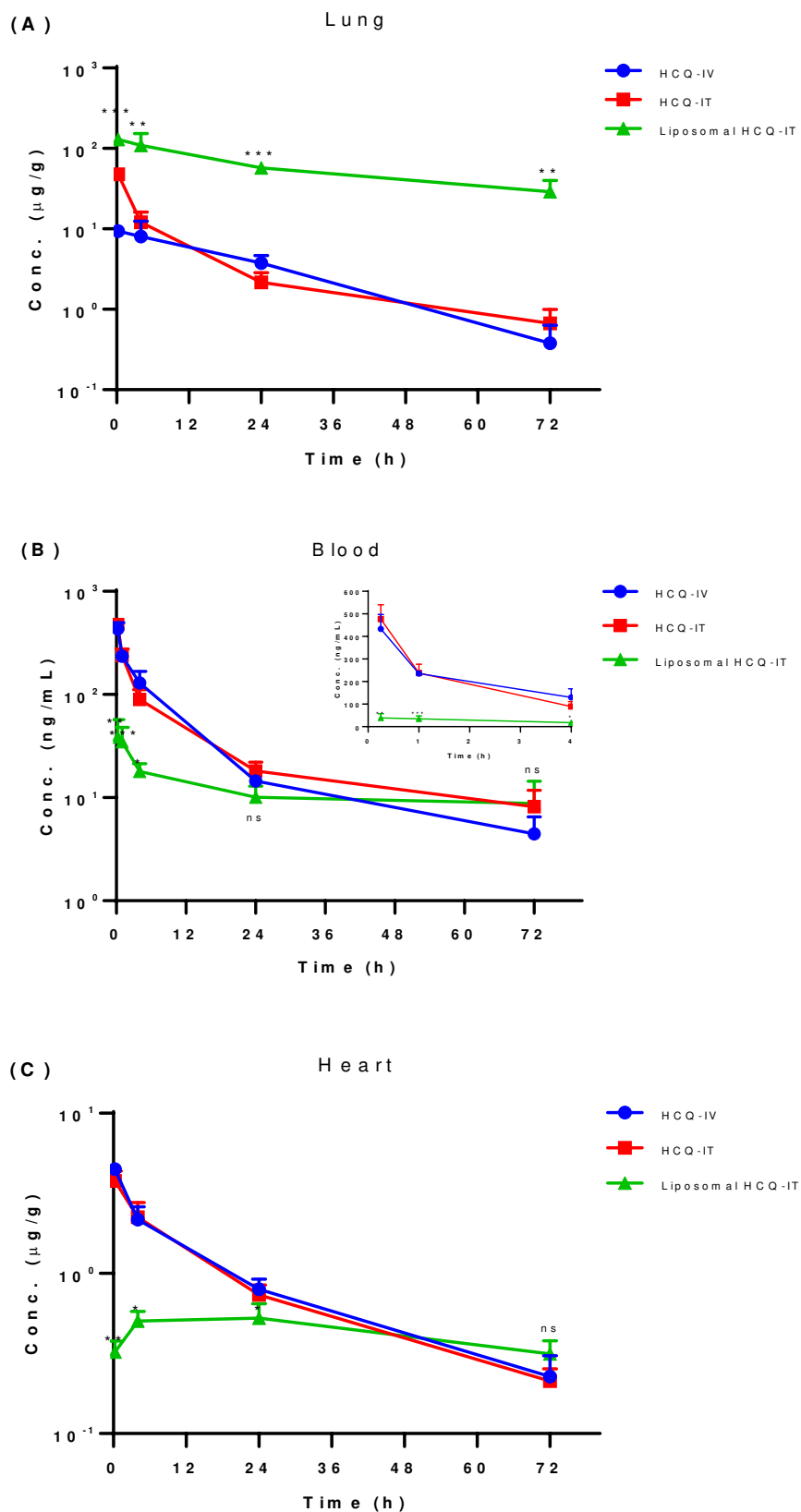


FIG 1 Mean concentration-time profiles of HCQ in rat lung (A), blood (B), and heart (C) after a single administration of liposomal HCQ through intratracheal delivery (IT) or HCQ through intravenous (IV) or IT. The inset graph (B) showed mean concentration-time profiles of HCQ in 0.25 to 4 hours. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; ^{ns} $P > 0.05$ compared to HCQ-IV.