J Antimicrob Chemother 2022; **77**: 1005–1011
https://doi.org/10.1093/jac/dkac008 Advance Access publication 8 February 2022

Journal of Antimicrobial Chemotherapy

Chloroquine-susceptible and -resistant *Plasmodium falciparum* strains survive high chloroquine concentrations by becoming dormant but are eliminated by prolonged exposure

Johan Ursing [1,2,3]*, Rasmus Johns[4], Berit Aydin-Schmidt[4], Carla Calçada[5,6], Poul-Erik Kofoed[1,7], Najia Karim Ghanchi[8], Maria Isabel Veiga [5,6] and Lars Rombo[9,10]

[1]*Projecto de Saúde de Bandim, Indepth Network, Bissau, Guinea-Bissau;* [2]*Department of Clinical Sciences, Karolinska Institutet, Stockholm, Sweden;* [3]*Department of Infectious Diseases, Danderyd Hospital, Stockholm, Sweden;* [4]*Department of Microbiology, Tumor and Cell biology, Karolinska Institutet, Stockholm, Sweden;* [5]*Life and Health Sciences Research Institute (ICVS), School of Medicine, University of Minho, Braga, Portugal;* [6]*ICVS/3B's - PT Government Associate Laboratory, Braga/Guimarães, Portugal;* [7]*Department of Paediatrics and Adolescent Medicine, Lillebaelt Hospital, University Hospital of Southern Denmark, Kolding, Denmark;* [8]*Department of Pathology and Laboratory Medicine, Aga Khan University, Karachi, Pakistan;* [9]*Centre for Clinical Research, Region Sörmland, Eskilstuna, Sweden;* [10]*Unit of infectious Diseases, Uppsala University, Uppsala, Sweden*

*Corresponding author. E-mail: johan.ursing@regionstockholm.se

Received 7 October 2021; accepted 24 December 2021

Background: *Plasmodium falciparum* strains that are resistant to standard-dose chloroquine can be treated by higher chloroquine concentrations maintained for a longer time *in vivo*.

Objectives: To determine the relative importance of chloroquine concentrations versus exposure time for elimination of chloroquine-susceptible and -resistant *P. falciparum in vitro*.

Methods: Chloroquine-susceptible (3D7) and -resistant (FCR3) strains were exposed *in vitro* to 1, 2, 4, 8, 16 or 32 times their respective 90% inhibitory chloroquine concentrations for 3, 5, 7 or 14 days and then followed until recrudescence, or not, by 42 days after the end of exposure.

Results: Exposure to chloroquine appeared to eliminate susceptible and resistant parasites, leaving small pyknotic apparently dead parasites. Chloroquine-susceptible and -resistant parasites recrudesced after 3 and 5 days of chloroquine exposure. Recrudescence occurred in one out of four 7 day exposure series but not after 14 days exposure. The median time to recrudescence was 13 to 28 days with a range of 8 to 41 days after the end of exposure. Time to recrudescence after the end of exposure increased with duration of exposure for susceptible and resistant strains ($P < 0.001$). Time to recrudescence did not correlate with concentrations greater than $1\times$ IC_{90}.

Conclusions: Chloroquine-susceptible and -resistant *P. falciparum* probably become dormant. Elimination of dormant parasites is primarily dependent upon the duration of chloroquine exposure. Exposure to effective drug concentrations for 7 days eliminates most parasites *in vitro*. The results support *in vivo* data indicating that elimination of chloroquine-resistant *P. falciparum* correlates with Day 7 chloroquine concentrations.

Introduction

Malaria causes an estimated 409000 deaths and 229 million episodes of illness each year.[1] Resistance to the currently recommended artemisinin-based combination therapy has developed and is spreading.[1–4] Our understanding of drug resistance and potential countermeasures needs to be improved and new treatment options need to be continuously evaluated. Data from Guinea-Bissau indicating that chloroquine-resistant *Plasmodium falciparum* can be eliminated by higher and well-tolerated chloroquine doses are therefore of considerable interest.[5–10]

In Guinea-Bissau, there was a linear correlation between Day 7 chloroquine concentrations and *in vivo* treatment outcome. This peaked at 96% adequate clinical and parasitological response when *P. falciparum* that were resistant to standard-dose chloroquine were treated with up to double the standard dose of chloroquine.[10] Moreover, parasite densities of chloroquine-susceptible and -resistant *P. falciparum* decreased

at the same rate *in vivo* when treated with double the standard dose of chloroquine.[10] Attained concentrations were thus sufficient to kill susceptible and resistant *P. falciparum*. Concurrent with routine use of well-tolerated triple standard-dose chloroquine, split into 2–3 daily doses for 5 days, the frequency of chloroquine-resistant *P. falciparum* was low and stable at 25% in Guinea-Bissau until replaced by artemether/lumefantrine.[8,11,12] Resistance to standard-dose chloroquine did thus not confer a selective advantage when high-dose chloroquine was routinely used and highly resistant parasites did not become prevalent. Consequently, the PCR-corrected Day 28 adequate clinical and parasitological response of double-dose chloroquine taken as two daily doses for 3 days was 95% in 2008.[9]

The principal mechanism of *P. falciparum* resistance to standard-dose chloroquine appears to be removal of the drug from its site of action by the *P. falciparum* chloroquine resistance transporter (PfCRT).[13,14] Higher chloroquine concentrations can overcome this mechanism, probably accounting for decreasing parasite densities when 'resistant' *P. falciparum* were treated with chloroquine.[15] Despite apparent *in vivo* elimination of *P. falciparum*, treatment failures occurred in children with low Day 7 chloroquine concentrations infected with parasites that were resistant to standard-dose chloroquine.[9,10] This is generally considered to be the result of submicroscopic levels of *P. falciparum* that survive treatment.[16] A less-explored aspect of parasite survival is the ability of *P. falciparum* to become dormant when exposed to multiple different antimalarials.[16–25] This study aimed to determine the relative importance of concentration versus time for the elimination of chloroquine-susceptible and -resistant *P. falciparum*.

Methods

Parasite strains and cultivation

P. falciparum strains 3D7 (chloroquine susceptible) and FCR3 (chloroquine resistant) were used in this study. Their chloroquine susceptibility phenotypes are matched by allelic differences in well-known modulators of chloroquine response. Specifically, the position 72–76 haplotypes in the *P. falciparum* chloroquine resistance transporter gene (*pfcrt*) are CVMNK and CVIET in 3D7 and FCR3, respectively. Both strains have a single copy of the *P. falciparum* multidrug resistance gene 1 (*pfmdr1*) and the allelic 86, 184, 1034, 1042, 1246 haplotypes are NYSND and YYSND for 3D7 and FCR3, respectively. Both strains were maintained in malaria culture medium RPMI-1640 (Gibco®/Invitrogen™) supplemented with 2 g/L sodium bicarbonate (Gibco®/Invitrogen™), 2 mM L-glutamine (Gibco®/Invitrogen™), 10 μg/mL gentamicin (Gibco®/Invitrogen™), at ∼5% haematocrit with human O positive erythrocytes and 10% human AB positive serum according to standardized methods.[26] The erythrocytes were donated by the Department of Transfusion Medicine at Karolinska University Hospital, washed twice with PBS and a third time with malaria culture medium. Aliquots were stored in 15 mL tubes at 4°C for up to 3 weeks. The 3D7 and FCR3 strains were kindly provided by the late D. Walliker, Department of Animal and Population Genetics, University of Edinburgh, UK.

In vitro drug susceptibility assays

The IC_{90} for the chloroquine-susceptible parasite strain 3D7 and the chloroquine-resistant strain FCR3 were assessed by measuring *P. falciparum* histidine-rich protein 2 (PfHRP2) using a double-site sandwich ELISA followed by non-linear regression analysis, as described previously.[27] Briefly, ring-stage parasites were incubated at 37°C with 0.05% starting parasitaemia and 1.5% haematocrit across a range of chloroquine diphosphate (Sigma–Aldrich) concentrations. After 72 h of incubation, cell lysis for ELISA analysis was performed by freeze/thawing the parasites. At least six independent assays were performed for each strain. Chloroquine IC_{90} concentrations were assessed in both strains prior to the start of each series of experiments and were consistently found to be 60 (95% CI 45–73) and 1000 (95% CI 946–1193) nmol/L for 3D7 and FCR3, respectively. Chloroquine IC_{90} concentrations were also assessed in strains that recrudesced. Chloroquine IC_{90} concentrations remained unchanged but the data were lost prior to the preparation of this manuscript and can therefore not be presented.

Experimental assays to determine the time-versus-concentration dependency of parasite elimination

Unsynchronized cultures of chloroquine-susceptible (3D7) and -resistant (FCR3) *P. falciparum* strains were diluted to approximately 1% parasitized erythrocytes and placed in 6-well culture plates with 5% haematocrit and a total culture volume of 5 mL (Figure 1). The 3D7 and FCR3 strains were then exposed to 1, 2, 4, 8, 16 or 32 times their respective chloroquine IC_{90} values for 3, 5, 7 or 14 days. Follow-up was for 42 days after the end of exposure or until recrudescence, which was defined as exponential growth at ≥1% parasitized erythrocytes. The 6-well culture plates were kept in candle jars and incubated at 37°C. Thin smears were examined to determine the proportion of parasitized erythrocytes and the presence or absence of pyknotic parasites during treatment and follow-up by counting the number of infected erythrocytes per 2000 erythrocytes. The frequency of pyknotic parasites was not determined as the frequency was very low.

Four series (A, B, C and D) of experiments were done as outlined below and in Figure 1. Parasites were maintained in continuous culture in between series.

(A) 3D7 and FCR3 clones were exposed to 1, 2, 4, 8, 16 or 32× IC_{90} for 3, 5 and 7 days.
(B) 3D7 and FCR3 clones were exposed to 1, 2, 4, 8, 16 or 32× IC_{90} for 3, 5, 7 and 14 days.
(C) 3D7 and FCR3 clones were exposed to 1, 2, 4, 8, 16 or 32× IC_{90} for 7 and 14 days.
(D) The Series C experiment was run in duplicate.

The 3, 5, 7 or 14 days of drug exposure were initiated by pipetting off all RPMI medium from the cultures in the 6-well plates and replacing the removed medium with complete medium containing chloroquine. The chloroquine concentrations in the complete medium were 1, 2, 4, 8, 16 or 32 times the 3D7 and FCR3 strains' respective IC_{90} values. Chloroquine-containing medium was removed once daily and replaced with fresh chloroquine-containing medium for the duration of exposure (3, 5, 7 or 14 days). Chloroquine-containing medium was removed at the end of exposure and cultures were washed in PBS and centrifuged three times at 1500 rpm for 2 min. After washing, the cultures were placed in new 6-well plates with complete chloroquine-free medium.

In Series A and B, the sampling procedure was as follows: medium was removed and 2 μL of the remaining erythrocyte-rich mixture were taken to make a thin smear for Giemsa staining. Fresh medium (with or without chloroquine) was added. The culture was swirled to create a suspension. Two hundred microlitres of the suspension was taken for PfHRP2 ELISA measurements.[27] Sampling was done after removal of old medium and addition of fresh medium to minimize the effect of remaining PfHRP2 from dead *P. falciparum* in the medium. Sampling for microscopy and PfHRP2 analysis was done daily (Days 0–7) and then every second day until the first signs of parasite growth, after which daily sampling was again done.

Series B included a 14 day exposure due to growth after exposure for 3, 5 and 7 days in Series A. As no growth was detected after 7 and

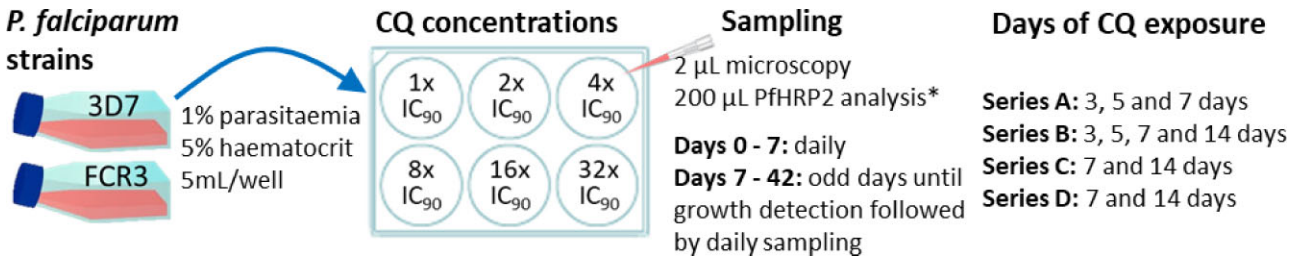

Figure 1. Experimental design. CQ, chloroquine. This figure appears in colour in the online version of *JAC* and in black and white in the print version of *JAC*.

14 days exposure in Series B, strains were only exposed for 7 and 14 days in Series C and D. To minimize the potential effects of sampling in Series C and D, no samples for PfHRP2 analyses were taken and sampling for microscopy only was done daily (Days 0 to 5) and then with approximately 72 hourly intervals until the end of follow-up.

To compensate for losses due to sampling and to provide fresh erythrocytes for parasites growing later during the follow-up, medium (with or without chloroquine) and haematocrit were adjusted to the initial conditions after each sampling.

Statistics

Time to recrudescence, PfHRP2 OD values, exposure time and chloroquine concentrations were analysed as continuous variables and parasite strain as a dichotomous variable. Medians, 95% CIs and IQRs of continuous variables were estimated using quantile regression with bootstrapping (100 repeats). Quantile regressions with bootstrapping (100 repeats) were used to assess the correlation between chloroquine concentrations and duration of exposure with time to recrudescence and PfHRP2 concentrations.

Results

The effect of chloroquine concentration and exposure time on parasite recrudescence

The times from the end of chloroquine exposure until recrudescence, or not, are shown in Table 1 and Figures S1 and S2 (available as Supplementary data at *JAC* Online). Recrudescence did not occur after 14 days of chloroquine exposure and only in one of four series of experiments after 7 days of exposure, irrespective of the drug concentration and strain. The median times to recovery for chloroquine-resistant (FCR3) and -susceptible (3D7) strains after the end of exposure to chloroquine for 3, 5 and 7 days were 13 versus 15 ($P=0.09$), 19 versus 28 ($P=0.01$) and 21 versus 27 ($P=0.92$) days, respectively. Exposure time was significantly linked to time to recrudescence for chloroquine-susceptible ($P<0.001$) and chloroquine-resistant ($P<0.001$) *P. falciparum*.

Time until recrudescence of chloroquine-resistant parasites (FCR3) was not significantly affected by chloroquine concentrations (1–32× IC_{90}), irrespective of whether each exposure time (3, 5 or 7 days) was assessed separately or exposure times were pooled. Recovery time increased significantly with increasing chloroquine drug concentrations in chloroquine-susceptible parasites (3D7) exposed for 3 ($P=0.002$) and 5 days ($P=0.003$) but not 7 days. However, the significance of this correlation disappeared if the 1× IC_{90} exposure was not included in the calculation. IC_{90} values did not correlate with recovery time when chloroquine-resistant and -susceptible strain data were pooled.

Several important additional points should be highlighted. In the 1× IC_{90} exposure in Series A, recrudescence occurred faster after 3 and 5 days of chloroquine exposure compared with higher concentrations (Table 1, Figures S1 and S2). The proportion of

Table 1. Time to *P. falciparum* recrudescence, defined as exponential growth at ≥1% parasitized erythrocytes, after chloroquine exposure

Clone	3D7 (chloroquine susceptible)				FCR3 (chloroquine resistant)			
Exposure time (days)	3	5	7	14	3	5	7	14
Experimental series	A, B	A, B	A, B, C, D	B, C, D	A, B	A, B	A, B, C, D	B, C, D
1× IC_{90}	8 (5, 10)	16 (14, 18)	(26, NR, NR, NR)	(NR, NR, NR)	13 (12, 13)	20 (21, 19)	(21, NR, NR, NR)	(NR, NR, NR)
2× IC_{90}	15 (14, 15)	26 (23, 28)	(26, NR, NR, NR)	(NR, NR, NR)	13 (12, 13)	18 (18, 18)	(21, NR, NR, NR)	(NR, NR, NR)
4× IC_{90}	16 (15, 16)	28 (24, 32)	(27, NR, NR, NR)	(NR, NR, NR)	13 (12, 13)	17 (16, 18)	(20, NR, NR, NR)	(NR, NR, NR)
8× IC_{90}	16 (15, 17)	32 (28, 35)	(27[a], NR, NR, NR)	(NR, NR, NR)	13 (12, 14)	23 (17, 29[b])	(21, NR, NR, NR)	(NR, NR, NR)
16× IC_{90}	17 (16, 18)	32 (30, 34)	(29, NR, NR, NR)	(NR, NR, NR)	14 (12, 16)	23 (18, 28[b])	(30, NR, NR, NR)	(NR, NR, NR)
32× IC_{90}	17 (17, 17)	33 (32, 34)	(41, NR, NR, NR)	(NR, NR, NR)	16 (14, 18)	29 (30, 28[b])	(36, NR, NR, NR)	(NR, NR, NR)
Median (95% CI) RT	15 (13–17)	28 (23–33)	27 (26–39)	NR	13 (11–15)	19 (12–25)	21 (20–35)	NR

Data show median time to recrudescence (RT), in days, followed by each experimental series recovery time in brackets. NR denotes no recrudescence during 42 days of follow-up.

[a]Reached 0.7% then growth failed.

[b]Reached 1%–2.5% parasitized erythrocytes, then growth failed.

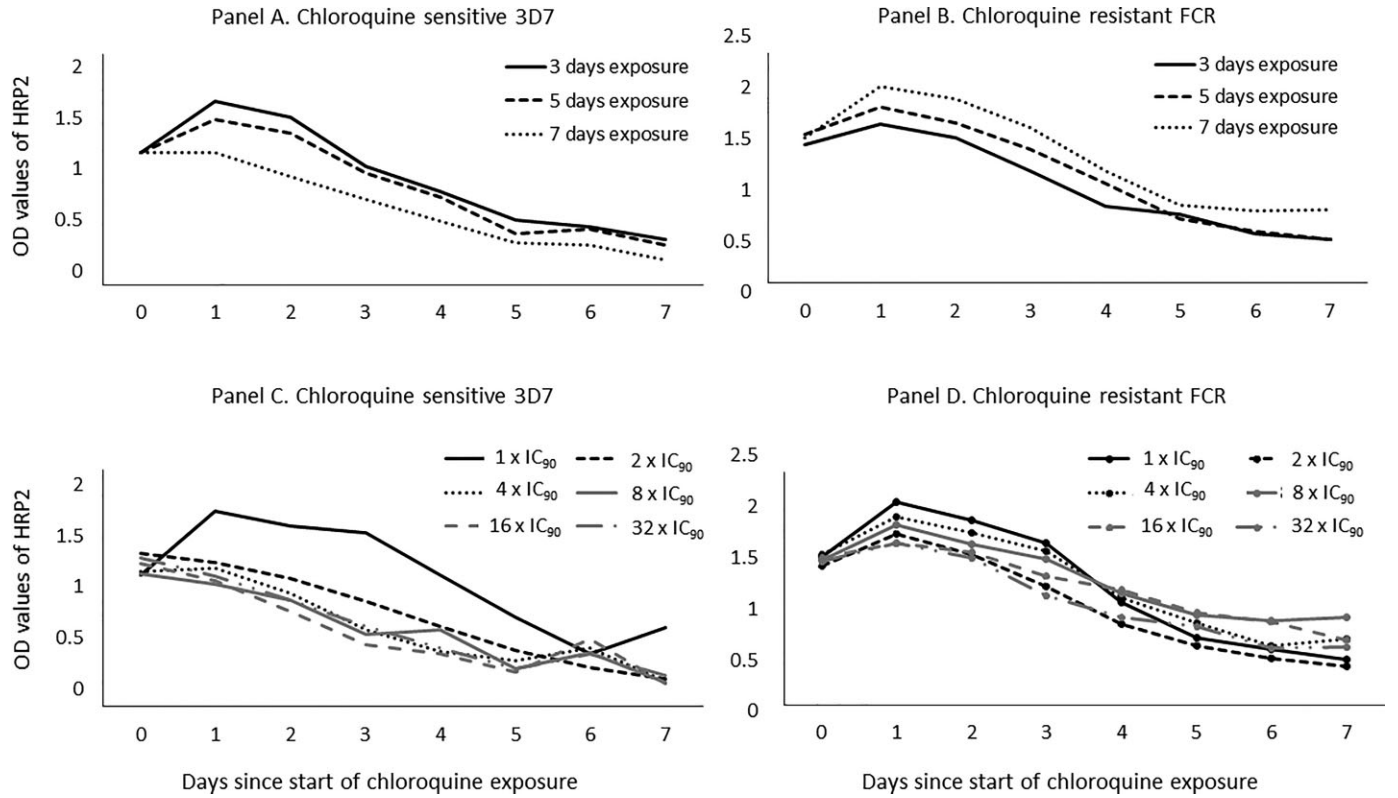

Figure 2. PfHRP2 levels in chloroquine-susceptible (3D7) and -resistant (FCR) *P. falciparum* during and after exposure to 1–32× IC_{90} for 3, 5 or 7 days.

3D7-parasitized erythrocytes at the start of Series A was 1.5% compared with 0.9%–1.1% for Series B and C, possibly contributing to this difference. Series A should also be highlighted as both clones recrudesced after 7 days of exposure. This also occurred in a pilot study in which 3D7 parasites were exposed to 1, 2, 4 and 8× IC_{90} concentrations. Finally, at the extreme end of the spectrum, the chloroquine-susceptible strain recrudesced 41 days after the end of exposure to 32× IC_{90} for 7 days.

The effect of concentration and exposure time on parasite clearance

PfHRP2 levels in chloroquine-susceptible (3D7) and -resistant (FCR3) *P. falciparum* during and after exposure to 1–32× IC_{90} for 3, 5 or 7 days are shown in Figure 2. PfHRP2 increased between Days 0 and 1 ($P<0.01$) and then steadily decreased ($P<0.001$) from Days 1 to 7 in chloroquine-resistant parasites. PfHRP2 decreased ($P<0.001$) between Days 0 and 7 in chloroquine-susceptible parasites exposed to more than 1× IC_{90}, whereas PfHRP2 increased between Days 0 and 1 and then decreased in susceptible parasites exposed to 1× IC_{90}. The decrease continued in both susceptible and resistant parasites irrespective of exposure for 3, 5 or 7 days. The rate of decrease was not affected by the concentration of chloroquine that was used ($P=0.45$).

Dormancy

When monitored during and after drug exposure, both chloroquine-susceptible and -resistant parasites appeared to die, leaving only small pyknotic apparently dead parasites. The pyknotic parasites remained in the erythrocytes and the frequency gradually decreased over time. The exact frequency could not be reliably determined as pyknotic parasites were very rare. The first signs of resurgence appeared to be pyknotic parasites developing a very thin layer of presumably cytoplasm that gradually increased in size until a recognizable live parasite developed (Figure 3). The process was as previously described by Teuscher *et al.*[22]

Discussion

This study was prompted by *in vivo* studies showing that chloroquine-resistant malaria can be overcome by the use of higher total doses of chloroquine.[5,6,8–10] The study aimed to determine the relative importance of chloroquine concentrations versus exposure time for elimination of chloroquine-susceptible and -resistant *P. falciparum*. A 42 day follow-up was used to match the *in vivo* follow-up recommended for detection of recrudescent *P. falciparum*.[28,29] Treatment with chloroquine most probably induced dormancy or selected parasites in a dormant stage, in line with previous studies and other antimalarials.[16–25] Dormancy occurred in both chloroquine-susceptible and -resistant *P. falciparum*, matching findings in artemisinin-susceptible and -resistant *P. falciparum*.[24] Strikingly, and to our knowledge previously not shown *in vitro*, parasites recrudesced up to 41 days after the end of treatment. Dormancy is thus a pathway of probably underestimated importance by which

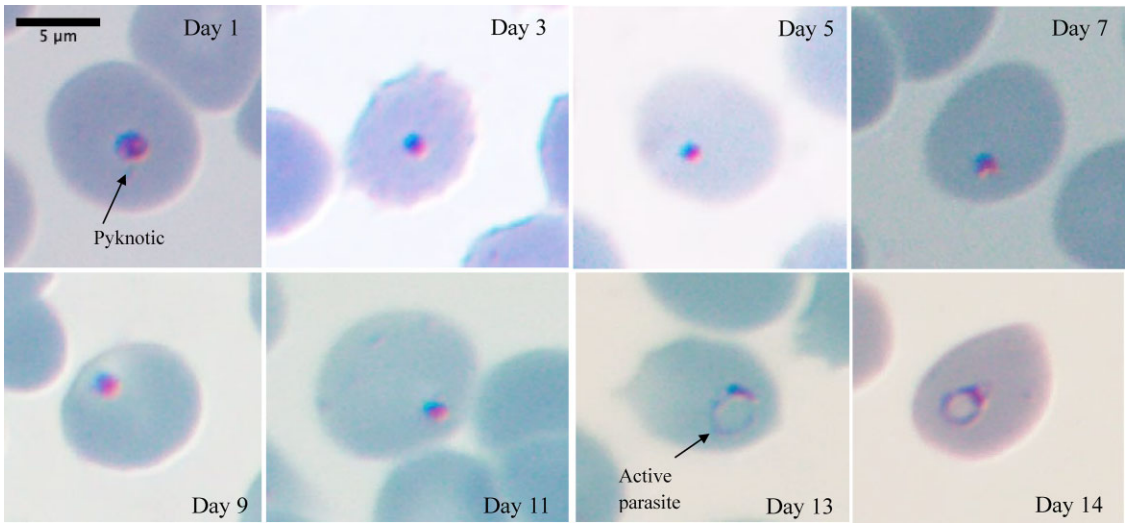

Figure 3. Representative images of pyknotic and recovering 3D7 *P. falciparum* strain after the end of exposure to 2× IC_{90} of chloroquine for 3 days. Giemsa-fixed parasites were analysed microscopically every day after 3 days of exposure. Twelve days after removing chloroquine, viable parasites were observed in the culture. Single images were obtained using a ×100 objective lens. Microscopy was performed using an Olympus BX61 microscope equipped with a visible light laser, and the images were recorded with a digital camera (DP70). This figure appears in colour in the online version of *JAC* and in black and white in the print version of *JAC*.

P. falciparum strains can survive chloroquine therapy even though they are susceptible to attained drug concentrations. As most antimalarials appear to induce dormancy it appears to be an innate survival mechanism that must be overcome if treatment is to be successful.

All cultures recovered after 3 and 5 days of drug exposure, irrespective of chloroquine concentrations. Recrudescence only occurred in one out of four series (plus a pilot study) after 7 days of exposure and did not occur after 14 days of exposure, suggesting that 7 days of drug exposure was sufficient to eliminate the majority of dormant *P. falciparum*. This matches *in vivo* data indicating that minimal parasiticidal drug concentrations must be maintained for four 48 h cycles and that Day 7 quinoline concentrations are predictive of treatment outcome when treating susceptible and resistant *P. falciparum*.[10,30] Time over minimal parasiticidal concentration is thus the primary determinant for treating both chloroquine-susceptible and -resistant *P. falciparum*.

The chloroquine concentration that needs to be maintained for at least 7 days only has to be above IC_{90}. The *in vitro* IC_{90} of 1000 nmol/L for chloroquine-resistant *P. falciparum* in this study does not automatically apply *in vivo*. However, parasite densities of both susceptible and resistant *P. falciparum* decreased at the same rate in children with whole-blood chloroquine concentrations of approximately 3000 nmol/L.[9,31] Moreover, chloroquine-resistant parasites were eliminated and 96% of children cured when Day 7 whole-blood chloroquine concentrations were 1600 nmol/L and recrudescence was not seen when the Day 7 concentrations were greater than 1900 nmol/L *in vivo*.[10] Moreover, similar time-dependent but concentration-independent parasite elimination was shown with *Plasmodium berghei* in a mouse model.[25] *In vitro* and *in vivo* data thus match and it is probable that blood chloroquine concentrations of 2000–3000 nmol/L for at least 8 days should be effective for *in vivo* treatment of chloroquine-resistant *P. falciparum*.

The concentrations above were well tolerated and attained in children taking double- or nearly triple-dose chloroquine split into two daily doses for 3 and 5 days, respectively. Median peak concentrations in adults taking standard-dose chloroquine were 3400 (range 1400–5600) nmol/L and the median steady-state chloroquine concentration in patients treated for rheumatoid arthritis was approximately 2000–2500 nmol/L.[32,33] The 2000–3000 nmol/L range is thus within this very well-tolerated concentration range, indicating that this is a realistic treatment option.

A common concern is that the use of higher doses will rapidly select highly resistant *P. falciparum*. However, the prevalence of *P. falciparum* resistant to standard-dose chloroquine was low and stable at approximately 25% and highly resistant *P. falciparum* were not detected during the decades that high-dose chloroquine was used in Guinea-Bissau.[12,34] Routine use of high-dose chloroquine thus apparently countered the spread of *P. falciparum* resistant to standard-dose chloroquine, probably by elimination of dormant *P. falciparum* that consequently lost much of their selective advantage. Thus, maintaining parasiticidal chloroquine concentrations for at least 7 days may delay the development of resistance, in line with previous suggestions.[30]

The increase of PfHRP2 (parasite biomass) during the first 24 h in chloroquine-susceptible *P. falciparum* exposed to 1× IC_{90}, as opposed to the decrease seen after exposure to 2× IC_{90} and higher concentrations, is probably related to the method by which IC_{90} was established, which utilized a lower proportion of parasitized erythrocytes and a 72 h exposure period. After the first 24 h, PfHRP2 decreased, irrespective of whether susceptible or resistant *P. falciparum* were exposed to 3, 5 or 7 days of

parasiticidal chloroquine concentrations. Time to recrudescence increased with increasing exposure time, suggesting that the number of surviving, presumably dormant, parasites decreased with exposure time. This is contrary to findings in *P. berghei*-infected mice in which time to recrudescence was approximately 5 days, irrespective of exposure time. However, the proportion of infections that recrudesced decreased with increasing exposure time in the mice.[25]

Chloroquine-resistant *P. falciparum* (FCR3) exposed for 5 days recovered a median 9 days faster compared with chloroquine-susceptible *P. falciparum* (3D7) in two independent series, indicating that this was a real difference. A similar but non-significant 2 day difference was seen after 3 days of exposure. A larger proportion of chloroquine-resistant parasites becoming dormant could explain this. As suggested previously, different parasite densities and/or proportions of different parasite stages likely to become dormant at the start of treatment could contribute to different numbers of dormant parasites.[25] Alternatively, chloroquine-resistant parasites might be better at becoming dormant or recover faster. For example, when chloroquine-susceptible and -resistant parasites were treated with high-dose chloroquine, the *in vivo* parasite densities decreased at the same rate. However, slide positivity decreased more slowly in chloroquine-resistant compared with -susceptible parasites, suggesting that a subpopulation was able to survive for longer.[31] Moreover, artemisinin-resistant F32-ART parasites recrudesced faster compared with artemisinin-susceptible F32-Tanzania parasites when exposed to artemisinin for the same time and concentration *in vitro*.[24]

During the first 24 h, PfHRP2 increased in the chloroquine-resistant strain (FCR3), indicating increasing parasite biomass, whilst PfHRP2 decreased in the chloroquine-susceptible strains (3D7). Artemisinin-induced dormant parasites down-regulated most metabolic pathways, with the exception of fatty acid and pyruvate metabolic pathways, enabling survival.[35] When these pathways were inhibited, recrudescence was delayed.[35,36] A possible explanation for the difference in PfHRP2 is that chloroquine-resistant parasites survived for longer by similarly altering their metabolic pathways thereby reducing the speed by which they were eliminated by chloroquine and enabling a larger proportion of parasites to become dormant. This could account for the slower *in vivo* elimination of a subset of chloroquine-resistant parasites and the more rapid recrudescence discussed in the previous paragraph.[31] However, the current experiment used unsynchronized parasites that may well account for the differences seen.

The main limitations of the study were the use of unsynchronized parasites, use of only two strains and not separating pyknotic parasites to prove that they recover. Unsynchronized parasites were used to mimic *in vivo* conditions but this affects comparisons between strains, as discussed above.[37] A study on various synchronized stages of parasites, several different strains and a range of parasitized erythrocytes would be valuable but these were beyond the scope of this study.

To summarize, both chloroquine-susceptible and -resistant *P. falciparum* appear to become dormant when exposed to chloroquine. Elimination of dormant parasites is dependent upon $T_{>MIC}$ and most dormant parasites are eliminated after 7 days of exposure to effective drug concentrations *in vitro*. This matches *in vivo* data indicating that higher but well-tolerated chloroquine concentrations can effectively treat *P. falciparum* that are resistant to standard-dose chloroquine.

Funding

This work was supported by the Anthony Cerami and Ann Dunne Foundation for World Health (http://acadforworldhealth.org/) award 20102011, Svenska Läkaresällskapet, Stockholm County Council (clinical researcher position to J.U., award number 20160597), the Portuguese Fundação para a Ciência e Tecnologia (FCT) (UIDB/50026/2020 and UIDP/50026/2020) and FCT contract funding 2020.03113.CEECIND to M.I.V.

Transparency declarations

All authors: no reported conflicts of interest. All authors have submitted the ICMJE form for disclosure of potential conflicts of interest. Conflicts that the editors consider relevant to the content of the manuscript have been disclosed.

Author contributions

J.U., R.J., B.A-S., P-E.K., M.I.V. and L.R. designed the study. J.U., R.J., B.A-S., C.C., N.K.G. and M.I.V. performed the experiments. J.U. did the statistical analyses. J.U., P-E.K. and L.R. wrote the first draft. All authors helped with the preparation of the manuscript and confirmed the final version.

Supplementary data

Figures S1 and S2 are available as Supplementary data at *JAC* Online.

References

1 WHO. World Malaria Report 2020. 2020. https://www.who.int/publications/i/item/9789240015791.

2 Imwong M, Hien TT, Thuy-Nhien NT *et al.* Spread of a single multidrug resistant malaria parasite lineage (PfPailin) to Vietnam. *Lancet Infect Dis* 2017; **17**: 1022–3.

3 Imwong M, Suwannasin K, Kunasol C *et al.* The spread of artemisinin-resistant *Plasmodium falciparum* in the Greater Mekong Subregion: a molecular epidemiology observational study. *Lancet Infect Dis* 2017; **17**: 491–7.

4 Phyo AP, Nkhoma S, Stepniewska K *et al.* Emergence of artemisinin-resistant malaria on the western border of Thailand: a longitudinal study. *Lancet* 2012; **379**: 1960–6.

5 Kofoed P-E, Lopez F, Johansson P *et al.* Treatment of children with *Plasmodium falciparum* malaria with chloroquine in Guinea-Bissau. *Am J Trop Med Hyg* 2002; **67**: 28–31.

6 Kofoed P-E, Ursing J, Poulsen A *et al.* Different doses of amodiaquine and chloroquine for treatment of uncomplicated malaria in children in Guinea-Bissau: implications for future treatment recommendations. *Trans R Soc Trop Med Hyg* 2007; **101**: 231–8.

7 Ursing J, Eksborg S, Rombo L *et al.* Chloroquine is grossly under dosed in young children with malaria: implications for drug resistance. *PLoS One* 2014; **9**: e86801.

8 Ursing J, Kofoed P-E, Rodrigues A *et al.* Chloroquine is grossly overdosed and overused but well tolerated in Guinea-Bissau. *Antimicrob Agents Chemother* 2009; **53**: 180–5.

9 Ursing J, Kofoed P-E, Rodrigues A *et al.* Similar efficacy and tolerability of double-dose chloroquine and artemether-lumefantrine for treatment

of *Plasmodium falciparum* infection in Guinea-Bissau: a randomized trial. *J Infect Dis* 2011; **203**: 109–16.

10 Ursing J, Rombo L, Bergqvist Y *et al.* High-dose chloroquine for treatment of chloroquine-resistant *Plasmodium falciparum* malaria. *J Infect Dis* 2016; **213**: 1315–21.

11 Jovel IT, Kofoed PE, Rombo L *et al.* Temporal and seasonal changes of genetic polymorphisms associated with altered drug susceptibility to chloroquine, lumefantrine, and quinine in Guinea-Bissau between 2003 and 2012. *Antimicrob Agents Chemother* 2015; **59**: 872–9.

12 Ursing J, Kofoed P-E, Rodrigues A *et al.* No seasonal accumulation of resistant *P. falciparum* when high-dose chloroquine is used. *PLoS One* 2009; **4**: e6866.

13 Fidock DA, Nomura T, Talley AK *et al.* Mutations in the *P. falciparum* digestive vacuole transmembrane protein PfCRT and evidence for their role in chloroquine resistance. *Mol Cell* 2000; **6**: 861–71.

14 Martin RE, Marchetti RV, Cowan AI *et al.* Chloroquine transport via the malaria parasite's chloroquine resistance transporter. *Science* 2009; **325**: 1680–2.

15 Summers RL, Dave A, Dolstra TJ *et al.* Diverse mutational pathways converge on saturable chloroquine transport via the malaria parasite's chloroquine resistance transporter. *Proc Natl Acad Sci U S A* 2014; **111**: E1759–67.

16 White NJ. Malaria parasite clearance. *Malar J* 2017; **16**: 88.

17 Codd A, Teuscher F, Kyle DE *et al.* Artemisinin-induced parasite dormancy: a plausible mechanism for treatment failure. *Malar J* 2011; **10**: 56.

18 Hoshen MB, Na-Bangchang K, Stein WD *et al.* Mathematical modelling of the chemotherapy of *Plasmodium falciparum* malaria with artesunate: postulation of 'dormancy', a partial cytostatic effect of the drug, and its implication for treatment regimens. *Parasitology* 2000; **121**: 237–46.

19 LaCrue AN, Scheel M, Kennedy K *et al.* Effects of artesunate on parasite recrudescence and dormancy in the rodent malaria model *Plasmodium vinckei*. *PLoS One* 2011; **6**: e26689.

20 Nakazawa S, Kanbara H, Aikawa M. *Plasmodium falciparum*: recrudescence of parasites in culture. *Exp Parasitol* 1995; **81**: 556–63.

21 Nakazawa S, Maoka T, Uemura H *et al.* Malaria parasites giving rise to recrudescence *in vitro*. *Antimicrob Agents Chemother* 2002; **46**: 958–65.

22 Teuscher F, Gatton ML, Chen N *et al.* Artemisinin-induced dormancy in *Plasmodium falciparum*: duration, recovery rates, and implications in treatment failure. *J Infect Dis* 2010; **202**: 1362–8.

23 Thapar MM, Gil JP, Björkman A. *In vitro* recrudescence of *Plasmodium falciparum* parasites suppressed to dormant state by atovaquone alone and in combination with proguanil. *Trans R Soc Trop Med Hyg* 2005; **99**: 62–70.

24 Witkowski B, Lelievre J, Barragan MJ *et al.* Increased tolerance to artemisinin in *Plasmodium falciparum* is mediated by a quiescence mechanism. *Antimicrob Agents Chemother* 2010; **54**: 1872–7.

25 Nakazawa S. *Plasmodium berghei* NK65: studies on the effect of treatment duration and inoculum size on recrudescence. *Exp Parasitol* 2005; **111**: 59–63.

26 Trager W, Jensen JB. Human malaria parasites in continuous culture. *Science* 1976; **193**: 673–5.

27 Noedl H, Bronnert J, Yingyuen K *et al.* Simple histidine-rich protein 2 double-site sandwich enzyme-linked immunosorbent assay for use in malaria drug sensitivity testing. *Antimicrob Agents Chemother* 2005; **49**: 3575–7.

28 White NJ. The assessment of antimalarial drug efficacy. *Trends Parasitol* 2002; **18**: 458–64.

29 WHO. Assessment and Monitoring of Antimalarial Drug Efficacy for the Treatment of Uncomplicated Malaria. 2003. https://apps.who.int/iris/handle/10665/68453.

30 Barnes KI, Watkins WM, White NJ. Antimalarial dosing regimens and drug resistance. *Trends Parasitol* 2008; **24**: 127–34.

31 Ursing J, Rombo L, Eksborg S *et al.* High-dose chloroquine for uncomplicated *Plasmodium falciparum* malaria is well tolerated and causes similar QT interval prolongation as standard-dose chloroquine in children. *Antimicrob Agents Chemother* 2020; **64**: e01846–19.

32 Mzayek F, Deng H, Mather FJ *et al.* Randomized dose-ranging controlled trial of AQ-13, a candidate antimalarial, and chloroquine in healthy volunteers. *PLoS Clin Trials* 2007; **2**: e6.

33 Augustijns P, Geusens P, Verbeke N. Chloroquine levels in blood during chronic treatment of patients with rheumatoid arthritis. *Eur J Clin Pharmacol* 1992; **42**: 429–33.

34 Ursing J, Schmidt BA, Lebbad M *et al.* Chloroquine resistant *P. falciparum* prevalence is low and unchanged between 1990 and 2005 in Guinea-Bissau: an effect of high chloroquine dosage? *Infect Genet Evol* 2007; **7**: 555–61.

35 Chen N, LaCrue AN, Teuscher F *et al.* Fatty acid synthesis and pyruvate metabolism pathways remain active in dihydroartemisinin-induced dormant ring stages of *Plasmodium falciparum*. *Antimicrob Agents Chemother* 2014; **58**: 4773–81.

36 Peatey CL, Chavchich M, Chen N *et al.* Mitochondrial membrane potential in a small subset of artemisinin-induced dormant *Plasmodium falciparum* parasites *in vitro*. *J Infect Dis* 2015; **212**: 426–34.

37 Carlsson AM, Ngasala BE, Dahlstrom S *et al.* *Plasmodium falciparum* population dynamics during the early phase of anti-malarial drug treatment in Tanzanian children with acute uncomplicated malaria. *Malar J* 2011; **10**: 380.