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

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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 × IC90.

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 409,000 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. Attributed 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. 

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.

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). Higher chloroquine concentrations can overcome this mechanism, probably accounting for decreasing parasite densities when ‘resistant’ *P. falciparum* were treated with chloroquine. 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. This is generally considered to be the result of submicronic levels of *P. falciparum* that survive treatment. A less-explored aspect of parasite survival is the ability of *P. falciparum* to become dormant when exposed to multiple different antimalarials. 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 CVMK 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 YYSND and YYYSND 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. 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 IC₉₀ 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. 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 IC₉₀ 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 IC₉₀ concentrations were also assessed in strains that recrudesced. Chloroquine IC₉₀ 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 IC₉₀ 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 erythrocyths. 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 × IC₉₀ for 3, 5 and 7 days.

(B) 3D7 and FCR3 clones were exposed to 1, 2, 4, 8, 16 or 32 × IC₉₀ for 3, 5, 7 and 14 days.

(C) 3D7 and FCR3 clones were exposed to 1, 2, 4, 8, 16 or 32 × IC₉₀ 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 IC₉₀ 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. 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
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 PHRP2 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, PHRP2 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 PHRP2 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 ($1–32 \times IC_{90}$), 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 $1 \times IC_{90}$ 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 $1 \times IC_{90}$ 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 $\geq 1\%$ 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 |
| $1 \times IC_{90}$ | 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) |
| $2 \times IC_{90}$ | 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) |
| $4 \times IC_{90}$ | 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) |
| $8 \times IC_{90}$ | 16 (15, 17) | 32 (28, 35) | (27, NR, NR, NR) | (NR, NR, NR) | 13 (12, 14) | 23 (17, 29) | (21, NR, NR, NR) | (NR, NR, NR) |
| $16 \times IC_{90}$ | 17 (16, 18) | 32 (30, 34) | (29, NR, NR, NR) | (NR, NR, NR) | 14 (12, 16) | 23 (18, 28) | (30, NR, NR, NR) | (NR, NR, NR) |
| $32 \times IC_{90}$ | 17 (17, 17) | 33 (32, 34) | (41, NR, NR, NR) | (NR, NR, NR) | 16 (14, 18) | 29 (30, 28) | (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.

aReached 0.7% then growth failed.
bReached 1%–2.5% parasitized erythrocytes, then growth failed.
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.5,8-10 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 2. PfHRP2 levels in chloroquine-susceptible (3D7) and -resistant (FCR) P. falciparum during and after exposure to 1–32× IC90 for 3, 5 or 7 days.
**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 IC90. The *in vitro* IC90 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 *vivo* 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× IC90, as opposed to the decrease seen after exposure to 2× IC90 and higher concentrations, is probably related to the method by which IC90 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 treatment.
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 chloroquinesusceptible 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 downregulated 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 similarily 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.

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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., R.J., B-A.S., P-E.K., M.I.V. 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.

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