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KINETIC MODELLING OF THE RESPONSE OF *PLASMODIUM FALCIPARUM* TO CHLOROQUINE AND ITS EXPERIMENTAL TESTING *IN VITRO*

IMPLICATIONS FOR MECHANISM OF ACTION OF AND RESISTANCE TO THE DRUG

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Abstract—The antimalarial mode of action of chloroquine (CQ) has been investigated in great detail in recent years, but the overall mechanism is still controversial. Instead of further probing the molecular aspects of partial reactions, a model based on the weak base properties of CQ and its ΔpH-driven accumulation in acid parasite compartments has been devised, and the integrated response of the parasite to the drug under different experimental conditions has been assayed to verify the validity of the model. Factors such as inoculum size (parasitemia · hematocrit) and medium pH were altered using CQ-sensitive (FCC_1) and -resistant (FCR_3, VNS) isolates of *Plasmodium falciparum*. Experimental results were in full agreement with the predictions of the model, implying that therapeutic concentrations of CQ do not raise the pH of the food vacuole, i.e. that alkalinization of the acid parasite compartments is an insufficient explanation for the antimalarial activity of CQ, and that there is no need to invoke an active QC efflux pump to explain drug resistance. Calculations based on the model and the experimental data demonstrate that resistance to CQ is correlated with higher pH and/or higher resistance of the intracellular target to the drug concentration in the parasite food vacuole. The data also have implications for the design and interpretation of *in vitro* CQ inhibitory tests.

It has been recognised that the chloroquine (CQ) accumulates to very high levels in malaria-infected red blood cells. Although it was first suggested that this accumulation was due to drug binding to ferriprotoporphyrin IX, a product of hemoglobin digestion by the parasite [1], it was subsequently demonstrated that CQ in fact simply accumulates in acidic parasite compartment(s) by virtue of its weak base properties [2]. In this and later experiments, the major acidic compartment (the central food vacuole of *Plasmodium falciparum*) has been identified and its pH has been determined directly using a fluorescent pH probe targetted to the food vacuole [2, 3]. However, these techniques required the disruption of the infected cell in order to circumvent the quenching of emitted fluorescence of the pH probe by host cell hemoglobin. The invasive handling of the cells raises doubts about the validity of the value for vacuolar pH thus obtained and its response to pharmacologically relevant CQ concentrations. An alternative approach that avoids disruption of cellular integrity is necessary to address basic questions about the mechanism of action of, and resistance to, CQ.

In this report we present a model based on the basic assumption that CQ accumulates exclusively in acid parasite compartment(s) as a diprotic weak base, and test it using the ultimate response of the parasite to the drug. Using parasite strains with different sensitivity to CQ, we could show that the model is valid and illuminate possible explanations for drug resistance.

MATERIALS AND METHODS

General culture methods. Cultures of the Vietnam Smith (VNS), FCR_3 and FCC_1 isolates of *P. falciparum* were maintained in candle jars [4]. Drug effects were determined by a modification [5] of the method of Desjardins *et al.* [6]. Wells in a 96-well plastic microtiter plate (Flow Laboratories, Inc., Rickmansworth, U.K.) received a total volume of 200 μL RPMI-1640 (Grand Island Biological Co., Long Island, NY) containing 10% (v/v) pooled human A^+ serum, 25 mM HEPES (Sigma Chemical Co., St Louis, MO), 25 mM $NaHCO_3$, 1–2 μCi [^{3}H]hypoxanthine (10 Ci/mmol, New England Nuclear, Boston, MA) and various concentrations of chloroquine diphosphate (Sigma) and parasitized erythrocytes.

Microtiter plates were kept at 37° in candle jars. Wells were harvested 24 or 48 hr after initial drug

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exposure onto glass fiber filters using a Bellco Microharvester. Radioactivity incorporated into parasite nucleic acids, trapped on these filters, was determined by liquid scintillation spectrometry. Drug effects are expressed as per cent reduction in radioactivity in drug-containing wells compared to identical, but drug-free, control wells. IC_{50} values are the CQ concentrations resulting in 50% reduction of incorporation, determined as described by Desjardins *et al.* [6].

Determination of the inoculum effect. Parasites in O^+ human erythrocytes were synchronized to the ring stage (age 0–8 hr) with sorbitol [7]. Wells received parasitized erythrocytes in the following combinations: 1% hematocrit (ht) at 0.5, 1, 3 or 6% parasitemia; 2% parasitemia at 5% ht; or 3% parasitemia at 0.5, 2, or 5% ht.

Measurement of the influence of medium pH. The FCR_3 strain was grown in a medium identical to that described above except that the $NaHCO_3$ concentration was reduced to 10 mM; this variation has no effect on parasite growth in culture (data not shown). The pH of the medium was adjusted to 6.0, 6.5, 7.0, 7.5 and 8.0, after which the media were sterilized by filtration through a 0.22 μm filter. Within 30–40 min of gassing these media with a mixture of 90% N_2, 5% O_2 and CO_2, the pH changed to 6.8, 6.9, 7.2, 7.45 and 7.75, respectively, and remained unaltered at least through 24 hr. Exposure to CQ occurred as described above beginning with ring stage parasites at 1% parasitemia, 1% ht.

Theoretical model for CQ accumulation as affected by inoculum size. The accumulation of CQ in infected cells is best described by the accumulation ratio L, which is defined as the intracellular concentration divided by the extracellular concentration. This is given by:

$$L = \frac{x/h}{(1-x)/(1-h)} \tag{1}$$

where x is the fractional amount of drug taken into the cell and h is the fractional volume of the parasitized cells and equals ht · parasitemia. We make here the explicit assumption that non-infected cells accumulate negligible amounts of drug compared to infected cells [8]. Since $h \ll 1$,

$$x = \frac{L \cdot h}{1 + L \cdot h}. \tag{2}$$

This means that the extracellular concentration of the drug, $(1-x)/(1-h)$, should decrease with increasing inoculum size. Consequently, the intracellular concentration of the drug, which determines its effect, should fall as well, and higher extracellular drug concentrations are needed to elicit an inhibitory effect as inoculum size increases. This should be reflected in an increase of IC_{50} (I) with the inoculum size h. If both L and the intracellular drug concentration eliciting the effect are constant, then the final extracellular concentration (C) which produces 50% inhibitory effect, i.e., that found after cells have accumulated the drug, should also be constant. These assumptions hold true only if there is no binding of CQ to intracellular receptors, the driving force for drug accumulation (the pH gradient between the acid compartment and the extracellular medium) remains unaltered at all extracellular CQ concentrations used and there is no active transport of CQ at any membrane crossed by the drug. Hence,

$$C = I \cdot (1-x). \tag{3}$$

Combining Eqns 2 and 3,

$$I = \frac{1}{1 - L \cdot h/(1 + L \cdot h)} \tag{4}$$

and

$$I = C + C \cdot L \cdot h. \tag{5}$$

This equation describes exactly the dependence of IC_{50} on h (=ht · parasitemia) and should be observed experimentally if the model is valid. Once the model is validated by experimental results, the derived value of L could be used for the calculation of the pH difference between the food vacuole, which is the drug accumulating compartment, and the extracellular medium. For this purpose, we assume that most if not all the drug concentrates inside the vacuole, which occupies 3.2% of the infected cell volume [2]. Since

$$L = \frac{[CQ]_I}{[CQ]_o} = \frac{[H^+]_I^2}{[H^+]_o^2} \tag{6}$$

we get

$$\Delta pH = 0.5 \cdot \log(L/0.032) \tag{7}$$

and the vacuolar drug concentration at IC_{50} is given by:

$$[CQ]_I = C \cdot 10^{2 \cdot \Delta pH}. \tag{8}$$

RESULTS

Multiplying parasitemia by ht generates a unitless number which reflects the amount of parasites present in the culture, termed here the inoculum size. To clarify this term, it is rather trivial to show that, at 1% ht and 1% parasitemia, the inoculum size will be 1.16×10^6 parasites/mL if we assume that the volume of erythrocytes in culture is 86 μm^3 and the small fraction of infected cells does not change ht. Incorporation of [^{3}H]hypoxanthine in drug-free wells was linearly related to inoculum size (Fig. 1),

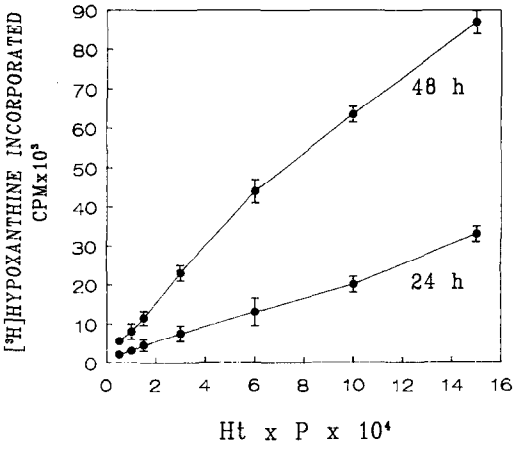

Fig. 1. [^{3}H]Hypoxanthine incorporation in the VNS strain of *P. falciparum* as a function of initial parasitemia · ht. Points are means of six observations; bars represent SD.

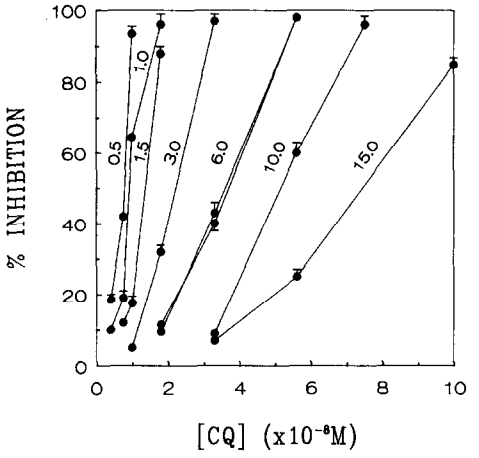

Fig. 2. Concentration–response curves for CQ as a function of initial parasitemia·ht. Data are for the FCC_1 strain of *P. falciparum*. Points are means of six observations; bars represent SD. Data are presented as per cent inhibition of [^{3}H]hypoxanthine incorporation compared to the appropriate drug-free control. Numbers besides the lines are the absolute values of parasitemia·ht (e.g., 2% ht·3% parasitemia = 6).

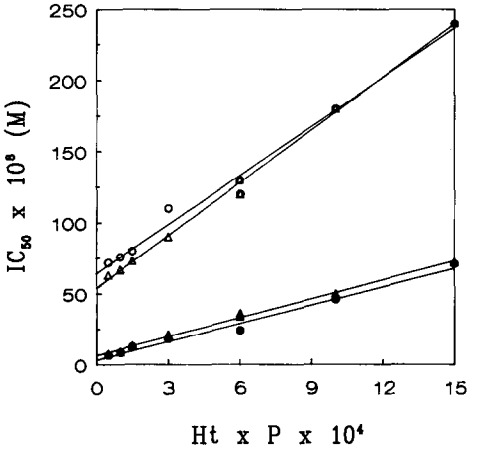

Fig. 3. Dependence of IC_{50} on inoculum size. IC_{50} values were derived from graphs such as those presented in Fig. 2. (●) FCC_1 at 24 hr; (▲) FCC_1 at 48 hr; (○) VNS at 24 hr; (△) VNS at 48 hr. Results of the regression lines are given in Table 1.

indicating that the viability of the parasites is independent of inoculum size over 48 hr in culture. Similar results were obtained with the FCC_1 and the FCR_3 strains (data not shown). Concentration–response curves for CQ showed that inhibition of parasite growth varied with inoculum size (Fig. 2). Statistically, those slopes were not different at 24 and 48 hr of cultivation. Similar results were obtained with the VNS and FCR_3 strains, though the intercepts (but not the slopes) were of course different, since these parasites are less susceptible to CQ (data not shown). Interestingly, the inoculum size of 6 was reached in two ways: 1% ht, 6% parasitemia and 2% ht and

Table 1. Regression parameters of IC_{50} vs ht·parasitemia × 10^4 lines

	FCC_1		VNS		FCR_3
Parameter	24 hr	48 hr	24 hr	48 hr	24 hr
y-Intercept	$3.31 \pm 1.9 \times 10^{-9}$	$6.48 \pm 0.91 \times 10^{-9}$	$5.40 \pm 0.20 \times 10^{-8}$	$6.44 \pm 0.40 \times 10^{-8}$	$4.04 \pm -0.67 \times 10^{-9}$
Slope	$4.29 \pm 0.27 \times 10^{-5}$	$4.44 \pm 0.13 \times 10^{-5}$	$1.23 \pm 0.03 \times 10^{-4}$	$1.15 \pm 0.06 \times 10^{-4}$	$1.34 \pm 0.15 \times 10^{-3}$
Coefficient of correlation	0.987	0.998	0.998	0.993	0.992

Values are mean ± SD. Data were calculated from graphs similar to that shown in Fig. 3. The factor of 10^4 applied to the ht·parasitemia relationship simply reflects the fact that both parasitemia and ht are expressed as percentages; this factor was retained for these calculation.

Table 2. Calculated distribution of chloroquine

	Strain				
	FCC_1		VNS		FCR_3
Parameter*	24 hr	48 hr	24 hr	48 hr	24 hr
C (nM)	3.31	6.48	54.03	64.40	4.04
L	12,967	6846	2285	1780	3327
ΔpH	2.80	2.67	2.47	2.37	2.51
$[CQ]_I$ (mM)	1.34	1.39	4.71	3.59	0.42

* Parameters, as described in Materials and Methods, indicate the following: C = the extracellular CQ concentration at equilibrium required for 50% inhibition of viability at a limitingly small inoculum size; L = accumulation ratio, ΔpH = difference between the pH of the food vacuole and the medium; $[CQ]_I$ = vacuolar CQ concentration at equilibrium associated with a 50% inhibition of viability.

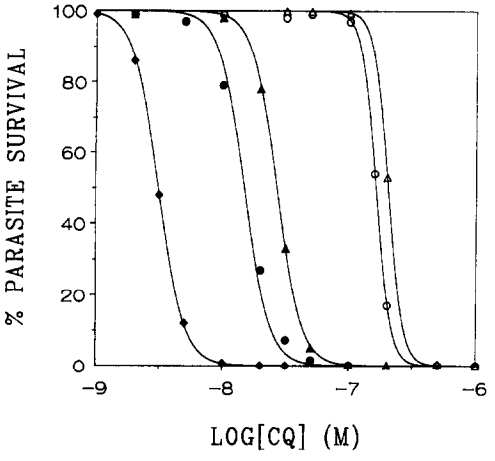

Fig. 4. Effect of extracellular pH on the susceptibility of FCR_3 to CQ. Synchronously grown cultures were subcultured at the ring stage at 1% parasitemia and 1% ht in media adjusted to various pH values, containing different concentrations of CQ. Parasite viability was estimated 24 hr later by measuring the amount of [^{3}H]hypoxanthine incorporated. Results shown are the means of four independent experiments. Curves from left to right were obtained with the following initial medium pH values: 8.0, 7.5, 7.0, 6.5 and 6.0. Correlation of non-linear regression analysis were ≥0.96.

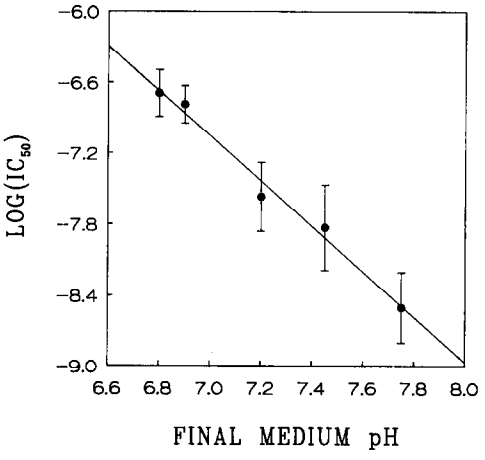

Fig. 5. Dependence of IC_{50} on extracellular pH. IC_{50} values derived from curves shown in Fig. 4, were plotted against the final medium pH measured in the culture (see Materials and Methods). The vertical bars represent SD of the means. Parameters of linear regression analysis: y-intercept = 6.35 ± 0.95; slope = −1.91 ± 0.13; r = 0.993.

3% parasitemia. The concentration–response curves were identical, implying that the relatively small amounts of CQ that accumulate in non-infected cells are immaterial to the drug effect, as we have assumed in devising the analytical model. IC_{50} values did not differ significantly at 24 or 48 hr when asynchronous cultures were compared to cultures initiated with ring stage parasites (results not shown).

When the IC_{50} values are graphed against inoculum size, a linear relationship is obtained (Fig. 3), as predicted by the model. No significant differences were noted in the absolute CQ potency or in the dependence of IC_{50} on inoculum size when incubations proceeded for 24 or 48 hr, although the maximum degree of inhibition was uniformly greater in the longer exposure. The regression parameters for these lines from the three strains used are presented in Table 1.

As described in Materials and Methods, these data were used to calculate the vacuolar concentrations of CQ at the IC_{50} and the pH gradient between the food vacuole and the extracellular medium. Results of these calculations are presented in Table 2.

Incorporation of [^{3}H]hypoxanthine by parasites was unaffected over 24–48 hr by the range of medium pH used in these experiments (data not shown). However, as can be seen in Fig. 4, medium pH profoundly influenced susceptibility to CQ: the inhibitory effect of this drug is directly proportional to medium pH. The data shown were obtained with the FCR_3 strain at 24 hr; similar results were obtained with the FCC_1 and VNS strains at 24 and 48 hr (data not shown). IC_{50} is logarithmically dependent on external pH (Fig. 5). The y-intercept of this line is 6.35 ± 0.95 and the slope is −1.91 ± 0.13 (r = 0.993), not significantly different from the theoretical slope of 2 expected from the model.

DISCUSSION

Investigations on the antimalarial mode of action of CQ over the last two decades have focused on the details of drug uptake and accumulation (see Refs 9 and 10 for reviews). The picture emerging from these studies can be summarized as follows: CQ penetrates rapidly ($T_{1/2}$ of equilibration <7 sec [8]) into cells as a free base by free diffusion. Although it has been previously suggested that it does so as a monoprotonated species through a facilitated transport system [11] recent evidence clearly demonstrates that CQ enters into red blood cells by simple diffusion of the free base [12]. It is generally assumed that the protonated species of CQ cannot cross membranes, although the free base achieves equal concentration throughout the cell. Thus, the drug accumulates in different compartments of infected cells (barring any specific binding to intracellular receptors) as a function of the pH of the respective compartment, which obviously determines the concentration of the non-permeating species of the drug. In other words, the driving force for drug accumulation is the pH gradient between the accumulating compartment and the extracellular medium [2], and, as CQ is mainly diprotonated at physiological pH and below, accumulation will be directly related to the square of the pH gradient. Hence, very small pH changes can result in vast changes in accumulation. Due to its acid pH, the parasite food vacuole seems to be the organelle where most of the drug accumulates. There is at present no agreement concerning the actual effect of CQ inside the food vacuole. While some investigators have argued that CQ raises the vacuolar pH [3], others have shown that this is not the case for CQ concentrations in the therapeutic range [13]. Whichever is the truth, CQ demonstrably inhibits the digestion of host cell cytosol [14, 15].

In the present work, we have developed a theoretical model which integrates the assumptions mentioned above and tested its validity by monitoring the inhibitory action of CQ on the parasite. Whenever a functional model is designed, it must consist of the minimal number of parameters necessary to describe the behaviour of the system. If the experimental results are fully compatible with the predictions of the model, there is obviously no need to invoke additional parameters or components. In developing this model, we have used the observation that infected erythrocytes are able to accumulate CQ to very high levels, thereby demonstrably depleting the medium of the drug [2, 8]. In order to compensate for drug depletion, which itself is a function of the inoculum size [16], the initial drug concentration must be increased as inoculum size increases in order to attain lethal intracellular levels [2, 8, 16]. This results in an increase in the observed value of IC_{50} as inoculum size increases. Since uninfected erythrocytes also accumulate CQ [8, 16], this factor could have also influenced the relationship between inoculum size and IC_{50}. However, the linearity of IC_{50} vs inoculum size over a 10-fold range in h (as expected by the model), argues otherwise, in agreement with the fact that infected cells accumulate ≥1000-fold more CQ than uninfected cells [8, 16].

Differences between the CQ-sensitive FCC_1 isolate and the CQ-resistant isolate VNS were found in the extent to which the inoculum size influenced the IC_{50}. For FCC_1, the IC_{50} shifted by about 10-fold over a 30-fold range of inoculum size. The corresponding shift for VNS was about 3.5-fold. The significant difference in the linear relationship found between inoculum size and IC_{50} reflects the basis of CQ resistance in *P. falciparum* [8, 16]. As described by the model, the slopes and intercepts of this relationship can be used to calculate the pH gradient between the parasite's food vacuole, the vacuolar [CQ] at IC_{50} and the extracellular [CQ] at limitingly low inoculum size. With these calculations, one finds that the pH gradient in FCC_1 is larger than that in the CQ-resistant strains FCR_3 and VNS (Table 2), in agreement with our previous work which encompassed a much larger selection of strains [8]. The physiological basis of these pH differences remains to be established.

Although the larger pH gradient results in a greater capacity to accumulate CQ, it is not the sole parameter determining drug resistance. As we have shown before [8], the vacuolar concentration of CQ at IC_{50} ($[CQ]_I$) in VNS compared to FCC_1 and FCR_3 (Table 2) suggests that susceptibility of the actual drug target to CQ is in the order $FCR_3 > FCC_1 >$ VNS. Thus, drug resistance is directly related to $[CQ]_I$ and inversely to the accumulation ratio, resulting in higher extracellular [CQ] at steady state for the resistant strain at limitingly low inoculum size.

That the susceptibility to CQ depends on extracellular pH has already been demonstrated previously [17] and corroborates the model's assumption that the pH gradient drives CQ accumulation to reach the critical toxic concentration inside the food vacuole. Moreover, the dependence of IC_{50} on medium pH serves an additional test for the validity of the model. Consider the following reasoning: CQ as a diprotic base accumulates inside the acidic parasite food vacuole according to the following relationship:

$$\frac{[CQ]_{fv}}{[CQ]_o} = \frac{[H^+]_{fv}^2}{[H^+]_o^2} \tag{9}$$

where the subscripts fv and o represent the food vacuole and the extracellular medium, respectively. From this equation one gets directly the following expression:

$$\log[CQ]_o = \log[CQ]_{fv} + 2 \cdot pH_{fv} - 2 \cdot pH_o. \tag{10}$$

Assuming, as we did in devising the model, that the $[CQ]_{fv}$ causing 50% inhibition of parasite growth is constant, as is pH_{fv} (irrespective of pH_o), the two first terms of Eqn 10 are also constant and $\log[CQ]_o$ should show a linear dependence on pH_o with a slope of −2. As IC_{50} is obviously equal to $[CQ]_o$, the linear relationship we found between $\log IC_{50}$ and pH_o (Fig. 5), with a slope of −1.91, is fully compatible with the model and its underlying basic assumptions. Furthermore, using the y-intercept of this line (6.35, e.g. when $pH_o = 0$) and the value of $[CQ]_{fv}$ for FCR_3 (Tables 1 and 2), we calculate from Eqn 10 a pH_{fv} of 4.86. This value is very close to the value of 4.89 calculated from ΔpH of 2.51 (Table 2) and pH_o = 7.4.

The internal consistency of the data obtained from two different types of experiments, e.g. IC_{50} dependence on inoculum size and on pH_o, supports the basic assumptions used to devise the model. Moreover, these results suggest that CQ does not exert its antimalarial effect by raising the vacuolar pH and thereby inhibiting hydrolases involved in digestion of host cell cytosol, as has been recently suggested [3]. The internal consistency of the data also precludes the need to assume any active transport of CQ to explain drug resistance [18, 19]. Our analysis is clearly compatible with the assumption that pH_{fv} is constant and not affected by the drug. The derived value of pH_{fv} is somewhat lower than that obtained with pH fluorescent probes in the absence of CQ [2, 3], probably because it was derived from unperturbed cells. The present derivation of pH_{fv} is also advantageous to the derivation from the directly determined accumulation ratio [8] which may yield erroneously low pH values due to possible intracellular binding of CQ, since it reflects the vacuolar concentration of free (mostly diprotonated) CQ. Commensurately, one must conclude that the latter is the toxic form of CQ.

Finally, the present results and their analysis underscore two major problems encountered with *in vitro* testing of CQ susceptibility. One is the demonstrable dependence of IC_{50} on inoculum size. Obviously, comparative studies of CQ susceptibility of various strains should be done under identical conditions of ht and parasitemia and pH_o, preferably at the lowest achievable h value and at physiological pH. Another is the discrepancy observed between CQ sensitivity of *P. falciparum in vitro* vs *in vivo*. If depletion of CQ from the medium during *in vitro* tests lowers the observed antimalarial effect of CQ (see also Refs 20 and 21), one would expect that parasites would be more resistant to CQ in culture where the drug is in limited supply than *in vivo*, where essentially unlimited drug is available and infection does not significantly alter blood concentrations [22]. Serum [CQ] during prophylactic therapy ranges from $>1\ \mu M$ to $\approx 0.3\ \mu M$ (day 1 vs day 7, [23]), and, during therapy, concentrations of at least $5\ \mu M$ are attained [24]. Highly CQ-resistant strains are unafffected even by such high [CQ] *in vivo*, while all strains are susceptible to drug concentrations below $0.5\ \mu M$ *in vitro* [8, 25, 26]. When one adds the complicating factor of CQ depletion in culture, it is obvious that CQ sensitivity is much greater *in vitro* than *in vivo*. The reasons for this discrepancy are unknown, but it should be noted that it does not extend to pyrimethamine [27]. The phenomenon of drug depletion in culture also removes the justification of equating initial drug concentrations *in vitro* with blood concentrations [28, 29]. Instead, one should try to find the proper correlation between blood concentrations *in vivo* and the extracellular medium concentrations at limitingly small inoculum size *in vitro*, which can be easily derived from our experimental procedure and theoretical model.

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