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KINETIC MODELLING OF CHLOROQUINE UPTAKE BY MALARIA-INFECTED ERYTHROCYTES

ASSESSMENT OF THE FACTORS THAT MAY DETERMINE DRUG RESISTANCE

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Abstract—The antimalarial chloroquine, by virtue of its weak base properties, concentrates in the acidic compartment(s) of the intraerythrocytic parasite. Drug accumulation is essential for it to exert its pharmacological activity. Drug resistance has been thought to result from insufficient acidification of drug-accumulating organelle(s), (due to weakened proton pump activity and/or proton leak) or to result from the action of the recently suggested active efflux drug pump. In this work we have devised a kinetic model which takes into account the various processes that have been postulated to account for acidification and drug fluxes. Using this model to analyse the time-course of chloroquine uptake and the steady-state levels of drug accumulation, in strains of *Plasmodium falciparum* which display variable drug resistance, we demonstrate that drug resistance is compatible with the existence of a weakened proton pump in the resistant parasite strains. Consistent with recent molecular studies that show no correlation between the presence of the multidrug efflux pump gene and the phenotypic expression of chloroquine resistance, our analysis fails to detect any such pump activity. We also show that analysis of drug efflux kinetics cannot distinguish between the possible modes of drug resistance.

Despite the fact that falciparum malaria is gradually becoming resistant to chloroquine (CQ), the drug is still a first line choice for prophylaxis and radical cure. Very little is known about the antimalarial mode of action of CQ, or the reasons for drug resistance [1–3]. The recent demonstration that CQ-resistant *Plasmodium falciparum* parasites can be rendered sensitive to CQ in the presence of various other drugs [4–6], underscores the need for further probing of the mode of action of CQ and the possible reasons for drug resistance.

Malaria-infected erythrocytes accumulate CQ to very high levels, mostly in the acidic [7, 8] food vacuole of the parasite [9]. The mechanism of accumulation depends on the diffusion of the unprotonated drug into the various compartments of the malaria-infected erythrocyte [10]. In each of these compartments the free base becomes protonated to an extent that is determined by the compartment's pH and by the pKa of the base, according to the Henderson–Hasselbalch equation. Hence, the pH gradient across the membrane of any particular cellular compartment is the driving force for the accumulation of CQ in this compartment. The total concentration of the drug in each compartment is the algebraic sum of the free and the protonated base and obviously will be higher as a compartment is more acidic. This paradigm has received wide experimental support in recent years [7, 8, 11]. Since drug resistant parasites accumulate less drug, it was further suggested that the pH of the accumulating compartment is more acidic in the drug-susceptible parasites than in their drug-resistant congeners [12].

Chloroquine-sensitive parasites release pre-accumulated CQ with a half-time substantially longer than do CQ-resistant strains [13]. Krogstad and his colleagues concluded that the failure of the resistant parasites to accumulate the drug might result from enhanced (active) efflux [13]. They have also shown that verapamil, diltiazem, vinblastine and daunomycin, compounds that reverse CQ resistance in *in vitro* cultures of *P. falciparum*, increase the ability of CQ-resistant parasites to accumulate CQ and increase the half time of CQ efflux. These same drugs are known to reverse drug resistance in multidrug resistant (MDR) cancer cells, by restoring cellular drug levels to those found in their drug sensitive progenitors [14–16]. It was therefore suggested that reversers of CQ resistance in malaria parasites may inhibit an active drug efflux pump, as has been suggested for their effect on the extrusion of anticancer drugs from cancer cells with the multidrug resistance phenotype.

That such extrapolation is probably unwarranted is underscored by several fundamental differences that exist between the phenomenology of CQ-resistance and reversal in malaria parasites and the events observed in MDR cancer cells:

(1) Anticancer drugs permeate into cells relatively slowly ($t_{1/2} > 1$ hr [16]) and it is possible therefore to assume that an efflux pump could extrude them at a rate sufficient to keep their cellular levels low. However, the half-time of equilibration of quinoline-containing drugs in malaria-infected cells (IRBCs) is at least two orders of magnitude shorter [12, 17, 18] and it is hard to envisage a pump that would be able to cope with such high flux rates.

(2) Both MDR cancer cells and CQ-resistant parasites accumulate less drug than their sensitive counterparts. However, upon metabolic deprivation, the cellular drug concentrations in MDR cancer cells reach those that are found in the drug-susceptible parent cell line [19, 20]. In fact, it was this observation that initiated the concept of an ATP-driven drug efflux pump. Metabolic deprivation of malaria parasites, however, invariably results in *lower* levels of drug accumulation [21, 22].

(3) Drugs which reverse resistance in MDR cancer cells, restore cellular drug concentrations to levels found in the drug sensitive parent line. In CQ-resistant malarial parasites they restore CQ-sensitivity but the cellular CQ concentrations are still at least one order of magnitude lower than those found in the susceptible strains [13].

(4) While MDR in cancer cells are cross-resistant to structurally and functionally unrelated drugs, drug-resistant malaria parasites are not [23–25]. Thus, multidrug resistance as it is known in cancer cells, has no analogy in malaria parasites.

It seems, therefore, that the outlined phenomenological differences do not warrant a direct mechanistic extrapolation from one type of organism to the other. We attempt, however, in the present work to develop a kinetic model that might indeed help identify the operational presence of the putative MDR pump in malaria parasites. Since this model suggests that the time course of drug uptake and the drug accumulation ratios are the needed parameters for such distinction, we analyse such experimental data obtained with various *P. falciparum* strains. We show that the time course of drug uptake in sensitive and resistant strains is not consistent with an MDR pump, but rather with the decreased activity of the vacuolar proton pump.

Kinetic theory

We shall analyse the following rather general model for the uptake of CQ. We assume that (i) unprotonated CQ freely crosses the limiting membrane of the food vacuole, (ii) the monoprotonated derivative crosses this membrane only with difficulty and (iii) the diprotonated form crosses this membrane not at all. It follows [26] that CQ (largely in the diprotonated form) is concentrated within the parasite's food vacuole, according to the square of the prevailing concentration gradient for proton across the vacuole/parasite–cytoplasm membrane. Very rapidly after adding CQ to a suspension of parasites, CQ will be taken up into the food vacuole according to the *initial* pH gradient. This CQ will titrate some of the protons present within the vacuole, raising the internal pH and reducing further uptake of CQ. The inwardly-driving proton pump, that we assume keeps the pH within the food vacuole low, continues to operate, however. As a result, the pH falls and more CQ enters, titrating this recently-entered proton. A sequence of the entry of proton, its titration by the accumulating CQ, followed by further entry of proton occurs, until an eventual steady-state distribution of proton and hence of CQ is reached according to the appropriate kinetic parameters of the transport of protons and of CQ and its derivatives, as we proceed to analyse in detail.

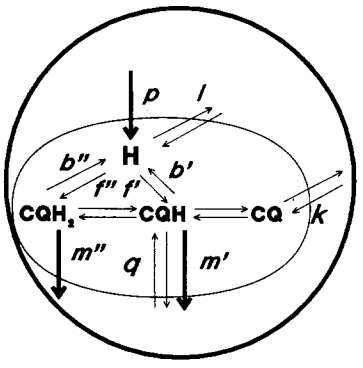

Fig. 1. Scheme for the accumulation of chloroquine within the vacuole of a malaria parasite. The heavy outer circle represents the parasite, the ellipse its vacuole. The heavy arrows represent one-way fluxes carried by either the proton pump, p or the two forms of the MDR pump, m' and m''. The light double arrows represent chemical equilibria between various forms of chloroquine, protonated or unprotonated, b being breakdown and f association constants or represent transmembrane diffusional fluxes of protons, l or free chloroquine k, or of CQH, q. See text.

Time course of uptake of CQ

Consider first the time course of accumulation of proton (refer to Fig. 1 in what follows). Let [Q] be the concentration of free CQ, [QH] the concentration of the singly protonated form, $[QH_2]$ the concentration of the doubly protonated form, $[H]_i$ the concentration of free proton within the parasite food vacuole, $[H]_0$ its concentration in the cytoplasm of the parasite. The proton pump operates with a rate constant p, pumping protons inwards from the cytoplasm. Protons in free form leak in and out of the vacuole according to the prevailing concentrations of free proton and a rate constant l. Protons bound in the form QH leak in and out of the vacuole according to the prevailing concentrations of QH and a rate constant q. In addition, a pump or carrier of some type, conveying the property of MDR, removes drug in the form of QH or of QH_2 and thus carries protons out of the vacuole with rate constants m' for the form QH and m'' for the form QH_2. Pumping of unprotonated drug seems very unlikely since it would be particularly inefficient in view of the extremely high permeability of this species.

The concentration QH is given by $[Q][H]/K_1 + [Q][H]/K_2$ where K_1 and K_2 are the two dissociation constants of QH, in its two monoprotonated forms. For convenience, we write this expression as $[Q][H]/K'$, where $1/K' = (1/K_1 + 1/K_2)$. The concentration of QH_2 is given by $[Q][H]^2/K_1K_2$. Again, for convenience we write K'' for K_1K_2. In these formulations, [H] is the proton concentration within or without the vacuole, as the case may be.

The instantaneous rate of accumulation of protons, $d[H]_i/dt$, is given by the difference between its influx and efflux or by:

$$d[H]_i/dt = [(p + l + q[Q]/K')[H]_0 - (l + q[Q]/K' + m'[Q]/K')[H]_i - m''[Q][H]_i^2/K'']. \quad (1)$$

Most of the protons that enter the vacuole, however, will not remain free but will become bound to intravacuolar bases, either those naturally present there or those composed of the basic groups on the entered CQ. Let B represent an intravacuolar base. Then the amount of proton bound to it at an intravacuolar proton concentration of $[H]_i$ will be $[BH]_i = [B][H]_i/K_{Bi}$ where K_{Bi} is the dissociation constant of the base. Similarly, the amount of proton bound to the two bases of the choloroquine will be $[Q][H]_i/K'$, where as before we write $1/K' = (1/K_1 + 1/K_2)$. Protons thus partition between free and bound protons according to the function free/bound = $[H]_i/([H]_i + \Sigma([B][H]_i/K_{Bi} + [Q][H]_i/K')$. At high base concentration, relative to concentration of free proton, the amount of intravacuolar free proton becomes negligible compared to that bound and the partition function becomes $1/(\bar{B} + \bar{Q})$, where $\bar{B}$ and $\bar{Q}$ represent the values of $\Sigma[B]$ and of $[Q]$, divided by the appropriate dissociation constants. To find the true net rate of accumulation of intravacuolar protons we have to multiply the instantaneous rate given in equation (1) by the partition function $1/(\bar{B} + Q)$ obtaining, finally, after rearranging terms:

$$d[H]_i/dt = [(p + l + q[Q]/K')[H]_0 - (l + q[Q]/K' + m'[Q]/K')[H]_i - m''[Q][H]_i^2/K'']/(\bar{B} + \bar{Q}).$$

Note that the right-hand of this equation is of the general form $(a + bx + cx^2)/(\bar{B} + \bar{Q})$. Rearranging and then integrating this equation yields the result that:

$$(1/Z^{1/2})\ln[(2cx + b - Z^{1/2})/(2cx + b + Z^{1/2})] = t/(\bar{B} + \bar{Q})$$

where $Z = (b^2 - 4ac)$. The steady-state distribution of protons is reached when $d[H]_i/dt$ is zero, or where

$$[H]_{i,ss} = (-b + Z^{1/2})/2c \quad (2)$$

and the half-time to reach this steady-state is given by

$$t_{1/2} = (\bar{B} + \bar{Q})(1/Z^{1/2}) \times \ln[(b + Z^{1/2})/(b + 3Z^{1/2})] \quad (3)$$

where we have taken into account the partition function $1/(\bar{B} + \bar{Q})$.

Note here an important conclusion: the parameter c appears as, or transforms into, a square root in the denominator both in the expression for the steady-state distribution of protons and in the expression for the half-time to reach this steady-state. Consider immediately an implication of this conclusion: If the steady-state distribution of drug is reduced by the imposition of an MDR pump, operating on the form QH_2, so that the rate constant m'' for drug pumping is *increased*, the value of c in both equations (2) and (3) will be increased. The steady-state distribution of protons and hence of CQ will be reduced, but so will the half-time for the reaching this steady-state. The same conclusions would be reached if the MDR pump was acting on the unprotonated species Q, because such a pump (like the others) can only act as the concentration of the pumped form rises in the cell, and hence $t_{1/2}$ is affected.

In contrast, consider a model in which such a QH_2-acting pump does not operate, i.e. m'' and hence c is zero. Then the appropriate differential equation is of the form $dx/dt = (a + bx)$, with integral $(1/b)\ln(a + bx)$, steady-state distribution (at $dx/dt = 0$)

$$[H]_{i,ss} = -a/b \quad (4)$$

and half-time to reach this steady-state of

$$t_{1/2} = (\bar{B} + \bar{Q})(1/b)\ln(1/2). \quad (5)$$

Now, one way to reduce the steady-state distribution is by altering the effectiveness of the proton pump, i.e. by reducing the parameter p in equation (1). This has the effect of reducing the term a in the generalized equation. The half-time will now be unchanged (compare equation 5), in spite of the lowering in the steady-state distribution concentration (compare equation 4). The steady-state distribution can be reduced instead by increasing the value of the parameter b in the general equation. The steady-state distribution is reduced but so is the half-time. An increase in b can be achieved either by increasing the leak for protons l, or the leak of the form QH, q being the appropriate rate constant, or by increasing the effectiveness of the MDR pump operating on the form QH, with rate constant m'. In all these cases, the steady-state distribution of protons and hence of CQ will be reduced but the half-time will decrease in strict parallel with the reduction in the steady-state distribution.

This analysis provides us with an appropriate tool for distinguishing between various mechanisms of operation of drug resistance in the malaria parasite: Measure the time course of uptake of the drug in sensitive and resistant strains, and also the steady-state distribution of the drug. In cases where drug resistance is associated with a reduced concentration of CQ in the parasite, compare the extent of reduction of the steady-state distribution with any change of the half-time to reach this steady-state. Only if the mechanism of reduction of drug concentration arises from a reduction of effectiveness in the pump for protons will there be no change in the half-time in the face of a reduction in the steady-state concentration. We later demonstrate that, in quite a few instances, the concentration of CQ in a resistant strain of parasite is greatly reduced as compared with sensitive strains, but we have found no instance in which, in our hands, the half-time of uptake of the drug is lower in resistant than in sensitive strains. We find, therefore, no kinetic grounds for assuming any mechanism for reducing the level of CQ in the parasite other than by the reduction of the effectiveness of the vacuolar proton pump.

Steady-state distribution of CQ

It is convenient to consider separately two limiting cases, the first where any drug resistance pump operates only on the form QH, the second where such a pump overwhelmingly operates on the form QH_2. In the first case, we saw that the steady-state distribution of protons, DR_{ss}, is given by $-a/b$ in the general equation which can be expanded to:

$$DR_{ss} = \frac{[H]_i}{[H]_0} = \frac{p + l + q[Q]/K'}{l + q[Q]/K' + m'[Q]/K'}. \quad (6)$$

We remember that the steady state distribution of CQ is equal to the square of DR_{ss}, and hence, all the predictions that are made for protons, are equally valid for CQ. The right-hand side of this expression has the following characteristics: It reduces to unity as l or q are increased indefinitely, i.e. as the vacuolar membrane is made increasingly leaky to free protons or to proton bound as QH. It goes to zero as m' is increased indefinitely, i.e. as the MDR pump is made increasingly effective. It goes to unity also as p is reduced, i.e. as the proton pump is rendered increasingly ineffective, and as [Q] is increased indefinitely, i.e. as the proton pump is overwhelmed by the addition of CQ.

In the case where an MDR pump operating on the form QH_2 is overwhelmingly effective, we obtain by substituting in equation (2), with $b = 0$:

$$DR_{ss} = \sqrt{\frac{p + l + q[Q]/K}{m''[Q]/K''}}. \quad (7)$$

This expression goes to zero as m'' is increased indefinitely, i.e. as the MDR pump is made increasingly effective. It again goes to unity as [Q] is increased indefinitely, i.e. as the proton pump is overwhelmed by the addition of CQ.

Half-time to reach the steady-state

Again, it is convenient to take the two limiting cases as above. When any MDR pump operates only on the form QH we have:

$$t_{1/2} = \frac{(\bar{B} + \bar{Q}) \ln(1/2)}{l + q[Q]/K' + m'[Q]/K'}. \quad (8)$$

The half-time is independent of the pump rate p but can increase with increasing [Q] or can decrease according to the relative values of $\bar{B}$ and $1/q$ or $1/m'$. If $\bar{B}$ is small relative to $1/q$ and $1/m'$, $t_{1/2}$ will initially increase with increasing [Q], but will eventually reach a limiting value given by $\ln(1/2)/(q + m')$. In contrast, if $\bar{B}$ is large, $t_{1/2}$ will initially fall but reaches the same limit as [Q] increases indefinitely. We shall see that the data on the uptake of chloroquine into the malaria parasite is consistent with the former case, in that $t_{1/2}$ initially increases as the concentration of CQ increases.

In the case where an MDR pump, operating on the form QH_2, is overwhelmingly present, the half-time is given by:

$$t_{1/2} = \frac{(\bar{B} + \bar{Q}) \ln(1/3)}{\sqrt{4(p + l + q[Q]/K')(m''[Q]/K'')}}. \quad (9)$$

This, as we saw above, decreases as the MDR pump becomes more effective but always increases, and does so without limit, as [Q] increases.

RESULTS AND DISCUSSION

We first illustrate the predictions of the model. Figure 2A and B display the predictions in two different ways: In Fig. 2A we plot drug uptake against time as a fraction of the steady-state level of the sensitive strain. The upper curve depicts a sensitive strain, the middle curve is for a resistant strain in which an MDR pump is operating, and the lower curve, a resistant strain for which the proton pump has reduced activity. It is obvious from the shape of the curves that the initial rate of uptake is identical for the sensitive, and the MDR pump-containing, strain. Since the final level of drug accumulation in the latter is so much reduced, clearly here the half time of uptake is far shorter. This point is made much clearer in Fig. 2B, where we plot the fractional filling (with respect to the final steady-state level in each case) as a function of time. Here the kinetics for the sensitive strain and for that resistant strain containing a weakened proton pump are identical, while the MDR-containing strain displays a much more rapid time course.

We have previously studied in detail the uptake of CQ into erythrocytes infected with strains of *Plasmodium falciparum* that display various sensitivity to CQ [12]. Figure 3 plots drug uptake vs time in those strains in the form of Fig. 2A. The medium CQ concentration was 1×10^{-7} M. In these experiments the level of hematocrit (*ht*) varied bewtween 1 and 2% and the parasitemia (p) between 5 and 15%. The total cellular [CQ] of infected cells was calculated assuming that essentially all the CQ that disappeared from the bathing medium accumulated in infected cells. Hence, $[CQ]_i = \Delta CQ_0/(ht \cdot p)$, where ΔCQ_0 is the amount of drug that disappeared from the bathing medium. Since the pH of the host cell and parasite's cytosols are substantially higher that that of the food vacuole [7], it is justified to assume that essentially all the drug will be found in the food vacuole. Three of the strains are sensitive to CQ, IC_{50} values being 1.55×10^{-8} M for FCN, 3.7×10^{-8} M for I_2 and 1.65×10^{-8} for FCR_8. The three other strains are resistant to CQ, IC_{50} values being 2.5×10^{-7} M for FCR_7, 1.8×10^{-7} M for VNS and 2.7×10^{-7} M for FCR_{3TC}. Obviously the CQ accumulation levels are higher in the sensitive strains than in the resistant strains, but the rate of uptake seems to be lower. Such behavior is in accordance with the prediction of the model in which drug resistance arises from reduced activity of the proton pump.

This point is made clearer when we present some of these data in the form of Fig. 2B. Figure 4A compares fractional filling in FCN, a sensitive strain and in the resistant FCR_7 at two concentrations of CQ. The uptake curves at each particular concentration are virtually superimposable. This accords with the predictions of the weakened proton pump model of Fig. 2B. The value of $t_{1/2}$ clearly increases with drug concentration in accordance with the prediction of equation (5) of the theoretical section. Figure 4B compares another pair of strains, the sensitive I_2 and the resistant FCR_{3TC}. The situation here is not as clear as in Fig. 4A, but it is obvious that the $t_{1/2}$ of the resistant strain FCR_{3TC} is certainly not shorter than that of I_2. The presented data are certainly not in accordance with the predictions of an MDR pump model.

In contrast to our studies, there exist another published [13] pair of partial time courses of CQ uptake into a sensitive (Haiti 135) and a resistant strain (Indochina I). Here the initial uptake curves

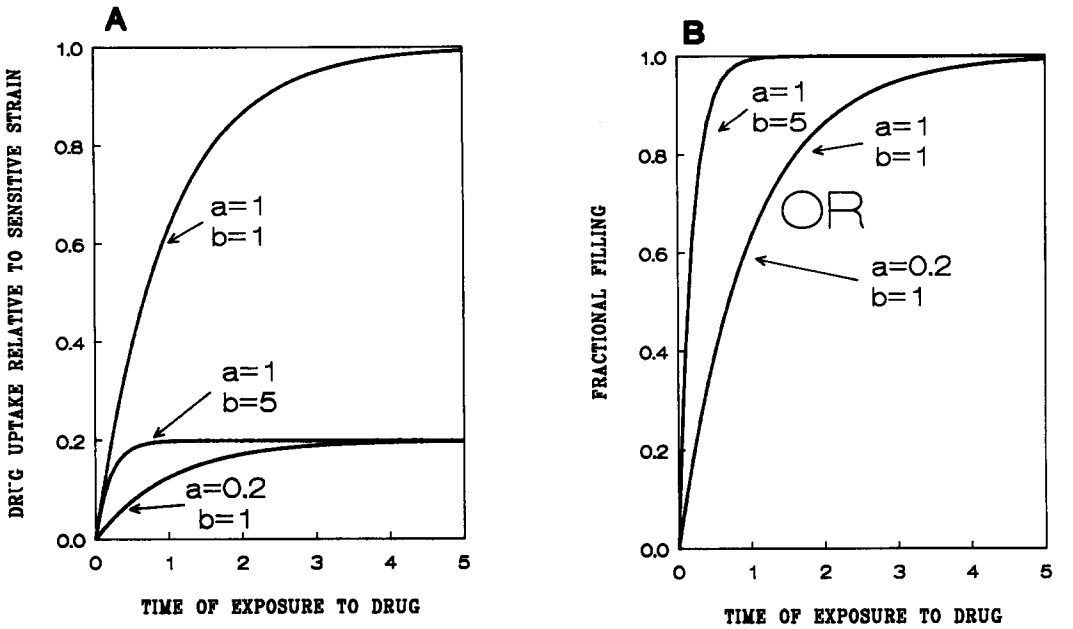

Fig. 2. Theoretical time courses of uptake of chloroquine for drug-sensitive and various models of drug-resistant parasites. In all cases parameters a and b are as used in the generalized equations (2)–(5) where a is the premultiplier of the term in H_0 in the full equation (1) and b the premultiplier of the term in H_i. (Thus a is reduced in a cell drug resistant owing to a weakened proton pump and b increased if resistance is due to any mechanism that produces an increased efflux of accumulated chloroquine.) The three cases depicted are where $a = b$, where $a = 0.2$ and $b = 1$, and where $a = 1$ and $b = 5$. In (A), the "data" are plotted as amount of drug accumulated (with the sensitive cell set at a steady-state level of unity) against time. In (B), each case is separately normalized to its own steady state level, lower of course in the drug-resistant cases.

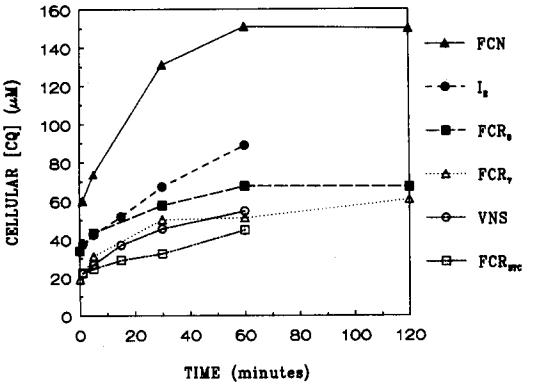

Fig. 3. Uptake of chloroquine into human erythrocytes infected by various sensitive and resistant strains of *Plasmodium falciparum*, plotted as a function of time, from an external concentration of 10^{-7} M. The data were originally presented in derived form in Geary *et al.* [12]. Strains FCN, I_2, and FCR_8 are chloroquine sensitive; FCR_7, VNS, and FCR_{3TC} are resistant.

plotted in the form of Fig. 2A are superimposable, although the steady-state levels of uptake are very different. Such results would be compatible with an MDR pump. These results would suggest that there could be more than one mechanism of drug resistance, but it would be advisable before making this conclusion, to repeat the study of these two strains under the conditions of Geary *et al.* [12].

Results of CQ efflux from drug sensitive and resistant strains have been interpreted as supporting the existence of an MDR pump [13]. The data show that the $t_{1/2}$ of efflux from CQ-preloaded sensitive strain is far greater than that from the resistant strain. The $t_{1/2}$ values are indeed directly proportional to the steady-state levels of accumulation of the drug. As is shown in the Appendix, this is the result to be expected of any mode of drug resistance, whether this occurs as a result of the presence of a weakened proton pump or of an MDR pump acting on the monoprotonated or the diprotonated forms of the drug. It appears that efflux experiments do not provide information that allows one to distinguish between the different possible mechanisms for drug resistance. In particular the results of drug efflux do not rule out the weakened proton pump model which is the model supported by the influx data.

It seems that attempts to correlate CQ-resistance with the expression of the putative MDR-pump, support our conclusion against such relationship. *P. falciparum* contains at least two genes (*pfmdr*1 and *pfmdr*2) that were shown to be related to the mammalian MDR gene that codes for the P-glycoprotein, a membrane protein involved in efflux pumping of drugs in cancer cells [26]. Originally, some correlation was suggested as existing between CQ resistance and gene amplification or transcription [27]. Subsequently, it was demonstrated that the two

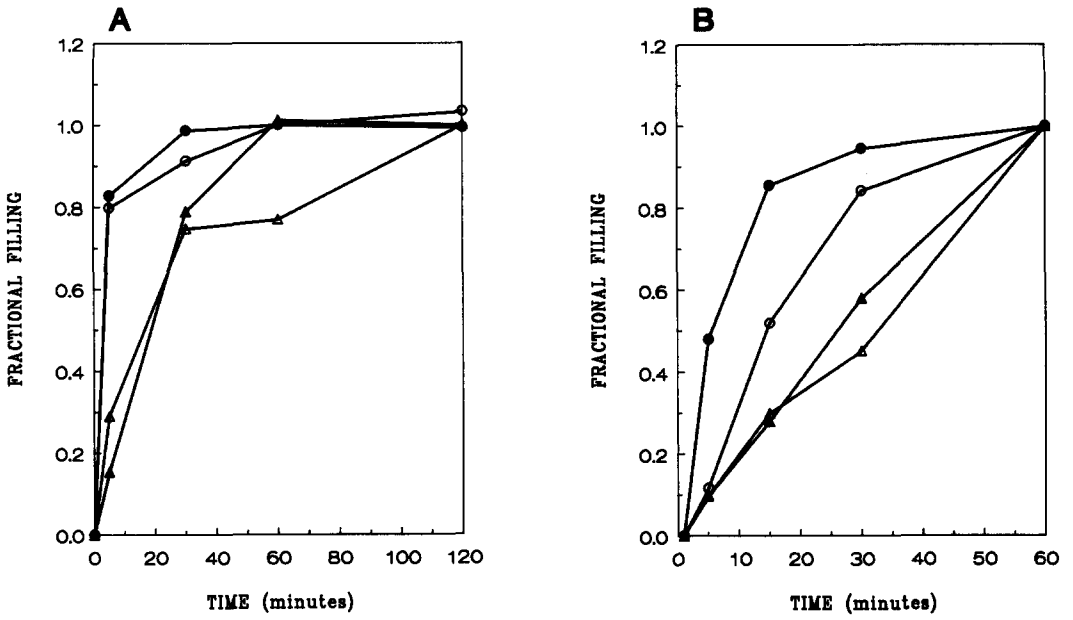

Fig. 4. Uptake of chloroquine as a function of time and concentration. Four paired examples of the strains depicted in Fig. 3, but now plotted on the ordinate in terms of fractional filling, i.e. as a fraction of the steady state level of the drug. In (A) are plotted the data for strains FCN (filled symbols) and FCR_7 (empty symbols), in (B), the data for I_2 (filled symbols) and FCR_{3TC} (empty symbols). The external level of chloroquine was 10^{-7} (triangles) and 10^{-8} M (circles).

genes are present in both CQ-sensitive and -resistant parasite strains, but those in the latter are mutated. The joint conclusion that can be reached from two more recent studies [28, 29] is that *pfmdr* mutations are insufficient (and possibly unimportant) in conferring the CQ-resistant phenotype. It could be of interest to analyse the kinetics of chloroquine uptake and steady-state distribution in strains of known genotype with respect to drug resistance. If our analysis is correct, that drug resistance is at least in some cases associated with a weakened proton pump, it seems important to explore the genetic basis of this low activity.

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APPENDIX

The kinetics of CQ efflux

Figure 1 illustrates the equilibria between the unprotonated and the various protonated forms of CQ. We shall assume that all these forms are able to cross the vacuolar membrane either on their own or else aided by an MDR pump, according to the rate constants denoted on the figure. When free CQ leaves the vacuole, the forms QH and QH_2 forms dissociate to yield further free CQ, liberating protons. These then leave the vacuole. The overall rate of efflux of CQ will be limited by whichever efflux rate is slower, that of free CQ or that of proton. We assume, first, that the efflux of CQ is rate-limiting for the overall effiux.

In this case, writing $d[C]_{tot}/dt$ for the instantaneous rate of overall efflux of CQ, we have, using the symbolism of the Theory section above:

$$d[C]_{tot}/dt = -(k[Q] + (m' + q)[QH] + m''[QH_2])$$
$$= -[Q](k + (m' + q)[H]_i/K' + m''[H]_i^2/K''). \quad (A1)$$

Now,

$$[C]_{tot} = [Q] + [QH] + [QH_2]$$
$$= [Q](1 + [H]_i/K' + [H]_i^2/K''). \quad (A2)$$

Rearranging,

$$\frac{d[C]_{tot}}{[C]_{tot}} = -\frac{k + (m' + q)[H]_i/K' + m''[H]_i^2/K''}{1 + [H]_i/K' + [H]_i^2/K''}\,dt = -K dt \quad (A3)$$

where we define K as the ratio on the right-hand side of equation (A3). Integrating this differential equation between the limits $t = 0$ and $t = t$, we obtain

$$[C]_{tot,\,t=t} = [C]_{tot,\,t=0} \exp(-Kt) \quad (A4)$$

from which we derive the half-time of efflux as $t_{1/2} = \ln 2/K$. Expanding the solution for K we can write

$$t_{1/2} = \frac{0.693(1 + [H]_i/K' + [H]_i^2/K'')}{k + (m' + q)[H]_i/K' + m''[H]_i^2/K'} \quad (A5)$$

We consider three limiting cases in turn, for all of which we can with little error assume that the pH within the vacuole is sufficiently low that only the term in H_i^2 in the numerator is significant:

(i) Let the constants $(m' + q)$ and m'' (multiplied by the appropriate term in H_i) be small with respect to k, i.e. let most of the efflux occur by the simple diffusion of CQ. Then $t_{1/2} = 0.693[H]_i^2/K''/k$. Since on every model we assume that the parameter k is independent of whether we are dealing with a sensitive or resistant strain of parasite, $t_{1/2}$ will be given by a constant times $[H]_i^2/K''$. We showed in the Introduction that the total concentration of CQ within the vacuole depends strictly on the square of $[H]_i$. Thus $t_{1/2}$ and the degree of accumulation of CQ will be proportional to one another, as is found experimentally (see Results and Discussion).

(ii) Let the term $m'[H]_i/K'$ be the dominant term in the numerator of equation (A5), i.e. let most of the efflux occur in the form of QH, either by an MDR pump or a leak. Then, $t_{1/2} = 0.693[H]_i K'/(m'K'')$. But on this model, equation (6) of the text shows that $[H]_i$ is proportional to the reciprocal of the value of the parameter m'. Thus $t_{1/2}$ is again proportional to $[H]_i^2$, as we showed for case (i) above. The same conclusion follows: the extent of accumulation of CQ will be proportional to the $t_{1/2}$ for its efflux.

(iii) Finally, if we assume that an MDR pump, operating only on QH_2, dominates the efflux of CQ, i.e. that only the term in $[H]_i^2/K''$ is significant in the denominator of equation (A5), we have that $t_{1/2} = 0.693/m''$. On this model, equation (7) of the main text shows that the proton concentration within the vacuole will be inversely proportional to the square root of m''. Hence the accumulation of CQ will be proportional to the square of this or to m'' itself. Once

again, $t_{1/2}$ and the extent of accumulation of CQ are directly proportional to one another.

Efflux kinetics do not, therefore, allow a distinction to be made between the three classes of model for the effect of the resistance mechanism on accumulation of CQ.

A similar treatment on the assumption that it is the rate of efflux of free proton that limits the loss of total CQ, yields the result that $t_{1/2}$ lies between ln2 divided by $(l + m'[Q])(\bar{B} + \bar{Q})$ and ln4 divided by $(l + m'[Q])(\bar{B} + \bar{Q})$, where l, m', $\bar{B}$ and $\bar{Q}$ are defined in the Theory section above. In contrast to the experimental findings, $t_{1/2}$ is independent of the state of the parasite (resistant or sensitive) for the cases where there is no MDR pump or where this pump operates only on the form QH_2 (since the relevant kinetic parameters describing the kinetics of flow of those processes appear nowhere in the expression for $t_{1/2}$). For the case where resistance operates on the form QH, by increasing the parameter m', $t_{1/2}$ *does* depend on the state of the parasite but depends inversely on m' and not $(m')^2$. Experimentally, the extent of accumulation of CQ depends on the square of $[H]_i$ and hence on the inverse square of m', in contrast to the predictions of this model. Thus all forms based on the assumption that it is the efflux of proton that limits the rate of egress of CQ are ruled out by the data.