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RAPID CHLOROQUINE EFFLUX PHENOTYPE IN BOTH CHLOROQUINE-SENSITIVE AND CHLOROQUINE-RESISTANT *PLASMODIUM FALCIPARUM*

A CORRELATION OF CHLOROQUINE SENSITIVITY WITH ENERGY-DEPENDENT DRUG ACCUMULATION

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Abstract—Recent reports suggest that lower levels of chloroquine accumulation in chloroquine-resistant isolates of *Plasmodium falciparum* are achieved by energy-dependent chloroquine efflux from resistant parasites. In support of this argument, a rapid chloroquine efflux phenotype has been observed in some chloroquine-resistant isolates of *P. falciparum*. In this study, no relationship was found between chloroquine sensitivity and the rate of [^{3}H]chloroquine efflux from four isolates of *P. falciparum* with a greater than 10-fold range in sensitivity to chloroquine. All the isolates tested displayed the rapid efflux phenotype, irrespective of sensitivity. However, chloroquine sensitivity of these isolates was correlated with energy-dependent rate of drug accumulation into these parasites. Verapamil and a variety of other compounds reverse chloroquine resistance. The reversal mechanism is assumed to result from competition between verapamil and chloroquine for efflux protein translocation sites, thus causing an increase in steady-state accumulation of chloroquine and hence a return to sensitivity. Verapamil accumulation at a steady-state is increased by chloroquine, possibly indicating competition for efflux of the two substrates. Increases in steady-state verapamil concentrations caused by chloroquine were identical in sensitive and resistant strains, suggesting that similar capacity efflux pumps may exist in these isolates. These data suggest that differences in steady-state chloroquine accumulation seen in these isolates can be attributed to changes in the chloroquine concentrating mechanism rather than the efflux pump. It seems likely that chloroquine resistance generally in *P. falciparum*, results at least in part from a change in the drug concentrating mechanism and that changes in efflux rates *per se* are insufficient to explain chloroquine resistance.

For reasons of cost, safety and efficacy, chloroquine has been the mainstay of malaria chemotherapy and propylaxis for over 40 years. There are four species of *Plasmodium* which are pathogenic to man, and of these *P. falciparum* is the species responsible for the majority of the fatalities. Cases of *P. falciparum* malaria resistant to chloroquine treatment first arose in the late 1950s [1] and since then, chloroquine-resistant infections have become widespread. The site of action of chloroquine in the malaria parasite is thought to be the food vacuole [2–4], and other studies indicate that the food vacuole is also the site of accumulation of the drug [5–8]. The mechanism of chloroquine resistance in *P. falciparum* is unresolved, but appears to be associated with lower levels of drug accumulation [7–11].

An enhanced efflux capacity for chloroquine has been observed in some strains of chloroquine-resistant but not chloroquine-sensitive parasites [11]. This has been proposed as the mechanism by which lower steady-state levels of the drug and hence reduced sensitivity are maintained in these parasites [11, 12].

Enhanced efflux of chloroquine in *P. falciparum* has been attributed to an active efflux pump similar to that found in the membranes of multidrug resistant cancer cells, despite a number of discrepancies between the two phenotypes [13, 14]. Drug efflux from cancer cells is mediated by a P-glycoprotein (PGp‖) in the cell membrane and the drug resistance phenotype in these cells has been linked with over-expression of PGp, the level of PGp expression usually indicating the degree of resistance [15].

It has been demonstrated that the level of expression of a homologue of mammalian PGp in *P. falciparum* is independent of the chloroquine resistance phenotype [16]. In addition, results from a genetic cross of chloroquine-sensitive and -resistant *P. falciparum* clones inferred that chloroquine resistance was not linked to *mdr*-like genes in the parasite [17]. These results suggest that the PGp homologue identified in malaria parasites is not the principal determinant of drug sensitivity.

An alternative hypothesis to explain the lower accumulation of chloroquine in resistant *P. falciparum* is that of a reduced force for drug uptake [9, 18]. This hypothesis is supported by a number of mathematical models [18–20] and some experimental data [9].

We have measured rates of chloroquine accumu-

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‖ Abbreviations: PGp, P-glycoprotein; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-(2-ethanesulphonic acid).

lation and efflux in four strains of *P. falciparum* with a greater than 10-fold range in sensitivity to the drug in an attempt to assess the relative importance of these two factors in the determination of chloroquine resistance.

A common feature of the phenotypes of chloroquine resistance in *P. falciparum* and multidrug resistance in cancer cells is the ability of verapamil (and other chemosensitizing agents) to reduce drug efflux rates and promote a shift in dose–response in the direction of drug sensitivity [21–25].

In cancer cell lines, the resistance reversing action of verapamil (and other chemosensitizing drugs) has been fairly well characterized. The chemosensitizing agent is thought to compete with the anti-tumour drug for a limited number of translocation sites on the PGp molecules [15]. The resistance reversal mechanism in *P. falciparum* is not so well characterized, although it is clear that verapamil and other chemosensitizing agents [10, 11] can cause resistant parasites to accumulate more chloroquine. It is questionable, however, as to whether the extent of extra chloroquine accumulation produced by these agents is sufficient to explain the sensitivity shift.

We have addressed the question of substrate competition for efflux in *P. falciparum* by examining the effect of chloroquine on the accumulation of radio-labelled verapamil to steady-state, in both chloroquine-sensitive and chloroquine-resistant isolates. In addition, we have examined the energy dependency of accumulation of both verapamil and chloroquine in sensitive and resistant parasites, in order to determine the relative importance of accumulation and efflux rates in the maintenance of steady-state drug concentrations.

MATERIALS AND METHODS

Parasite strains, maintenance and preparation. Four strains of *P. falciparum* were used in this study. 3D7 was cloned from the NF5 strain which was isolated from an airport worker in Amsterdam. The T9-94 and T9-96 clones, and the K1 strain originated in Thailand.

Parasites were maintained in continuous culture using an adaptation of the method of Jensen and Trager [26]. Cultures consisting of a 2.5% suspension of infected O positive erythrocytes, at a parasitaemia of 0.1–15% in complete medium (RPMI 1640, 25 mM HEPES buffer, 23 mM $NaHCO_3$ and 10% human AB serum) were maintained in sealed sterile plastic flasks at 37°, and gassed with an atmosphere of 3% O_2, 4% CO_2 and 93% N_2. Cultures were synchronized by selective sorbitol lysis [27], 48 hr before use.

In vitro *sensitivity assays.* Sensitivity of all the isolates to chloroquine alone and chloroquine plus verapamil was assessed. Drug activity was monitored by its ability to reduce the incorporation of [^{3}H]-hypoxanthine into the nucleic acids of the parasite, in a standard microdilution assay [28]. To measure the effect of verapamil on the sensitivity of the parasites to chloroquine, the drug was added to the assay system at fixed concentrations of 1 and 5 μM. Stock solutions of chloroquine and verapamil were made up in 50% ethanol:water. Concentrations of ethanol in the assay system were always less than 0.01%. IC_{50} and IC_{90} values were interpolated from either log, or log-probit transformations of the dose–response curve.

Measurement of chloroquine accumulation. Cultures of highly synchronized trophozoites were suspended in complete medium (parasitaemia and haematocrit are given in results). Accumulation experiments were initiated by the addition of [^{3}H]-chloroquine (NEN, Boston, MA, U.S.A. 69.2 Ci/mM). Accumulation was halted at the desired time point, by centrifugation (12,000 rpm for 10–20 sec) of a 0.5 mL aliquot of cell suspension in a 1.5 mL microcentrifuge tube containing 0.3 mL dibutyl phthalate. After centrifugation, the cells containing labelled chloroquine were sedimented below the layer of dibutyl phthalate and separated from the medium containing the remaining labelled chloroquine, which remained above the layer of dibutyl phthalate. The cell pellet was removed from the centrifuge tube by cutting off the tip of the centrifuge tube (containing the cell pellet) with a scalpel blade. The tube tip containing the cell pellet was placed in a plastic scintillation vial. The cells were lysed by vortex mixing the pellet with 200 μL distilled water for 1–2 min. The lysate was digested with 200 μL NCS tissue solubilizer (BDH, Poole, U.K.) for 4 hr at 37°, decolourized with 25 μL 30% H_2O_2 and acidified with 25 μL glacial acetic acid. Scintillation fluid (3.5 mL) (LKB 'Optiphase safe') was added to each tube and the tubes counted on a scintillation counter (LKB Rackbeta 1219). To check the extent of depletion of labelled chloroquine, an aliquot of medium was removed, placed in a plastic scintillation minivial, 4 mL scintillation fluid was added and the vials were counted in the liquid scintillation counter.

The accumulation ratio (the ratio of intracellular to extracellular drug λ) was calculated from the amount of drug in the parasitized cells and the amount of drug in an equivalent volume of incubation medium at the end of each experiment.

The values for intracellular pH were calculated from the equation:

$$pH_{fv} = pH_{out} - \log(\lambda/0.032)$$

for monobasic verapamil,
or

$$pH_{fv} = pH_{out} - \log(\lambda/0.032)^{1/2}$$

for dibasic chloroquine.

The food vacuole is assumed to occupy 3.2% of the volume of the infected cell [7]. The above calculation is based on the following assumptions: only the unprotonated species of chloroquine can pass through membranes, the drug is accumulated exclusively in the food vacuole of the parasite and no active transport of chloroquine is taking place across the vacuole membrane.

Measurement of chloroquine efflux. Culture suspensions were loaded with [^{3}H]chloroquine as described above for periods of 5 or 60 min. At t_0 (for efflux measurement) the cell pellets containing pre-accumulated [^{3}H]chloroquine were suspended in complete medium without chloroquine, warmed to 37° in narrow-necked culture flasks. Although it is preferable to measure efflux into an infinite

volume, practical considerations dictate otherwise and in order to maintain compatibility with previously published work [12], haematocrit of the suspension was equivalent to that of the loading suspension. Aliquots (0.5 mL) of the suspension were removed at the required time point, and the cell pellets were obtained and processed for counting as described above.

The effect of verapamil on efflux rate of chloroquine from the cells was assessed as follows. The cell pellet containing pre-accumulated [^{3}H]-chloroquine was suspended in complete medium containing unlabelled chloroquine at the same concentration as that of [^{3}H]chloroquine in the loading medium. Verapamil was added, at a concentration of 5 μM and the cell suspension was incubated for 5 min at 37°. The suspension was centrifuged, the supernatant removed and the pellet suspended (at t_0 for efflux measurement) in complete medium containing verapamil at 5 μM, but no chloroquine. The cell pellets were obtained at the required time points and processed as described above. This experimental approach is similar to that reported previously [11]. The effect of verapamil on efflux rate of chloroquine was also assessed without the equilibration period. Results of these experiments (not shown) indicated that verapamil does not have any effect on rate of chloroquine efflux from the parasites without the 5 min equilibration period. The addition of unlabelled chloroquine during this period was carried out in order to minimize [^{3}H]chloroquine loss.

To calculate the rate of efflux, the amount of chloroquine in the cell pellet at equilibrium (see Results) was subtracted from the amount of chloroquine at the previous time points. The amount of chloroquine in the cell pellet at each time point was then expressed as the percentage of the amount at t_0. After log transformation of the data, the slopes were calculated by linear regression and the half times to equilibrium calculated from the slopes.

Metabolic inhibition experiments. Steady-state levels of chloroquine accumulation were measured as described above, with the following modifications. Two groups of cells were incubated for 60 min in physiological saline buffered to pH 7.4 with 25 mM HEPES and 23 mM $NaHCO_3$ at 37°, containing [^{3}H] chloroquine. The first group of cells were incubated in the above medium containing glucose at 1 mg/mL and the second group of cells were incubated in the above medium, substituting 10 mM sodium azide for glucose. Cells in the second group were considered to be energy depleted, as sodium azide is an inhibitor of oxidative phosphorylation [29], and malaria parasites require an external source of glucose for glycolysis [12]. Such a protocol has been used by many workers to energy deplete cells in similar transport experiments [15, 24, 25]. After 60 min (a time sufficient to achieve steady-state), the cell pellets were harvested and processed as for the standard accumulation experiments.

The effect of metabolic inhibition on the steady-state accumulation of [^{3}H]verapamil in the parasites was measured in the same way. The concentration of verapamil in the medium was 5 μM, consisting of 1 μCi/mL [^{3}H]verapamil (66 Ci/mmol; NEN) made up to a total concentration of 5 μM with unlabelled verapamil. The effect of chloroquine at a concentration of 10^{-7} M on verapamil accumulation was also assessed.

Precautions taken in flux studies. Parasitaemias of less than 7% were used for all flux studies to avoid any problems of loss of viability in high parasitaemia cultures. Accumulation and efflux of label in an identical volume of uninfected red cells was measured at each time point and under each experimental condition. These counts were subtracted from the corresponding counts from the pellet containing parasites. In the case of the chloroquine studies, counts due to uninfected red cells were less than 10% of the counts due to infected cells except for the initial two time points in the accumulation studies, when uninfected cells accounted for no more than 25% of the total counts. In the case of the verapamil flux studies, uninfected red cells accounted for approximately 60% of the total accumulation. All groups were normalized for starting radioactivity before subtracting red cell radioactivity. The groups were then normalized for parasitaemia before calculation of flux rates.

Accumulation and efflux experiments were performed in narrow-necked flasks, which were gassed before each experiment with the same atmosphere used for the cultures. Under these conditions, there was no measurable increase in pH of the cell medium suspension over the duration of the experiments.

Statistics. Data points presented are means of at least three observations; error bars, when shown, represent standard deviations. Significance of differences between means, when quoted, was calculated to 99% confidence limits, using the Wilcoxon rank test. Significance of differences in log-transformed efflux slopes was calculated to 99% confidence limits using analysis of variance (assuming a common intercept).

RESULTS

Table 1 shows the sensitivity of the four isolates to chloroquine, as IC_{50} values, with IC_{90} values in parentheses. Minimum, maximum and typical values for each isolate, obtained from at least six sensitivity assays are shown. There is a greater than 10-fold range in chloroquine sensitivity in the four isolates. Assays were performed at a haematocrit of 5% and a parasitaemia of 1%. This inoculum size is expected to influence the sensitivity values slightly [18],

Table 1. Sensitivity of the strains to chloroquine

Strain	3D7	T9-96	T9-94	K1
Minimum	26 (50)	46 (85)	80 (150)	250 (750)
Maximum	42 (100)	70 (120)	114 (220)	500 (2000)
Typical	33 (75)	56 (90)	100 (200)	340 (1000)

Values are expressed in nanomoles of chloroquine per litre of medium. Normal figures are IC_{50} values, figures in parentheses are IC_{90} values.

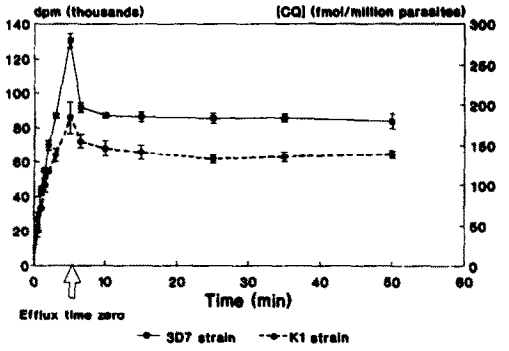

Fig. 1. Accumulation and efflux of [^{3}H]chloroquine for the 3D7 (■) and K1 (●) strains.

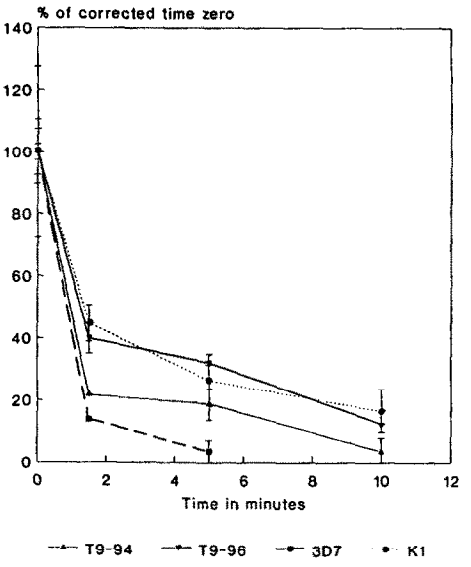

Fig. 2. Efflux of [^{3}H]chloroquine to equilibrium (expressed as percentage of t_0 radioactivity, after subtraction of equilibrium radioactivity) for the T9-94 (▲), T9-96 (▼), 3D7 (■) and K1 (●) strains.

Table 2. Rates of chloroquine accumulation with fit statistics and typical IC_{50} values

Strain	Rate of chloroquine accumulation (fmol/million parasites/min)	R^2 (N)	Typical IC_{50} (nM)
3D7	46.5	99.2 (18)	33
T9-96	34.4	91.1 (18)	56
T9-94	38.3	96.4 (18)	100
K1	28.6	94.5 (18)	340

tending to underestimate rather than overestimate the range of sensitivity of the isolates.

Chloroquine flux studies

Data in Fig. 1 represent accumulation and efflux of chloroquine for the most sensitive (3D7) and the most resistant (K1) strains (normalized to 4% parasitaemia, 1.5% haematocrit, [chloroquine] = 4 nM). Levels of uptake were significantly different at times of 1 min and later. Accumulation was measured over a period of 5 min to enable accurate estimation of uptake rates. The 5 min time point on this graph represents both the end point of accumulation measurement and the starting point of efflux measurement.

Efflux of chloroquine from all the strains tested was biphasic, consisting of an initial rapid phase of decline followed by a phase where the chloroquine concentration remained invariant over the remaining time course. We assumed that this second phase represented the establishment of an equilibrium between efflux and accumulation.

Efflux rate to equilibrium was calculated as described. Table 3 shows each strain with its corresponding efflux rate (half-time to equilibrium), fit statistic and steady-state concentration of chloroquine after establishment of efflux equilibrium. Linear–linear efflux curves for all the strains are shown in Fig. 2; log transformation of the Y-axis data was used to calculate the rates of efflux. The preferred way to measure efflux is into an infinitely large extracellular volume. This was not done because of the practical limitations of the experimental system: the limited volume capacity of the centrifuge tubes used dictates that haematocrits have to be relatively high (>1%) to obtain reasonable radioactivity counts without using high concentrations of labelled chloroquine or high parasitaemias. The use of a microcentrifuge is essential because of the fast sampling times necessary.

Despite the limited accuracy of fit, in no way could efflux of drug from any of the strains be described as being similar to the slow efflux reported by Krogstad *et al.* [11, 12] for chloroquine-sensitive strains. There was no significant difference in rate of chloroquine efflux from the K1, T9-94 and T9-96 strains; however, the rate of chloroquine efflux from the 3D7 strain was significantly greater than the rates of chloroquine efflux from the other strains. Rank order of increasing resistance of these isolates to chloroquine is 3D7, T9-96, T9-94, K1. Rank order of increasing chloroquine efflux rate is K1, T9-96, T9-94, 3D7. There is no correlation, therefore, between increasing efflux rate and increasing resistance. If anything, increasing chloroquine efflux rates are correlated with an increase in sensitivity to the drug (r = 0.65).

Accumulation rate was also calculated using linear regression and the results are shown in Table 2. Rate of chloroquine accumulation (expressed as femtomoles of chloroquine per million parasites per minute) together with fit statistics and typical IC_{50} value are shown, for each strain. There was a correlation between decreasing accumulation rate and increasing resistance (r = 0.75).

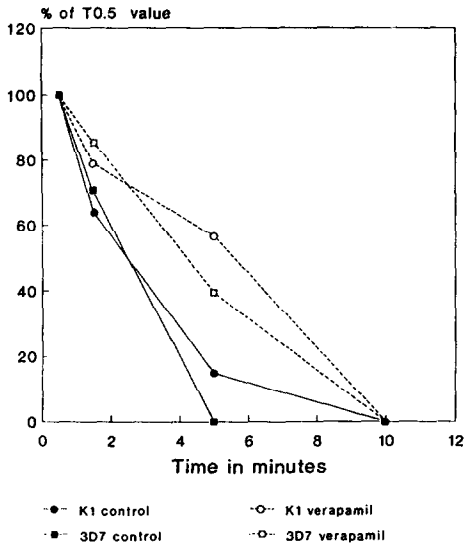

Fig. 3. Efflux of [^{3}H]chloroquine to equilibrium (expressed as percentage of $t_{0.5}$ radioactivity, after subtraction of equilibrium radioactivity) for the 3D7 strain in the presence (□) and absence (■) of verapamil and the K1 strain in the presence (○) and absence (●) of verapamil.

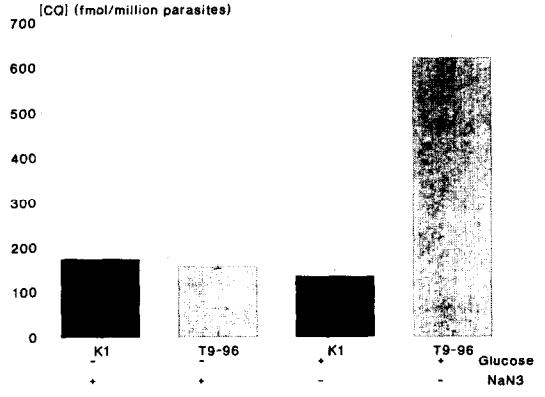

Fig. 4. Energy-dependence of [^{3}H]chloroquine accumulation for T9-96 and K1 strains.

Verapamil, after a 5 min pre-incubation and at a concentration of 5 μM, caused a significant reduction in the rate of chloroquine efflux from all of the strains (2–12-fold). Of the four isolates tested, only the K1 strain exhibited an increase in sensitivity to chloroquine in the presence of verapamil (IC_{50} of chloroquine = 340 nM, IC_{50} of chloroquine plus verapamil at 5 μM = 85 nM). Figure 3 shows the effect of verapamil on chloroquine efflux (corrected to percentage of starting radioactivity after subtraction of equilibrium radioactivity) from the most sensitive (3D7) and most resistant (K1) strains.

Metabolic deprivation

The energy dependence of chloroquine accumulation in sensitive (T9-96) and resistant (K1) strains is shown in Fig. 4 (parasitaemia 3.2%, haematocrit 1.25%, [chloroquine] = 3.6 nM). Steady-state chloroquine accumulation in the resistant strain is significantly higher under conditions of metabolic deprivation than under control conditions. In contrast, there is a 4-fold decrease in steady-state chloroquine concentrations in the sensitive strain under conditions of metabolic deprivation compared to control conditions.

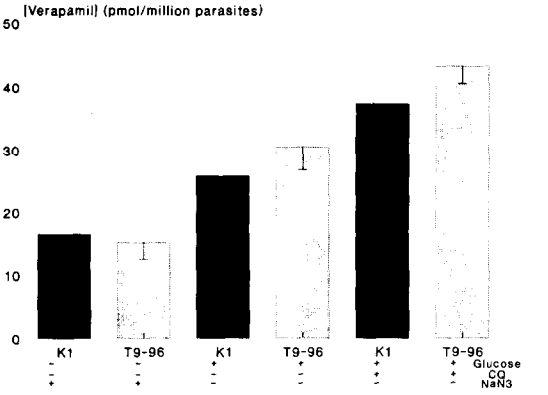

Fig. 5. The effect of chloroquine at 10^{-7} M on energy-dependent [^{3}H]verapamil accumulation for T9-96 and K1 strains.

Figure 5 shows the effect of energy depletion and the effect of chloroquine at 10^{-7} M on steady-state verapamil accumulation in the same strains (parasitaemia 3.2%, haematocrit 1.25%, [verapamil] = 5 mM). Energy depletion causes a decrease in steady-state verapamil accumulation in both chloroquine-sensitive and -resistant parasites, although because of the wide variation this increase is not significant in the resistant parasites. Under control culture conditions, chloroquine-sensitive parasites appear to accumulate more verapamil than do chloroquine-resistant parasites, although this difference is not significant.

Chloroquine at 10^{-7} M produced a significant increase in steady-state verapamil accumulation in both chloroquine-sensitive and chloroquine-resistant parasites of 42% and 43% over the control groups, for the T9-96 and K1 strains, respectively.

DISCUSSION

Krogstad *et al.* [11] have reported that chloroquine-resistant strains of *P. falciparum* release pre-accumulated chloroquine 40–50 times more rapidly than chloroquine-sensitive strains [11]. In addition, they have demonstrated that efflux of chloroquine is energy dependent [12], and have suggested that chloroquine-resistant parasites possess a rapid efflux phenotype (efflux half-time of approximately 2 min), absent from sensitive strains, which is responsible for lower steady-state accumulation levels and hence lower sensitivity to the drug.

As an alternative hypothesis, other workers have postulated that chloroquine resistance in *P. falciparum* results from a higher pH or lower chloroquine buffering capacity in the food vacuole

Table 3. Rates of chloroquine efflux, expressed as half-time to equilibrium with fit statistic, and steady-state chloroquine levels at efflux equilibrium

Strain	$T_{\frac{1}{2}}$ to equilibrium (min)	R^2 (N)	Steady-state [chloroquine] (fmol/million parasites)
3D7	1.13	85.1% (9)	178 ± 19
T9-96	3.79	89.6% (12)	183 ± 7
T9-94	2.29	79.0% (12)	138 ± 13
K1	4.15	77.4% (12)	134 ± 4

of resistant parasites; hence, a weaker force for chloroquine accumulation [9, 18, 19]. This could be caused by a weakened proton pump in the food vacuole membranes of resistant parasites. In support of this, we have demonstrated that a specific vacuolar proton pump inhibitor (at sub-lethal concentrations) can reduce chloroquine accumulation and reduce sensitivity of the parasites to chloroquine [30].

Given that rapid efflux of chloroquine in *P. falciparum* is defined by an efflux half-time of approximately 2 min (vs 75 min in the absence of the rapid efflux phenotype) [11, 12], all the isolates in this study, both resistant and sensitive, display the rapid efflux phenotype (Table 3). We found no significant difference in efflux rates of chloroquine from three of the four isolates used in this study (Fig. 2). No rank order correlation between increasing chloroquine resistance and increasing efflux rate of the drug was found. Additionally, the most sensitive isolate (3D7) demonstrated the fastest rate of efflux. This outcome is at odds with the rapid efflux hypothesis.

These findings are substantiated by the data presented in Fig. 3, which indicate that verapamil can decrease the rate of efflux of chloroquine from both chloroquine-resistant and -sensitive parasites. If verapamil is inhibiting efflux of chloroquine by acting as a competing substrate, we would expect an increase in steady-state accumulation of verapamil in the presence of chloroquine. Indeed, the results presented in Fig. 5 demonstrate a significant increase in steady-state verapamil accumulation in the presence of chloroquine, in both chloroquine-resistant (K1) and -sensitive (T9-96) strains. Moreover, the increase in steady-state verapamil accumulation is almost identical in resistant (43%) and sensitive (42%) parasites, implying that the efflux pump of sensitive and resistant parasites has a similar capacity. The results presented in Fig. 5 also demonstrate that accumulation of verapamil by *P. falciparum* is an energy-dependent process.

Verapamil is a monoprotic weak base (pK_a = 9.2). Accumulation of verapamil, like chloroquine [5], could be driven by energy-dependent acidification of the food vacuole. The parasite food vacuole is the site of chloroquine accumulation [2–4] and presumably also the site of accumulation of other weak bases. If, as has been suggested, chloroquine-resistant parasites have a higher vacuolar pH than do chloroquine-sensitive parasites, higher levels of verapamil accumulation would be expected in chloroquine-sensitive vs-resistant parasites. Given the assumptions stated in the materials and methods and assuming a vacuolar pH of 5 for the sensitive strain and 5.3 for the resistant strain, we would expect the verapamil accumulation ratios to be 8.03 and 4.02, respectively. The measured values are 62 and 52. This large discrepancy could be caused by significant accumulation of verapamil in the cell cytosol (the expected ratios assume that all the drug is accumulated in the food vacuole). If this is happening, the increased verapamil accumulation produced by chloroquine could reflect increased accumulation of verapamil in the cell cytosol as well as in the food vacuole. It is important to emphasize that differences in verapamil accumulation for a given pH change in the food vacuole would be small compared to differences in accumulation of a diprotic weak base like chloroquine (diprotic weak bases are expected to accumulate in acid compartments to the square of the concentration of monoprotic weak bases [31]). Making the same assumptions for accumulation of chloroquine, expected accumulation ratios would be 2019 for the T9-96 strain and 507 for the K1 strain. The actual values obtained were 1708 and 431, corresponding to vacuolar pH values of 5.04 and 5.34, respectively. These pH values are calculated on the assumption that there is no active transport of chloroquine out of the parasite. If active efflux of drug is taking place, the pH of the vacuoles is likely to be more acidic than these estimates.

Differential chloroquine accumulation of our isolates would go some way towards explaining the selective resistance reversal effects of verapamil: a large (4–5-fold) increase in the intracellular chloroquine to verapamil ratio would be expected in chloroquine-sensitive vs-resistant strains (for a constant extracellular chloroquine to verapamil ratio). We concede that, if this were the case, some small changes in chloroquine accumulation and chloroquine sensitivity would be expected in sensitive strains (in the presence of verapamil). It is important to emphasize that this does not happen: chloroquine-sensitive parasites do not exhibit sensitivity shifts or increases in chloroquine accumulation in the presence of verapamil. Furthermore, verapamil-induced increases in chloroquine accumulation, seen in resistant parasites, seem to be insufficient to explain shifts in sensitivity *per se*. These questions are currently being addressed in our laboratory.

If the variation in sensitivity to chloroquine of our isolates cannot be explained by their respective efflux

rates, does the rate of accumulation of chloroquine by these isolates furnish an improved explanation? Rate of accumulation of chloroquine by these isolates is much more closely correlated to their chloroquine sensitivity than rate of efflux (Table 2). However, the correlation is not perfect: the T9-94 isolate has a slightly greater rate of chloroquine accumulation than the more sensitive T9-96 isolate (which achieves higher steady-state levels), suggesting an additional factor in the determination of chloroquine sensitivity.

These results are similar to those of Geary *et al* [9], who demonstrated that some resistant strains required higher intravacuolar chloroquine concentrations to kill them than sensitive strains. These findings suggested some changes in the site specificity of chloroquine in some strains of resistant parasites, as well as changes in the chloroquine concentrating mechanism.

The energy dependency of chloroquine accumulation in sensitive and resistant parasites is represented in Fig. 4. Similar data have been reported by other workers for chloroquine-sensitive and -resistant strains of *P. falciparum* [12] and rodent malaria [32, 33]. The data for *P. falciparum* have been interpreted as providing evidence for selective energy-dependent chloroquine efflux in resistant parasites. We offer an alternative interpretation of this data, based on levels of chloroquine accumulation and rates of efflux obtained in this study. We intimate that the capacity of the efflux pump to remove chloroquine from the sensitive (T9-96) parasites is swamped by the stronger force for accumulation in these parasites. It is only when the accumulation force has been reduced to the levels seen in the resistant (K1) strain that the efflux pump has any influence (as detected in this type of experiment) on steady-state chloroquine levels.

It is noteworthy that only a small pH change (approximately 0.3 pH units) is necessary to account for differences in accumulation of and presumably sensitivity to chloroquine displayed by the strains of *P. falciparum* used in this study. Such a small pH change is well within the pH optima reported for the parasite's haem polymerase system, which is thought to be the site of action of chloroquine [4].

The data presented here support the view that changes in chloroquine accumulation force, rather than changes in efflux rate, are responsible for chloroquine resistance, at least in these isolates. We suggest that the apparent equivalent efflux capacity of both resistant and sensitive isolates only becomes relevant in the determination of steady-state drug levels when the accumulation force is reduced. We acknowledge the difficulty in extrapolation of data obtained from a small number of isolates, given the likely multigenic nature of chloroquine resistance [14, 34]. Nevertheless, we feel that chloroquine resistance generally in *P. falciparum* could be determined, at least in part, by changes in the force for accumulation of the drug.

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