0026-895X/98/010170-10$3.00/0

MOLECULAR PHARMACOLOGY, **54:**170–179 (1998).

Access to Hematin: The Basis of Chloroquine Resistance

PATRICK G. BRAY, MATHIRUT MUNGTHIN, ROBERT G. RIDLEY, and STEPHEN A. WARD

Department of Pharmacology and Therapeutics (P.G.B., M.M., S.A.W.), The University of Liverpool, Liverpool L69 3BX, UK, and Hoffmann-La Roche (R.G.R.), Pharmaceuticals Division, Pharma Research Preclinical, CH-4070 Basel, Switzerland

Received January 9, 1998; Accepted March 31, 1998

This paper is available online at http://www.molpharm.org

ABSTRACT

The saturable uptake of chloroquine by parasites of *Plasmodium falciparum* has been attributed to specific carrier-mediated transport of chloroquine. It is suggested that chloroquine is transported in exchange for protons by the parasite membrane Na^+/H^+ exchanger [*J Biol Chem* **272:**2652–2658 (1997)]. Once inside the parasite, it is proposed that chloroquine inhibits the polymerization of hematin, allowing this toxic hemoglobin metabolite to accumulate and kill the cell [*Pharmacol Ther* **57:**203–235 (1993)]. To date, the contribution of these proposed mechanisms to the uptake and antimalarial activity of chloroquine has not been assessed. Using sodium-free medium, we demonstrate that chloroquine is not directly exchanged for protons by the plasmodial Na^+/H^+ exchanger. Furthermore, we show that saturable chloroquine uptake at equilibrium is due solely to the binding of chloroquine to hematin rather than active uptake: using Ro 40–4388, a potent and specific inhibitor of hemoglobin digestion and, by implication, hematin release, we demonstrate a concentration-dependent reduction in the number of chloroquine binding sites. An equal number of chloroquine binding sites are found in both resistant and susceptible clones, but the apparent affinity of chloroquine binding is found to correlate with drug activity ($r^2 = 0.93$, $p < 0.0001$). This completely accounts for both the reduced drug accumulation and activity observed in resistant clones and the "reversal" of resistance produced by verapamil. The data presented here reconcile most of the available biochemical data from studies of the mode of action of chloroquine and the mechanism of chloroquine resistance. We show that the activity of chloroquine and amodiaquine is directly dependent on the saturable binding of the drugs to hematin and that the inhibition of hematin polymerization may be secondary to this binding. The chloroquine-resistance mechanism regulates the access of chloroquine to hematin. Our model is consistent with a resistance mechanism that acts specifically at the food vacuole to alter the binding of chloroquine to hematin rather than changing the active transport of chloroquine across the parasite plasma membrane.

Current theory suggests that chloroquine kills parasites by preventing the detoxification of hematin inside the parasite food vacuole (Slater, 1993). Heme, which is released as a byproduct of hemoglobin digestion, is oxidized rapidly to hematin and sequestered into hemozoin or malarial pigment by an autocatalytic mechanism (Dorn *et al*., 1995). Chloroquine inhibits the polymerization process *in vitro* and is proposed to do the same *in vivo*, causing a build up of free hematin or hematin/chloroquine complex that would ultimately kill the parasite (Slater, 1993; Sullivan *et al*., 1996). Alternatively, it has been proposed that weakly basic chloroquine accumulates to high levels in the acid food vacuole by a proton-trapping mechanism (Yayon *et al*., 1985). Thus, chloroquine could kill parasites in its own right by direct inhibition of vacuolar enzymes such as phospholipase (Ginsburg and Geary, 1987) or proteinase (Vander Jagt *et al*., 1987).

A proportion of the total chloroquine taken up by *Plasmodium falciparum* is saturable at nanomolar drug concentrations, and early studies hinted that this saturable component is important for drug activity (Fitch, 1970). There also is a component of chloroquine uptake that is nonsaturable in the submicromolar range (Fitch, 1970). Recent evidence suggests that nonsaturable uptake can be attributed to low affinity chloroquine binding to plentiful cytosolic proteins (Menting *et al*., 1997; Dorn *et al*., 1998). The contribution of both saturable and nonsaturable uptake to the antimalarial activity of chloroquine is unknown.

Chloroquine readily forms a complex with free hematin *in vitro* (Chou *et al*., 1980), and it was originally suggested that saturable uptake into cells is due to the binding of chloroquine to hematin inside the food vacuole. Chloroquine/hematin binding clearly occurs to some extent *in situ*. Radiolabeled quinolines are found to be associated with hemozoin after prolonged incubation of infected cells with sublethal concentrations of drug (Sullivan *et al*., 1996), and intracellular quinoline/hematin interactions have been detected by photoacoustic spectroscopy (Balasubramanian *et al*., 1984). However, the contribution of chloroquine/hematin binding to the saturable cellular uptake and the importance of this binding for the antimalarial activity of the drug have not been determined. Accordingly, the hematin binding hypothesis has been challenged on quantitative grounds, and it was suggested that the uptake of chloroquine is determined mainly by the titration of protons inside the food

This work was supported by a Research Program Grant from The Wellcome Trust.

vacuole (Ginsburg and Geary, 1987; Yayon *et al*., 1985). In this case, the observed saturation kinetics would reflect saturation of the vacuolar proton pump rather than hematin binding (Ginsburg and Stein, 1991). More recently, the saturation kinetics of chloroquine uptake have been attributed to a carrier-mediated chloroquine transport process: Based on the ability of 5-(*N*-ethyl-*N*-isopropyl)amiloride, a specific inhibitor of Na^+/H^+ antiport, to competitively inhibit chloroquine uptake, it was suggested that chloroquine is directly transported by the parasite plasma membrane Na^+/H^+ exchanger, in place of sodium and in exchange of protons (Sanchez *et al*., 1997). In a modification of this theory, it was later suggested that chloroquine is carried through the Na^+/H^+ exchanger in a burst of self-stimulated sodium/proton exchange (Wünsch *et al*., 1998).

Using a two-component model, we have shown that the saturable component of chloroquine uptake is solely responsible for the antimalarial activity of the drug. Further studies have focused on the nature of saturable chloroquine uptake in the context of the theories outlined above. We used Ro 40–4388, a potent and specific inhibitor of plasmepsin I (Moon *et al*., 1997), to assess the importance of chloroquine/hematin binding. Plasmepsin I acts on native hemoglobin, cutting the Phe33—Leu34 bond of the α chain, which unfolds the hemoglobin tetramer and allows the release of heme (Gluzman *et al*., 1994). We believe that Ro 40–4388 allows chloroquine/hematin binding to be differentiated from alternative uptake mechanisms that may operate inside intact cells. This inhibitor will inhibit the release of heme by blocking the first step in the degradation of hemoglobin but is not likely to affect the Na^+/H^+ exchanger or the proton gradient. Binding parameters have been measured in chloroquine-susceptible and -resistant strains in the presence or absence of resistance modulators such as verapamil. The differences in the cellular accumulation and activity of chloroquine in susceptible and resistant strains, together with the effects of verapamil, can be fully described by the chloroquine-hematin binding parameters. Analysis of the data indicates that there is no change in the rate of hemoglobin digestion or heme sequestration in resistant strains. Instead, the chloroquine-resistant parasite has evolved a mechanism to reduce the access of chloroquine to hematin.

Materials and Methods

Parasites. The chloroquine susceptible 3D7, T9–96, and HB3 clones were obtained from Prof. D. Walliker, Edinburgh University (Edinburgh, UK). The K1 chloroquine-resistant strain (cloned in-house) was obtained from Dr, D. C. Warhurst, London School of Hygiene and Tropical Medicine (London, UK), and the TM5 and TM6 chloroquine-resistant clones were obtained from Dr. P. Tan-Ariya, Mahidol University (Bangkok, Thailand). Parasites were maintained in continuous culture using standard techniques (Desjardins *et al*., 1979).

Sensitivity assays. Sensitivity of all the strains to chloroquine, daunomycin, primaquine, and verapamil was determined by measuring the ability of serial dilutions of drugs to inhibit the incorporation of radiolabeled [^{3}H]hypoxanthine into parasite nucleic acids (Desjardins *et al*., 1979). Chloroquine assays were performed in the absence or presence of 5 μM verapamil or 1.5 μM primaquine, at the lowest practicable inoculum size (0.5% parasitemia, 1% hematocrit). IC_{50} values were calculated for each assay using the four-parameter logistic method (Grafit; Erithacus Software, Kent, UK), and values presented are the mean of five sensitivity assays, unless otherwise stated.

Accumulation of radiolabeled drugs. Radiolabeled [^{3}H]chloroquine (specific activity, 50.4 Ci/mmol), [^{3}H]verapamil (specific activity, 66 Ci/mmol), and [^{3}H]daunomycin (specific activity, 4 Ci/mmol) were purchased from DuPont-New England Nuclear (Boston, MA). Radiolabeled [^{3}H]amodiaquine (specific activity, 106.5 mCi/mmol) was synthesized as described previously (Hawley *et al*., 1996a). Radiolabeled [^{14}C]primaquine (specific activity, 85 mCi/mmol) was a gift from Prof. W. Peters.

Chloroquine accumulation into all the strains was measured as described previously (Bray *et al*., 1996) over a range of extracellular chloroquine concentration of 1–5000 nM. In addition, the three chloroquine-resistant strains were incubated over the same chloroquine concentration range in the presence of 5 μM verapamil, the K1 clone in the presence of 5 μM daunomycin or 1.5 μM primaquine, and the HB3 strain in the presence of 5 μM Ro 40–4388. The cellular accumulation ratio was calculated as the ratio of chloroquine in the cell pellet to that in a similar volume of medium at equilibrium. Total uptake (the sum of saturable and nonsaturable uptake) was calculated by multiplying the accumulation ratio by the equilibrium chloroquine concentration in the medium.

In addition, chloroquine accumulation was measured at a single fixed initial external concentration of 10 nM in the presence of a range of concentrations of Ro 40–4388 or leupeptin. Nonsaturable uptake was estimated using a single external chloroquine concentration of 10 μM and subtracted from total uptake. The influence of sodium on the steady state chloroquine uptake was measured over 1 hr at a fixed external chloroquine concentration of 1 nM using buffer [1.2 mM $CaCl_2$, 5.4 mM KCl, 0.8 mM $MgCl_2$, 1 mM K_2HPO_4, 5.5 mM glucose, buffered to pH 7.4 with 10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid] containing 122.5 mM sodium chloride, 122.5 mM choline chloride, or 122.5 mM *N*-methyl-D-glucamine. Parallel incubations were performed in the presence of verapamil (10 μM) or daunomycin (5 μM). Uptake of radiolabeled [^{3}H]amodiaquine (10 nM), [^{14}C]primaquine (1 μM), [^{3}H]daunomycin (10 nM), and [^{3}H]verapamil (10 nM) was measured in the presence or absence of Ro 40–4388. Accumulation was measured over 1 hr, a time sufficient to reach steady state. Inoculum size for the drug accumulation experiments was the same as that used for the sensitivity assays, and whenever possible the same batch of culture was used for both the accumulation experiments and the parallel sensitivity assays. Under the conditions of low inoculum size used for chloroquine (0.5% parasitaemia, 1% hematocrit), there was no significant depletion of chloroquine from the medium. For reasons of lower specific activity and/or lower parasite specific drug accumulation, higher inoculum sizes were used to measure the uptake of the other radiolabeled drugs. Significant medium depletion was encountered with amodiaquine (25–35%) and daunomycin (20–30%). This was corrected by using the cell-to-medium ratio. For all the experiments and for all the conditions used, counts attributable to an equal volume of uninfected red cells were subtracted from the total.

Modeling saturable and nonsaturable chloroquine uptake and the relationship with antimalarial activity. Total uptake versus external chloroquine concentration data were fitted by computer to eq. 1 using an iterative procedure (Grafit). The slope of the nonsaturable component was determined graphically and checked by using this value as an initial estimate for iteration. The amount of nonsaturable uptake at each concentration was calculated by multiplying the external concentration by the slope of the nonsaturable uptake component. Saturable uptake at each concentration was calculated by subtracting nonaturable uptake from total uptake at each concentration.

Hybrid drug uptake curves can be described by the following equation, which superimposes a rectangular hyperbola onto a straight line:

$$[TD] = [BD] + m \cdot [ED]$$

or

$$[TD] = \frac{[ED] \cdot Cap}{[ED] + K_d} + m \cdot [ED] \quad (1)$$

where [TD]is the total drug concentration inside the parasites, [BD]is the concentration of bound drug, [ED]is the external drug concentration, Cap is the capacity or concentration of binding sites, K_d is the dissociation constant of the chloroquine binding site, and m is the slope of the nonsaturable component of drug uptake. This equation assumes the simplest case of ligand binding at sites with similar binding characteristics.

We developed a model relating drug accumulation to activity using the above relationship and based on the following assumptions: (1) Over the range of external concentrations used and for a given isolate, the internal concentration of chloroquine available for binding (both ionized and unionized) is directly proportional to the external concentration of drug for a given medium pH. (2) Drug activity is determined by the extent of saturable uptake only, and nonsaturable uptake is assumed to be nonspecific and similar for all strains. (3) The amount of chloroquine bound to hematin at IC_{50} is the amount of drug required to kill 50% of the parasite population and is the same for all strains regardless of the actual IC_{50}. (4) The chloroquine/hematin binding displays Michaelis-Menten kinetics.

Subtracting the linear component m·[ED]from eq. 1, the saturable component (corresponding to chloroquine-hematin binding) can be arranged as:

$$[BD] = Cap - \frac{[BD] \cdot K_d}{[ED]} \quad (2)$$

Chloroquine resistance is associated with reduced drug accumulation, and two hypotheses to explain chloroquine resistance are currently favored. It is proposed that resistant parasites posses a weakened proton gradient driving chloroquine uptake (Ginsburg and Stein, 1991). Alternatively, it has been proposed that resistant parasites have an energy-dependent drug efflux pump (Krogstad *et al.*, 1987). Both of these processes would reduce the internal concentration of drug available to bind to the receptor for a given external chloroquine concentration. From eq. 2, any such change would be expected to increase the measured (apparent) value of the dissociation constant (K_d), whereas the measured value for Cap will not be changed. Although the apparent K_d will be higher, the actual binding affinity will be unchanged.

In addition, we used the two-component accumulation model to examine the relationship between chloroquine accumulation and activity. Eq. 1 can be rearranged to give:

$$[TD] = [BD] + m \cdot [ED] \quad (3)$$

Plotting total drug concentration at IC_{50} ([TD]) against IC_{50} ([ED]) will give a linear plot of slope m and a *Y* intercept of bound drug at IC_{50}. Total drug concentration is seen to increase along with IC_{50} due to the greater proportional contribution of the nonsaturable component to total uptake as the IC_{50} increases.

The accumulation ratio is the ratio of drug concentration in the parasites to drug concentration in the medium and can be described for this model by:

$$AR = \frac{[BD] + m \cdot [ED]}{[ED]}$$

or

$$AR = \frac{[BD]}{[ED]} + m \quad (4)$$

Plotting accumulation ratio at IC_{50} against the reciprocal of IC_{50} (1/[ED]) will give a linear relationship of slope [BD]and *Y* intercept m. This reciprocal relationship predicts that relatively small increases in drug accumulation ratio will have large effects on the IC_{50} of resistant strains, providing a potential explanation for the chemosensitization effects of verapamil that cannot be explained in terms of simple modulation of whole-cell chloroquine transport (Bray *et al.*, 1994).

Association of preloaded bound chloroquine with hematin-containing cell debris after cell lysis. Cells infected with HB3 or K1 strain parasites (5% parasitemia, 2% hematocrit), synchronized at the trophozoite stage were loaded with [^{3}H]chloroquine in complete medium, under gas, for 30 min at 37°. The loaded cells then were separated from the medium by centrifugation, and the cell pellet was lysed by quick freezing in liquid nitrogen, followed by thawing at 5–7°C. This procedure was repeated, and the cell debris was diluted into 50 volumes of distilled water buffered to pH 7.4 with 5 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (temperature, 5–7°C) containing antimalarial drugs or chemosensitizers at the appropriate concentrations and shaken vigorously for 20 sec. Samples were centrifuged in a microcentrifuge (12,000 × *g* for 5 min at 4°); a sample of the supernatant was taken for measurement, and the remainder was discarded. The cell debris was processed for scintillation counting as described previously (Bray *et al.*, 1994). The water space associated with the cell debris was estimated by using ^{14}C-inulin, and the pellet counts were corrected accordingly.

Under these conditions and in the time taken to perform the experiments (7 min), ~50% of the initial saturable uptake of the intact cells was retained in the pelleted debris. We assume that membrane integrity has been completely disrupted in this procedure because (1) parallel preparations in which the distilled water contained 2% Triton X-100 retained exactly the same amount of chloroquine, and (2) cell debris did not exhibit significant accumulation when the drug was added after lysis (data not shown). Like Fitch and Chevli, (1981), who described a similar procedure using erythrocytes infected with *Plasmodium berghei*, we observed that the association of chloroquine with cell debris is transient ($t_{1/2}$ = ~15 min at 5–7°C). At higher temperatures, the loss of bound chloroquine from the pellet was much more rapid (data not shown).

Results

Chloroquine is not transported as a simple sodium substitute by the parasite Na^+/H^+ exchanger. Based on the inhibition of saturable chloroquine uptake by EIPA (a specific blocker of the plasma membrane Na^+/H^+ exchanger), it recently has been proposed that chloroquine is actively imported into the parasite via the Na^+ binding domain of the Na^+/H^+ exchanger, instead of sodium and in exchange of protons (Sanchez *et al.*, 1997). If this hypothesis is correct, removal of competing substrate (sodium) should increase chloroquine accumulation. In fact, chloroquine accumulation is decreased significantly when sodium in the medium is replaced by choline or *N*-methyl-D-glucamine (Fig. 1), suggesting that chloroquine is not simply substituted for sodium as a substrate for proton exchange. Furthermore, phenotypic differences in the accumulation of chloroquine by chloroquine-susceptible and -resistant clones are largely maintained in sodium-free buffer. For example, the HB3 chloroquine-susceptible clone takes up three to four times more chloroquine than the K1 chloroquine-resistant clone in sodium-free buffer, and clone-specific differences in chloroquine accumulation due to chemosensitizers such as verapamil and daunomycin are maintained in sodium-free buffer (Fig. 1).

Saturable chloroquine binding characteristics of resistant and susceptible strains. We found that all six strains tested exhibited both saturable and nonsaturable components of chloroquine accumulation. Fig. 2, *top*, shows representative derived saturable accumulation data from experiments using the K1 chloroquine-resistant clone and the HB3 chloroquine-susceptible clone.

Examination of Fig. 2 suggests that there are differences in

the chloroquine binding parameters of the two clones. The saturable equilibrium binding of chloroquine to both chloroquine-susceptible and -resistant clones can be described by the Michaelis-Menten equation, which was applied using an iterative least-squares method (Roberts, 1977). The results for all the strains are presented in Table 1. The saturable binding capacity is the same in resistant and susceptible strains, with mean values of 33.93 μM for the chloroquine-susceptible strains and 33.13 μM for the chloroquine-resistant strains. There is little difference in the nonsaturable accumulation ratio; the mean for chloroquine-resistant strains is 220 versus 288 for chloroquine-susceptible strains. The major difference between susceptible and resistant strains is in the apparent K_d value of saturable chloroquine binding, with mean values of 22.1 and 177 nM, respectively. Verapamil caused a 4-fold average decrease in the apparent K_d value of resistant strains (mean value, 43 nM) but was without effect on either the measured capacity or the amount of nonsaturable uptake. Verapamil was not found to significantly affect the accumulation of chloroquine by any of the chloroquine-susceptible strains (data not shown).

Analysis of the data in Fig. 2, *top*, using a Hill plot (Fig. 2, *bottom*) revealed Hill coefficients of 1.015 ± 0.036 for HB3, 0.964 ± 0.023 for K1 without verapamil, and 0.950 ± 0.020 for K1 in the presence of 5 μM verapamil. These data indicate that *P. falciparum* has a receptor with a single chloroquine binding site and that verapamil acts specifically to increase the apparent affinity of chloroquine binding at this single binding site. We have also examined the effects of other potential resistance modulators, including daunomycin and primaquine (Table 1). It can be seen that both daunomycin and primaquine act in the same way as verapamil, in that the affinity of the saturable chloroquine accumulation is significantly increased, whereas nonsaturable chloroquine accumulation is totally unaffected. Primaquine also produces a marked "resistance reversal" effect that is in line with the effect of the drug on saturable chloroquine accumulation. Unfortunately, it was not possible to test the resistance reversing potential of daunomycin due to the high inherent antimalarial activity of this compound (Table 2).

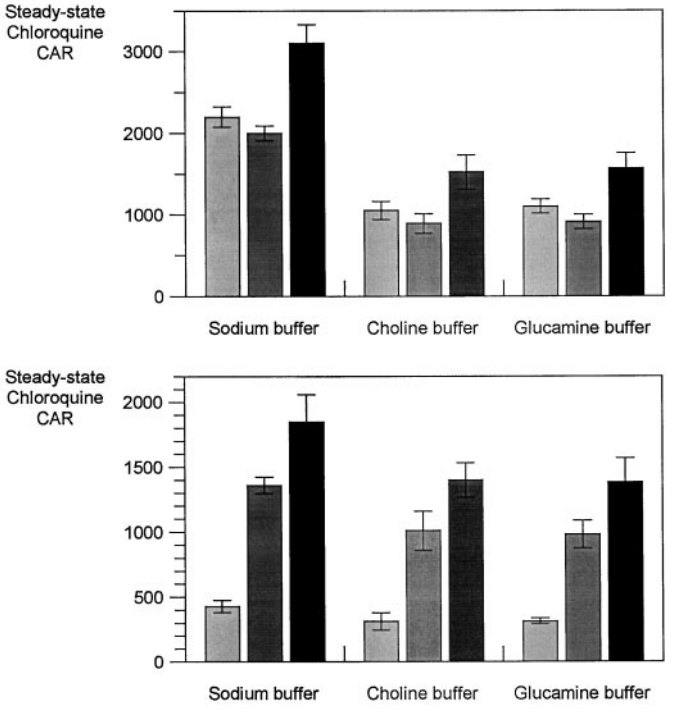

Fig. 1. Effect of medium sodium on steady state chloroquine accumulation. Steady state cellular accumulation ratio of [^{3}H]chloroquine in the HB3 clone (*top*) or the K1 clone (*bottom*), determined in medium containing sodium, choline, or *N*-methyl-D-glucamine in the absence of chemosensitizers (*light shading*) or the presence of 10 μM verapamil (*intermediate shading*) or 5 μM daunomycin (*dark shading*). Data represent mean ± standard deviation values of single observations from 15 separate experiments.

The relationship of chloroquine accumulation and activity. Fig. 3, *top*, depicts the relationship between total cellular chloroquine accumulation (measured at external concentrations equivalent to the IC_{50}) and activity (taken as the IC_{50}) for all the strains in the presence or absence of verapamil. The relationship is nonlinear and can be fitted to a double-exponential curve as illustrated. It is clear that neither the degree of resistance nor the reversal of resistance can be adequately explained by differences in the simple total cellular accumulation of chloroquine.

On the other hand, the activity of chloroquine is well correlated with the apparent K_d value of saturable binding (r^2 = 0.93, $p < 0.0001$, Fig. 3, *bottom*). Furthermore, the decrease in the chloroquine IC_{50} brought about by verapamil is reflected in a corresponding decrease in the apparent K_d value of saturable chloroquine binding. Because the extent of chlo-

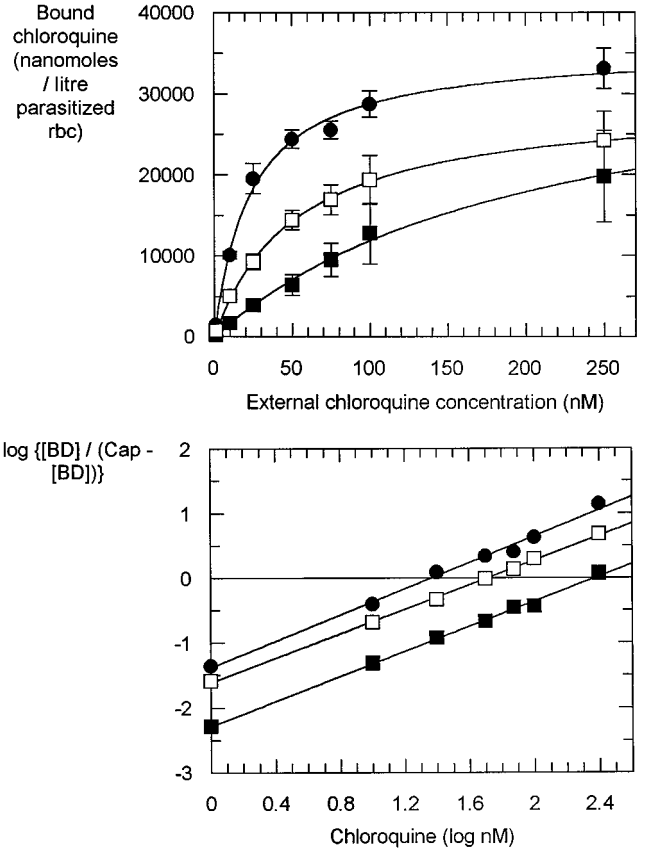

Fig. 2. Characterization of the saturable equilibrium binding of chloroquine. *Top*, equilibrium binding of [^{3}H]chloroquine was determined as a function of the external chloroquine concentration. Data are presented for the HB3 (CQS, ●) and the K1 (CQR, ■) clones. Also plotted are data for the K1 clone in the presence of 5 μM verapamil (□). Data represent mean ± standard deviation values of observations from three to five separate experiments. *Bottom*, Hill plot of the data presented in Fig. 2, *top*.

roquine resistance and the extent of resistance reversal can be fully explained by changes in the apparent affinity of chloroquine binding, we believe that (1) the fundamental difference between chloroquine-susceptible and -resistant strains is the apparent affinity of the saturable binding component and (2) the resistance reversing effect of verapamil is due to an increase in the apparent affinity of saturable chloroquine binding.

As outlined in Materials and Methods, we used two simple methods of linearizing the relationship of drug accumulation and activity to validate the two-component model. If drug accumulation is measured at IC_{50}; then, both methods allow the calculation of the amount of drug that must be bound to the receptor to kill 50% of the parasites on average. Both methods also allow the calculation of the average nonsaturable component of chloroquine uptake. Because the ratio of saturable to nonsaturable chloroquine accumulation is assumed to vary between chloroquine-resistant and -susceptible strains and because linearization techniques will weight either resistant or susceptible strains, we have chosen a method that weights data from resistant strains and a method that weights data from susceptible strains. Method 1 (eq. 4) weights data from susceptible strains and is used to plot data in Fig. 4, *top*. The amount of drug bound to the receptor at IC_{50} is given by the slope as 13.46 ± 0.74 μM. This figure is in good agreement with the value of 14.33 ± 1.58 μM obtained from the *Y*-intercept of Fig. 4, *bottom*, plotted by method 2 (eq. 3), which weights data from chloroquine-resistant strains. It also can be seen that the amount of nonsaturable drug accumulation is similar using the two methods, giving accumulation ratios of 272 ± 34 and 248 ± 17 by methods 1 and 2, respectively. In both cases, the data fit the model well: least-squares linear regression $r^2 = 0.98$ for method 1, and least-squares linear regression $r^2 = 0.97$ for method 2. The good fit of the data to the model and the high degree of internal consistency suggest that nonsaturable uptake also is nonspecific in that the antimalarial activity of chloroquine is determined solely by the saturable component.

Displacement of transiently bound chloroquine. After lysis and centrifugation, pellets of cell debris were dark brown (implying the presence of hemozoin/hematin), contained no hemoglobin, and by light microscopy contained no intact erythrocytes or parasites. Cell debris from parasitized erythrocytes contained ~50% of the radioactivity associated with saturable chloroquine binding to intact infected erythrocytes, with concentrations several hundredfold greater than can be explained by the water space. In contrast, cell debris from preloaded uninfected erythrocytes contained no more chloroquine than that due to the water space. The nonsaturable component of chloroquine accumulation seen with intact infected cells was completely lost when using the cell debris. The radiolabeled chloroquine recovered reflected only the saturable component of uptake into intact cells (Fig. 5). The apparent K_d value of the chloroquine bound to the cell debris (21. 56 nM) was very similar to the apparent K_d value of chloroquine bound to intact cells (21.0 nM, Table 1), although the capacity was reduced to about half of that measured with intact cells (17.3 ± 2.9 μM versus 34.0 ± 12 μM). The inability to recover all the saturable radioactivity is due to the transient nature of binding, which decreases progressively with time (data not shown).

These data support the hypothesis that *P. falciparum* parasites contain a saturable chloroquine binding site, as demonstrated previously for *P. berghei* parasites (Fitch and Chevli, 1981). It is quite clear that the binding cannot be attributed to either proton trapping or active transport.

We tested the ability of antimalarials and chemosensitizers to displace the [^{3}H]chloroquine bound to cell debris. Fig. 6, *top*, represents data for the CQS clone HB3, and Fig. 6, *bottom*, represents data for the CQR clone K1. Because of the transient nature of chloroquine binding, the data are only semiquantitative and reflects the ability of drugs to displace bound [^{3}H]chloroquine in the fixed time required for suspension and centrifugation of the cell debris (7 min) rather than the establishment of a true equilibrium. Nevertheless, it is clear that [^{3}H]chloroquine binding is specific, as evidenced by the ability of nanomolar concentrations of nonradiolabeled chloroquine and amodiaquine to displace it. Furthermore, the concentrations of these drugs required for half-maximal displacement of [^{3}H]chloroquine (15–30 nM) were very similar for preparations from CQS and CQR clones, suggesting that the receptor itself is unchanged in drug-resistant strains. On the other hand, much higher concentrations of chemosensitizers had no significant effect on the amount of bound chloroquine, indicating that these compounds increase the apparent affinity of chloroquine binding by an indirect mechanism that is dependent on the integrity of parasite membranes.

Chloroquine accumulation is reduced by an inhibitor of plasmepsin I. Data presented in Fig. 7 for the HB3 clone show that chloroquine accumulation by intact parasitized erythrocytes is markedly reduced by the plasmepsin I inhibitor Ro 40–4388 in a concentration-dependent manner. A similar effect was seen with the K1 chloroquine-resistant

TABLE 1

Comparison of the antimalarial activity and saturable binding characteristics of chloroquine in the presence and absence of verapamil

Saturable binding parameters were determined as described in Experimental Procedures. Errors indicated for capacity and apparent K_d are standard errors of fitting data to eq. 2. IC_{50} values are mean values of five experiments, each performed in triplicate; errors indicate the standard deviation.

Strain and protocol	Capacity	Apparent K_d	IC_{50}	Nonsaturable accumulation ratio
	μM	nM external	nM	
HB3 (pH 7.4)	34.0 ± 1.2	21.0 ± 2.4	13.0 ± 2.6	300
HB3 (pH 7.7)	29.3 ± 0.9	8.2 ± 1.3	N.D.	560
3D7	38.1 ± 2.8	25.9 ± 5.7	13.0 ± 4.0	297
T9-96	29.7 ± 1.0	19.5 ± 2.3	15.0 ± 3.5	278
K1	37.0 ± 8.1	215.0 ± 80.0	210.0 ± 38.0	250
K1 + VP	35.2 ± 5.5	67.0 ± 20.0	47.0 ± 12.0	248
K1 + PQ	30.5 ± 2.9	86.4 ± 14.1	84.8 ± 9.0	245
K1 + DAU	35.2 ± 2.3	30.2 ± 5.3	N.D.	251
TM5	32.9 ± 9.7	178.0 ± 73.8	112.0 ± 27.0	200
TM5 + VP	30.8 ± 2.5	35.0 ± 7.2	30.4 ± 14.0	203
TM6	29.5 ± 5.3	139.0 ± 38.0	128.0 ± 33.0	210
TM6 + VP	31.3 ± 5.6	27.8 ± 12.8	33.0 ± 14.0	209

TABLE 2

Lack of cross resistance of chloroquine and resistance modulators

The chloroquine data represent mean ± standard deviation of data from five separate sensitivity assays, each performed in triplicate. Data for the other drugs represents mean values of two separate sensitivity assays, each performed in triplicate.

Strain	CQ IC_{50}	VP IC_{50}	PQ IC_{50}	DAU IC_{50}
	nM	μM	μM	nM
K1	210 ± 38	16.7	2.04	310
TM6	128 ± 33	13.8	2.03	257
TM5	112 ± 27	21.3	5.13	395
1952	30 ± 5.8	20.1	4.3	420
HB3	13 ± 2.6	5.05	23.1	365

clone (data not shown). In contrast, leupeptin, a nonspecific cysteine proteinase inhibitor, did not affect chloroquine accumulation. These results suggest that a large proportion of chloroquine accumulation by CQS strains could be due to the binding of chloroquine to hematin because plasmepsin I is the enzyme responsible for the initial cleavage of hemoglobin, so the inhibition of this enzyme also would be expected to inhibit the supply of hematin.

Data presented in Fig. 8 demonstrate that the effect of Ro 40–4388 is specific for chloroquine and amodiaquine, drugs that have been shown to bind hematin with high affinity (Chou *et al*., 1980) and displace [^{3}H]chloroquine bound to cell debris (Fig. 6). The proteinase inhibitor has no effect on the accumulation of [^{14}C]primaquine, [^{3}H]daunomycin, or [^{3}H]verapamil, which are weak bases like chloroquine and amodiaquine, but do not bind to hematin and do not displace chloroquine bound to cell debris (Sugioka and Suzuki, 1991; Gabay *et al*., 1994; Fig. 6). The specificity of Ro 40–4388 for hematin binding drugs argues against a vacuolar pH increase caused by disruption of vacuolar function, which would reduce the accumulation of all weak base drugs.

Further support for a specific effect of Ro 40–4388 is presented in Fig. 9, a Scatchard plot of saturable equilibrium chloroquine binding of infected red blood cells in the presence or absence of Ro 40–4388. According to the model outlined in Materials and Methods, changes in the pH gradient would be expected to alter the apparent affinity of saturable binding and not the number of binding sites (exactly what is observed when the pH gradient is altered experimentally; see Table 1). It is clear that the proteinase inhibitor causes a marked and significant reduction in the number of binding sites (given by the *X*-axis intercept) from 34.6 ± 2.04 μM to 13.52 ± 0.44 μM but has no significant effect on the apparent affinity of binding (K_d calculated from the slopes as 17.1 ± 2.76 nM in the absence of the inhibitor and 15.48 ± 1.4 nM with the inhibitor present).

Data presented in Table 1 suggested that resistance modulators specifically increase the saturable binding of chloroquine. If this is true and the site of saturable binding is hematin, then the enhanced accumulation of chloroquine due to verapamil should be inhibitable by Ro 40–4388. It can be seen from Fig. 10 that this is indeed the case. It is clear that the verapamil effect is completely abrogated in the presence of the protease inhibitor, suggesting that verapamil acts specifically to produce increased binding of chloroquine to hematin. All of the data presented above indicate that the chloroquine-resistance mechanism of *P. falciparum* is specific for drugs that bind to hematin. This argument is reinforced by a lack of cross-resistance of chloroquine with other drugs that interact with the resistance mechanism but do not bind to hematin (Table 2). We measured the susceptibility of a panel of strains that differ by ~16-fold in their susceptibility to

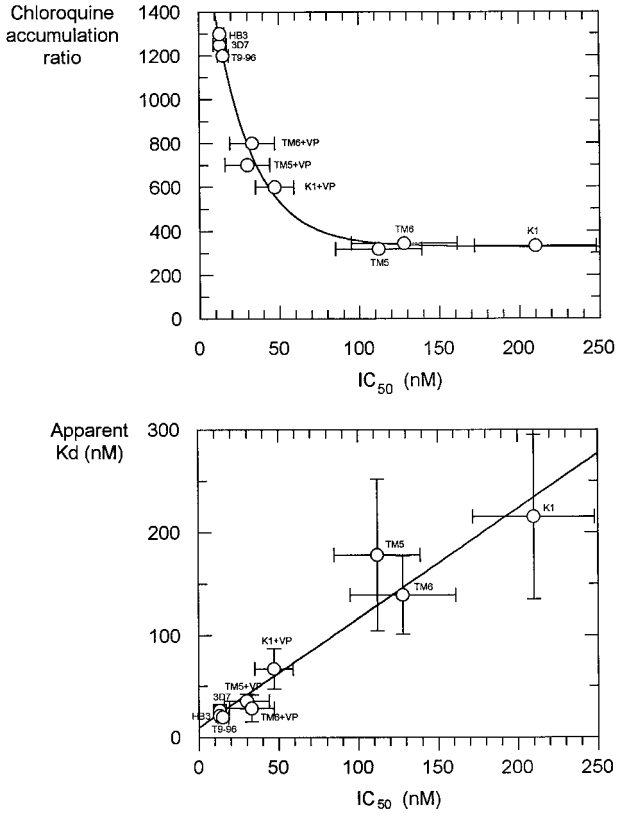

Fig. 3. The activity of chloroquine is linearly related to the apparent receptor K_d rather than total cellular chloroquine accumulation. *Top*, total cellular accumulation ratio at IC_{50} was interpolated from graphs of chloroquine accumulation versus external chloroquine concentration. *Line*, fit of the data to the double exponential equation: $y = I_1 \cdot e^{-k1 \cdot x} + I_2 \cdot e^{-k2 \cdot x}$, where I_1 = initial value 1 (at $x = 0$) = 335, k_1 = rate 1 = 0.0001, I_2 = initial value 2 (at $x = 0$) = 1606, and k_2 = rate 2 = 0.0416. This equation has no theoretical justification but has a suitable mathematical form to allow a smooth curve to run through the data. IC_{50} values are mean values of five independent experiments. *Bottom*, apparent affinity data were taken from Table 1. *Line*, least-squares linear regression fit to the data.

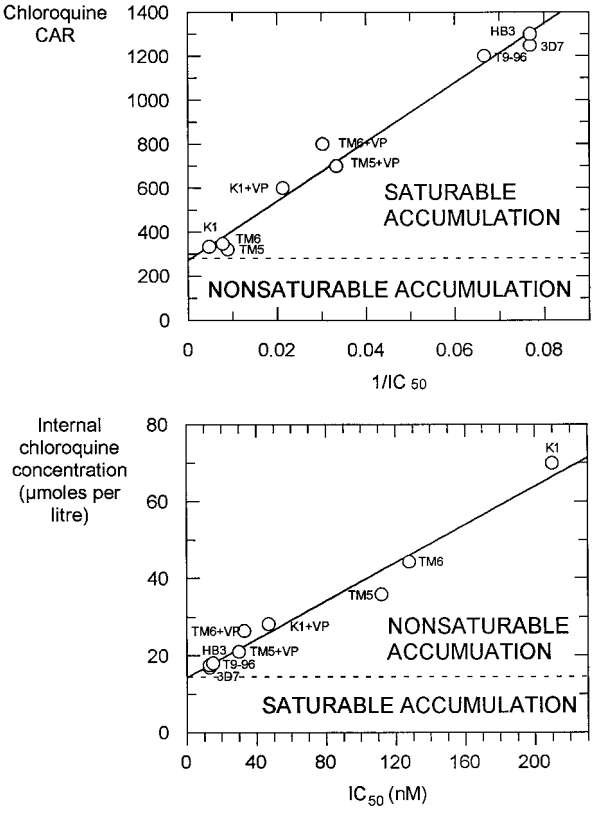

Fig. 4. Validation of the two-component model. *Top*, data from three CQS strains and three CQR strains with and without 5 μM verapamil were fitted to eq. 4. *Line*, least-squares linear regression fit to the data. *Bottom*, data from three CQS strains and three CQR strains with and without 5 μM verapamil were fitted to eq. 3. *Line*, least-squares linear regression fit to the data.

chloroquine and found no cross-resistance with verapamil (r^2 = 0.128), daunomycin (r^2 = 0.372), or primaquine (r^2 = 0.449).

Discussion

Data presented in this report provide evidence that the saturable component of chloroquine accumulation is responsible for the antimalarial activity of the drug [i.e., the K_d value of saturable uptake and the IC_{50} are well correlated (Fig. 3, *bottom*)]. Although the binding of chloroquine to hematin is an extremely persuasive explanation for saturable chloroquine accumulation, there are at least two other theories that deserve serious consideration.

First, we considered that the proton gradient into the food vacuole drives the uptake of weakly basic antimalarials (Yayon *et al.*, 1985). It has been amply demonstrated that the vacuolar pH gradient is important for the accumulation of chloroquine and related drugs (Yayon *et al.*, 1985; Geary *et al.*, 1990; Bray *et al.*, 1992a; Martiney *et al.*, 1995; Hawley *et al.*, 1996c). It also has been demonstrated that these drugs titrate protons inside the food vacuole and raise the vacuolar pH (Krogstad *et al.*, 1985; Yayon *et al.*, 1985; Ginsburg *et al.*, 1989). Therefore, it is quite possible that the saturable chloroquine accumulation observed with intact cells could stem from a saturation of the vacuolar proton pumping capacity (Ginsburg and Stein, 1991) rather than a saturation of chloroquine-hematin binding, and this possibility was considered in the current work.

We also have considered the possibility that both the saturability and the specificity of chloroquine accumulation are attributable to a drug importer or permease in the parasite (Warhurst, 1986). In support of this theory, it was proposed recently that the parasite Na^+/H^+ exchanger is responsible for saturable chloroquine uptake (Sanchez *et al.*, 1997). Two transport mechanisms have been proposed: (1) chloroquine could be an alternative substrate for the exchanger, substituting for sodium in direct exchange for protons (Sanchez *et al.*, 1997), or (2) chloroquine could be carried through the exchanger in a burst of sodium/proton exchange stimulated by chloroquine itself (Wünsch *et al.*, 1998).

Sodium-free buffer produces a rapid and reversible acidification of the parasite cytosol by ≥0.5 pH unit (Bosia *et al.*, 1993). The cytosolic pH of the parasite in sodium buffer is at most 0.2 pH unit higher than the pH of the host cell cytosol (Wünsch *et al.*, 1998). Therefore, in sodium-free buffer containing 1 nM chloroquine, there is a huge molar excess of protons available to drive chloroquine uptake by a proton exchange mechanism (from values for cytosolic buffer capacity given in Wünsch *et al.*, 1998, a gradient of 0.3 pH unit will provide ~50 mmol of protons for every nmol of chloroquine). In a situation of direct chloroquine/proton exchange, this would predict 5×10^7-fold drug accumulation. In fact, chloroquine accumulation in sodium-free medium is >4 orders of magnitude lower than this prediction (Fig. 1). Furthermore, instead of being enhanced by sodium-free buffer compared with sodium buffer (as might be expected if a competing substrate is removed), chloroquine accumulation actually is reduced significantly (Fig. 1). These data indicate that chloroquine is not a substrate for direct stoichiometric proton exchange. Nevertheless, a significant amount of chloroquine is taken up in the absence of sodium by both chloroquine-susceptible and -resistant parasites (Fig. 1). This uptake is saturable, stimulated by chemosensitizers, and uniquely de-

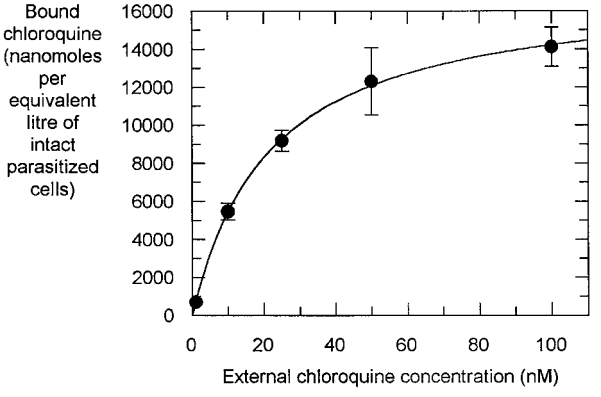

Fig. 5. Saturable chloroquine binding associated with cell debris. Cells infected with the HB3, CQS clone were preloaded with [^{3}H]chloroquine for 1 hr, at 37°, in the presence of increasing concentrations of nonradiolabeled chloroquine as indicated. Cells were lysed, and the associated radioactivity was counted as described in Experimental Procedures. Values are mean ± standard deviation of duplicate observations from three separate experiments.

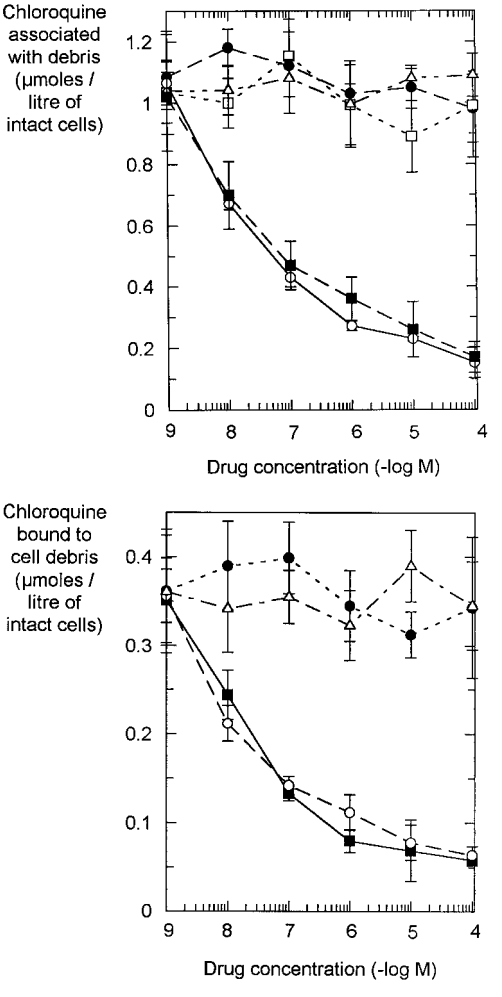

Fig. 6. Displacement of chloroquine bound to cell debris from cells preloaded with [^{3}H]chloroquine. Cell debris was exposed to verapamil (●), primaquine (□), daunomycin (△), amodiaquine (○), or nonradiolabeled chloroquine (■ at the concentrations indicated. *Top*, data for the HB3, CQS clone. *Bottom*, data for the K1, CQR clone. Results represent mean ± standard deviations of three independent experiments.

pendent on the availability of hematin (Bray PG, Mugthin M, and Ward SA. Verapamil-sensitive chloroquine resistance is not determined by the Na^+/H^+ exchanger in *Plasmodium falciparum*, manuscript in preparation). With regard to the second proposed active uptake mechanism outlined above, it is difficult to see how this saturable chloroquine uptake could in any way be attributed to a burst of rapid sodium/hydrogen exchange on stimulation of the Na^+/H^+ exchanger. Finally, the specific chloroquine accumulation/chemosensitization phenotype of chloroquine-susceptible and -resistant clones is preserved in sodium-free medium (Fig. 1), suggesting that the chloroquine-resistance phenotype may be attributable to mutation of proteins other than the Na^+/H^+ exchanger. We believe that these data cast doubt on the involvement of direct chloroquine transport through the parasite Na^+/H^+ exchanger in both the uptake of chloroquine by the parasite and in the mechanism of chloroquine resistance. However, in keeping with the large body of evidence showing that pH gradients influence the accumulation of chloroquine (see above), we acknowledge that any of the pH regulation mechanisms of the parasite (including the Na^+/H^+ exchanger) may be indirectly involved in the uptake of chloroquine.

The techniques and new inhibitor used in this study have, for the first time, allowed discrimination between the proposed mechanisms of drug accumulation in *P. falciparum*. The substantial amount of saturable chloroquine accumulation associated with the cell debris (Figs. 5 and 6) can only reflect chloroquine binding to hematin rather than proton trapping or active uptake, both of which require a high degree of membrane integrity. Much of the heme/chloroquine complex undoubtedly will be present in the supernatant as well as the cell debris, but our results are in line with the demonstrated ability of hematin and hematin aggregates to bind chloroquine while adsorbed to cell debris (Chou *et al.*, 1980).

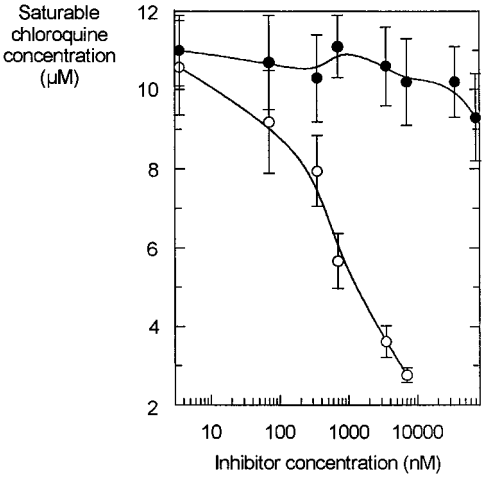

Fig. 7. The effect of increasing concentrations of the protease inhibitors leupeptin and Ro 40–4388 on the accumulation of chloroquine by the HB3 clone of *Plasmodium falciparum*. Drug accumulation was measured over 1 hr after a 5-min preincubation with the protease inhibitors leupeptin (●) or Ro 40–4388 (○). Values are mean ± standard deviation of single observations from five separate experiments.

Ro 40–4388 is a potent and specific inhibitor of plasmepsin I (Moon *et al.*, 1997), the *P. falciparum* aspartic proteinase that is responsible for the initial cleavage of hemoglobin and release of free heme (Francis *et al.*, 1994). Therefore, our proteinase inhibitor studies have established that the saturable chloroquine accumulation is dependent on the initial cleavage of hemoglobin and release of heme (Figs. 7 and 9). The lack of effect of this inhibitor on the accumulation of other weak base drugs that do not bind hematin argues against a nonspecific effect on the vacuolar pH (Fig. 8). Finally, it is difficult to see how this specific aspartic proteinase inhibitor could reduce the capacity of the proposed chloroquine importer. Although we acknowledge the importance of the pH gradient for the overall process of chloroquine accumulation, we propose that the saturable chloroquine accumulation that is responsible for the specific activity of chloroquine (Fig. 3) is attributable solely to chloroquine-hematin binding.

Free hematin exists only transiently *in situ*, and the amount available for drug binding is difficult to measure. Accordingly, the hematin binding hypothesis has been questioned on quantitative grounds when the rate of hemoglobin digestion was found to be insufficient to account for the large total amount of chloroquine accumulated by infected cells (Ginsburg and Geary, 1987). These calculations were made using measurements of whole-cell accumulation. No attempt was made to distinguish saturable accumulation from nonsaturable accumulation. This distinction is critical as the major proportion of chloroquine accumulation in resistant strains is nonsaturable (i.e. not bound to hematin) (Fig. 4, *bottom*). In fact, using published estimates of the rate of hemoglobin digestion (Ginsburg and Geary, 1987), we calculate that only ~4% of the heme turnover would need to be available to bind chloroquine to account for our measurements of saturable uptake, provided we assume that most of the hemoglobin digestion occurs in the mid to late trophozoites. These data are in agreement with the small effect of chloroquine on the polymerization of hematin that is observed *in situ* as the parasites are killed (Meshnick, 1996). The important question remains as to how interaction of chloroquine with hematin leads to parasite death. Our data are consistent with a suggestion that the formation of chloroquine/hematin complexes, rather than a build-up of free

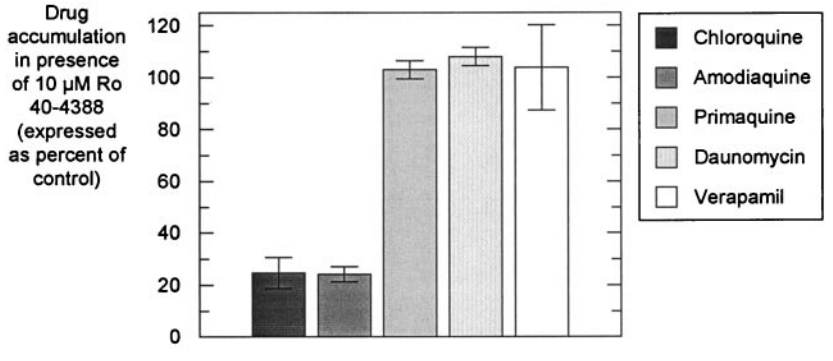

Fig. 8. The effect of Ro 40–4388 is specific for weak base drugs that bind to hematin. Accumulation of radiolabeled drugs was measured over 1 hr after a 5-min preincubation with 10 μM Ro 40–4388. *Histogram bars*, mean ± standard deviation values of single observations from five separate experiments.

hematin, is the prime cause of cell killing and that hematin polymerization is only a secondary consequence of this. We are currently investigating a hypothesis that enhanced lipid solubility of the chloroquine/hematin complex (Ward SA, unpublished observations) allows it to escape from the food vacuole along a concentration gradient. Once the vacuolar membrane has been crossed, the elevated pH and lower hematin concentrations in the cytosol would make polymerization much less likely, and the hematin or drug/hematin complex then could interact with a wide range of vital cellular targets.

In terms of the mechanism of chloroquine resistance, the capacity of chloroquine-hematin binding is the same for both chloroquine-susceptible and -resistant strains and is not altered by modulators of chloroquine resistance (Table 1). These observations are incompatible with chloroquine resistance mediated by a mechanism of reduced production of heme, accelerated sequestration of hematin, or peroxide-mediated decomposition of heme (Fitch, 1989). Thus, several theories to explain chloroquine resistance can now be ruled out. Our data demonstrate that chloroquine resistance is associated with reduced apparent affinity of chloroquine-hematin binding rather than changes in capacity (Table 1). It is difficult to envisage a mechanism by which the true affinity of hematin for chloroquine could be altered by the parasite. Consequently, our data suggest that resistant parasites have evolved a mechanism to reduce the accessibility of hematin rather than alter its structure (Fig. 6).

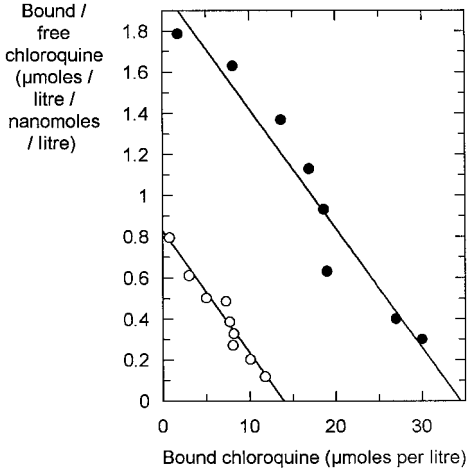

Fig. 9. Scatchard plot of saturable equilibrium chloroquine binding to hematin. Binding was measured in the presence (○) or absence (●) of 5 µM Ro 40–4388 using the HB3 chloroquine-susceptible clone. Saturable chloroquine binding was determined as described in Experimental Procedures. Values are mean of single observations from three separate experiments. *Lines*, least-squares linear regression fit to the data points.

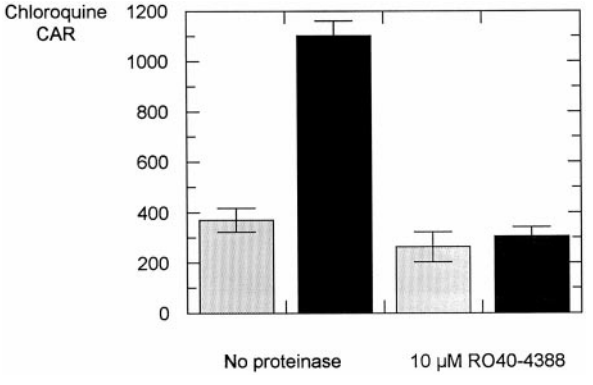

Fig. 10. Ro 40–4388 inhibits the verapamil effect. The K1 clone parasites were preincubated in the presence or absence of 10 µM Ro 40–4388 for 5 min. Samples from each group were incubated for 1 hr with 1 nM [^{3}H]chloroquine in the presence (*dark shading*) or absence (*light shading*) of 5 µM verapamil. *Histogram bars*, mean ± standard deviation of single observations from five separate experiments.

It was largely due to the publication of many data demonstrating pH-dependent uptake and proton trapping that the chloroquine-hematin binding hypothesis has fallen from favor (Ginsburg and Geary, 1987). However, because the pH gradient plays a role in concentrating the amount of chloroquine at the site of hemoglobin degradation, we believe that the two hypotheses are compatible. The apparent affinity of chloroquine binding can be readily altered by manipulating the pH gradient from outside or inside. For example, the apparent affinity is more than doubled by increasing the medium pH by 0.3 pH unit (Table 1). A widely supported model of chloroquine resistance predicts that resistant parasites have an elevated vacuolar pH (Ginsburg and Stein, 1991). Mathematical models suggest that increased vacuolar pH will reduce the rate of uptake of chloroquine (Ferrari and Cutler, 1991; Ginsburg and Stein, 1991). In support of this, it would be fair to say that the majority of kinetic studies measure a reduced rate of chloroquine accumulation into chloroquine-resistant compared with chloroquine-susceptible strains (Ginsburg and Stein, 1991; Bray *et al.*, 1992a, 1994, 1996; Martiney *et al.*, 1995; Sanchez *et al.*, 1997; Wünsch *et al.*, 1998). Such observations are entirely compatible with our interpretation of the data in this report. Because heme is being released and hematin is sequestered at a fixed rate, any process that reduces the rate of uptake of chloroquine would reduce the relative amount of drug available to bind hematin per unit time. This would reduce the apparent affinity and extent of saturable drug accumulation at steady state.

The "resistance reversing" effects of verapamil pose problems for all the current theories of chloroquine resistance. First, it is difficult to see how verapamil could decrease vacuolar pH as required by the pH model, and second, the increased cellular chloroquine accumulation brought about by verapamil is much too small to explain resistance reversal by simple inhibition of rapid efflux (Bray *et al.*, 1994). The lack of an unifying hypothesis to account for both chloroquine resistance and resistance reversal prompted the proposal that verapamil-sensitive chloroquine resistance may be multifactorial (Ginsburg and Stein, 1991; Bray *et al.*, 1994; Sanchez *et al.*, 1997). Our data correlate both chloroquine resistance and the verapamil effect with the apparent affinity of chloroquine/hematin binding, suggesting a common mechanism (Fig. 3). Although our data do not rule out the involvement of multiple genes in the process of chloroquine resistance, our findings are in accord with a single genetic locus for the verapamil effect and chloroquine resistance phenotype, as demonstrated in a genetic cross (Wellems *et al.*, 1991). At the molecular level, it is possible that the candidate resistance protein CG2 (Su *et al.*, 1997) alters the cellular drug distribution directly by pumping drug out of the infected cell (Krogstad *et al.*, 1987). It is more likely however, given the data presented here, that such a protein acts specifically at the target site, either by altering *trans*-vacuolar ion gradients or possibly by binding to free hematin itself and reducing the accessibility to chloroquine.

The chloroquine-hematin binding hypothesis was first proposed in the 1960s by Macomber *et al*., (1967) and later championed by Fitch (1989). Although compelling, the hypothesis was never proved, and many other target molecules were proposed (Ginsburg and Geary, 1987). The data presented here finally provide good evidence in support of the original hypothesis. The unparalleled success of chloroquine, before the evolution and spread of resistance, is testimony to what can be achieved by targeting the process of hemoglobin catabolism in the parasite. Our data suggest that *P. falciparum* is uniquely vulnerable to hematin binding drugs in that it cannot alter the quantity or the nature of the hematin target. Instead, the parasite has evolved in a way to reduce the accessibility of hematin to some of these drugs. Regardless of how such resistance is achieved at the molecular level, it is evident that the resistance mechanism can be overcome by relatively simple alterations of the basic 4-aminoquinoline structure (Bray *et al*., 1996; De *et al*., 1996; Hawley *et al*., 1996b; Ridley *et al*., 1996). For these reasons, we believe that great priority must be given to the development of novel hematin-binding agents.

Acknowledgments

We thank Profs. Hagai Ginsburg and W. D. Stein for helpful discussion of the data herein.

References

Balasubramanian D, Mohan Rao C, and Panjipan B (1984) The malaria parasite monitored by photoacoustic spectroscopy. *Science (Washington DC)* **223:**828–830.

Bosia A, Ghigo D, Turrini F, Nissani E, Pescarmona GP, and Ginsburg H (1993) Kinetic characterization of Na^+/H^+ antiport of *Plasmodium falciparum* membrane. *J Cell Physiol* **154:**527–534.

Bray PG, Boulter MK, Ritchie GY, and Ward SA (1994) Relationship of global chloroquine transport and reversal of resistance in *Plasmodium falciparum*. *Mol Biochem Parasitol* **63:**87–94.

Bray PG, Hawley SR, Mungthin M, and Ward SA (1996) Physicochemical properties correlated with drug resistance and the reversal of drug resistance in *Plasmodium falciparum*. *Mol Pharmacol* **50:**1559–1566.

Bray PG, Howells RE, Ritchie GY, and Ward SA (1992a) Rapid chloroquine efflux phenotype in both chloroquine-sensitive and chloroquine-resistant *Plasmodium falciparum*: a correlation of chloroquine sensitivity with energy-dependent drug accumulation. *Biochem Pharmacol* **44:**1317–1324.

Bray PG, Howells RE, and Ward S (1992b) Vacuolar acidification and chloroquine sensitivity in *Plasmodium falciparum*. *Biochem Pharmacol* **43:**1219–1227.

Chou AC, Chevli R, and Fitch CD (1980) Ferriprotoporphyrin IX fulfils the criteria for identification as the chloroquine receptor of malaria parasites. *Biochemistry* **19:**1543.

De D, Krogstad FM, Cogswell FB, and Krogstad DJ (1996) Aminoquinolines that circumvent chloroquine resistance in *Plasmodium falciparum*. *Am J Trop Med Hyg* **55:**579–583.

Desjardins RE, Canfield J, Haynes D, and Chulay JD (1979) Quantitative assessment of antimalarial activity *in vitro* by a semi-automated microdilution technique. *Antimicrob Agents Chemother* **16:**710–718.

Dorn A, Stoffel R, Matile H, Bubendorf A, and Ridley RG (1995) Malarial haemozoin *beta*-haematin supports haeme polymerization in the absence of protein. *Nature (Lond)* **374:**269–271.

Dorn A, Vippagunta SR, Matile H, Bubendorf A, Vennerstrom JL, and Ridley RG (1998) A comparison and analysis of several ways to promote hematin (heme) polymerisation and an assessment of its initiation *in vitro*. *Biochem Pharmacol* **55:**737–747.

Ferrari V and Cutler DJ (1991) Simulation of kinetic data on the influx and efflux of chloroquine by erythrocytes infected with *Plasmodium falciparum*: evidence for a drug-importer in chloroquine-sensitive strains. *Biochem Pharmacol* **42:**S167–S179.

Fitch CD (1970) *Plasmodium falciparum* in owl monkeys: drug resistance and chloroquine binding capacity. *Science (Washington DC)* **169:**289–290.

Fitch CD (1989) *Malaria and the Red Cell 2.* pp 45–52, Alan R. Liss, New York.

Fitch CD and Chevli R (1981) Sequestration of the chloroquine receptor in cell-free preparations of erythrocytes infected with *Plasmodium berghei*. *Antimicrob Agents Chemother* **19:**589–592.

Francis SE, Gluzman IY, Oksman A, Knickerbocker A, Mueller R, Bryant ML, Sherman DR, Russell DG, and Goldberg DE (1994) Molecular characterization and inhibition of a *Plasmodium falciparum* aspartic hemoglobinase. *EMBO J* **13:**306–317.

Gabay T, Krugliak M, Shalmiev G, and Ginsburg H (1994) Inhibition by antimalarial drugs of haemoglobin denaturation and iron release in acidified erythrocytes: possible mechanism of their antimalarial effect. *Parasitology* **108:**371–381.

Geary TG, Divo AD, Jensen JB, Zangwill M, and Ginsburg H (1990) Kinetic modeling 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. *Biochem Pharmacol* **40:**685–691.

Ginsburg H and Geary TG (1987) Current concepts and new ideas on the mechanism of action of quinoline-containing antimalarials. *Biochem Pharmacol* **36:**1567–1576.

Ginsburg H and Stein WD (1991) Kinetic modeling of chloroquine uptake by malaria-infected erythrocytes: assessment of the factors that may determine drug-resistance. *Biochem Pharmacol* **41:**1463–1470.

Ginsburg H, Nissani E, and Krugliak M (1989) Alkalinization of the food vacuole of malaria parasites by quinoline drugs and alkylamines is not correlated with their antimalarial activity. *Biochem Pharmacol* **38:**2645–2654.

Gluzman IY, Francis SE, Oksman A, Smith CE, Duffin KL, and Goldberg DE (1994) Order and specificity of the *Plasmodium falciparum* hemoglobin degradation pathway. *J Clin Invest* **93:**1602–1608.

Hawley SR, Bray PG, O'Neill PM, Park BK, and Ward SA (1996a) The role of drug accumulation in 4-aminoquinoline antimalarial potency: the influence of structural substitution and physicochemical properties. *Biochem Pharmacol* **52:**723–733.

Hawley SR, Bray PG, O'Neill PM, Naisbitt DJ, Park BK and Ward SA (1996b) Manipulation of the n-alkyl substituent in amodiaquine to overcome the verapamil-sensitive chloroquine resistance component. *Antimicrob Agents Chemother* **40:**2345–2349.

Hawley SR, Bray PG, Park BK, and Ward SA (1996c) Amodiaquine accumulation in *Plasmodium falciparum* as a possible explanation for its superior antimalarial activity over chloroquine. *Mol Biochem Parasitol* **80:**15–25.

Krogstad DJ, Gluzman IY, Kyle DE, Oduola AMJ, Martin SK, Milhous WK, and Schlesinger PH (1987) Efflux of chloroquine from *Plasmodium falciparum*: mechanism of chloroquine resistance. *Science (Washington DC)* **235:**1283–1285.

Krogstad DJ, Schlesinger PH, and Gluzman IY (1985) Antimalarials increase vesicle pH in *Plasmodium falciparum*. *J Cell Biol* **101:**2302–2309.

Macomber PB, Sprinz H, and Tousimis AJ (1967) Morphological effects of chloroquine on *Plasmodium berghei* in mice. *Nature (Lond)* **214:**937–939.

Martiney JA, Cerami A, and Slater AFG (1995) Verapamil reversal of chloroquine resistance in the malaria parasite *Plasmodium falciparum* is specific for resistant parasites and independent of the weak base effect. *J Biol Chem* **270:**22393–22398.

Menting JGT, Tilley L, Deady LW, Ng K, Simpson RJ, Cowman AF, and Foley M (1997) The antimalarial drug, chloroquine, interacts with lactate dehydrogenase from *Plasmodium falciparum*. *Mol Biochem Parasitol* **88:**215–224.

Meshnick S (1996) Is haemozoin a target for antimalarial drugs? *Ann Trop Med Parasitol* **90:**367–372.

Moon RP, Tyas L, Certa U, Rupp K, Bur D, Jacquet C, Matile H, Loetscher H, Grueninger-Leitch F, Kay J, Dunn BM, Berry C, and Ridley RG (1997) Expression and characterisation of plasmepsin I from *Plasmodium falciparum*. *Eur J Biochem* **244:**552–560.

Ridley RG, Hofheinz W, Matile H, Jacquet C, Dorn A, Masciadri R, Jolidon S, Richter WF, Guenzi A, Girometta MA, Urwyler H, Huber W, Thaithong S, and Peters W (1996) 4-Aminoquinoline analogs of chloroquine with shortened side-chains retain activity against chloroquine-resistant *Plasmodium falciparum*. *Antimicrob Agents Chemother* **40:**1846–1854.

Ridley RG, Dorn A, Vippagunta SR, and Vennerstrom, JL (1997) Haematin (haem) polymerisation and its inhibition by quinoline antimalarials. *Ann Trop Med Parasitol* **91:**559–566.

Roberts DV (1977) Simple enzyme catalysed reactions, in *Enzyme Kinetics* (Elmore DT, Leadbetter AJ, and Schofield K, eds) Cambridge University Press, Cambridge, UK.

Sanchez CP, Wünsch S, and Lanzer M (1997) Identification of a chloroquine importer in Plasmodium falciparum: differences in import kinetics are genetically linked with the chloroquine-resistant phenotype. *J Biol Chem* **272:**2652–2658.

Slater AFG (1993) Chloroquine: mechanism of drug-action and resistance in *Plasmodium falciparum*. *Pharmacol Ther* **57:**203–235.

Su X, Kirkman LA, Fujioka H, and Wellems TE (1997) Complex polymorphisms in an ~330 kDa protein are linked to chloroquine-resistant *P. falciparum* in Southeast Asia and Africa. *Cell* **91:**593–603.

Sugioka Y and Suzuki M (1991) The chemical basis for the ferriprotoporphyrin IX-chloroquine complex induced lipid peroxidation. *Biochim et Biophys Acta* **1074:**19–24.

Sullivan DJ Jr, Gluzman IY, Russell DG, and Goldberg DE (1996) On the molecular mechanism of chloroquine's antimalarial action. *Proc Natl Acad Sci USA* **93:**11865–11870.

Vander Jagt DL, Hunsaker LA, and Campos NM (1987) Comparison of proteases from chloroquine-sensitive and chloroquine-resistant strains of *Plasmodium falciparum*. *Biochem Pharmacol* **36:**3285–3291.

Warhurst DC (1986) Antimalarial schizontocides: why a permease is necessary. *Parasitol Today* **2:**331–334.

Wellems TE, Walker-Jonah A, and Panton LJ (1991) Genetic mapping of the chloroquine resistance locus on *Plasmodium falciparum* chromosome 7. *Proc Natl Acad Sci USA* **88:**3382–3386.

Wünsch S, Sanchez CP, Gekle M, Groβe-Wortmann L, Wiesner J, and Lanzer M (1998) Differential stimulation of the Na^+/H^+ exchanger determines chloroquine uptake in *Plasmodium falciparum*. *J Cell Biol* **140:**335–345.

Yayon A, Cabantchik ZI, and Ginsburg H (1985) Susceptibility of human malaria parasites to chloroquine is pH dependent. *Proc Natl Acad Sci USA* **82:**2784–2788.

Send reprint requests to: Dr. Stephen. A. Ward, Department of Pharmacology and Therapeutics, The University of Liverpool, Liverpool L69 3BX, UK. E-mail: saward@liv.ac.uk