Evidence for a Substrate Specific and Inhibitable Drug Efflux System in Chloroquine Resistant *Plasmodium falciparum* Strains†

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ABSTRACT: The mechanism underpinning chloroquine drug resistance in the human malarial parasite *Plasmodium falciparum* remains controversial. By investigating the kinetics of chloroquine accumulation under varying-trans conditions, we recently presented evidence for a saturable and energy-dependent chloroquine efflux system present in chloroquine resistant *P. falciparum* strains. Here, we further characterize the putative chloroquine efflux system by investigating its substrate specificity using a broad range of different antimalarial drugs. Our data show that preloading cells with amodiaquine, primaquine, quinacrine, quinine, and quinidine stimulates labeled chloroquine accumulation under varying-trans conditions, while mefloquine, halofantrine, artemisinin, and pyrimethamine do not induce this effect. In the reverse of the varying-trans procedure, we show that preloaded cold chloroquine can stimulate quinine accumulation. On the basis of these findings, we propose that the putative chloroquine efflux system is capable of transporting, in addition to chloroquine, structurally related quinoline and methoxyacridine antimalarial drugs. Verapamil and the calcium/calmodulin antagonist W7 abrogate stimulated chloroquine accumulation and energy-dependent chloroquine extrusion. Our data are consistent with a substrate specific and inhibitible drug efflux system being present in chloroquine resistant *P. falciparum* strains.

Chloroquine, once the mainstay of malaria therapy in endemic countries, has lost its efficacy in the field due to the spread of resistant *Plasmodium falciparum* strains (*1*). Alternative drugs that could replace chloroquine are not commonly available. In an effort to revitalize chloroquine, its mode of action and the mechanism underpinning resistance to it have been intensely investigated, yet these underlying processes are still not fully understood.

Chloroquine's antimalarial activity appears to be linked to the parasite's heme metabolism (*2*). During intraerythrocytic development, *P. falciparum* degrades the hemoglobin of the host erythrocyte within the parasite's acidic food vacuole. Heme released in the process is presumably detoxified through crystallization to inert hemozoin (*3*, *4*). Other heme detoxification pathways that have been considered include peroxidative decomposition (*5*) and glutathione-dependent degradation (*6*). Chloroquine accumulates in the food vacuole to millimolar concentrations and is believed to form complexes with heme, resulting in a buildup of toxic heme adducts that intercalate into lipid bilayers and eventually destroy them (*2*).

Central to an understanding of chloroquine resistance in *P. falciparum* are the observations that chloroquine resistant (CQR)[1] strains accumulate less drug and have higher apparent binding constants for chloroquine, as compared to chloroquine sensitive (CQS) strains (*7*, *8*). On the basis of these findings, a simple binding model has been proposed, suggesting that CQR strains have a lower affinity for chloroquine to heme (diminished binding model), possibly due to the intervention of a heme binding protein that controls access of chloroquine to its intracellular binding site (*7*). However, numerous other models, including altered chloroquine transport (*9*, *10*) or changes in prevailing pH gradients that affect either partitioning of the diprotic weak base chloroquine (partitioning model) (*11*) or heme turnover rates (*12*), are also fully consistent with a reduced apparent binding constant for chloroquine (*9*). Each of these models has its advocates.

Studies investigating the kinetics of chloroquine accumulation have mostly relied on zero-trans or steady-state procedures (*7*, *8*, *13*), which provide accurate determinations of apparent binding constants, but only limited insight into the underlying mechanism. More discriminatory are infinite-trans and varying-trans conditions under which the transport of solute under investigation is assessed when defined concentrations of it are present on both sides of the cell membrane (*14*). If a transporter, be it a simple reversible carrier or an outwardly directed carrier, such as a pump or a secondary active transporter, plays a role in the movement of a given solute across the membrane, varying-trans

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[1] Abbreviations: CQ, chloroquine; CQS, chloroquine sensitive; CQR, chloroquine resistant; mdr, multi-drug resistance; SEM, standard error of the mean.

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conditions may reveal stimulated net uptake of this solute (*14*). Passive diffusion via a channel cannot show this phenomenon, nor is stimulated solute uptake observed under varying-trans conditions if the solute is merely held in the cell because of binding to an intracellular site (*14*). Stimulated solute uptake under varying-trans conditions is, therefore, an intrinsic property of a carrier or a pump (*14*).

We employed the varying-trans procedure to investigate chloroquine accumulation in *P. falciparum*-infected erythrocytes and observed a marked difference between the CQS and CQR strains that were examined (*9*). In CQS strains, accumulation of labeled chloroquine was inhibited continuously as the concentration of the preloaded cold chloroquine increased (*9*). This finding is consistent with proposed simple binding models for chloroquine accumulation, in which labeled chloroquine that enters the cell competes with preloaded cold chloroquine for intracellular binding sites. In contrast, in CQR strains, the level of accumulation of labeled chloroquine first increased with an increase in the concentration of preloaded chloroquine and then decreased again (*9*). This finding is not readily reconcilable with a simple binding model, and in accordance with accepted theories in transport kinetics (*14*), we interpreted this phenomenon as evidence of a membrane transport process that, in CQR strains, expels chloroquine. We proposed that, at low concentrations, the preloaded cold chloroquine competes with the labeled chloroquine, which entered the cell by simple diffusion, for transport by the efflux system, thereby blocking efflux of labeled chloroquine and hence increasing the net entry of label (*9*). At higher levels of preloaded cold chloroquine, the postulated efflux system is already fully blocked and the preloaded cold chloroquine, by competing for binding to intracellular sites, reduces the level of accumulation of labeled chloroquine.

If the stimulated chloroquine accumulation is indeed due to a drug efflux system, then one might expect the transporter and, hence, the stimulation phenomenon to have a substrate specificity and to be inhibitable. Here we have investigated the substrate specificity of stimulated chloroquine accumulation by preloading cells with different antimalarial drugs in a varying-trans procedure. Other data show that stimulated chloroquine accumulation is sensitive to verapamil and the calcium/calmodulin antagonist W7. Our data provide further evidence for a substrate specific and inhibitible efflux system as a mechanism contributing to chloroquine resistance in *P. falciparum*.

EXPERIMENTAL PROCEDURES

Chemicals. [^{3}H]Chloroquine (23 Ci/mmol) and [^{3}H]hypoxanthine (17.9 Ci/mmol) were obtained from Amersham International. [^{3}H]Quinine (20 Ci/mmol) was purchased from American Radiolabeled Chemicals, Inc. Chloroquine, 2-deoxyglucose, verapamil, artemisinin, pyrimethamine, daunorubicin, quinacrine, amodiaquine, quinidine, quinine, cyclosporin A, and prazosin were obtained from Sigma, and W7 was from Calbiochem. Halofantrine, mefloquine, XR9576, and ONT-093 were gifts from GaxoSmithKline, Roche, Xenova, and Ontogen, respectively.

P. falciparum Strain and Cultures. P. falciparum clones HB3 and Dd2 were cultured as described previously (*15*) and synchronized using the sorbitol method (*16*). The genotypes of the strains used were confirmed by microsatellite analysis of the PfRRM marker as described previously (*9, 17*). Dd2 is a chloroquine resistant strain (IC_{50} = 137.0 ± 11.0 nM), while HB3 is a chloroquine sensitive strain (IC_{50} = 16.0 ± 1.0 nM). The IC_{50} values for other antimalarial drugs are as follows (Dd2/HB3): 17.0 ± 1.7 and 10.0 ± 6.2 nM for amodiaquine, 2.5 ± 1.2 and 2.1 ± 1.1 nM for artemisinin, not determined and 5.2 ± 1.0 nM for halofantrine, 9.4 ± 1.0 and 21.0 ± 5.0 nM for mefloquine, 880.8 ± 252.0 and 1054 ± 351 nM for primaquine, 221.0 ± 17.0 and 59.0 ± 6.4 nM for quinine, and 53.0 ± 7.2 and 17.8 ± 1.8 nM for quinidine. The means ± the standard error of the mean of at least four independent determinations are shown.

Varying-Trans Procedure. The varying-trans procedure has been described in full detail previously, although described there using the term "trans-stimulation" (*9*). Data were fitted merely for illustrative purposes using the equations previously described (*9*). Where indicated, verapamil (30 μM) or W7 (10 μM) was added during the probing phase.

Energy Dependence of Verapamil Effects on Chloroquine Flux. Magnet-purified trophozoite-infected erythrocytes were resuspended in glucose- and bicarbonate-free RPMI 1640 supplemented with 25 mM HEPES-Na and 2 mM glutamine and containing 43 nM [^{3}H]chloroquine at 37 °C, as described previously (*9*). When incubated in this buffer, parasites quickly exhaust their internal ATP pool due to glucose starvation, as previously shown (*9*). After incubation for 20 min, the reaction mixture was split in three. One aliquot had no additives, and to the other three was added 11 mM glucose, 30 μM verapamil, or both. At the indicated time points, aliquots were removed and the concentrations of intracellular and extracellular chloroquine determined (*9*).

RESULTS

Exploring the Substrate Specificity of the Putative Chloroquine Transporter. The varying-trans procedure we have used to investigate the basis of chloroquine accumulation consists of two steps. The first is a preloading phase during which cells are incubated with different concentrations of cold chloroquine or other compounds suspected to interfere with chloroquine accumulation (preloading ensures that cells have defined intracellular concentrations of the relevant compound). After preloading, cells are washed and transferred to a medium containing a defined amount of radiolabeled chloroquine (probing phase). The amount of label taken up is then analyzed as a function of time or at a fixed time point.

We have applied the varying-trans procedure to explore the substrate specificity of the putative chloroquine efflux system previously described for CQR *P. falciparum* strain Dd2 (*9*). Instead of loading the cells with chloroquine, here we loaded them with amodiaquine, quinacrine, quinine, or quinidine, and then determined the amount of labeled chloroquine accumulation after incubation for 4 min. For each of these compounds, as the concentration of the preloaded compound is increased, the characteristic rise and fall of the 4 min chloroquine accumulation levels is seen in Dd2 cells (Figure 1). This is similar in nature to the previously described stimulation effect observed when Dd2 cells were preloaded with chloroquine (*9*) and, assuming the

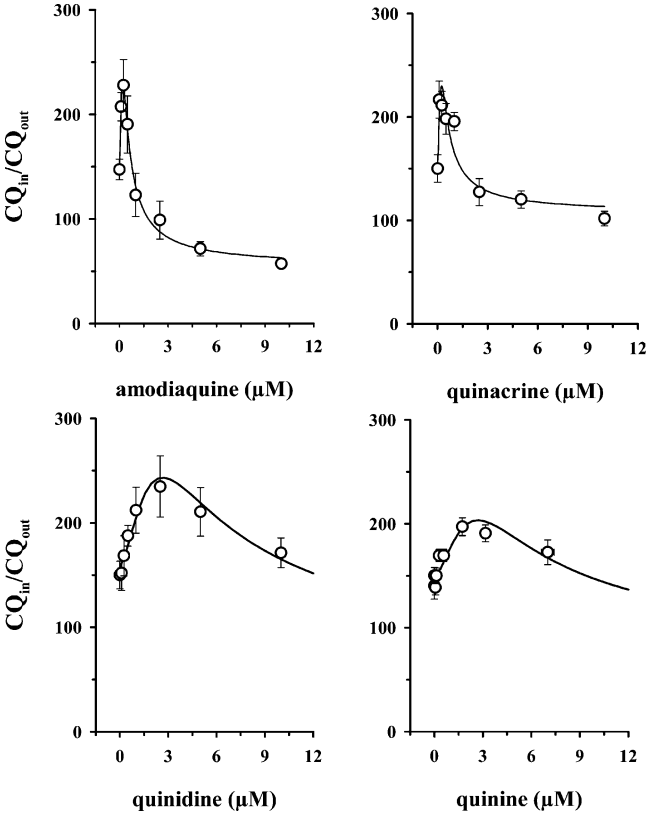


FIGURE 1: Effect of preloading CQR *P. falciparum* strain Dd2 with different unlabeled antimalarial drugs on labeled chloroquine accumulation. The level of chloroquine accumulation, given as the ratio of the intracellular vs the extracellular chloroquine concentration (CQ_{in}/CQ_{out}), is analyzed as a function of the extracellular concentration of the respective drug during preloading. The smooth curves were obtained by curve fitting of the data points, using a previously described equation (*9*), which assumes that CQR strains possess an outwardly directed transporter that expels chloroquine. The mean ± SEM of four or more independent determinations is shown.

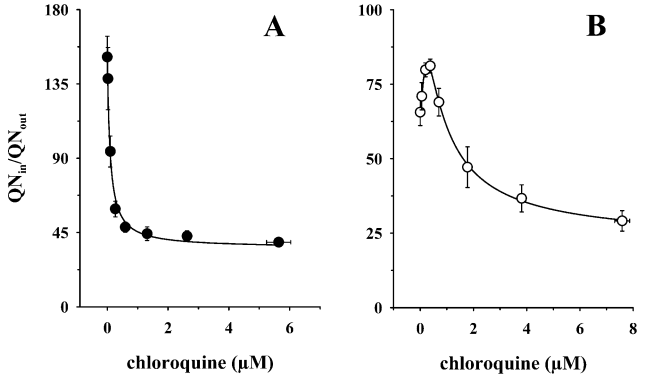


FIGURE 2: Effect of preloading CQS *P. falciparum* strain HB3 (A) and CQR strain Dd2 (B) with unlabeled chloroquine on labeled quinine accumulation. The level of quinine accumulation, given as the ratio of the intracellular vs extracellular quinine concentration (QN_{in}/QN_{out}), is analyzed as a function of the extracellular chloroquine concentration during preloading. Fitting the data points of HB3 was performed using a previously described equation (*9*), which assumes a simple binding model. Curve fitting the data points of Dd2 was performed as described in the legend of Figure 1. The mean ± SEM of four or more independent determinations is shown.

presence of a chloroquine efflux carrier in Dd2, would suggest that these drugs are substrates and/or inhibitors of the putative efflux system. It is also apparent from Figure 1 that the characteristics of the curves differ, which may indicate differences in the apparent binding constants of the proposed efflux system for the different drugs, with that for quinidine and quinine being higher than that for chloroquine, amodiaquine, and quinacrine.

Since quinine, among other drugs that were tested, is apparently able to alter the efflux of chloroquine by the putative chloroquine transporter, we thought it would be interesting to extend these observations by conducting the reverse experiment, that is, to test whether quinine itself can be transported. To this end, we loaded CQS and CQR cells with different concentrations of chloroquine and assessed the accumulation of labeled quinine. Figure 2 depicts the result of this test. CQS strain HB3 showed no stimulation of quinine accumulation upon preloading cells with chloroquine (Figure 2A). There is merely a diminution of accumulation, consistent with previous data suggesting that chloroquine and quinine compete for intracellular sites that, in this case, bind quinine (*2*, *18*−*20*). In CQR strain Dd2, however, quinine accumulation is stimulated by low concentrations of preloaded intracellular chloroquine (Figure 2B). Only at higher levels of preloaded cold chloroquine is there now the expected diminution of quinine accumulation caused, by our hypothesis, by competition between chloroquine and quinine for common intracellular binding sites. On the basis of these data, it would appear that the putative chloroquine transporter conveys to the parasite some ability to export quinine, in addition to chloroquine.

Other antimalarial drugs that we tested in the Dd2 cells behaved in a manner very different from that of the four described above. For mefloquine and halofantrine, the level of chloroquine accumulation decreased continuously with an increase in the concentration of the preloaded drug (Figure 3). This is the picture shown by chloroquine itself in CQS strains (*9*). Pyrimethamine and artemesinin appear to have little or no effect on chloroquine accumulation, at least in the concentration range that was studied (Figure 3). Finally, daunorubicin and, to a far smaller degree, primaquine (Figure 3) caused an increase in the level of chloroquine accumulation as the preloaded drug concentration was increased. Interestingly, no decline in the extent of chloroquine accumulation is observed at higher preloaded drug concentrations, suggesting that these drugs act on the putative chloroquine transporter, but do not compete with chloroquine for binding to intracellular sites.

Effect of Verapamil on Stimulated Chloroquine Accumulation. Verapamil, the classical inhibitor of the human MDR1 P-glycoprotein, reverses chloroquine resistance by increasing the net level of chloroquine accumulation in CQR strains (Figure 4A) (*21*). We asked whether verapamil exerts its sensitizing function by inhibiting the proposed chloroquine efflux system. We reasoned that the stimulation phenomenon seen in the CQR strains should disappear when the transporter is blocked by verapamil. To this end, we performed the standard varying-trans procedure, using HB3 and Dd2. Cells were preloaded with different concentrations of chloroquine, washed, and then incubated in medium containing labeled chloroquine and verapamil at a concentration of 30 μM, which is the most effective concentration for a 5 min incubation period with labeled chloroquine (Figure 4A). As shown in Figure 4C, the stimulation phenomenon is lost in Dd2 in the presence of verapamil. Now the curve falls with

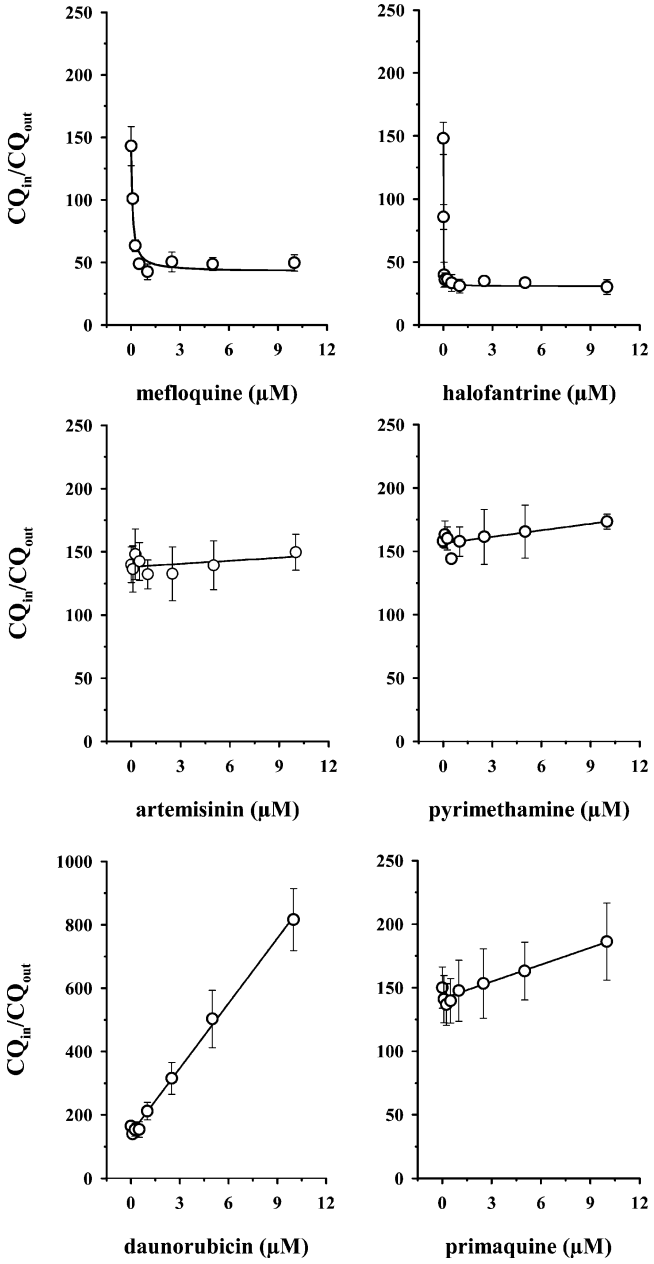


FIGURE 3: Effect of preloading CQR strain Dd2 with different unlabeled drugs on labeled chloroquine accumulation. The level of chloroquine accumulation, given as the ratio of the intracellular vs extracellular chloroquine concentration (CQ_{in}/CQ_{out}), is analyzed as a function of the extracellular concentration of the respective drug during preloading. Fitting the mefloquine and halofantrine data points was performed, using a previously described equation (*9*), which assumes a simple binding model. All the other data points were fitted by linear regression. The mean ± SEM of four or more independent determinations is shown.

each increased concentration of preloaded intracellular chloroquine, very similar to the situation observed in CQS strain HB3 (Figure 4B).

We have previously demonstrated that addition of glucose to glucose-starved *P. falciparum*-infected erythrocytes led to accumulation of chloroquine in the CQS strain, but to its efflux from the cell in the CQR strain (*9*). We used those data in that earlier study as part of our argument that a chloroquine efflux system is present in the resistant strains. We wondered if this differential behavior of the CQR and CQS strains would persist in the presence of verapamil.

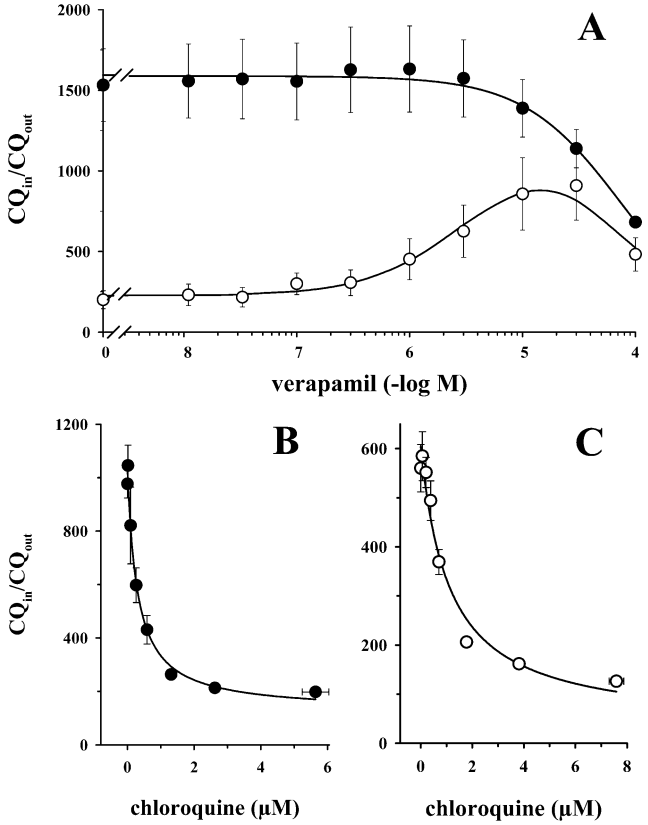


FIGURE 4: Effect of verapamil on chloroquine accumulation. (A) Chloroquine accumulation, given as the ratio of the intracellular vs extracellular chloroquine concentration (CQ_{in}/CQ_{out}), was determined after 5 min in the presence of different concentrations of verapamil. (B and C) Varying-trans procedure in the presence of 30 μM verapamil using CQS strain HB3 (B) and CQR strain Dd2 (C). The mean ± SEM of four or more independent determinations is shown: (○) CQR strain Dd2 and (●) CQS strain HB3.

Figure 5 depicts experiments in which both HB3 (Figure 5A) and Dd2 (Figure 5B) were first starved by withholding glucose and then allowed to take up labeled chloroquine over the course of 20 min. At that point, glucose (11 mM) was added, in the absence (white circles) or in the presence of 30 μM verapamil (gray circles). In the controls, the cells were left in the absence of glucose (black circles) or in the presence of 2-deoxyglucose (11 mM) (white triangles). As shown in our earlier study, addition of glucose in the absence of verapamil led to the increased level of accumulation of chloroquine in the CQS strain, but to its loss in the CQR strain. In the presence of verapamil, however, chloroquine accumulated above the levels seen in the absence of glucose, in both strains, and to a comparable extent. The glucose-stimulated chloroquine level reached by the CQS cells was lower in the presence than in the absence of verapamil, consistent with the data depicted in Figure 4A. Thus, in the presence of verapamil, the starved CQR and CQS cells behave almost identically when given glucose, suggesting that the efflux of chloroquine induced by adding glucose to starved CQR cells is indeed brought about by an inhibitable transporter. 2-Deoxyglucose could not replace glucose in these experiments (compare white circles with white triangles in Figure 5), providing further support to the concept that both chloroquine accumulation and chloroquine efflux are energy-dependent processes.

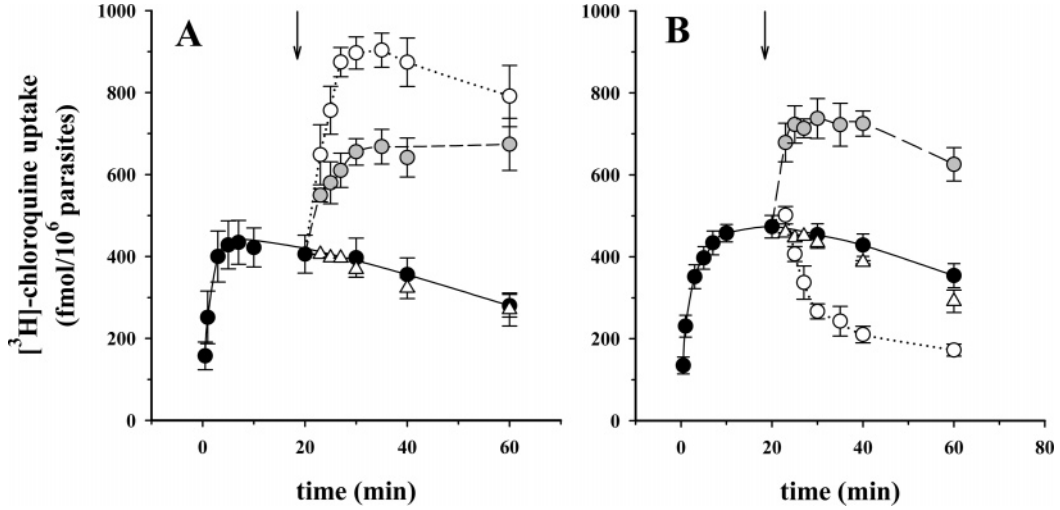


FIGURE 5: Dependence of chloroquine flux on energy and verapamil in *P. falciparum.* Temporal changes in radiolabeled chloroquine accumulation were monitored in CQS strain HB3 (A) and CQR strain Dd2 (B) in glucose-free medium (●) or after addition of either glucose (○), 2-deoxyglucose (△), or glucose and verapamil (gray circles). An arrow indicates the time point of addition of either glucose or glucose and verapamil. The means ± standard error of six independent determinations are shown.

Effect of Other Human MDR1 Inhibitors on Stimulated Chloroquine Accumulation. We next investigated whether other inhibitors of the human *mdr1*-encoded P-gp drug efflux pump modulate chloroquine accumulation in *P. falciparum*. From the broad range of available modulators, we selected cyclosporin A, prazosin, the calcium/calmodulin antagonist W7, XR9576, and ONT-093. For each substance, we performed a response curve, using both HB3 and Dd2, by determining the amount of labeled chloroquine that accumulated within 5 min in the presence of increasing concentrations of P-gp modulator (from 0 to 100 μM) (data not shown). The only substance that substantially sensitized chloroquine accumulation in a concentration-dependent manner in CQR strain Dd2 was W7 (Figure 6A), with an optimal concentration of 10 μM. To test whether W7 acts on the putative chloroquine efflux system, we investigated the effect of W7 (10 μM) on chloroquine accumulation using the varying-trans procedure. As seen in Figure 6C, W7 abrogated the stimulation phenomenon in Dd2. Now the curve descends with each increased concentration of preloaded chloroquine, very similar to the situation observed in CQS strain HB3 (Figure 6B). In the presence of 10 μM W7, the zero-preloaded chloroquine control value in Dd2 is comparable to that of HB3 (Figure 6B,C), consistent with the response curve (Figure 6A).

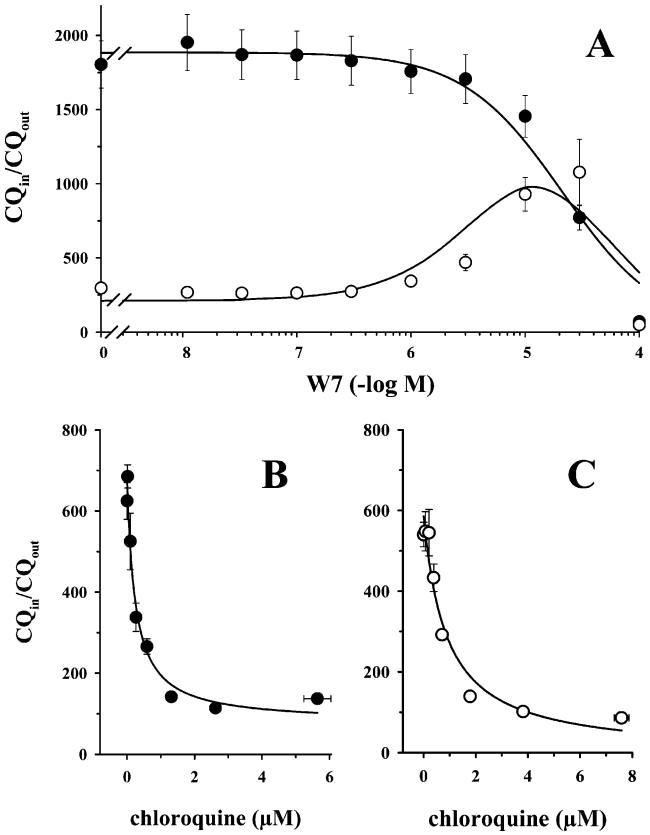


FIGURE 6: Effect of W7 on the kinetics of chloroquine accumulation. (A) The level of chloroquine accumulation, given as the ratio of the intracellular vs extracellular chloroquine concentration (CQ_{in}/CQ_{out}), was determined after 5 min in the presence of different concentrations of W7. (B and C) Varying-trans procedure in the presence of 10 μM W7 using CQS strain HB3 (B) and CQR strain Dd2 (C). The mean ± SEM of four or more independent determinations is shown: (○) CQR strain Dd2 and (●) CQS strain HB3.

DISCUSSION

In a previous study, we have shown that CQR strains, but not CQS strains, demonstrate stimulated chloroquine accumulation in a varying-trans procedure, when cells were preloaded with cold chloroquine (*9*). The literature describes several examples of trans-stimulated solute uptake (*14*). Importantly, in each case, it was found to be linked to a transport-mediated process, although the type of transporter and the underlying cause bringing about stimulated uptake may differ (*14*). For example, the human erythrocyte glucose transporter shows stimulated uptake when loaded with substrate in trans (*14*). In the case of this simple symmetrical carrier, substrate in trans reorients the glucose transporter such that the transport cycle is accelerated and the movement of the labeled substrate from cis to trans accelerated (*14*). Alternatively, stimulated uptake may result from trans substrate saturating an outwardly directed transporter (pump or secondary active transporter), thereby reducing the rate of efflux of the incoming labeled substrate (*14*). Given that stimulated chloroquine accumulation was strictly energy-

dependent, we interpreted our data as evidence for a saturable and energy-dependent outward-directed chloroquine transporter (pump or secondary active carrier) present in CQR strains (*9*). The data in this paper provide further evidence in favor of this model.

Stimulated chloroquine accumulation exhibits substrate specificity. The phenomenon is found in CQR strain Dd2, when chloroquine is substituted, as the preloaded compound, with amodiaquine, quinacrine, quinidine, quinine, daunorubicin, and primaquine. In comparison, preloading cells with mefloquine, halofantrine, pyrimethamine, and artemisinin failed to stimulate chloroquine accumulation. Completely inert in our assay are pyrimethamine and artemisinin. These two antimalarial drugs target pathways other than heme detoxification, and cross resistance to chloroquine has not been described (*22*). Thus, both pyrimethamine and artemisinin can be considered as controls for our assay.

Interestingly, most of the substances capable of stimulating chloroquine accumulation share the quinoline ring present in chloroquine. This includes chloroquine's side chain analogue amodiaquine, the 8-aminoquinoline derivative primaquine, and the arylamino alcohols quinine and quinidine. Quinacrine also shares some overall structural similarities with chloroquine, including the same side chains and a quinoline building block which is contained within the methoxyacridine ring. Clearly structurally distinct from chloroquine is the anthracycline derivative daunorubicin, an anticancer drug that is not used as an antimalarial, but which has been shown to interfere with the unwinding activity of a DEAD-box DNA helicase in *Plasmodium cynomolgi* (*23*).

The only investigated quinoline derivative not capable of stimulating chloroquine accumulation was mefloquine. Mefloquine has ring substitutions in positions not found in the other quinoline derivatives that were tested, which may explain its failure to stimulate chloroquine accumulation. However, mefloquine appears to compete with chloroquine for common intracellular binding sites, consistent with previous reports (*2*, *18*, *19*, *24*), as shown by the decline in the level of chloroquine accumulation with each increased increment of preloaded mefloquine. Similarly, the phenanthrene derivative halofantrine does not stimulate, but rather inhibits, chloroquine accumulation, again consistent with halofantrine and chloroquine competing for common intracellular binding sites (*19*, *24*).

Previous studies have demonstrated that amodiaquine, quinacrine, quinidine, and quinine also bind to heme (*2*, *5*, *18*, *19*, *25*−*27*) and, thus, are expected to compete with chloroquine for binding in the varying-trans procedure. The decline in the respective curves following the stimulation effect would be consistent with such a competition model. Can a model in which chloroquine merely competes with these compounds for common intracellular binding sites also account for the stimulation phenomenon? It is hard to see how the stimulation effect can be reconciled with such a model, unless one postulates that low concentrations of heme-bound compounds increase the affinity of chloroquine for heme while high concentrations have the opposite effect. There is, however, no evidence in the literature to suggest that binding to heme of chloroquine, or any of the other antimalarial drugs capable of stimulating chloroquine accumulation, diverges from a simple binding model. It is also difficult to explain by a binding and competition model why only some of the compounds that bind to heme are capable of stimulating chloroquine accumulation, while others, such as mefloquine and halofantrine, do not show this effect.

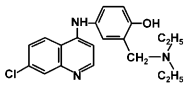
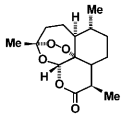
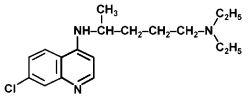
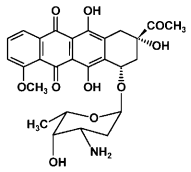
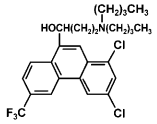
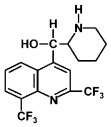
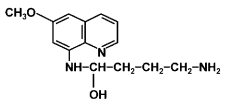
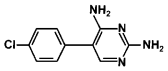
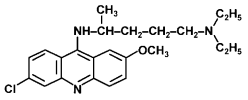
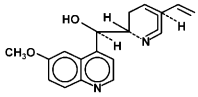
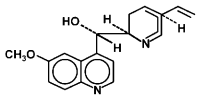
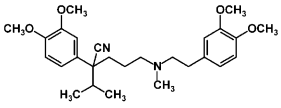
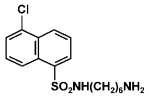
Is it possible that changes in food vacuolar pH caused by the various preloading compounds account for the outcome of the varying-trans procedure? Chloroquine and most of the compounds that have been investigated are lipophilic diprotic or monoprotic weak bases believed to cross membranes largely in the unprotonated form by passive diffusion but to accumulate, following protonation, to high concentrations in acidic compartments, such as the parasite's food vacuole (*11*, *28*, *29*). Protonation of compounds entering the food vacuole as a free base may alter the vacuolar pH. As the pK_a values differ among the compounds that have been investigated, they may affect the food vacuolar pH to different degrees. While it is possible that some of the compounds affect food vacuolar pH during preloading (*30*), this does not appear to influence the outcome of the varying-trans procedure as there is no apparent correlation between a substance's pK_a values (Table 1) and its ability to stimulate chloroquine accumulation.

Thus, the substrate specific stimulation of chloroquine accumulation cannot be easily explained by the pK_a values of the preloaded compound or a model in which chloroquine merely competes with other compounds for intracellular binding sites. Stimulated chloroquine accumulation, however, would be a prediction of a membrane-bound transporter. On the basis of these considerations, we favor the model that such compounds stimulate chloroquine accumulation by saturating and/or blocking a putative chloroquine efflux system. For quinine, a radiolabeled compound is commercially available, allowing us to test this interpretation. If quinine is transported by the putative chloroquine transporter, then, in the reverse of the varying-trans experiment, we should find stimulation of quinine accumulation by preloaded chloroquine. This is indeed what we observed (Figure 2). The concept of an efflux system capable of extruding structurally related antimalarial drugs would be consistent with the reported reduced susceptibility of some CQR strains to amodiaquine, quinine, and quinidine (*31*−*35*). In comparison, chloroquine responsiveness and mefloquine and halofantrine responsiveness appear to be inversely correlated (*33*, *36*, *37*). That mefloquine and halofantrine failed to stimulate chloroquine accumulation would suggest that these two antimalarial drugs do not interact with the putative chloroquine efflux system.

To what extent the putative chloroquine efflux system may contribute to quinine resistance is not yet clear. That some inheritable determinants of drug response are shared was recently confirmed in the analysis of a genetic cross between Dd2 and HB3 (*38*). For example, polymorphisms within *pfcrt* encoding a putative food vacuolar transmembrane protein seem to be associated with both chloroquine and quinine resistance (*38-40*). Another possibly shared drug resistance gene is *pfmdr1* encoding a homologue of the human P-glycoprotein-associated multi-drug resistance efflux pump (*38*, *41*).

Daunorubicin and, to a lesser extent, primaquine show an interesting behavior in that they stimulate chloroquine accumulation over the entire concentration range that was tested, suggesting that they saturate and/or block the putative chloroquine efflux pump but do not compete with chloro-

Table 1: pK_a Values and Chemical Structures for Antimalarial Drugs and Other Compounds (*46*)

Compound	Structure	pK_{a1}	pK_{a2}
amodiaquine		8.1	7.1
artemisin		-	-
chloroquine		10.18	8.38
daunorubicin		10.3	-
halofantrine		~ 8-9	-
mefloquine		8.6	< 2
primaquine		10.4	3.2
pyrimethamine		7.34	-
quinacrine		9.7	7.8
quinidine		8.58	4.42
quinine		8.58	4.12
verapamil		8.9	-
W7		-	-

quine for binding to heme. Consistent with this conclusion, primaquine does not inhibit heme detoxification (*19*); its mode of action is distinct from that of chloroquine (*22*).

A second line of evidence in favor of a chloroquine efflux system is the responsiveness of the stimulation phenomenon to verapamil and the calcium/calmodulin antagonist W7. Our original suggestion that chloroquine is transported out of CQR cells by an energy-dependent efflux system was initiated by our finding that addition of glucose to starved cells led to further accumulation of labeled chloroquine in the CQS cells but to a loss of chloroquine in the CQR cells (*9*). This distinction between the CQR and CQS cells is

totally abrogated if the glucose is added to cells in the presence of the verapamil (Figure 5). Now both HB3 and Dd2 accumulate comparable concentrations of labeled chloroquine, consistent with the inhibition of an outwardly directed chloroquine transporter. Moreover, in the presence of verapamil or W7, stimulated chloroquine accumulation disappears in CQR strain Dd2, concurrent with an increase in the level of total chloroquine uptake. This is again consistent with inhibition of an efflux system, but is not easily ascribed to the diminished binding or the partitioning model. Our interpretation of these data concurs with other studies which have found that the sensitizing action of verapamil is independent of weak base processes but is associated with the inhibition of a chloroquine efflux system (*10*, *21*, *42*−*44*). That W7, an established calcium/calmodulin antagonist, blocks the putative chloroquine efflux system might suggest that calcium-dependent interactions are important for chloroquine resistance. Our finding that, apart from verapamil and W7, none of the other P-gp inhibitors affect chloroquine accumulation in CQR strain Dd2 suggests that the putative chloroquine transporter is not the parasite's homologue of MDR1, consistent with previous reports (*41*, *44*).

All in all, our studies strongly support the existence of an efflux transporter as a contributing factor to chloroquine resistance in the malaria parasite. Direct identification of this putative transporter is still needed for final acceptance of this model. In a recent development, evidence has been presented in favor of PfCRT binding to chloroquine, earmarking PfCRT as a prime candidate for the proposed efflux system (*45*).

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