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MOLECULAR PHARMACOLOGY, 50:1559–1566 (1996).

Physicochemical Properties Correlated with Drug Resistance and the Reversal of Drug Resistance in *Plasmodium falciparum*

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Received February 8, 1996; Accepted August 26, 1996

SUMMARY

At high molar excess, verapamil can selectively increase the accumulation and cytotoxicity of structurally dissimilar natural product drugs in many multidrug-resistant tumor cell lines. Such concentrations of verapamil are also capable of increasing the accumulation and activity of chloroquine in chloroquine-resistant strains of the human malaria parasite *Plasmodium falciparum*. Despite such similarities, it is not clear why chloroquine-resistant *P. falciparum* is often susceptible to closely related compounds such as amodiaquine, whereas cancer cells are cross-resistant to many structurally unrelated drugs. For 13 aminoquinoline and aminoacridine compounds, relative drug resistance was negatively correlated with lipid solubility at physiological pH ($r^2 = 0.90$, $p < 0.0001$). The ability of verapamil (5 μM) to reverse drug resistance was also negatively correlated with lipid solubility ($r^2 = 0.88$, $p < 0.0001$). Furthermore, molar refractivity was weakly correlated with relative drug resistance ($r^2 = 0.46$, $p < 0.05$) and reversal of drug resistance ($r^2 = 0.52$, $p < 0.005$). Verapamil increases chloroquine accumulation by resistant parasites, a mechanism suggested to account for its selective chemosensitization effect. We show that the initial rate of chloroquine accumulation by resistant parasites is increased by verapamil. This effect of verapamil is abolished when deoxy-glucose is substituted for glucose. Therefore, verapamil produces an energy-dependent increase in the permeability of resistant parasites to chloroquine. For a panel of four chloroquine-resistant and two chloroquine-susceptible isolates, the effect of verapamil on the accumulation of chloroquine and monodesethyl amodiaquine was found to be correlated ($r^2 = 0.96$, $p < 0.001$). Verapamil chemosensitization was also correlated for the two drugs ($r^2 = 0.92$, $p < 0.005$), suggesting a common mechanism. In summary, the degree of drug resistance and the extent of verapamil chemosensitization for a particular drug seem to be dependent on general physical features such as lipid solubility and molar refractivity rather than on closely defined structural parameters. These studies provide insight into this important resistance mechanism of malaria parasites and may provide direction for the development of new drugs that are effective against resistant parasites.

Chemotherapy in the treatment of malaria patients is limited by resistance of *Plasmodium falciparum* to CQ. Parasite resistance to CQ shows some phenotypic similarities to MDR of human cancer cells.

Both types of cells exhibit reduced drug accumulation *in vitro* compared with susceptible cells (1–5). In addition, both types of resistant cells can be chemosensitized *in vitro* by a high molar excess of some structurally unrelated drugs, such as the calcium channel blocker VP (5, 6).

Originally, drug resistance in cancer cells was thought to be associated with an enhanced rate of efflux of the anticancer drug in resistant cell lines (7, 8). In the case of *P. falciparum*, initial research suggested that CQ resistance was also associated with an enhanced rate of efflux from resistant strains (3). However, more recent work with other strains indicates that CQ resistance may be more closely correlated with a reduced rate of drug accumulation (4, 9, 10). It is interesting to note that these observations are not necessarily incompatible with the concept of a MDR-like mechanism for CQ resistance: recent reviews of work from cancer researchers indicate that a reduced rate of drug accumulation is commonly found in resistant cells (5, 11).

Despite the similarities outlined above, there are two important differences between cancer cell MDR and malaria CQ resistance that are difficult to reconcile with a P-glycoprotein-mediated resistance mechanism. First, resistance of MDR cancer cells is mediated by increased expression of MDR protein, and selectivity of the resistance profile may be influenced by mutation of this gene (5). However, resistance to CQ cannot be satisfactorily correlated with levels of expression or mutation of any of the known *mdr*-like genes of the parasite (12, 13). Second, MDR cells display cross-resis-

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

ABBREVIATIONS: CQ, chloroquine; DCQ, monodesethyl chloroquine; BDCQ, bidesethyl chloroquine, AQ, amodiaquine; DAQ, monodesethyl amodiaquine; AMOPYR, amopyraquine; NTB-AQ, *N-tert*-butyl amodiaquine; PYR, pyronaridine, QN, quinine; VP, verapamil; VBL, vinblastine; GLU, glucose; DOG, 2-deoxy-D-glucose; AEI, activity enhancement index; RF, resistance factor; MR, molar refractivity; MDR, multidrug resistance; HPLC, high performance liquid chromatography; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

tance to a large variety of structurally and functionally distinct agents. In contrast, CQ-resistant malaria parasites are often susceptible to other antimalarial agents, even drugs of the same chemical class, such as AQ (14).

Previous studies of cross-resistance of CQ with other 4-aminoquinoline drugs and their metabolites may provide important clues to the mechanistic basis of drug resistance in malaria parasites. Although CQ-resistant isolates are less susceptible to AQ *in vitro* (15), a much greater level of cross-resistance apparently exists between CQ and DAQ, the major plasma metabolite of AQ (16). Similarly, BDCQ is significantly less potent than CQ against CQ-resistant strains, whereas comparable activity is maintained against CQ-susceptible strains (15). Dealkylation of the terminal nitrogen of the side chain might be expected to alter physicochemical properties of these drugs, such as pK and lipid solubility (15). It is interesting to note that physical parameters such as lipid solubility and MR have been shown to be important in drug resistance and chemosensitization of cancer cells (17, 18). For these reasons, we examined the relationship of drug resistance and the reversal of drug resistance to lipid solubility and MR through the use of 13 structurally related aminoquinoline and aminoacridine drugs. In addition, the energy dependence and kinetics of the effect of VP on CQ uptake were investigated to shed light on the mechanism of resistance reversal.

Materials and Methods

Parasites. Six isolates of *P. falciparum* were used in the study: the 3D7 and HB3 CQ-susceptible isolates and K1, PH3, Indochina, and V1S CQ-resistant isolates. The K1, 3D7, and HB3 isolates were provided by Prof. D. Walliker (Edinburgh University, Scotland, UK); the PH3 and Indochina isolates were provided by Prof. M. Hommel (Liverpool School of Tropical Medicine, Liverpool, UK); and the V1S strain was supplied by Dr. J. Hyde (University of Manchester Institute of Science and Technology, Manchester, UK). Two alleles of the *pfmdr1* gene (the parasite homologue of the mammalian MDR gene) have been linked to CQ resistance (19). The 3D7 and PH3 isolates have no *pfmdr1* mutations (19, 20). The K1 and Indochina isolates have the *K1 CQ*-resistance allele. HB3 has one of the three amino acid changes associated with the *7G8 CQ*-resistance allele, and it was suggested that mutations of a second gene are required for the CQ-resistant phenotype (19). The *pfmdr1* status of V1S is unknown, although this isolate was cloned from the V1 strain, which has the *K1* allele (19).

Cultures. Strains were cultivated *in vitro* using O^+ human erythrocytes suspended in RPMI 1640 medium containing 25 mM HEPES, 23 mM $NaHCO_3$, and 10% human AB serum. Cultures were kept at 37° under an atmosphere of 93% N_2/4% CO_2/3% O_2. Cultures were synchronized using sorbitol at ring stage at least 48 hr before use.

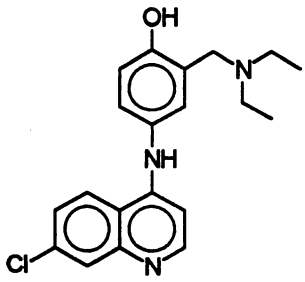
Drugs. CQ, AQ, mepacrine, QN, VP, and VBL were purchased from Sigma Chemical (Poole, Dorset, UK). CQ-pyrollidinyl [7-chloro-4-(4′-pyrollidino-1′-methylbutamino)quinoline], DCQ, and BDCQ were obtained from The Walter Reed Army Institute (Washington, D.C.). DAQ, bidesethyl AQ, and AMOPYR were obtained from Parke Davis (Hampshire, UK). PYR was a gift from Dr. David Warhurst (London School of Hygiene and Tropical Medicine, London, UK). [^{3}H]AQ (106 μCi/mM), AQ-mepacrine [6-chloro-2-methoxy-9-(3′-diethylamino methyl-4′-hydroxyanilino)acridine], and NTB-AQ [7-chloro-4-(3′-*tert*-butylamino methyl-4′-hydroxyanilino) quinoline] were synthesized in-house. Chemical structures of all the drugs are shown in Fig. 1. Radiolabeled [^{3}H]CQ (69 Ci/mM) was obtained from DuPont-New England Nuclear (Boston, MA).

Measurement of drug susceptibility. Susceptibility of the parasites to the drugs was determined in the absence and presence of 5 μM VP as follows: Ring stage cultures at 1% parasitemia/1% hematocrit were exposed to serial dilutions of drugs with and without the fixed concentration of VP under the same gas atmosphere as the continuous cultures. After 24 hr in culture, [^{3}H]hypoxanthine was added at a concentration of 50 μCi/ml. After an additional 24 hr in culture, the cells were harvested with an Automash cell harvester, and the filter mats were dried and counted by liquid scintillation counting. IC_{50} values were calculated using the four-parameter logistic method (GraFit; Erithacus Software, Kent, UK).

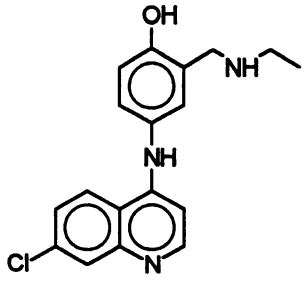
Preparation of radiolabeled DAQ. Radiolabeled DAQ was prepared from [^{3}H]AQ (106 μCi/mM) as described previously (21). Briefly, microsomes were prepared from human livers as described previously (22). Five milligrams of microsomal protein was preincubated with an NADPH-regenerating system (10 μM glucose-6-phosphate, 0.5 mM $NADP^+$, 20 units of glucose-6-phosphate dehydrogenase) for 2 min. The reaction was initiated by the addition of 10 μCi of (100 μM) [^{3}H]AQ, incubated for 1 hr at 37°, and terminated by the addition of two volumes of acetonitrile. Metabolites were extracted using a conditioned SepPak C18 cartridge (Waters, Watford, UK) (21), and [^{3}H]DAQ was purified to 97% by HPLC. HPLC conditions were as follows: A μBondapak C18 column (300- × 3.7-mm i.d., 10 μm; Waters) was connected to a Spectra Physics SP 8800 pump. Radiolabeled analytes were determined on-line with a Radiomatic A250 Flo-one β-radioactive flow detector.

Determination of log D and MR. Distribution of drugs between lipid and aqueous phases at pH 7.2 and 4.5 was determined using a modification of the procedure of Zamora *et al.* (18). Drugs were added at a concentration of 10^{-4} M to 0.1 M phosphate buffer, pH 7.2, or 0.1 M sodium acetate buffer, pH 4.5. The drug solutions were vigorously shaken with buffer-saturated HPLC grade 1 octanol for 20 min. The phases were separated by centrifugation (600 × *g* for 10 min), and the absorbance of each phase was measured at the maximum for each drug (340 nm for the 4-aminoquinolines, 333 nm for QN, and 425 nm for the acridine compounds). The drug concentration in each phase was determined by comparing the absorbance with standard curves prepared in buffer and octanol. The distribution coefficient *D* is the concentration of drug in the octanol phase divided by the concentration of drug in the aqueous phase. The $\log_{10}$ D was used as a measure of lipid solubility. MRs were calculated using the CLOG P module of the Medchem program (version 3.55) (C-qsar Software; Biobyte, Claremont, CA). The additive substituent constant method was used.

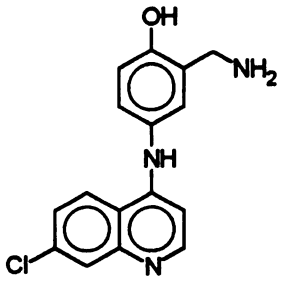
Drug uptake studies. Accumulation of [^{3}H]CQ was measured at 1% parasitemia/1% hematocrit. Accumulation of [^{3}H]DAQ was measured at 5% parasitemia/1% hematocrit to compensate for lower specific activity of this radiolabel. Accumulation of DAQ was linear (r = 0.97 for the 3D7 strain) over the range of inoculum size 1–10 (inoculum size = parasitemia × hematocrit) calculated through inoculum effect analysis [see Bray *et al.* (23)]. Parasites were incubated with drug at 37° or at room temperature in phosphate-buffered saline, pH 7.4, in the presence of GLU or DOG at 5 mM. Incubations were also performed in the absence or presence of VP or VBL at a concentration of 5 μM. At the desired time point, samples were removed, centrifuged through silicon oil (12,000 × *g* for 1 min), and processed for scintillation counting as described previously (10). The initial external concentration of CQ was 1 nM, and the initial concentration of DAQ was 10 nM. In all experiments, counts resulting from a similar volume of uninfected erythrocytes were subtracted from the parasite pellet counts. Time zero blanks (values are given in table legends) were obtained through suspension of the parasites in ice-cold medium containing radiolabel and immediate centrifugation. The time zero values are assumed to result from initial surface binding, and these values were subtracted from the counts at the later time points. Initial accumulation rate data were analyzed using the GraFit program. To measure the apparent initial accumulation rate, data (t = 20–360 sec) were best fit (using reduced χ^2) to a



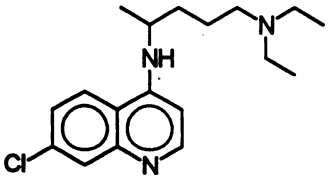
Amodiaquine



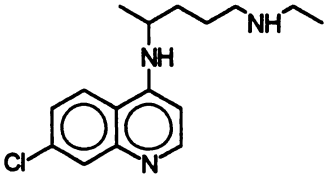
Monodesethyl amodiaquine



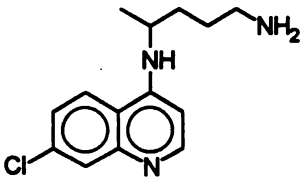
Bidesethylamodiaquine



Chloroquine



Monodesethyl chloroquine



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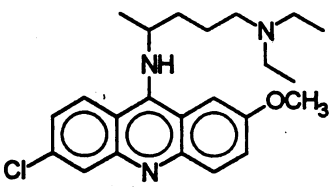
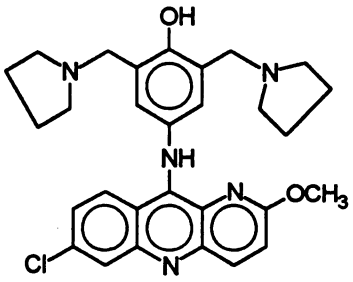
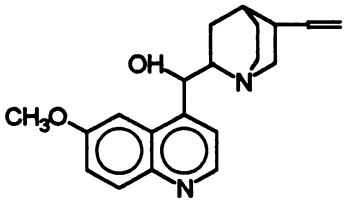


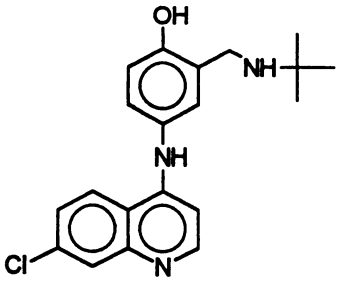
Mepacrine



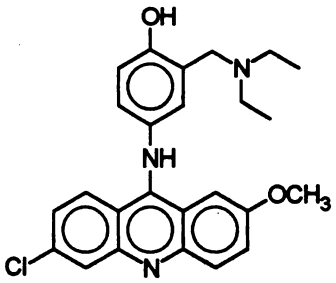
Pyronaridine



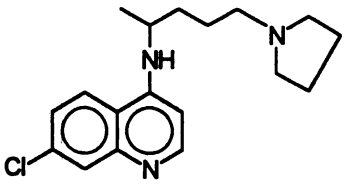
Quinine



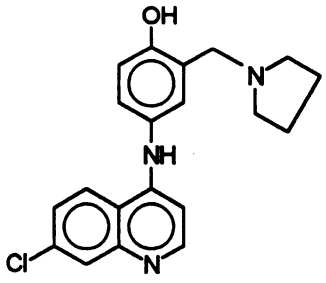
7-chloro-4-(3't-butylamino methyl-4'-hydroxyanilino) quinoline (N-t-butyl amodiaquine)



6-chloro-2-methoxy-9-(3'diethylamino methyl-4'-hydroxyanilino) acridine (Amodiaquine-mepacrine)



7-chloro-4-(4'-pyrollidino-1'-methyl butylamino) quinoline (Chloroquine-pyrollidinyl)



Amopyroquine

Fig. 1. Chemical structures of the compounds used in the study.

first-order rate equation: $CQ_t = CQ_\infty (1 - e^{-kt})$, where CQ_t is the amount of CQ accumulated at time t, CQ_∞ is the steady state accumulation of CQ, and k is the rate constant. The initial rate of CQ accumulation or linear portion of the curve is defined as $k \cdot CQ_\infty$ and was calculated with the computer based on the parameters generated by the fit. A sample of incubation medium was also removed and counted at each time point to calculate the cellular accumulation ratio (i.e., the ratio of the amount of labeled drug in the cells to the amount of labeled drug in a similar volume of medium). Significance of differences between groups was tested using the two-tailed Mann-Whitney U test.

Results

Correlation of physicochemical properties with drug resistance and its reversal. Thirteen related antimalarial drugs were screened for lipid solubility, MR, relative drug resistance (IC_{50} value of the K1 CQ-resistant strain compared with IC_{50} value of the HB3 CQ-susceptible strain), and VP effect (given as AEI). The data are presented in Table 1. Most of the compounds were found to be relatively lipid soluble at pH 7.2 (Table 1). At pH 4.5, all of the compounds were predominantly water soluble (with the exception of QN; data not shown). The general importance of lipid solubility is confirmed by the data presented in Fig. 2, which suggests a correlation of the relative lipid solubility or log D (at physiological pH) of the 13 related antimalarial drugs and the RF (i.e., relative resistance displayed by a CQ-resistant strain, K1, compared with a CQ-susceptible strain, HB3). The correlation coefficient of linear regression (r^2) in this case is 0.90. The relationship of log D (at physiological pH) to VP dose-response modification is depicted in Fig. 3. These two parameters were also found to be very well correlated (linear regression $r^2 = 0.88$). It seems that the loss of alkyl groups from the terminal nitrogen of the AQ or CQ side chain produces a significant change in the lipid solubility, presumably due to an alteration of charge on the terminal nitrogen atom. Such changes render the molecule more susceptible to the resistance mechanism, and the resistance reversal effect of VP is more pronounced with these compounds. Interestingly, there was no significant difference in the AEI for racemic CQ and its stereoisomers, indicating that the interaction of the drug with the resistance mechanism is not stereospecific (Table 1). There was no significant correlation of log D at pH 4.5 with either the RF or the VP effect (data not shown). Dealkylation will reduce molecular volume and MR. The parameter of MR was weakly correlated to RF (linear regression $r^2 = 0.46$) and VP effect (linear regression $r^2 = 0.52$), suggesting that molecular volume may play a role, albeit a less important role than lipid solubility, in substrate recognition by the resistance mechanism.

TABLE 1

Relationship of lipid solubility and MR to relative drug resistance (RF) and VP effect (AEI) for 13 aminoquinoline and aminoacridine antimalarials

The log AEI and log RF values are derived from mean IC_{50} values from three to five sensitivity assays; each assay was performed in triplicate.

Drug	Log D	Log AEI	Log RF	MR (Å^3)
CQ	0.045	0.732	1.551	9.57
−CQ	0.045	0.716	N.D.	9.57
+CQ	0.045	0.748	N.D.	9.57
DCQ	−0.373	0.927	1.844	8.64
BDCQ	−0.553	1.070	1.866	7.71
CQ-pyrollidinyl	0.361	0.660	0.885	9.29
AQ	2.60	0.034	0.292	10.38
DAQ	1.182	0.380	0.778	9.45
Bidesethyl AQ	0.346	0.722	1.034	8.52
NTB-AQ	1.781	0.146	0.260	10.38
AQ-mepacrine	2.458	−0.015	0.022	12.68
Mepacrine	1.337	0.346	0.724	11.87
AMOPYR	1.893	0.000	0.204	10.09
PYR	1.633	0.079	0.193	14.80
QN	1.830	0.580	0.398	9.48

N.D., not determined.

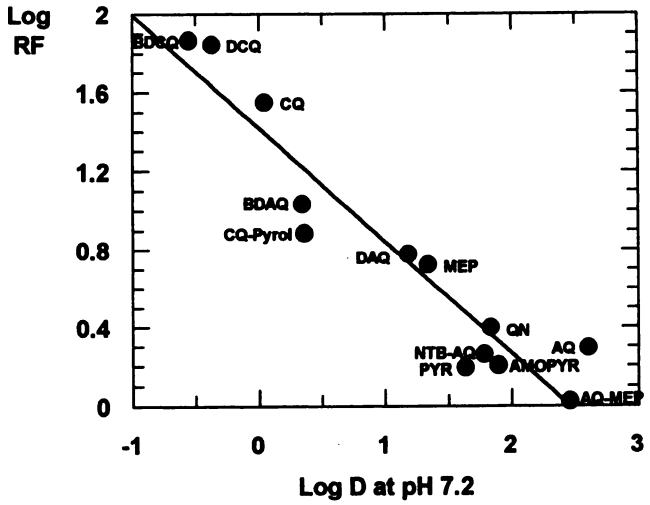


Fig. 2. Correlation of log D at pH 7.2 with log RF [the ratio of the drug IC_{50} value of the K1 (CQ-resistant) strain to the drug IC_{50} value of the HB3 (CQ-susceptible) strain]. *BDAQ*, bidesethyl AQ; *AQ-MEP*, AQ-mepacrine; *MEP*, mepacrine.

Effect of VP on the drug uptake process. Fig. 4 depicts the time course of uptake of CQ into K1 CQ-resistant parasites. The external CQ concentration used was 1 nM (~1/200 of the IC_{50} concentration of this drug against this isolate). Accumulation of CQ at steady state (not shown) is ~3.5-fold higher in the presence of 5 μM VP. It can be seen from the graph that this steady state increase can in large part be attributed to a 2.5-fold increase in the initial uptake rate of CQ in the presence of VP. The effects of VP on the accumulation of CQ reported in this study are greater than those of an earlier study that reported a 2.5-fold increase in CQ accumulation by the K1 strain at steady state (10). We have previously shown that chemosensitization effects are lost when serum protein levels are increased (24). Experiments reported here are performed in the absence of serum. We believe therefore that the increased VP effect is due to the absence of protein binding of VP, leading to greater concentrations of VP available to inhibit drug transport. The ability of VP to increase steady state CQ accumulation is abolished when DOG is substituted for GLU (Fig. 5). Similar results are obtained when 5 μM VBL is used to alter CQ concentration (Fig. 5).

Data presented in Table 2 compare the effect of VP on steady state accumulation of the 4-aminoquinoline antimalarials, CQ, AQ, and DAQ. These data, which were obtained from a panel of four CQ-resistant and two CQ-susceptible isolates, indicate that VP significantly increases the accumulation of DAQ as well as CQ in the four CQ-resistant isolates. In the case of the CQ-susceptible isolates, VP decreased CQ accumulation slightly, although the effect is not always significant. In the case of AQ, VP had no significant effect on

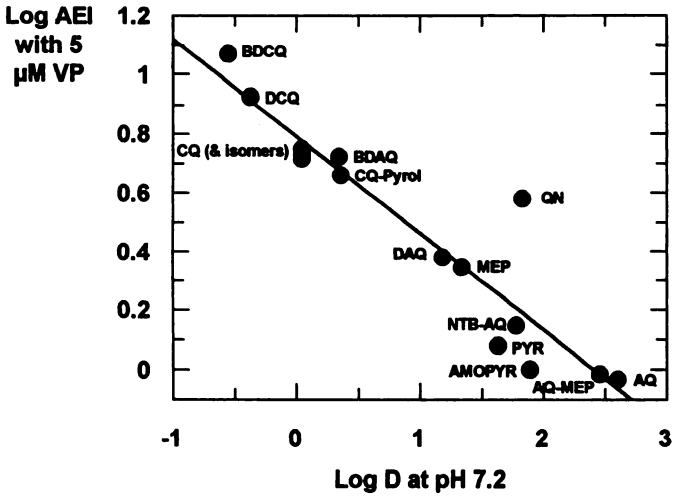


Fig. 3. Correlation of log D at pH 7.2 with log AEI [the ratio of the drug IC_{50} value of the K1 (CQ-resistant) strain in the absence of VP to the drug IC_{50} value in the presence of 5 μM VP]. *BDAQ*, bidesethyl AQ; *AQ-MEP*, AQ-mepacrine; *MEP*, mepacrine.

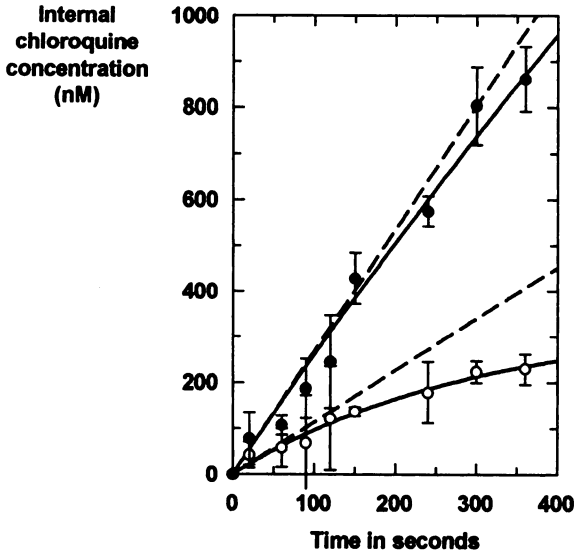


Fig. 4. Accumulation of [^{3}H]CQ by the K1 (CQ-resistant) strain in the (○) absence and (●) presence of 5 μM VP plotted against time. Dashed lines, apparent initial rates of accumulation calculated as described in Materials and Methods. Data are mean ± standard deviation values from three individual experiments. Values for time zero blanks were 69 and 73 in the absence and presence of VP, respectively.

drug accumulation by any of these isolates. In addition, accumulation of all three antimalarial drugs in the absence of VP was significantly reduced in the CQ-resistant compared with the CQ-susceptible isolates ($p < 0.01$). Fig. 6 shows the correlation of VP effect on the accumulation of CQ and DAQ by the isolates. It can be seen that the effect of VP on the accumulation of CQ is well correlated to its effect on DAQ accumulation ($r^2 = 0.96$). Finally, the degree of VP accumulation enhancement is 3.3-fold smaller for DAQ (mean for CQ-resistant isolates, 65%) than for CQ (mean for CQ-resistant isolates, 213%).

The effect of VP on drug susceptibility. Table 2 also shows the effect of VP on the IC_{50} value of the isolates to the three antimalarial drugs (values in parentheses are shown as percentage increase of drug susceptibility caused by 5 μM

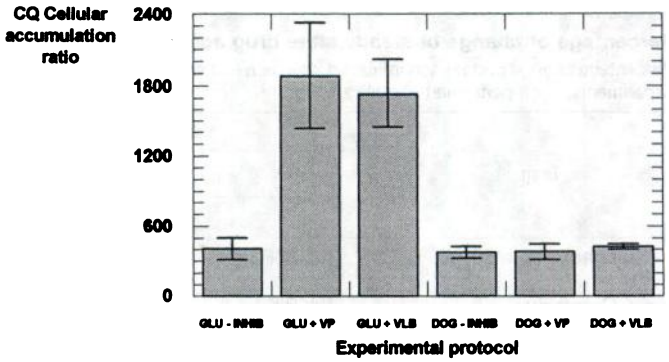


Fig. 5. A comparison of the effects of VP and VBL on the steady state accumulation of [^{3}H]CQ by the K1 (CQ-resistant) strain in medium containing either GLU or DOG. *Histogram* and *error bars*, mean ± standard deviation values from nine individual experiments.

VP). It can be seen that similar to the effects of VP on drug accumulation, there is a selective enhancement of activity of DAQ as well as CQ against CQ-resistant isolates, and this effect is again well correlated for these two drugs ($r^2 = 0.92$). In parallel with the effects of VP on drug accumulation noted above, the degree of VP dose modification in resistant isolates is 2.5-fold smaller for DAQ (mean, 156%) than for CQ (mean, 389%). In the case of AQ, there is no chemosensitization by VP in any of the isolates tested.

Discussion

The fact that CQ resistance in *P. falciparum* is not uniformly associated with cross-resistance to structurally similar drugs has posed problems for many of the current theories put forward to explain drug resistance in this parasite. It does seem likely, however, that resistance to 4-aminoquinoline and related drugs may be linked to reduced drug accumulation and as such may be unrelated to the mode of action of the drugs. We demonstrate here and in Bray *et al.* (23) that accumulation of CQ, AQ, and DAQ is significantly reduced in CQ-resistant isolates compared with CQ-susceptible isolates, confirming previous research with CQ and providing new information on AQ and DAQ (1–4). Two models have been suggested to account for reduced accumulation of this class of drug in resistant parasites. In the first model, resistance is achieved through the action of an efflux pump, which actively removes drugs from the cell in a manner analogous to that proposed for human P-glycoprotein (3). In the second model, drug accumulation is a passive process, which is driven by a proton gradient that is reduced in resistant parasites (9).

The efflux model is based in part on research that demonstrated that VP can selectively chemosensitize CQ-resistant parasites to CQ and selectively increase CQ accumulation by these isolates, supposedly through inhibition of a CQ "efflux pump" (3, 6). We report similar observations for DAQ (Table 2). Also, the close correlations of a VP effect with both drug accumulation (Fig. 6) and dose response for the two drugs strongly suggest that a similar mechanism is responsible for the effect of VP on both drug accumulation and activity. In resistant isolates, the apparent initial accumulation rate of CQ is increased 2.5-fold in the presence of VP (Fig. 4). This compares with an increase of 3.5-fold in the steady state accumulation of CQ caused by VP under the same conditions and suggests that much of this increase may be attributable

TABLE 2

Percentage of change of steady state drug accumulation and percentage of change of dose response due to VP

Percentage change of drug accumulation was derived from means of six to nine individual experiments. Percentage change of IC_{50} was derived from means of three experiments, each performed in triplicate.

Drug	Percentage change of steady state drug accumulation and activity due to 5 μM VP					
	CQR strains				CQS strains	
	K1	PH3	Indochina	V1S	HB3	3D7
CQ accumulation	+219%[a]	+117%[b]	+287%[a]	+231%[a]	−14.7%[c]	−36%[b]
CQ activity	+440%	+422%	+393%	+302%	−1%	−3.1%
AQ accumulation	−10.5%[c]	−8.5%[c]	−2.6%[c]	−25%[c]	−15%[c]	−3.5%[c]
AQ activity	−8.1%	−8.5%	−17.6%	−9.1%	−4.2%	−13.6%
DAQ accumulation	+63%[a]	+43%[b]	+92%[a]	+65%[a]	−24%[b]	−7%[c]
DAQ activity	+141%	+163%	+165%	+158%	+1%	+2%

[a] $p < 0.01$.
[b] $p < 0.05$.
[c] No significant difference.

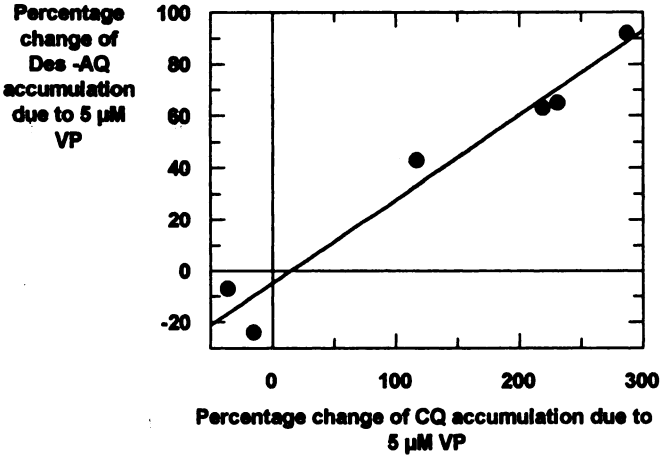


Fig. 6. Correlation of the effect of 5 μM VP on the accumulation of CQ and DAQ by a panel of six isolates with varying susceptibility to CQ. Data are mean values from six to nine individual experiments.

to an increased initial rate of uptake rather than an enhanced efflux of preaccumulated drug. There are two potential problems with our interpretation of the data: 1) a significant amount of CQ has already accumulated before our first sampling time. If drug can be removed from the cytosol by an efflux transporter (i.e., an enhanced rate of efflux), it is possible that this process is taking place before our first measurement. 2) The effects of VP are seen at a huge molar excess, which may call into question the specificity of inhibition of the putative pump.

Malaria parasites possess a saturable high affinity, low-capacity "receptor" for CQ and other aminoquinolines (1, 23, 25). This receptor is most likely situated inside the food vacuole, the site of most of the parasite-specific CQ accumulation (26). The receptor has a low capacity and is saturable at nanomolar external drug concentrations (1). A significant proportion of the total cellular CQ accumulated is bound to sites of lower affinity and may not contribute to antimalarial activity (1). It is possible that the resistance mechanism may act specifically to reduce high affinity binding to the putative receptor. We show that the effect of VP as a "resistance reverser" is ~2-fold greater than the effect of VP on drug accumulation (Table 2). These data confirm our earlier observations that the degree of global drug accumulation enhancement produced by VP is insufficient to explain the chemosensitization effects of the drug, even at very low drug concentrations, where the effect is maximal (10). These observations suggest that VP is inhibiting the resistance mechanism to specifically increase the component of CQ accumulation that is responsible for activity.

The effect of ATP withdrawal on the accumulation of CQ is shown in Fig. 5. In terms of the proposed efflux mechanism, it is difficult to interpret the apparent lack of energy-dependent CQ uptake in the absence of VP. If a P-glycoprotein-type efflux mechanism is responsible for reduced drug accumulation by CQ-resistant parasites, the reduction of ATP might be expected to increase drug accumulation in resistant cells, as demonstrated in MDR cancer cells (8). In malaria parasites, the situation is more complex because the uptake process is apparently also dependent on ATP, which is required to maintain the proton gradient necessary for the uptake of CQ (26). This obviously complicates the interpretation of such experiments in the parasite system as neither uptake nor efflux can be studied in isolation and it is possible that reduction of ATP could affect energy-dependent CQ uptake and efflux to the same extent.

Data presented in Fig. 5 show that the effects of VP and VBL (also a P-glycoprotein antagonist) are completely abrogated when DOG is substituted for GLU. It is possible that DOG is binding to the chemosensitizers and reducing drug availability and thus reducing inhibition of CQ transport. However, it is unlikely that DOG will bind with equal and high affinity to both of these structurally distinct agents. On the contrary, in addition to its inhibitory effects on glycolysis, DOG is known to bind ATP in an unusable form as 2-deoxy-D-glucose-6-phosphate (27). Therefore, we believe that these results most likely indicate that the component of drug resistance that is sensitive to VP is an ATP-dependent process.

The effectiveness of the resistance mechanism for the drugs used in this study seems to depend more on physicochemical properties than a tightly defined structural requirement (Figs. 1–3). A similar negative correlation of lipid solubility with degree of anticancer drug resistance and chemosensitization has been observed in MDR cancer cells; in a recent study of anthracycline analogs, both the degree of

resistance and the degree of dose modification obtained with VP were negatively correlated with increasing drug hydrophobicity (17). In addition to providing an explanation for the puzzling patterns of cross-resistance in this parasite, our data may provide some parallels between drug resistance in *P. falciparum* and tumor cell MDR phenomena that is thought to result from ATP-dependent drug efflux. The accepted concept that drug accumulation is achieved as a balance between passive permeability of the membrane and the effect of an efflux pump could provide an explanation for our data. As the lipid solubility increases, drugs would be expected to enter the cell faster through increased passive membrane permeability. The putative pump would eventually be overwhelmed as it failed to cope with the enhanced rate of entry, resulting in a loss of drug resistance.

The exact mechanism of action of the P-glycoprotein responsible for tumor cell MDR is unknown, and many theories have been put forward to explain the lack of specificity of drug transport. One recent theory suggests that P-glycoprotein pumps water across the cell membrane, flushing out of the membrane any anticancer drugs that are present and thereby removing those drugs from the cell (28). Drawing parallels with drug resistance in *P. falciparum*, this is an attractive theory to explain the correlation of increasing water solubility with increasing cross-resistance and increasing VP effect. Molecules in the membrane that are more water soluble could be removed more efficiently by such a mechanism. Alternatively, an indirect effect of parasite P-glycoprotein on intracellular pH with consequent effects on the membrane potential (29) could also explain our results. The reduction of the electrochemical gradient into the food vacuole would lead to a greater reduction of accumulation of more highly charged, less-lipophilic drugs. Therefore, a greater loss of activity would be expected for the more-water-soluble drugs.

Although these concepts could account for the greater activity of AQ than CQ against CQ-resistant isolates, there remains some cross-resistance of CQ and AQ [see Bray *et al.* (23)]. VP is unable to improve the AQ dose response (Table 2), and these observations suggest that some component of the resistance mechanism is not sensitive to VP. With regard to the major metabolites of both CQ and AQ, it seems that both cross-resistance and VP effect are increased progressively as the drugs are dealkylated (Table 1). This observation has important implications for the selection of resistant parasites *in vivo* and suggests that quinoline-type drugs with a tertiary nitrogen in the side chain that is resistant to metabolic oxidation (e.g., AMOPYR and NTB-AQ) may have a pharmacological advantage over available drugs.

Studies of the known P-glycoprotein homologues of *P. falciparum* have presented a rather confusing picture. This parasite possesses two *mdr*-like genes termed *pfmdr1* and *pfmdr2* (30, 31). Resistance to the 4-aminoquinolines cannot be adequately correlated with overexpression or mutation of either gene (12, 13, 32, 33), although there is some recent evidence suggesting that drug movement can be mediated by the *pfmdr1* gene product (34). Indeed, CQ resistance has been mapped to a genetic locus on chromosome 7, an area of the parasite genome not associated with any known *mdr*-like genes (35). Taken together, these observations suggest that the mutation(s) responsible for CQ resistance do not directly involve the *mdr* genes of the parasite. Our previous studies have indicated significant MDR-like drug transport phenomena in CQ-susceptible as well as CQ-resistant isolates (4), and these observations are supported by data in Bray *et al.* (23). It is possible that mutations of other genes (e.g., genes controlling membrane phospholipid composition) could modulate the activity of the putative parasite efflux pump, allowing it to work more efficiently in resistant isolates. Anionic phospholipids are important membrane targets for cationic hydrophobic drugs such as doxorubicin and VP. In a recent study using a model membrane system, an increase in the amount of membrane anionic phospholipid resulted in enhanced binding of doxorubicin and VP (36). It is reasonable to speculate that such changes would also increase the binding of cationic antimalarial drugs such as CQ and AQ. It is interesting to note that CQ resistance has recently been correlated with increased acidic phospholipid content of infected cells (37). These changes may increase the efficiency of extraction of water-soluble molecules from parasite membranes by putative drug transporters, resulting in lower intracellular drug concentration.

The current studies provide new insights into the mechanism of aminoquinoline resistance in *P. falciparum* and help to explain reported cross-resistance patterns. Involvement of a P-glycoprotein-type transporter in the resistance mechanism is a possibility, although our data cannot differentiate between a direct or indirect drug interaction with any such transporter. Regardless of the molecular basis for resistance, the findings of this study should prove to be of value in the rational design and screening of new aminoquinoline/aminoacridine-type antimalarials. We predict that compounds with a log D at pH 7.2 of 1.8–3, containing a tertiary amino group 0.6–0.9 nm from the ring nitrogen of a quinoline or acridine ring, would have *in vitro* (and *in vivo*) activity in the nanomolar range and exhibit minimal cross-resistance with the CQ-resistant phenotype.

References

1. Fitch, C. D. Chloroquine resistance in malaria: a deficiency of chloroquine binding. *Proc. Natl. Acad. Sci. USA* **64**:1181–1187 (1969).
2. Verdier, F., J. Le Bras, F. Clavier, I. Hatin, and M. Blayo. Chloroquine uptake by *Plasmodium falciparum*-infected human erythrocytes during *in vitro* culture and its relationship to chloroquine resistance. *Antimicrob. Agents Chemother.* **27**:561–564 (1985).
3. Krogstad, D. J., I. Y. Gluzman, D. E. Kyle, A. M. J. Oduola, S. K. Martin, W. K. Milhous, and P. H. Schlesinger. Efflux of chloroquine from *Plasmodium falciparum: mechanism* of chloroquine resistance. *Science (Washington D. C.)* **235**:1283–1285 (1987).
4. Bray, P. G., R. E. Howells, G. Y. Ritchie, and S. A. Ward. Rapid chloroquine efflux phenotype in both chloroquine-sensitive and chloroquine-resistant *Plasmodium falciparum*. *Biochem. Pharmacol.* **44**:1317–1324 (1992).
5. Gottesman, M. M., and I. Pastan. Biochemistry of multidrug resistance mediated by the multidrug transporter. *Annu. Rev. Biochem.* **62**:385–427 (1993).
6. Martin, S. K., A. M. J. Oduola, and W. K. Milhous. Reversal of chloroquine resistance in *Plasmodium falciparum* by verapamil. *Science (Washington D. C.)* **235**:899–901 (1987).
7. Dano, K. Active outward transport of daunomycin in resistant Erlich ascites tumor cells. *Biochim. Biophys. Acta* **323**:466–483 (1973).
8. Skovsgaard, T. Mechanisms of resistance to daunorubicin in Erlich ascites tumor cells. *Cancer Res.* **38**:1785–1791 (1978).
9. Ginsburg, H., and W. D. Stein. Kinetic modelling of chloroquine uptake by malaria infected erythrocytes. *Biochem. Pharmacol.* **41**:1463–1470 (1991).
10. Bray, P. G., M. K. Boulter, G. Y. Ritchie, and S. A. Ward. Relationship of global chloroquine transport and reversal of chloroquine resistance in *P. falciparum*. *Mol. Biol. Parasitol.* **63**:87–94 (1994).
11. Roepe, P. D. The role of MDR protein in altered drug translocation across tumor cell membranes. *Biochim. Biophys. Acta* **1241**:385–406 (1995).
12. Cowman, A. F., S. Karcz, D. Galatis, and J. G. Culvenor. A P-glycoprotein homologue of *Plasmodium falciparum* is localised on the digestive vacuole. *J. Cell Biol.* **113**:1033–1045 (1991).
13. Wellems, T. E., L. J. Panton, I. Y. Gluzman, V. E. de Rosario, R. W. Gwadz,

A. Walker-Jonah, and D. J. Krogstad. Chloroquine resistance not linked to to mdr-like gene in a *Plasmodium falciparum* cross. *Nature (Lond.)*. **345:** 253–255 (1990).
14. Karcz, S., and A. F. Cowman. Minireview: similarities and differences between the multidrug resistance phenotype of mammalian tumour cells and chloroquine resistance in *Plasmodium falciparum*. *Exp. Parasitol.* **73:**233–240 (1991).
15. Geary, T. G., A. A. Divo, and J. B. Jensen. Activity of quinoline-containing antimalarials against chloroquine sensitive and resistant strains of *Plasmodium falciparum in vitro*. *Trans. R. Soc. Trop. Med. Hyg.* **81:**499–503 (1987).
16. Childs, G. E., E. F. Boudreau, W. K. Milhous, T. Wimonwattratee, N. Pooyindee, L. Pang, and D. E. Davidson, Jr. A comparison of the *in vitro* activities of amodiaquine and desethylamodiaquine against isolates of *Plasmodium falciparum*. *Am. J. Trop. Med. Hyg.* **40:**7–11 (1989).
17. Mülder, H. S., H. Dekker, H. M. Pinedo, and J. Lankelma. The P-glycoprotein-mediated relative decrease in cytosolic free drug concentration is similar for several anthracyclines with varying lipophilicity. *Biochem. Pharmacol.* **50:**967–974 (1995).
18. Zamora, J. M., H. L. Pearce, and W. T. Beck. Physical-chemical properties shared by compounds that modulate multidrug resistance in human leukemic cells. *Mol. Pharmacol.* **33:**454–462 (1988).
19. Foote, S. J., J. K. Thompson, A. F. Cowman, and D. J. Kemp. Amplification of the multidrug resistance gene in some chloroquine resistant isolates of *P. falciparum*. *Cell* **57:**921–930 (1989).
20. Haruki, K., P. G. Bray, S. A. Ward, M. Hommel, and G. Y. Ritchie. Chloroquine resistance of *Plasmodium falciparum*: further evidence for a lack of association with mutations of the *pfmdr1* gene. *Trans. R. Soc. Trop. Med. Hyg.* **88:**694 (1994).
21. Jewell, H., J. L. Maggs, A. C. Harrison, P. M. O'Neill, J. E. Ruscoe, and B. K. Park. Role of hepatic metabolism in the bioactivation and detoxification of amodiaquine. *Xenobiotica* **25:**199–217 (1995).
22. Purba, H. S., J. L. Maggs, M. L. Orme, D. J. Back, and B. K. Park. The metabolism of 17-*alpha*-ethinylestradiol by human liver microsomes: formation of catechol and chemically reactive metabolites. *Br. J. Clin. Pharmacol.* **23:**447–453 (1987).
23. Bray, P. G., S. R. Hawley, and S. A. Ward. 4-Aminoquinoline resistance of *Plasmodium falciparum*: insights from the study of amodiaquine uptake. *Mol. Pharmacol.* **50:**1551–1558 (1996).
24. Boulter, M. K., Bray, P. G., Howells, R. E., and Ward, S. A. The potential of desipramine to reverse chloroquine resistance of *Plasmodium falciparum* is reduced by its binding to plasma protein. *Trans. R. Soc. Trop. Med. Hyg.* **87:**303 (1993).
25. Foley, L., W. M. Deady, K. Ng, A. F. Cowman, and L. Tilley. Photoaffinity labeling of chloroquine binding proteins in *Plasmodium falciparum*. *J. Biol. Chem.* **269:**6955–6961 (1994).
26. Yayon, A., Z. I. Cabantchik, and H. Ginsburg. Susceptibility of human malaria parasites to chloroquine is pH dependent. *Proc. Natl. Acad. Sci. USA* **82:**2784–2788 (1985).
27. Detwiler, T. C. Effects of deoxyglucose on platelet metabolism. *Biochim. Biophys. Acta* **244:**303–310 (1971).
28. Zeuthen, T., and W. D. Stein. Cotransport of salt and water in membrane proteins: membrane proteins as osmotic engines. *J. Membr. Biol.* **137:**179–195 (1994).
29. Roepe, P. D., L. Yong Wei, J. Cruz, and D. Carlson. Lower electrical membrane potential and altered pH_i homeostasis in multidrug resistant (MDR) cells: further characterization of a series of MDR cell lines expressing different levels of P-glycoprotein. *Biochemistry* **32:**11042–11056 (1993).
30. Foote, S. J., Thompson, J. K., Cowman, A. F., and Kemp, D. J. Amplification of the multidrug resistance gene in some chloroquine-resistant isolates of *P. falciparum*. *Cell* **57:**921–930 (1989).
31. Wilson, C. M., A. E. Serrano, A. Wasley, M. P. Bogenshutz, A. H. Shankar, and D. F. Wirth. Amplification of a gene related to mammalian mdr genes in drug resistant *P. falciparum*. *Science (Washington D. C.)* **244:**1184–1186 (1989).
32. Wilson, C. M., Volkman, S. K., Thaithong, S., Martin, R. K., Kyle, D. E., Milhous, W. K and Wirth, D. F. Amplification of *pfmdr1* associated with mefloquine and halofantrine resistance in *Plasmodium falciparum* from Thailand. *Mol. Biol. Parasitol.* **57:**151–160 (1993).
33. Awad-el-Kariem, F. M., Miles, M. A., and Warhurst, D. C. Chloroquine resistant *Plasmodium falciparum* isolated from the Sudan lack two mutations in the *pfmdr1* gene thought to be associated with chloroquine resistance. *Trans. R. Soc. Trop. Med. Hyg.* **86:**587–589 (1992).
34. Volkman, S. K., Cowman, A. F., and Wirth, D. F. Functional complementation of the *ste6* gene of *Saccharomyces cerevisiae* with the *pfmdr1* gene of *Plasmodium falciparum*. *Proc. Natl. Acad. Sci. USA* **92:**8921–8925 (1995).
35. Wellems, T. E., A. Walker-Jonah, and L. J. Panton. Genetic mapping of the chloroquine resistance locus on *Plasmodium falciparum* chromosome 7. *Proc. Natl. Acad. Sci. USA* **88:**3382–3386 (1991).
36. Speelmans, G., R. W. H. M. Staffhorst, F. De Wolf and B. De Kruijff. Verapamil competes with doxorubicin for binding to anionic phospholipids resulting in increased internal concentrations and rates of passive transport of doxorubicin. *Biochim. Biophys. Acta* **1238:**137–146 (1995).
37. Shalmiev, G., and H. Ginsburg. The susceptibility of the human malaria parasite *Plasmodium falciparum* to quinoline-containing drugs is correlated to the lipid composition of the infected erythrocyte membranes. *Biochem. Pharmacol.* **46:**365–374 (1993).

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