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4-Aminoquinoline Resistance of *Plasmodium falciparum*: Insights from the Study of Amodiaquine Uptake

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SUMMARY

The relationship between antimalarial activity and drug accumulation of chloroquine and amodiaquine was evaluated with four chloroquine-resistant and two chloroquine-susceptible isolates of *Plasmodium falciparum*. Susceptibility of the strains to amodiaquine was correlated with susceptibility to chloroquine (r^2 = 0.96). Similarly, accumulation of amodiaquine was correlated with accumulation of chloroquine (r^2 = 0.94). Accumulation of both chloroquine and amodiaquine was significantly reduced in chloroquine-resistant isolates ($p < 0.005$). For the panel of isolates, the accumulation ratio of both drugs was inversely proportional to drug susceptibility (r^2 = 0.963 and 0.994 for amodiaquine and chloroquine, respectively). Time course studies highlighted a reduced initial rate of amodiaquine accumulation in chloroquine-resistant isolates compared with chloroquine-susceptible isolates, with no evidence of an enhanced drug efflux rate. Daunomycin, a modulator of parasite chloroquine transport, significantly increased steady state accumulation of both drugs in chloroquine-resistant isolates and, to a lesser extent, in chloroquine-susceptible isolates. Furthermore, daunomycin increased the initial rate of accumulation of amodiaquine in both chloroquine-resistant and chloroquine-susceptible isolates. Resistance to 4-aminoquinoline drugs is associated with reduced drug permeability rather than enhanced cellular exit of preaccumulated drug, and daunomycin seems to increase the permeability of parasites to aminoquinolines. A new model of 4-aminoquinoline resistance is proposed to take account of these and earlier observations.

In many parts of the world, the 4-aminoquinoline AQ is used in preference to CQ for the treatment of *Plasmodium falciparum* malaria (1). This is the direct consequence of extensive parasite resistance to CQ and the need for a cheap effective alternative. AQ is rapidly cleared *in vivo* to an active dealkylated metabolite, monodesethyl AQ (2). Therefore, any cross-resistance of CQ-resistant infections to AQ *in vivo* must be interpreted as cross-resistance between CQ and monodesethyl AQ (3).

The principal structural difference between CQ and AQ is that AQ possesses a phenolic substitution in the side chain. In view of the close structural similarity of the two drugs, some degree of cross-resistance might be expected. However, studies of cross-resistance between CQ and AQ *in vitro* have been inconclusive; some studies have demonstrated cross-resistance between the two drugs (4, 5), and others have not (6).

Investigations into parasite susceptibility to CQ have demonstrated the inability of CQ-resistant parasites to accumulate as much CQ as CQ-susceptible parasites (7–10).

Two theories have been proposed to explain these observations. The first, which is based on the ability of VP and other agents to "reverse" CQ resistance (11), consists of an enhanced energy-dependent CQ efflux capacity in the resistant parasite (12). The second theory is based on a reduced proton gradient into the food vacuole of the resistant parasite (13). Because of the weak base properties of CQ, the accumulation of the drug is driven by a proton gradient between the external environment and the acid food vacuole within the parasite (14).

As noted above, CQ and AQ are very similar in structure. In view of the current theories explaining CQ resistance, analysis of drug accumulation and its relationship to dose response may be expected to highlight parasite resistance mechanisms common to both drugs. Also, such studies will provide fundamental information, which is clearly lacking concerning AQ accumulation and activity. In view of the very high levels of AQ accumulation by CQ-resistant and CQ-susceptible isolates (7, 15), true antimalarial activity of AQ was assessed through analysis of the effect of inoculum size on the dose response (13).

Furthermore, to assess the relative importance of these factors in the control of parasite susceptibility to 4-aminoquinoline drugs, we used direct and indirect techniques that

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ABBREVIATIONS: HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; PRBC, parasitized red blood cells; CAR, cellular accumulation ratio; CQ, chloroquine; AQ, amodiaquine; VP, verapamil.

were designed to differentiate between reduced drug uptake and enhanced drug efflux.

Materials and Methods

Parasites. Six isolates of *P. falciparum* were used in the study: 3D7 and HB3 were CQ susceptible (IC_{50} = <70 nM), and K1, PH3, Indochina, and V1S were CQ resistant (IC_{50} = >70 nM). The K1, 3D7, and HB3 isolates were obtained from Prof. D. Walliker (Edinburgh University, Scotland, UK); the PH3 and Indochina isolates were obtained from Prof. Marcel Hommel (Liverpool School of Tropical Medicine, Liverpool, UK); and the V1S strain was supplied by Dr. John Hyde (University of Manchester Institute of Science and Techology, Manchester, UK).

Cultures contained a 1–5% suspension of O^+ erythrocytes in RPMI 1640 medium supplemented with 10% human AB serum containing 25 mM HEPES and 23 mM $NaHCO_3$ (complete medium). Cultures were gassed with an atmosphere of 93% N_2/4% O_2/3% CO_2.

Sensitivity assays. Drug sensitivity was determined by measuring the incorporation of [^{3}H]hypoxanthine into parasite nucleic acids (16). Cultures were exposed to serial dilutions of CQ or AQ for 48 hr in the absence or presence of fixed concentrations of VP as indicated. Initial inoculum size is indicated in the figure legends.

Drug accumulation. Radiolabeled [^{3}H]CG (specific activity, 69 Ci/mM) and radiolabeled [^{3}H]daunomycin (specific activity, 4 Ci/mM) were obtained from DuPont-New England Nuclear (Boston, MA). Radiolabeled [^{3}H]AQ (specific activity, 106 mCi/mM) was synthesized and purified to 97% by high performance liquid chromatography as described previously (15). Accumulation of CQ was measured at an inoculum size of 1 (inoculum size = percentage of parasitemia × percentage of hematocrit), which is equivalent to a fractional volume of PRBC of 0.0001. Accumulation of AQ was measured at an inoculum size of 3 to compensate for lower specific activity of this radiolabel. Parasites were incubated with drug at 37° or at room temperature under an atmosphere of 93% N_2/4% O_2/3% CO_2 in complete medium or in phosphate-buffered saline, pH 7.4, under atmospheric air in the absence or presence of glucose at 10 mM. Incubations were also performed in the absence or presence of VP or daunomycin, both added at time zero 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). Daunomycin accumulation was measured after incubating parasites with radiolabeled daunomycin (at an initial concentration of 43 nM) in complete medium under an atmosphere of 93% N_2/4% O_2/3% CO_2 at 37° for 60 min. Daunomycin accumulation was measured in the absence or presence of AQ or VP added at time zero at concentrations of 500 nM and 5 μM, respectively. In the case of incubations with radiolabeled CQ, the initial external concentration was 1 nM. In some experiments (data presented in Table 1), the initial external concentration of radiolabeled AQ was adjusted to give a steady state external AQ concentration equivalent to the IC_{50} value. This was done to enable analysis of the relationship of AQ accumulation and activity at external drug concentrations equivalent to the IC_{50} value. In all other experiments, the initial concentration of radiolabeled AQ was 10 nM. In all experiments, counts resulting from drug accumulation by a similar volume of uninfected erythrocytes were subtracted from the parasite pellet counts. The time course experiments were performed under atmospheric air because of the difficulty of maintaining the atmosphere of 93% N_2/4% O_2/3% CO_2 during rapid sampling. Exposure of complete medium to air can cause pH increases due to the high bicarbonate concentration, so instead phosphate-buffered saline with 10 mM glucose was used. The drug uptake process is probably not affected by the different culture conditions used for the time course experiments because steady state accumulation of AQ was the same when either protocol was used for the incubation: CARs measured over 1 hr at 37° in complete medium or phosphate-buffered saline were 4048 ± 1156 and 3885 ± 1951, respectively, for the HB3 strain; and 1892 ± 310 and 1996 ± 1404, respectively, for the K1 strain (values are taken from Tables 1 and 2). 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 28° or 37° time points. Initial accumulation rate data were analyzed using GraFit (Erithacus Software, Staines, UK). The initial accumulation data (t = 15–120 sec) were best fit (using reduced χ^2) to a first-order rate equation:

$$AQ_t = AQ_\infty (1 - e^{-kt})$$

where AQ_t is the amount of AQ accumulated at time t, AQ_∞ is the steady state accumulation of AQ, and k is the rate constant. The initial rate of AQ accumulation or linear portion of the curve is defined as $k \cdot AQ_\infty$ and was calculated with the computer on the basis of the parameters generated by the fit. The full time course of AQ uptake was analyzed according to the method of Ginsburg and Stein (14). This analysis is designed to highlight differences in the efflux of weak base antimalarials like CQ and AQ, for which the uptake process is thought to involve trapping of protonated drug inside acidic intracellular compartments. Any transport of charged drug out of the parasite across any of the parasite membranes will reduce the time taken to reach steady state; this is demonstrated most clearly when the data are normalized for steady state accumulation and plotted as fractional fill. To calculate the CAR, a sample of incubation medium was also removed and counted at each time point. The CAR is the ratio of the amount of labeled drug in the cells to the amount of labeled drug in a similar volume of medium. Tests of significance between groups used the two-tailed Mann-Whitney *U* test. The CAR values are presented as mean ± standard deviation, and the sample number is given in the figure legends.

Inoculum effect. The effect of increasing the initial fractional volume of PRBC in a sensitivity assay is to increase the amount of drug depleted from the medium in the sensitivity assay plates. The

TABLE 1

Relationship of dose-response (IC_{50}) and drug accumulation (CAR) for CQ and AQ

AQ IC_{50} values are derived from data from inoculum-effect experiments and are mean ± standard error. CQ IC_{50} values are measured directly and are mean ± standard error from of four experiments. Values for CQ and AQ CAR are measured directly. CAR values are mean ± standard error of at least three experiments for CQ and at least five experiments for AQ.

	Susceptible isolates		Resistant isolates			
	3D7	HB3	V1S	Indo	PH3	K1
AQ IC_{50} (nM)	2.4 ± 1.6 r^2 = 0.992	3.3 ± 1.1 r^2 = 0.998	6.4 ± 5.8 r^2 = 0.917	6.9 ± 2.0 r^2 = 0.964	10.5 ± 2.7 r^2 = 0.996	18.7 ± 4.5 r^2 = 0.984
AQ CAR	4368 ± 1931	4048 ± 1156	2616 ± 510	2310 ± 1836	2097 ± 1166	1892 ± 310
CQ IC_{50} (nM)	7.0 ± 1.6	9.75 ± 2.75	98.2 ± 19	83.3 ± 16	172 ± 39	238 ± 27
CQ CAR	2335 ± 695	1859 ± 50	435 ± 101	425 ± 93	491 ± 18	442 ± 37
$[AQ]_{int}$ at IC_{50} (μM)	10.3	13.5	16.7	15.9	21.9	35.3

$[AQ]_{int}$, intracellular AQ concentration.

TABLE 2

Effect of removal of glucose on the steady state accumulation of CQ and AQ

Values are mean ± standard error.

	HB3 Isolate			K1 Isolate		
	No glucose	Plus glucose	Percent Change	No glucose	Plus glucose	Percent Change
Mean AQ CAR	787 ± 251 (n = 7)	3885 ± 1951 (n = 7)	+394% $p < 0.001$	747 ± 200 (n = 4)	1996 ± 1407 (n = 4)	+167% $p < 0.05$
Mean CQ CAR	549 ± 327 (n = 3)	2459 ± 1095 (n = 3)	+347% $p < 0.05$	550 ± 250 (n = 3)	410 ± 66 (n = 3)	−25% N.S.

concentration of drug in the medium at steady state is therefore lower than the initial concentration, leading to an underestimation of dose response. We established experimentally that AQ accumulation of uninfected erythrocytes is small compared with that of infected cells: The mean CAR for AQ into uninfected RBC was 10.2 ± 1.6 (n = 15) compared with 1892 ± 310 for PRBC of the K1 isolate, the isolate that accumulates the least AQ (see Table 1). Inoculum effect experiments were performed with parasitemias in excess of 5%; therefore, the fraction of uptake resulting from uninfected erythrocytes at any inoculum size was always <10% of the total. If we assume that the extracellular AQ concentration at IC_{50} is constant, then the measured IC_{50} value should increase as inoculum size increases due to drug depletion from the medium by the parasite. Using a method developed to correct the CQ dose response for inoculum effect (13), we interpolated the AQ IC_{50} value at a fractional volume of zero (no medium depletion) from a graph of IC_{50} value versus fractional volume of PRBC. This value is called the "true IC_{50}."

Relationship of drug accumulation and activity. Studies of the uptake of 4-aminoquinoline drugs by malaria parasites have identified a two-component system. There is a saturable component of high affinity that is stimulated by glucose and a nonsaturable component of low affinity that is not stimulated by glucose (7, 17, 18). Curves of drug uptake versus external concentration can be simulated by superimposing a rectangular hyperbola onto a straight line, described by the equation:

$$[TD] = \frac{[ED] \cdot Cap}{[ED] + K_d} + m \cdot [ED]$$

where [TD] is the total concentration of drug taken up, [ED] is the concentration of drug in the external medium and is proportional to the concentration of drug available to bind the high affinity component, *Cap* is the capacity of the high affinity component, K_d is the apparent dissociation constant of the high affinity component, and m is the slope of the line describing the low affinity component.

It is clear that the saturable component of drug uptake is reduced in CQ-resistant malaria parasites, and CQ resistance has been attributed to a loss of high affinity uptake (17, 18). In our model of drug resistance, the nature and capacity of the high affinity binding site are unchanged, but the resistant parasite has a mechanism (e.g., an enhanced efflux capability) that would reduce intracellular drug concentration compared with the susceptible parasite. Higher external drug concentrations would be required to saturate high affinity uptake in resistant isolates, resulting in a higher apparent *Kd* value compared with susceptible isolates. It has been shown that low affinity drug uptake is similar in CQ-susceptible and CQ-resistant *P. falciparum* (7), suggesting that the resistance mechanism is without a significant effect on low affinity uptake. In this case, the proportion of total uptake at IC_{50} that is at low affinity would be greater in resistant parasites than in susceptible parasites (with the assumption that equivalent high affinity uptake is required at IC_{50}). If low affinity uptake is equivalent in all isolates, then based on the above relationship, the plot of total intracellular drug concentration at IC_{50} against external drug concentration at IC_{50} for our isolates should give a straight line with a slope of m (the low affinity CAR) and a y-intercept equivalent to the amount of high affinity uptake at IC_{50}. CAR is equivalent to total intracellular drug concentration divided by the extracellular drug concentration. From the above relationship, the following is derived:

$$CAR = \frac{\left([TD] = \frac{[ED] \cdot Cap}{[ED] + K_d} + m \cdot [ED]\right)}{[ED]}$$

The plot of CAR at IC_{50} against the reciprocal of [ED] at IC_{50} for our isolates will give a linear relationship if the amount of high affinity uptake at IC_{50} is the same. The slope of the line corresponds to the amount of high affinity uptake at IC_{50}, and the intercept corresponds to the low affinity CAR m.

Results

Susceptibility of the isolates to CQ and AQ. Fig. 1 represents the effect of increased inoculum size on the measured AQ IC_{50} in two CQ-susceptible and two CQ-resistant strains. Under assay conditions, the availability of drug is limiting, causing the measured IC_{50} to increase linearly with increasing inoculum size. The relationship between IC_{50} values for different parasite isolates (i.e., the cross-resistance pattern) will alter as a function of inoculum size if the degree of drug depletion (CAR) is not constant. It is clear from Fig. 1 that there is a decrease in measured cross-resistance from ~4-fold at a fractional volume of 0.0001 to ~2-fold at a fractional volume of 0.001. Apparent narrowing of the cross-resistance pattern is a consequence of greater AQ accumulation by CQ-susceptible isolates, resulting in a relatively larger increase in measured IC_{50} (compared with CQ-resis-

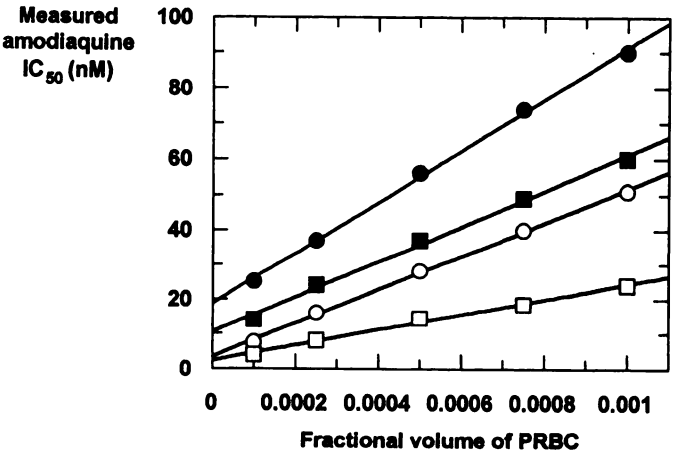

Fig. 1. The effect of increasing inoculum size (fractional volume of PRBC) on the measured dose response to AQ sensitivity as described in Materials and Methods using five different initial inoculum sizes. Measured AQ IC_{50} plotted against fractional volume of PRBC for CQ-susceptible isolates (○) HB3 and (□) 3D7 and for CQ-resistant isolates (●) K1 and (■) PH3.

tant isolates) for a given inoculum size. For these reasons, we interpolated the true IC_{50} value from the *y*-axis intercept, a theoretical inoculum size of zero.

True AQ IC_{50} values for all six isolates, interpolated from the inoculum effect plots, are given in Table 1 with correlation (r^2) and standard deviation values associated with the linear fit of the inoculum data. All isolates displayed greater susceptibility to AQ than to CQ. The CQ IC_{50} values presented in Table 1 were obtained from single inoculum size experiments (CQ assays were performed at a fractional volume of PRBC of 0.00005 to minimize the inoculum effect).

Although AQ was more active than CQ, it was still less effective against CQ-resistant isolates that against CQ-susceptible isolates. The correlation of measured CQ IC_{50} and interpolated AQ IC_{50} values for all the isolates is shown in Fig. 2A (linear regression $r^2 = 0.96$).

Accumulation of CQ and AQ. The CAR values of CQ and AQ for all of the isolates are shown in Table 1. These values were measured directly using radiolabeled drugs.

The accumulation of both CQ and AQ is significantly reduced in CQ-resistant parasites compared with CQ-susceptible parasites ($p < 0.005$). The reduction in CQ accumulation is 3–5-fold compared with 2-fold for AQ (Table 1). The AQ CAR is linearly related to the CQ CAR (linear regression $r^2 = 0.94$) and is shown in Fig. 2B. However, accumulation data for both drugs seem to fall into two discrete groups rather than following the distribution of drug susceptibility. Intracellular AQ concentration at IC_{50} was calculated by multiplying the true IC_{50} value by the CAR. It is evident that CQ-resistant isolates require higher internal AQ concentrations to kill them (Table 1 and Fig. 3A). Indeed, internal AQ concentration at IC_{50} is linearly related to IC_{50} (linear regression $r^2 = 0.98$). Based on the model outlined above, the low affinity CAR obtained from the slope of the graph was 1483 ± 80, and the amount of high affinity drug uptake at IC_{50} as given by the *y*-intercept was 7.07 ± 0.78 μM (see legend to Fig. 3A to identify low and high affinity accumulation values). AQ CAR is inversely proportional to IC_{50}, and this relationship is plotted in Fig. 3B (linear regression $r^2 =$

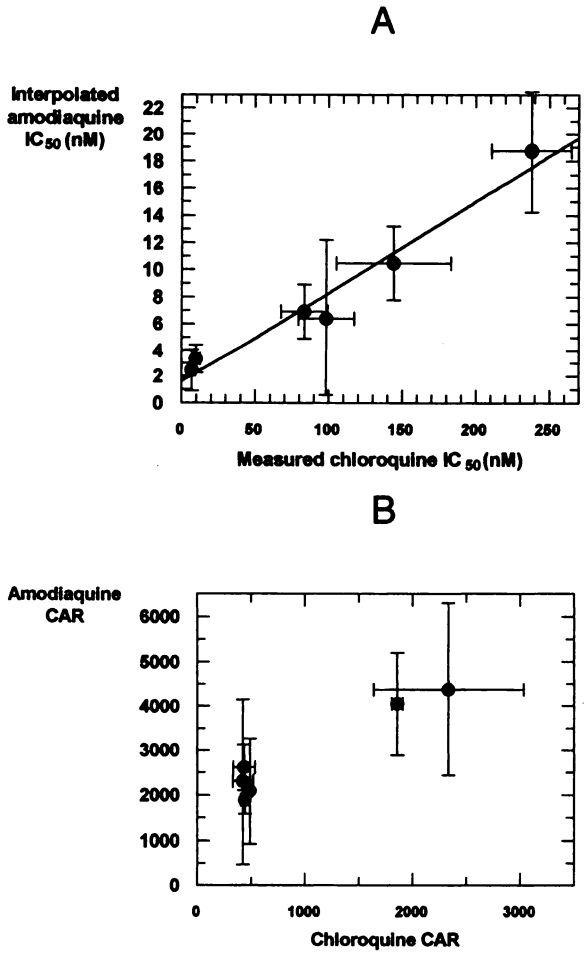

Fig. 2. Relationship of CQ and AQ IC_{50} and CQ and AQ accumulation. A, Correlation of AQ IC_{50} with CQ IC_{50} for six isolates of *P. falciparum* (IC_{50} values are mean ± standard deviation of four experiments for CQ and five experiments for AQ). B, Correlation of AQ cellular accumulation ratio (CAR) with CQ CAR for six isolates of *P. falciparum* (CAR values are mean ± standard deviation of at least three experiments for CQ and five experiments for AQ).

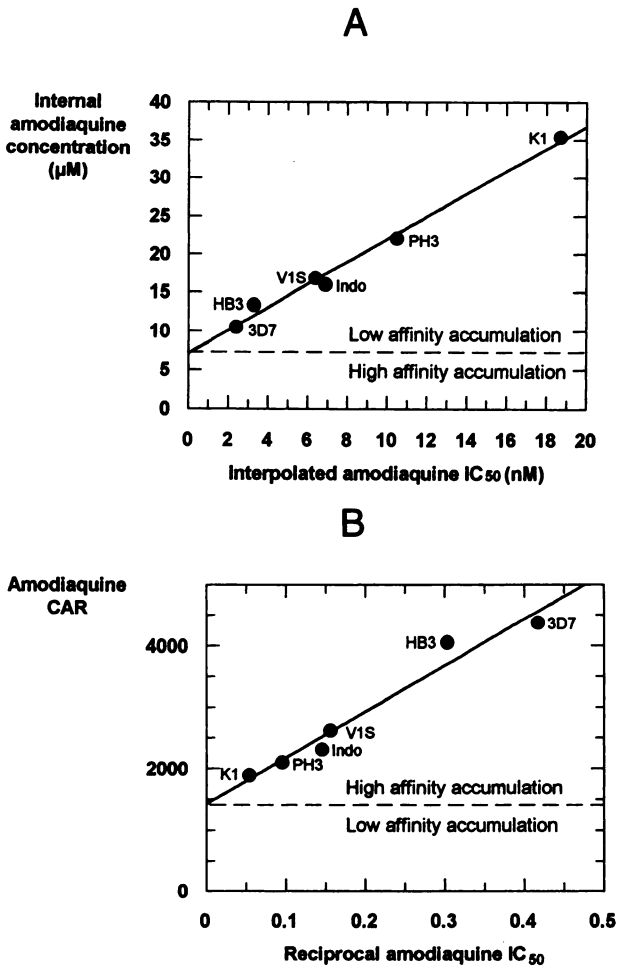

Fig. 3. Relationship of drug accumulation and activity at IC_{50} for AQ. A, Internal AQ concentration at an intracellular AQ concentration IC_{50} plotted against AQ IC_{50} for the six isolates of *P. falciparum*. *Values below the dotted line*, theoretical accumulation at high affinity; *values above the dotted line*, theoretical accumulation at low affinity. B, The relationship of reciprocal IC_{50} with AQ CAR for six isolates. *Values above the dotted line*, theoretical accumulation at high affinity; *values below the dotted line*, theoretical accumulation at low affinity. CAR values are mean of at least five experiments. IC_{50} values are interpolated from the inoculum effect graphs. The internal AQ concentration was calculated by multiplying the AQ CAR values by the interpolated AQ IC_{50} values in Table 1.

0.96) (see legend to Fig. 3B to identify low and high affinity accumulation values). The low affinity CAR obtained from the *y*-intercept was 1422 ± 170, and the amount of high affinity drug uptake at IC_{50} as given by the slope was 7.52 ± 0.73 μM. According to this analysis, resistant isolates have a greater proportion of drug uptake at low affinity than susceptible isolates. For the K1-resistant isolate, for example, 75–78% of uptake is at low affinity; for the 3D7-susceptible isolate, 32–34% of uptake is at low affinity (Fig. 3, A and B).

Analysis of the mechanism of reduced AQ accumulation. In contrast to the well-documented effects of VP on the dose response of the parasites to CQ (11) and the accumulation of CQ (9, 10), VP did not change the dose response to AQ [see Bray *et al.* (19)] or the steady state accumulation of the drug (Fig. 4A).

Data in Fig. 4A show the effects of daunomycin and VP on CQ and AQ accumulation. In contrast to VP, daunomycin increases the accumulation of both drugs by CQ-resistant and -susceptible isolates. VP increases CQ accumulation only in the CQ-resistant isolate, giving a 100–200% increase. For this isolate, daunomycin produced a 400% increase of CQ accumulation. Daunomycin also increased CQ accumulation by the CQ-susceptible isolate by 40% and, importantly, increased AQ accumulation by both the CQ-susceptible and -resistant isolates (by 150% and 270%, respectively). The effect of AQ (500 nM) and VP (5 μM) on the steady state accumulation of daunomycin is shown in Fig. 4B. Accumulation of daunomycin is significantly increased by AQ, and this effect is seen in both CQ-resistant and -susceptible isolates. On the contrary, VP has no effect on the steady state accumulation of daunomycin in these isolates. Unfortunately, it was not possible to test the ability of daunomycin to alter the dose response of the parasites to AQ because of the high inherent antimalarial activity of the drug (data not shown).

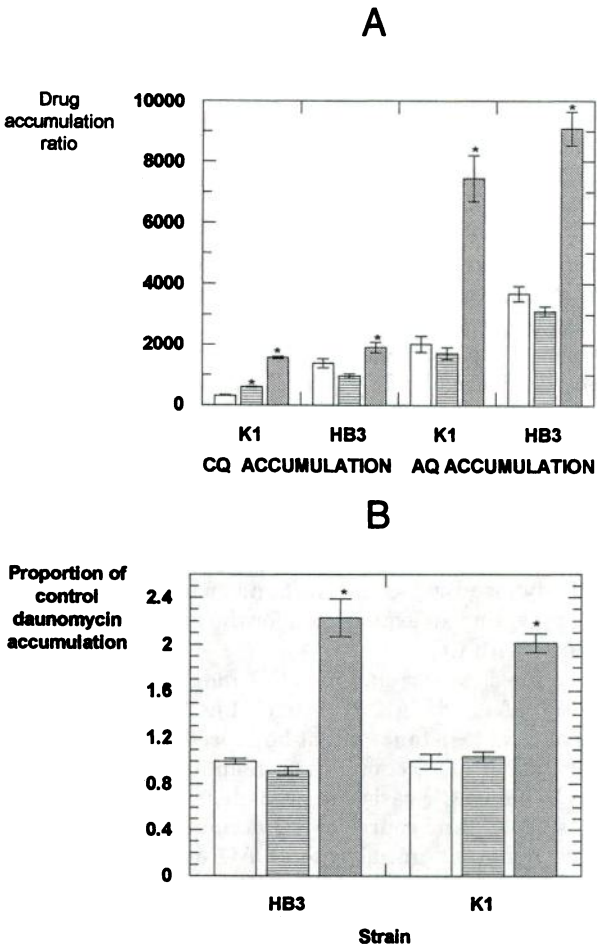

Fig. 4. Possible competition for transport of AQ and daunomycin in CQ-susceptible and -resistant isolates. A, Effect of VP and daunomycin (both inhibitors at 5 μM) on steady state accumulation of CQ and AQ in CQ-susceptible (HB3) and CQ-resistant (K1) isolates of *P. falciparum*. CQ or AQ accumulation was measured alone (*open bars*), with VP (*horizontally striped bars*), or daunomycin (*diagonally striped bars*). B, Effect of VP (5 μM) and AQ (500 nM) on steady state accumulation of daunomycin in CQ-susceptible (HB3) and CQ-resistant (K1) isolates of *P. falciparum*. Daunomycin accumulation was measured alone (*open bars*), with VP (*horizontally striped bars*), or with AQ (*diagonally striped bars*). Values are mean ± standard deviation of three experiments. *, $p < 0.001$.

The energy dependence of CQ and AQ accumulation was determined by measuring steady state drug accumulation in the absence or presence of glucose. In the case of CQ, the addition of glucose caused a 347% increase in the accumulation of CQ in the CQ-susceptible isolate. In contrast, the addition of glucose caused a 25% decrease in CQ accumulation by the CQ-resistant isolate (Table 2), although this effect was not significant ($p = 0.49$). In the case of AQ, however, the addition of glucose caused a significant increase in accumulated drug in both the CQ-susceptible and -resistant isolates (394% and 167%, respectively).

The initial rate of AQ accumulation is lower in the two CQ-resistant strains, K1 and PH3, compared with the CQ-susceptible strains, HB3 and 3D7 (Table 3). This rate was computed from a best fit to the initial time course data as described in Materials and Methods. Furthermore, the increased steady state AQ accumulation produced by daunomycin was accompanied by an increased apparent initial rate of accumulation of AQ in the presence of the drug (Table 3 and Fig. 5). The complete time course data for the four isolates are plotted as fractional fill in Fig. 6. The fractional fill curves for each isolate are essentially superimposable, showing that steady state AQ accumulation is reached at the same time in both CQ-susceptible and -resistant isolates. From the time course analysis, reduced AQ accumulation in CQ-resistant isolates seems to be due to a reduced apparent initial rate of AQ accumulation rather than to an enhanced rate of exit of drug from the parasites.

TABLE 3

Initial rate of uptake of AQ

Measured in HB3, 3D7, K1, and PH3 isolates at 37° and in HB3 and K1 isolates with and without daunomycin (5 μM) at 28°. Values are expressed as change in CAR per minute. Time zero CAR blanks were subtracted from data points as described in Materials and Methods. Values were 103, 63, 72, and 77 for K1, PH3, HB3, and 3D7 at 37° and 92, 97, 59, and 63 for K1 without daunomycin, K1 with daunomycin, HB3 without daunomycin, and HB3 with daunomycin at 28°.

Strain	Initial uptake rate at 37°	Initial uptake rate at 28°	
		Without daunomycin	With daunomycin
K1	347	159	435
PH3	403	N.D.	N.D.
HB3	562	356	575
3D7	610	N.D.	N.D.

N.D., not determined.

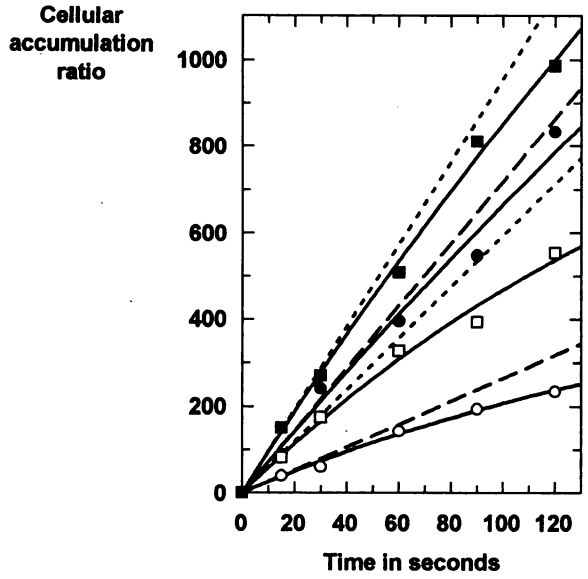

Fig. 5. Effect of daunomycin on the initial rate of uptake of AQ in CQ-susceptible and -resistant isolates. Initial rate of uptake of AQ by CQ-susceptible (HB3, □) and CQ-resistant (K1, ○) isolates of *P. falciparum* and the influence of daunomycin (5 μM, ■ and ●). *Dotted lines*, initial rates for CQ-susceptible isolate; *dashed lines*, initial rates for CQ-resistant isolate (values are mean of three separate experiments performed at 28°).

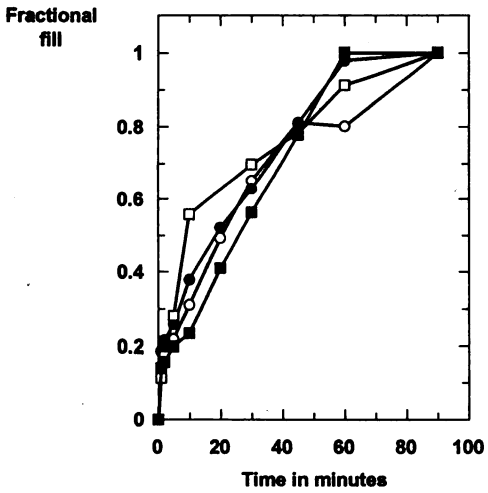

Fig. 6. Time course of AQ uptake plotted as "fractional fill." Uptake of AQ by CQ-susceptible isolates HB3 (○) and 3D7 (□) and CQ-resistant isolates K1 (●) and PH3 (■) isolates of *P. falciparum* plotted as fraction of steady state value (values are mean of three separate experiments performed at 37°).

Discussion

Studies of the uptake and activity of CQ in malaria parasites have repeatedly indicated that the difference in drug accumulation between CQ-susceptible and -resistant isolates is much smaller than the difference in drug susceptibility (7, 8, 10, 18, 20, 21). These observations have been confirmed and extended to include AQ (Table 1). For CQ, a 10–20-fold difference in susceptibility corresponds to only a 4–5-fold difference in accumulation. Similarly, for AQ, a 3–6-fold difference in susceptibility corresponds to a 2-fold difference in accumulation.

These data provide evidence for another source of drug resistance, possibly a reduction in target site sensitivity, which is additional to the observed reduction of drug accumulation by resistant isolates. If we assume that there is no difference in drug accumulation within groups of resistant or susceptible isolates, the 2-fold reduction in AQ accumulation seen in the group of resistant isolates (Fig. 2B) would have to be accompanied by a 1.5–3-fold decrease in target site sensitivity, as demonstrated by the greater internal AQ concentrations required to kill resistant parasites (Fig. 3A). Indeed, this explanation has previously been put forth to explain the relationship of CQ accumulation and activity in this parasite (21).

We offer the alternative explanation that only the proportion of the drug accumulated at high affinity has antimalarial activity and that the proportion of high affinity uptake is reduced in resistant isolates. This explanation is preferred because it takes into account the demonstrated saturable nature of 4-aminoquinoline drug accumulation by malaria parasites when the external drug concentration is in the nanomolar range (7, 17, 18). Furthermore, recent work from our laboratory has indicated that AQ accumulation is far in excess of what would be expected if weak base theory alone is used to predict accumulation and that accumulation is driven at least in part by intracellular drug binding (15). Finally, in our opinion, the inversely proportional relationship of AQ accumulation and activity (Fig. 3B) and good correlation of linear fit to this model (r^2 = 0.963) justify the use of this analysis.

The energy dependence of AQ accumulation is shown in Table 2. The absence of glucose results in a marked reduction in drug accumulation in both CQ-susceptible and -resistant isolates, confirming the observations of Fitch *et al.* (7). Energy-dependent AQ uptake is reduced ≥2-fold in CQ-resistant isolates compared with CQ-susceptible counterparts. These results could simply be due to a reduced energy-dependent AQ uptake force in CQ-resistant isolates. We recently observed energy-dependent high affinity binding of AQ in *P. falciparum* that is at least in part responsible for the high degree of AQ accumulated by parasites (15). Net efflux of AQ would be reduced compared with CQ because of greater lipophilicity [see Bray *et al.* (19)] and/or intracellular binding of AQ, providing an explanation for the greater activity of AQ compared with CQ.

Given the close correlation of CQ and AQ IC_{50} values (r^2 = 0.96, Fig. 2A) and CAR (r^2 = 0.94, Fig. 2B), similar mechanisms of drug resistance might be expected. We analyzed the time course of AQ accumulation in an attempt to clarify the means by which CQ-resistant parasites reduce accumulation. Results of the time course of AQ accumulation demonstrate reduced apparent initial rate of AQ accumulation in CQ-resistant isolates compared with CQ-susceptible isolates (Table 3 and Fig. 5). Fractional fill plots for the same isolates suggest that there is little change in the rate to equilibrium (Fig. 6). Any efflux of drug from the cytosol would be expected to increase the rate to equilibrium (14). Based on these graphs, there is no evidence of enhanced efflux of AQ from the cytoplasm of resistant parasites. If anything, steady state is reached sooner in the 3D7 strain, which is susceptible, than in the PH3 strain, which is resistant. Nevertheless, we cannot completely rule out a contribution of cytosolic efflux before our first samples because not all of the steady state differences can be explained by the initial uptake rates. There is a 57% reduction in steady state AQ accumulation

across the isolates (Table 1) but only a 43% reduction in the apparent initial accumulation rate (Table 3).

Results of the experiments using VP and daunomycin are intriguing. It is clear that VP did not increase the accumulation of AQ in resistant or susceptible isolates, which is in contrast with the significant effects of VP on the steady state accumulation of CQ by resistant isolates (Fig. 4A). Based on the remainder of the data from this study and from Bray *et al.* (19), the results suggest that there is a component of 4-aminoquinoline resistance that is related to reduced drug accumulation but not sensitive to VP. The effect of daunomycin is quite different. This drug increases steady state accumulation of AQ (and CQ) in both CQ-resistant and, to a lesser extent, CQ-susceptible isolates (Fig. 4A). One interpretation of these data is that malaria parasites possess a transport mechanism that is manifestly similar to that mediated by MDR protein in human cells, which is capable of reducing the cellular accumulation of 4-aminoquinolines. VP and daunomycin could exert their effects by inhibiting the action of the putative transporter. Because we can find no evidence of enhanced efflux of AQ from the cytoplasm of the resistant parasite (Fig. 6), the putative transporter would have to pump drug from within the membrane before it enters the cell, in a manner similar to the "hydrophobic vacuum cleaner" model proposed for human MDR (22, 23). Data in Table 3 and Fig. 5, which demonstrate that daunomycin increases the apparent initial rate of AQ accumulation, can be interpreted as support for this hypothesis. As a note of caution, it should be pointed out that the effects of daunomycin (and VP) are seen at significant molar excess. At these high concentrations (5 μM), both VP and daunomycin would likely be present in the membrane at much higher concentrations than the antimalarial drugs, casting doubt on the relative specificity of any proposed interactions with proteins in this domain.

Alternatively and by analogy with human MDR cells, 4-aminoquinoline drug resistance in this parasite may result from changes in intracellular pH or membrane potential with consequent altered partitioning rather from than actual pumping of drug (24). This is an attractive hypothesis, especially considering the weak base properties of these antimalarial agents. Accordingly, it has been suggested that resistance could stem from a rise in pH in the cytosolic compartment (25) or in the food vacuole of the parasite (13). VP and daunomycin could increase the rate of accumulation and possibly the pH-dependent binding of CQ and AQ by decreasing the compartmental pH. With this in mind, transfection studies of *pfmdr1*, the parasite homologue of the MDR gene of cancer cells, have implicated its gene product as having importance in compartmental acidification (26). Indeed, the subcellular localization and probable orientation of this protein seem to be inconsistent with a role involving direct drug pumping (27). Mutations of the *pfmdr1* gene result in a nonfunctional protein that could reduce vacuolar acidification and thereby diminish the pH gradient driving drug uptake (26). If this were so, other mutant alleles in addition to the two suggested would have to be involved; at least one of the resistant isolates used in this study (PH3) lacks both of the alleles suggested to be responsible for CQ resistance (28, 29). In any event, addressing the issues of direct drug pumping versus altered partitioning should provide fruitful avenues for further investigation.

We believe that this study provides important new data to add to the debate on 4-aminoquinoline resistance. We present evidence that AQ is subject to the same resistance mechanism as CQ; our high specific activity radiolabel has permitted lower drug concentrations to be used, which have revealed significantly reduced AQ accumulation by CQ-resistant parasites that has been undetected previously (7). It is reasonable to suggest that the extent of accumulation and therefore the activity of 4-aminoquinolines may be dependent on the affinity of an internal binding site for these drugs. The resistance mechanism is much less effective for AQ than for CQ. Analysis of the data in Bray *et al.* (19) suggests that this is due to the greater lipophilicity of AQ. A better understanding of the structural and physical features of this class of drug that influence both the receptor affinity and the degree of cross-resistance is clearly a priority.

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