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VACUOLAR ACIDIFICATION AND CHLOROQUINE SENSITIVITY IN *PLASMODIUM FALCIPARUM*

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Abstract—The antimalarial chloroquine concentrates in the acid vesicles of *Plasmodium falciparum* partially as a result of its properties as a weak base. Chloroquine-resistant parasites accumulate less drug than sensitive parasites. A simple hypothesis is that the intravacuolar pH of resistant strains is higher than that for sensitive strains, as a consequence of a weakened proton pump in the vacuoles of resistant strains, thereby explaining the resistance mechanism. We have attempted to test this hypothesis by the use of bafilomycin A1, a specific inhibitor of vacuolar proton pumping ATPase systems in plant cells, animal cells and microorganisms. Bafilomycin A1 significantly reduces uptake of [^{3}H]chloroquine into both chloroquine-sensitive and -resistant strains of *P. falciparum*, at concentrations of inhibitor which have no antimalarial effect. Additionally, chloroquine-resistant strains of *P. falciparum* are more sensitive to bafilomycin A1 than chloroquine-sensitive strains. The use of bafilomycin A1 in combination with chloroquine in the standard *in vitro* sensitivity assay, produced an apparent reduction in sensitivity of both strains to chloroquine. The reported data support the hypothesis that chloroquine resistance in *P. falciparum* is associated with increased vacuolar pH, possibly due to a weakened vacuolar proton pumping ATPase.

Chloroquine has been an important antimalarial since its introduction over 40 years ago. It is effective against all strains of *Plasmodium vivax*, *P. malariae* and *P. ovale*, and is still the drug of choice for *P. falciparum* infections, although its effectiveness against this parasite has declined steadily with the spread of resistance over the last 30 years.

The mechanism of chloroquine resistance and the mode of action of the drug are unclear, although it is known that high concentrations of the drug are accumulated in the acid vesicles of the parasite [1, 2], partially as a result of its weak base properties [3]. Actual accumulation may be greater than can be explained purely in terms of this hypothesis [4–6], suggesting an additional buffering component within the vacuole. Chloroquine accumulates to a greater extent in sensitive strains of malaria parasites compared to resistant strains [6–9]. To date, two main hypotheses have been proposed to explain this.

Krogstad *et al.* [7], showed that resistant strains of *P. falciparum* released preaccumulated [^{3}H]-chloroquine 40–50 times more rapidly than sensitive strains and concluded that the lower steady-state levels of the drug seen in resistant parasites could result from active efflux by a pump similar to that found to operate in the membranes of multi-drug resistant neoplastic cells (the so called MDR‖ pump). This theory was substantiated by the findings of Martin *et al.* [10], who demonstrated partial reversal of chloroquine resistance in *P. falciparum in vitro* with verapamil, a compound which inhibits the MDR efflux pump on multi-drug resistant neoplastic cells, thereby reversing resistance [11].

Alternatively, it has been suggested that the pH of the accumulating compartments in sensitive parasites are more acidic than those of the resistant parasites resulting in a stronger pH gradient producing greater chloroquine uptake [6]. Recent evidence indicates that parasite acid vesicles are analogous to mammalian lysosomes [12–15] and that their low pH is probably maintained by a proton pumping ATPase similar to that found in mammalian lysosomes [16–19]. It has consequently been suggested that the proposed higher pH of the resistant parasite vacuoles could arise as a result of a weakened proton pumping ATPase [20].

Summarizing the available evidence, there are three possible mechanisms (see Fig. 1) for chloroquine resistance in *P. falciparum:* (1) enhanced efflux of chloroquine from resistant parasites via a MDR type glycoprotein (Fig. 1a); (2) reduced accumulation of chloroquine due to a weakened proton pump in the vacuoles of resistant parasites (Fig. 1b); and (3) a combination of these two mechanisms (Fig. 1c).

The possibility of chloroquine resistance arising solely as a result of increased amounts of an MDR-type protein in the membranes of resistant parasites seems unlikely in view of the large amount of conflicting evidence from studies of cross-resistance with other antimalarials [21–24] and from studies on the genetic basis of resistance [25–28].

In this study, we have concentrated on the weakened proton pump hypothesis. Inhibitors have played a major role in the study of ion-translocating ATPases. There are three classes of ion-pumping ATPases, F_1F_0, E_1E_2 and vacuolar. Until recently,

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‖ Abbreviations: MDR, multidrug resistance; HEPES, *N*-2-hydroxyethylpiperazine-*N'*-[2-ethanesulphonic acid]; DMSO, dimethyl sulphoxide.

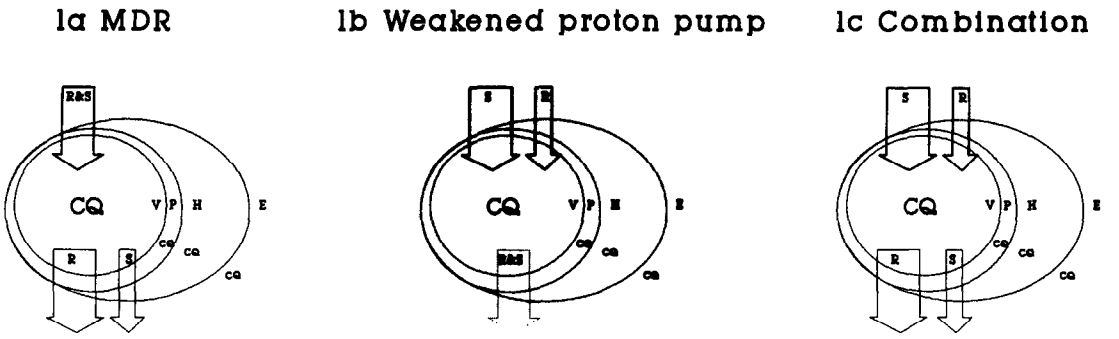

Fig. 1. Schematic diagram of the possible mechanisms of chloroquine resistance in *P. falciparum*. Chloroquine uptake (via the weak base effect) from the extracellular milieu (E), through the host (H), parasite (P), and into the vacuolar (V) compartments, is represented by the bold arrows. Chloroquine efflux (via MDR pump) is represented by the fine arrows. The broken arrow in (b) represents either no MDR pump, or MDR pumps of equal capacity in resistant and sensitive strains. Relative levels of uptake and efflux in sensitive (S) and resistant (R) strains are indicated by the size of the arrows. Possible intravacuolar buffering systems have been omitted for the sake of simplicity.

there have been no specific inhibitors of vacuolar ATPases, although specific inhibitors exist for the other classes.

Vacuolar ATPases hydrolyse ATP to generate a proton gradient which is used to acidify vacuoles [29].

Bafilomycin A1, a macrolide antibiotic, has recently been shown to be a specific and potent inhibitor of vacuolar ATPases from animal and plant cells and from microorganisms [30]. In this study we have assessed the antimalarial activity of bafilomycin A1 against chloroquine-sensitive and -resistant strains of *P. falciparum*. Further to this we have examined the effect of bafilomycin A1 at sub-lethal concentrations on uptake of [^{3}H]chloroquine by sensitive and resistant strains of *P. falciparum*. We have also examined the effect of this inhibitor on the *in vitro* sensitivity of the parasites to chloroquine. If the weakened proton pump hypothesis holds, we would expect this specific inhibitor to reduce chloroquine uptake and decrease chloroquine sensitivity, especially in sensitive parasites.

MATERIALS AND METHODS

Parasites. Two strains of *P. falciparum* were employed in this study: K1, chloroquine resistant and T9-96, chloroquine sensitive; both strains originated in Thailand.

Parasites were maintained in continuous culture by a modification of the method of Jensen and Trager [31]. The parasites were maintained in sealed flasks at 37° containing a 2–5% suspension of infected erythrocytes (at 0.1–15% parasitaemia) in complete medium (RPMI 1640 supplemented with 25 mM HEPES buffer, 23 mM $NaHCO_3$ and 10% human AB serum). The flasks were gassed prior to sealing with a mixture of 3% O_2, 4% CO_2 and 93% N_2. Parasites were synchronized by the method of Lambros and Vandenberg [32] by incubating mainly ring stage parasitized cells in 5% sorbitol (1:5 v/v) for 10 min at room temperature. Parasites were returned to culture for at least one growth cycle (48 hr) before use.

Sensitivity assays. In vitro sensitivity assays were performed as described by Desjardins *et al.* [33]. In this method inhibition of parasite growth by drugs is measured by monitoring incorporation of [^{3}H]-hypoxanthine into the nucleic acids of the parasite. Bafilomycin A1 stock solution was made up in dimethyl sulphoxide (DMSO) and the concentration determined spectrophotometrically [30]. Chloroquine stock solution was made up in 50% ethanol. The concentration of DMSO and ethanol in the final drug dilutions was always less than 1:1000. Assays were performed in 96-well microtitre plates, each well containing 100 μL complete medium without serum, and either a dilution of the drug being assayed or vehicle only, in the case of the controls. In combination assays, all wells were dosed with inhibitor at the same concentration (see Results). Infected erythrocytes were added at a starting parasitaemia of 1% and haematocrit of 5% and the plates were maintained at 37° in the same atmosphere as the culture flasks. After 24 hr, 0.5 μCi of [^{3}H]-hypoxanthine (Amersham, 17.2 Ci/mM) was added to each well. After a further 20 hr the plates were harvested by deposition of the infected cells on filter mats. The filter mats were dried, placed in polypropylene scintillation vials with 4 mL scintillation fluid (Optiphase "safe", LKB) and counted on a liquid scintillation counter (LKB 1219 Rackbeta). IC_{50} values were calculated by extrapolation of either the log dose–response curve or its probit transformation (depending on goodness of fit of transformation).

Chloroquine uptake. Cultures highly synchronized at trophozoite stage were used for the uptake studies, at a parasitaemia of 10–15%. Bafilomycin-treated groups were preincubated for 90 min in complete medium without serum containing 10^{-6} M (T9-96) or 5×10^{-7} M (K1) bafilomycin A1. Haematocrit of the suspension was approximately 2% and constant for a given experiment. The gas atmosphere used in

the uptake studies was the same as that used for culture maintenance. Experiments were initiated by the addition of [^{3}H]chloroquine (NEN, 69 Ci/mM) at a concentration of 10 nM. Chloroquine uptake was terminated at the required time point by centrifugation of the cell–medium suspension through a layer of dibutyl pthalate in a 1.5 mL microcentrifuge tube at 12,000 rpm for 20 sec. After centrifugation, the cells were sedimented below the dibutyl phthalate separated from the medium which remained above the pthalate layer. The centrifuge tips were cut off at the junction between the cell pellet and the dibutyl pthalate. The tips containing the cell pellets were placed in polypropylene scintillation vials and processed for scintillation counting as follows: cells were removed from the centrifuge tips and lysed by vortex mixing with 0.5 mL distilled water. The lysate was digested by the addition of 0.4 mL 1 M quaternary ammonium hydroxide in methanol (Scintran, BDH), followed by incubation for 4 hr at 37°. The contents of the vials were decolourized by the addition of 0.1 mL 30% hydrogen peroxide solution. Chemiluminescence was minimized by the addition of 0.1 mL glacial acetic acid and 3.5 mL scintillation fluid added. The tubes were then counted by liquid scintillation. A sample of medium was also taken from each centrifuge tube and counted, to check for excessive depletion of chloroquine from the incubation medium.

Assessment of parasite viability after 150 min exposure to bafilomycin A1. Viability of the parasites after bafilomycin treatment was assessed by an adaptation of the sensitivity assay method. Parallel groups of cultures, synchronized at trophozoite stage, were incubated in bafilomycin at a range of concentrations for the maximum times employed in the chloroquine uptake studies. Treated groups and controls were then washed in saline followed by centrifugation (600 *g* for 5 min). After a further two washes, cultures were suspended in complete medium, diluted to the appropriate parasitaemia with uninfected cells and pipetted into wells on a 96-well microtitre plate. [^{3}H]Hypoxanthine was added and the plates incubated, harvested and counted after 20 hr, as for the sensitivity assays.

RESULTS

The susceptibility to bafilomycin A1 of both strains of *P. falciparum* employed in the study was assessed. The assays were performed in the absence of serum for reasons outlined in the discussion. The effect of long-term (48 hr) and short-term (150 min) exposure was examined. Results of the 48 hr assays are summarized in Fig. 2. The range of concentration of bafilomycin A1 was 10^{-9} to 10^{-5} M for both strains. Data points are expressed as a percentage of the drug-free control value (points are means of data from two experiments, each experiment was performed in triplicate).

Chloroquine resistant strains of *P. falciparum* appear to be more sensitive to bafilomycin A1 than chloroquine sensitive strains. The IC_{50} of the drug against the chloroquine resistant K1 strain was 3.6×10^{-6} M compared with an IC_{50} against the sensitive strain of 3.2×10^{-5} M. Total exposure time of the bafilomycin A1 treated groups in the [^{3}H]-chloroquine uptake experiments (see Figs 3 and 4) was 150 min or less, consisting of a 90 min preincubation with bafilomycin A1, followed by measurement of [^{3}H]chloroquine uptake at times up to 1 hr. To avoid the complications of non-specific inhibitor toxicity influencing chloroquine transport, concentrations of inhibitor which had no effect on parasite viability were selected for use in the chloroquine uptake experiments. Results of the 150 min assays are summarized in Fig. 3, again the concentrations of bafilomycin A1 employed were the same for both strains ranging from 10^{-7} to 5×10^{-6} M. The IC_{50} value of the inhibitor against the K1 strain was 2.2×10^{-6} M. Using the data in Fig. 3, the highest concentrations of inhibitor which had no antimalarial effect were chosen for the chloroquine uptake experiments (concentrations of

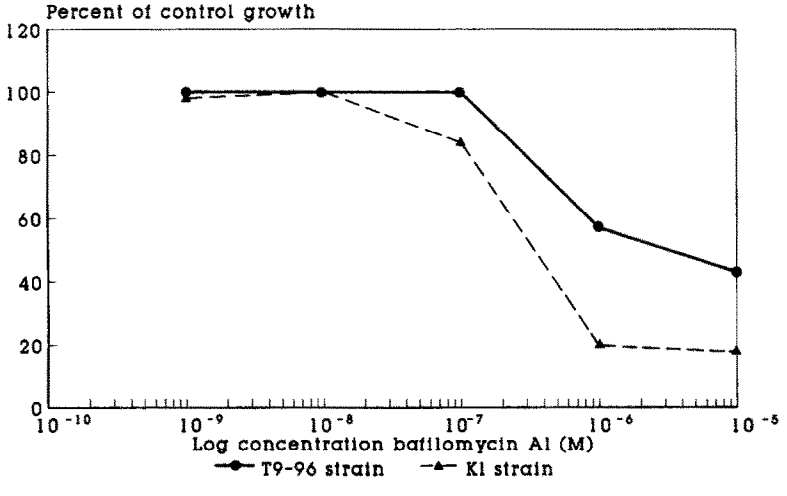

Fig. 2. Sensitivity of K1 (▲) and T9-96 (●) strains to bafilomycin A1 over 48 hr.

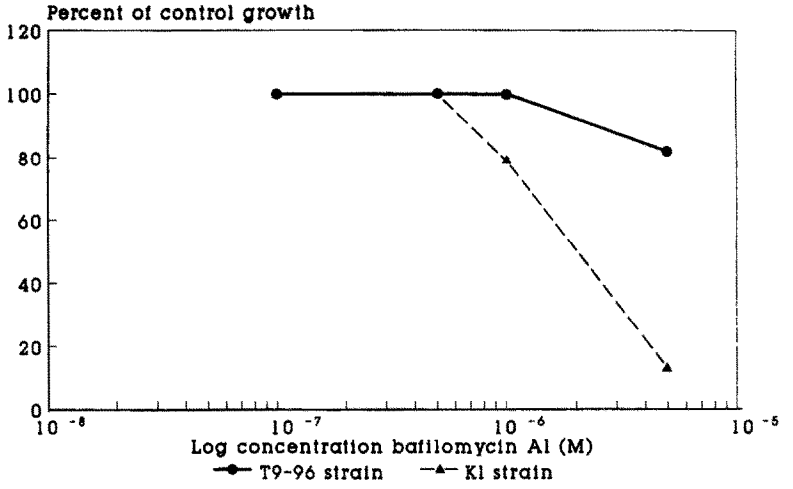

Fig. 3. Sensitivity of K1 (▲) and T9-96 (●) strains to bafilomycin A1 over 150 min.

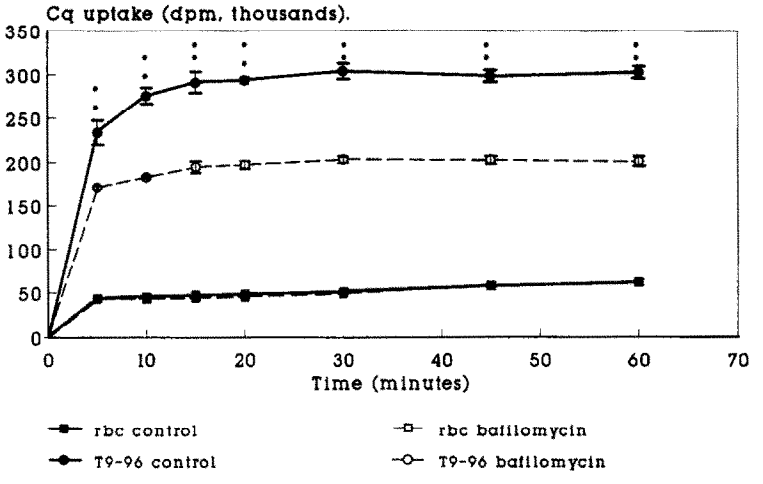

Fig. 4. Uptake of [^{3}H]chloroquine by parasitized (T9-96) erythrocytes of 1 hr in the presence (○) and absence (●) of bafilomycin A1. Also uptake of [^{3}H]chloroquine by unparasitized erythrocytes in the presence (□) and absence (■) of bafilomycin A1.

5×10^{-7} M in the case of the K1 strain and 10^{-6} M in the case of the T9-96 strain).

Uptake of [^{3}H]chloroquine versus time into parasitized erythrocytes of both strains was measured, together with uptake of [^{3}H]chloroquine into uninfected erythrocytes. In addition, the influence of bafilomycin A1 on chloroquine uptake into infected and uninfected erythrocytes was measured. Results of the [^{3}H]chloroquine uptake studies for the T9-96 strain and for uninfected erythrocytes are shown in Fig. 4. Uptake of chloroquine into infected and uninfected cells was measured at times of up to 1 hr, with and without bafilomycin A1 at a concentration of 10^{-6} M. Data points in the uptake graphs are expressed as mean dpm values (of five samples) with standard deviation. This data was analysed at each time point using the Wilcoxon Rank Test, the level of significance of difference is indicated by the number of asterisks (two asterisks indicate greater than 99% significance, one indicates 95%–99% significance).

Uptake in the bafilomycin A1 treated parasitized group was reduced to 60% of the control value (when corrected for uptake due to uninfected erythrocytes). Bafilomycin A1 had no effect on uptake of [^{3}H]chloroquine into uninfected erythrocytes (uninfected bafilomycin-treated erythrocytes were subjected to an identical preincubation in bafilomycin A1 before measurement of chloroquine uptake). The number of cells in the uninfected erythrocyte groups was calculated to be the same as the number of uninfected cells in the parasitized groups. Equilibrium of uptake was reached at 20 min. Rate of uptake of [^{3}H]chloroquine in the bafilomycin A1-treated infected group was lower than the control.

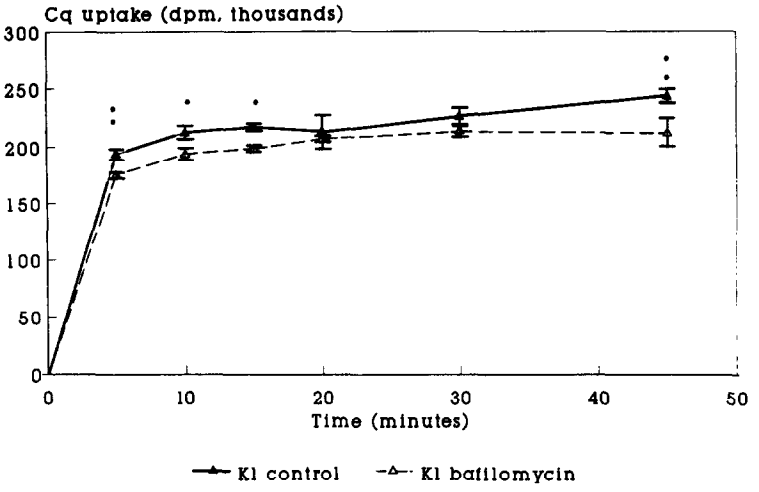

Fig. 5. Uptake of [^{3}H]chloroquine by parasitized (K1) erythrocytes over 45 min in the presence (△) and absence (▲) of bafilomycin A1.

Uptake of [^{3}H]chloroquine by parasitized (K1 chloroquine-resistant) erythrocytes over 45 min in the presence and absence of bafilomycin A1, at a concentration of 5×10^{-7} M is shown in Fig. 5. Uptake in the bafilomycin A1-treated group was reduced to 88% of the control value (corrected for uptake due to uninfected erythrocytes). Bafilomycin A1 had no effect on uptake of [^{3}H]chloroquine into uninfected erythrocytes (not shown). Equilibrium was reached at 15 min. Rate of uptake of [^{3}H]-chloroquine in the bafilomycin A1-treated infected group was lower than the control. Means of data for the bafilomycin-treated groups were significantly different from means of untreated group data at the 5, 10, 15, and 45 min time points. Depletion of chloroquine from the incubation medium was always less than 20%.

Figure 6 shows the [^{3}H]chloroquine uptake data for bafilomycin A1-treated and untreated parasites of both strains plotted as fractional filling. When plotted in this form, uptake at each time point is normalized to its own steady-state level (in this case, the corrected data from the final time point, was chosen as the steady-state level). All the curves are superimposable, indicating that fractional filling as a function of time was similar in the two strains tested, also fractional filling of bafilomycin A1-treated and untreated groups of the same strain was similar (see Discussion).

The influence of bafilomycin A1 on the sensitivity of both strains to chloroquine was assessed. Assays were performed over 48 hr, again in the absence of serum (the chloroquine IC_{50} values obtained in the absence of serum were almost identical to previous values obtained for these strains using serum in the assay system). The sensitivity of the T9-96 strain to chloroquine in the presence and absence of bafilomycin A1 is shown in Fig. 7. The range of concentrations of chloroquine was 10^{-9} to 5×10^{-6} M. Bafilomycin A1 was added at 0 and 24 hr, at a concentration of 10^{-6} M. Data points are

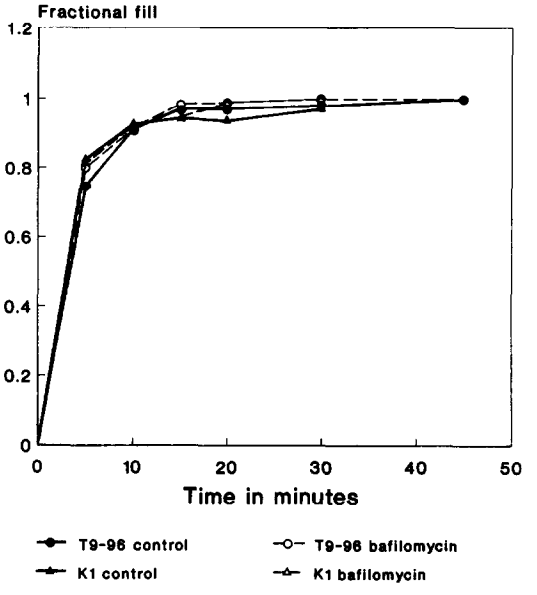

Fig. 6. Uptake of [^{3}H]chloroquine as measured by fractional filling of the steady-state level against time, for bafilomycin A1-treated (○) and untreated (●) chloroquine-sensitive, and bafilomycin A1-treated (△) and untreated (▲) chloroquine-resistant parasites.

expressed as a percentage of the drug-free control value (controls in the bafilomycin A1-treated group were also treated with the inhibitor).

The sensitivity to chloroquine of the bafilomycin A1-treated group was very much reduced, although exact quantification was not possible as a proper dose–response curve for this group could not be produced (see Discussion). The IC_{50} of chloroquine

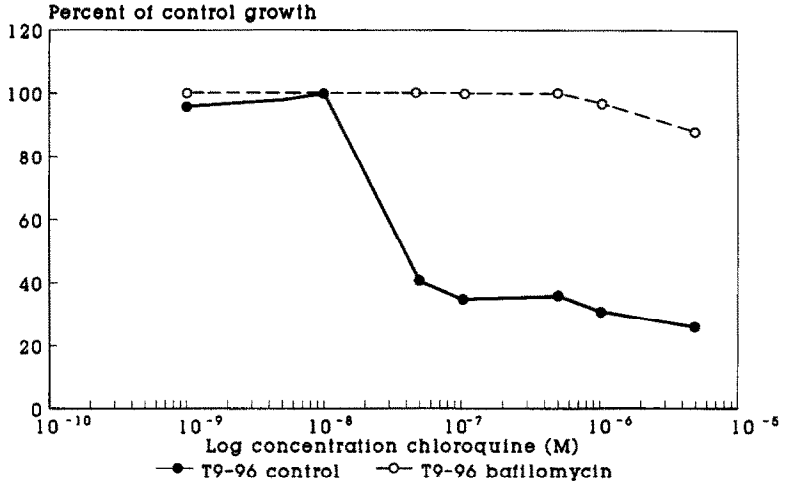

Fig. 7. Sensitivity of T9-96 strain to chloroquine in the presence (○) and absence (●) of bafilomycin A1.

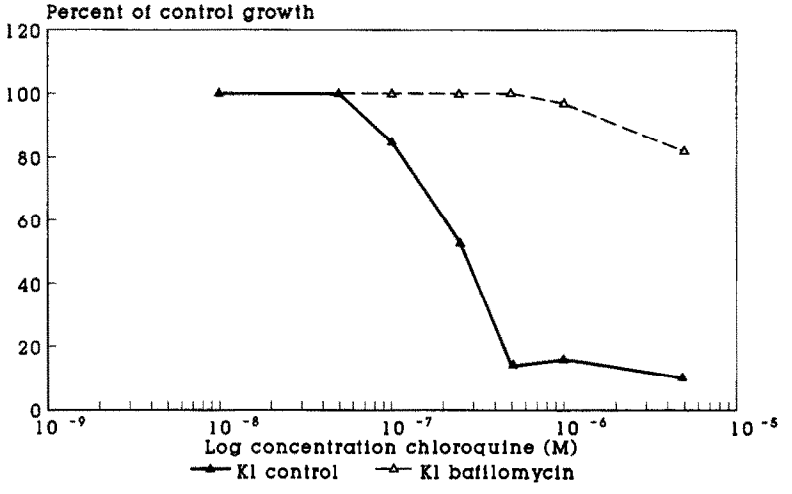

Fig. 8. Sensitivity of K1 strain to chloroquine in the presence (△) and absence (▲) of bafilomycin A1.

alone was 4×10^{-8} M. Comparison of the sensitivity of the K1 strain to chloroquine in the presence and absence of bafilomycin A1 is shown by Fig. 8. The range of concentrations of chloroquine was 10^{-8} to 5×10^{-6} M. Bafilomycin A1 was again added at 0 and 24 hr, at a concentration of 5×10^{-7} M. The sensitivity to chloroquine of the bafilomycin A1-treated group was again very much reduced and production of a complete dose–response curve for the bafilomycin-treated group was also impossible. The IC_{50} of chloroquine alone was 2×10^{-7} M.

DISCUSSION

A number of hypotheses have been proposed to explain the mode of action of chloroquine. Of these, raising of the parasite vacuolar pH (by the protonation of chloroquine accumulated by the weak base effect), to a level above the optimum for acid protease activity [3], is receiving the greatest attention. This would have the effect of inhibiting lysomal function and limiting growth. It is not difficult to envisage a hypothesis for chloroquine resistance based on the raising of vacuolar pH (as the mode of action of chloroquine), in the absence of any evidence demonstrating differences in buffering capacity between sensitive and resistant strains (as resistant parasites accumulate less chloroquine than sensitive). A resistance hypothesis based on a weakened proton pump and hence a higher vacuolar pH is clearly inconsistent with this proposed mode of action of chloroquine (resistant parasite vacuoles would be expected to be more easily alkalinized by chloroquine).

In support of this proposed mode of action of chloroquine, it is important to consider the findings of Krogstad *et al.* [12] that inhibitory external concentrations of chloroquine and other weak base antimalarials can raise the pH of the parasite food vacuole *in vitro*, however more recent work suggests that the antimalarial activity of quinoline drugs is not correlated with alkalinization of the food vacuole [6, 34]. Further to this, Yayon *et al.* [35] were able to demonstrate higher pH elevation by ammonium chloride (at concentrations which had little or no antimalarial effect) than by chloroquine at therapeutic concentrations. From the available data, we have concluded that raising vacuolar pH as the mode of action of chloroquine is not an essential component of a resistance hypothesis.

Development of resistance appears to be related to lower steady-state chloroquine levels in the resistant parasites [6, 7]. Resistance in *P. falciparum* based on reduced chloroquine accumulation in resistant parasites could occur as a result of one or more of the following mechanisms: an enhanced efflux of chloroquine from the vacuoles of resistant parasites, a change in the buffering capacity of the food vacuole in resistant parasites, or a higher pH in the food vacuole of the resistant parasite.

There is no clear evidence demonstrating differences in pigment [36] or any other factors which would affect buffering capacity between sensitive and resistant strains of *P. falciparum*. This, together with the fact that the MDR pump hypothesis is insufficient to explain chloroquine resistance, leaves room for the alternative hypothesis of a weakened proton pump.

We have addressed the issue of vacuolar pH and parasite sensitivity to chloroquine by the use of the proton pump inhibitor, bafilomycin A1. We were able to show that bafilomycin A1 has inherent antimalarial activity (Fig. 2), as assessed by 48 hr sensitivity test. We suggest that this effect is a result of inhibition of the vacuolar proton pumping system in the parasite, causing a rise in vacuolar pH sufficient to render acid proteases inoperable, resulting in death of the parasite.

Additionally, the chloroquine-resistant (K1) strain is more sensitive to bafilomycin A1 than the T9-96 chloroquine-sensitive strain, which would support the view that the proton pump in resistant parasites is already weakened, compared to that of the sensitive parasites. Interestingly, short-term (150 min) exposure to bafilomycin A1 appears to have similar antimalarial activity to long-term (48 hr) exposure (Figs 2 and 3), indicating that the effect of bafilomycin A1 is irreversible. Short-term (150 min) pre-exposure to bafilomycin A1 produced an irreversible decrease in sensitivity to chloroquine over 24 hr (at least two-fold) in both strains (results unpublished). These findings are in agreement with those of Bowman *et al.* [30], who found that bafilomycins irreversibly inhibit a V-type proton pumping ATPase from *Neurospora crassa*. Although the data can be interpreted as supporting the proton pump hypothesis, we cannot prove that the antimalarial action of bafilomycin A1 is not the result of a pharmacological action unrelated to the inhibition of a proton pumping ATPase. As a consequence, we investigated the effect of bafilomycin A1 on chloroquine uptake using inhibitor concentrations (10^{-6} M for the T9-96 strain and 5×10^{-7} M for the K1 strain) which have no antimalarial effect (see Fig. 3), in order to avoid any non-specific effects of this macrolide.

Uptake of [^{3}H]chloroquine over 1 hr into sensitive and 45 min into resistant strains of *P. falciparum* is significantly reduced (to 60% of control value in the case of the T9-96 strain and 88% of the control value in the case of the K1 strain, see Figs 4 and 5) in the presence of concentrations of bafilomycin A1 which have no effect on parasite viability over this time course. This effect could be a direct result of raising vacuolar pH, although no attempt has been made in this study to directly measure the effect of bafilomycin A1 on vacuolar pH. There are insufficient data points to accurately measure the rate of uptake. However, when uptake (calculated as fractional filling of steady-state level) is plotted against time (Fig. 6), curves for the T9-96 control and the bafilomycin-treated T9-96 groups are superimposable, as are the two curves for the K1 strain. Furthermore, the curves for the two strains are also superimposable. These findings are in agreement with the hypothesis of weakened proton pump activity, when applied to the mathematical model published by Ginsburg and Stein [20]. Briefly, according to this model, uptake differences arising from weakened proton pump activity will appear as superimposable fractional fill curves, whilst uptake differences arising from altered efflux via differing amounts of P-glycoprotein will appear as non-superimposable curves [20].

Preliminary investigations (unpublished) on the effect of verapamil on chloroquine accumulation in bafilomycin A1-treated cultures, indicate that verapamil can partially (approximately 50%) restore chloroquine uptake levels to control values (with a concentration of verapamil which reverses chloroquine resistance). These results indicate the possible presence of a functional efflux pump, but from our data, it is unclear if this MDR protein is present in larger amounts on the membranes of resistant parasites.

Results from the chloroquine sensitivity assays in combination with bafilomycin A1 (Figs 7 and 8) suggest a significant decrease in sensitivity to chloroquine of both strains, however results should be interpreted with a degree of caution for the following reasons: it was not possible to obtain consistent results in 48 hr sensitivity assays with medium containing serum. This was presumably due to irreversible binding of bafilomycin A1 to serum proteins. Assays were performed in the absence of serum; under these conditions, we were unable to get complete inhibition of growth even at concentrations of chloroquine as high as 5×10^{-6} M. Bearing this in mind, we believe that the reduction in sensitivity of both strains to chloroquine in the presence of bafilomycin A1 could be overestimated. The published figures are, however, typical of results obtained from at least five similar experiments. We suggest that the qualitative reduction in sensitivity to chloroquine of both strains in the presence of bafilomycin A1 could result from the inhibition of

the vacuolar acidification mechanism of the parasite. The concentrations of bafilomycin required to produce this effect are approaching the IC_{50} values of the inhibitor for each strain. It was not possible to produce this effect with lower inhibitor concentrations. This could be a reflection of a small dynamic range of pH manipulation in the parasite food vacuole. The effect of the inhibitor on chloroquine sensitivity was much more pronounced if the dose was split and given at 0 and 24 hr (data shown), or if a single dose was given at 24 hr (data not shown). The stage-specificity of bafilomycin A1 action is currently being assessed.

The data presented in this paper indicates that uptake of chloroquine and sensitivity of parasites to chloroquine can be influenced by an inhibitor specific to vacuolar ATPases. These findings clearly support the hypothesis that chloroquine resistance results from increased vacuolar pH due to a weakened vacuolar proton pump, although vacuolar pH in resistant parasites could also be raised by increased vacuolar membrane leakiness to protons and/or by P-glycoprotein activity [37]. Finally, it is not known at this point, if these effects are a direct result of raising vacuolar pH, but the results demonstrate the need for a greater understanding of vacuolar acidification mechanisms in these parasites.

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