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ALKALINIZATION OF THE FOOD VACUOLE OF MALARIA PARASITES BY QUINOLINE DRUGS AND ALKYLAMINES IS NOT CORRELATED WITH THEIR ANTIMALARIAL ACTIVITY

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Abstract—Quinoline-containing antimalarial drugs accumulate inside the acid food vacuole of the parasite where they inhibit the digestion of ingested host cell cytosol, and consequently, parasite growth. In order to verify whether this inhibition is caused by drug-induced alkalinization of the food vacuole, we investigated the accumulation of acridine orange (AO) as a vacuolar pH probe in intact *Plasmodium falciparum*-infected human erythrocytes as affected by the drugs chloroquine (CQ), 7H-quinoleine (7HQ), quinine (Q) and mefloquine (MQ). It was established by various criteria that AO accumulates primarily in the acid compartment(s) of the parasite as a function of the pH difference between it and the extracellular medium. This pH gradient was dissipated by the drugs in the rank order MQ > CQ > Q > 7HQ. The kinetics of vacuolar alkalinization and the concentration ranges at which it was observed imply that the monoprotic drugs MQ and Q exerted their effect mostly by translocating protons across the vacuolar membrane, i.e. they could cross the membrane as a protonated species, while the diprotic drugs CQ and 7HQ raised the vacuolar pH mostly by proton trapping. Similarly, hydrophobic alkylamines raised the vacuolar pH by proton translocation, while their relatively more polar congeners and ammonia did so by proton titration. However, the alkalinizing effect of each drug was observed at a concentration which was 1–2 orders of magnitude larger than the IC_{50} of its antimalarial effect. These results mean that vacuolar alkalinization is not the primary effect of antiparasitic action of quinoline antimalarials.

Quinoline-containing antimalarials such as chloroquine (CQ), quinine (Q) or mefloquine (MQ), are amphiphilic weak bases which accumulate to high levels in the acidic compartments of the malaria-infected erythrocyte, mostly in the parasite's food vacuole [1]. This accumulation is driven by the pH gradient existing between the vacuole and the extracellular medium [2–4], and CQ-resistant strains of the human malarial parasite *Plasmodium falciparum* have higher vacuolar pH (as calculated from the accumulation of CQ [5]) and accumulate less drug [5–7]. The primary effect of the drugs on the parasite is the inhibition of the process whereby the host cell cytosol is digested inside the food vacuole [8, 9]. Inhibition of digestion by the drugs could be due either to their direct interaction with the vacuole's acidic hydrolases, or to their effect on the vacuolar pH, raising it above the optimal range for hydrolase action. The latter mechanism was originally suggested by Homewood *et al.* [10].

Using malaria-infected erythrocytes where the food vacuole has been loaded with fluoresceinated dextran as a pH-sensitive probe, Krogstad *et al.* [4] showed that CQ, Q and MQ raise the vacuolar pH, and they contended that a mere increase of 0.3–0.5 pH units was sufficient to inhibit parasite growth. These authors further suggested that the specificity of the drugs against malarial parasites is due to the lower buffering capacity of the parasite's acidic compartment compared to that of sommatic cells [11]. However, assaying either methylamine [2] or CQ accumulation in intact infected cells [5], no evidence for alkalinization could be found at the therapeutic range of drug concentration. This discrepancy probably stems from the fact that the digitonin used by Krogstad *et al.* [4] to discard the host cell cytosol, led to demonstrable physiological alterations in the parasite, hence casting some doubt about the validity of their results.

The acceptance of the alkalinizing effect of antimalarials raises several important questions: (a) Why are these drugs more efficient than other weak bases in raising vacuolar pH that render them so specific as antimalarials? (b) What is the mechanism of pH alteration: is it by titration of vacuolar protons, as suggested for ammonia [12], or by back-flux of protonated drug, as could be the case for some hydrophobic weak bases [13]? (c) And most importantly, do these drugs affect the vacuolar pH at therapeutic drug concentrations?

The fundamental difference between the two modes of drug action suggested above underscores the necessity to measure the putative alkalinization effect of quinoline-containing drugs on intact malaria-infected erythrocytes, using alternative methodologies.

MATERIALS AND METHODS

Chemicals. Chloroquine, quinine, carbonylcyanide-*m*-chlorophenyl hydrazone (CCCP), *N*-ethyl

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maleimide (NEM) and acridine orange (AO) were from Sigma Chemical Co. (Poole, U.K.). Alkylamines were from BDH (Poole, U.K.), Fluka, Aldrich (Gillingham, U.K.) and Pierce. 7H-quinoleine and mefloquine were kindly provided by Dr S. Moreau and Dr T. G. Geary, respectively. All other chemicals were from best available grade. [^{3}H]Hypoxanthine (6 Ci/mmol) was obtained from the Radiochemical Centre (Amersham, U.K.). All solutions were prepared in deionized and glass distilled water and the osmolarities were determined on a Wescor osmometer.

Cultures. Cultures of *P. falciparum* (FCR3 uncloned strain from Gambia, obtained from Dr J. B. Jensen) were grown in culture flasks containing growth medium (RPMI-1640 from Gibco, Uxbridge, U.K. supplemented with 25 mM HEPES, 32 mM Na-bicarbonate, 10 mM glucose and 10% (v/v) heat-inactivated AB^+ or A^+ plasma) and either O^+ or A^+ washed human erythrocyte at 2–2.5% hematocrit. The growth medium was replaced daily and the cultures were gassed with a mixture of 90% N_2, 5% CO_2 and 5% O_2. Cells were normally harvested or subcultured when reaching 15–20% parasitemia (determined microscopically by thin blood smears stained with Giemsa). Cultures were synchronized according to Lambros and Vanderberg [14], using iso-osmotic alanine. Trophozoite and early schizont stages were routinely isolated by the gelatin flotation method as described elsewhere [15].

Effect of drugs on parasite growth. Synchronized cultures at the ring stage were resuspended in growth medium containing 15 mM Na-bicarbonate, to a 2% hematocrit and 0.5–1% parasitemia and distributed into wells of a 24-well microtiter plate (0.6 ml/well). The lower bicarbonate concentration used here compared to the 32 mM used for cultures was chosen since it was observed that the culture bottles were permeable to gas, resulting in increased medium pH in the course of cultivation. It was ascertained that this lower bicarbonate concentration did not compromise parasite growth. Inhibitors and [^{3}H]hypoxanthine (5–10 μCi/ml) were added to the indicated concentrations, the plate transferred to a glass desiccator gassed with the gas mixture and incubated at 37° for 48 hr. Cells were then transferred in triplicate samples to 96-well plates and harvested in a Cell Harvester (Dynatech). The filters were washed with distilled water, then dried for 2 hr at 60° and transferred into toluene-based scintillation fluid for counting of radioactivity. The IC_{50} values of inhibition of growth were calculated according to Desjardins *et al.* [16].

Effect on parasite acidic compartments. Cultures of parasitized cells which had reached the trophozoite stage (20–30% parasitemia) or were enriched in trophozoites and schizonts (85% parasitemia) by the gelatin flotation method [15] were washed in RPMI 1640 medium lacking bicarbonate and phenol red (spectroscopy medium, SM). Polycarbonate cuvettes were used for fluorescence measurements to minimize drug binding and it was ascertained by measurement of the drugs' own fluorescence that no appreciable binding did indeed occur. Acridine orange was added to a cuvette containing 3 ml SM, final concentration of 0.25–1 μM, and allowed to equilibrate. An aliquot of cells was suspended in the cuvette (0.2–0.8% hematocrit) which was placed in Spex Fluorolog II spectrofluorometer thermostatted at 37° with continuous stirring. Time-dependent changes in fluorescence were monitored at 495 nm excitation and 525 nm emission in a ratio mode. To check the effects of drugs, 1–10 μl aliquots were added from stock solutions. At the end of the experiment, NH_4Cl or CCCP were added to final concentrations of 25 mM and 20 μM, respectively, enough to release preaccumulated fluorophore. The pH difference between the parasite food vacuole and the extracellular medium, or the change in this gradient as affected by drugs, were calculated as described in the Results section.

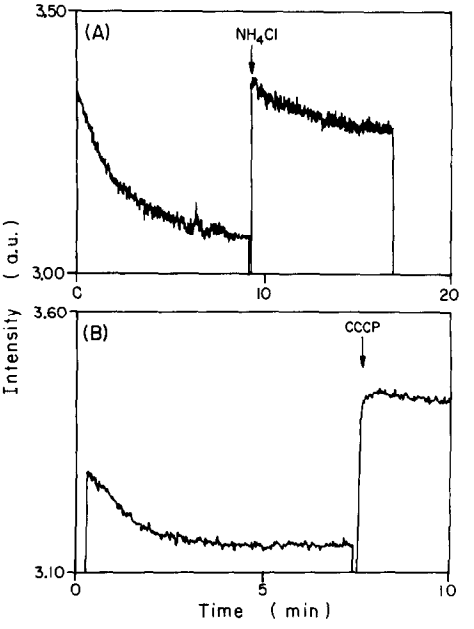

Fig. 1. Accumulation of acridine orange into malaria-infected erythrocytes. Erythrocytes infected with parasites at the trophozoite and schizont stages (0.25–0.4% hematocrit, 70–90% parasitemia) were incubated in RPMI-1640 medium at 37° in presence of 1 μM AO, and fluorescence intensity was monitored as a function of time. (A) Addition of 25 mM NH_4Cl. (B) Addition of 10 μM CCCP.

RESULTS

Accumulation of acridine orange in infected cells

Suspension of *P. falciparum*-infected erythrocytes in RPMI-1640 solutions containing AO resulted in a time-dependent quenching of AO fluorescence until a steady state was achieved (Fig. 1). The extent of quenching increased with the degree of parasitemia, cell concentration (results not shown) and with extracellular pH (see below). Non-infected erythrocytes accumulated negligible amounts of AO. Concurrently, infected cells from which the host cell cytosol has been released by means of Sendai virus-induced lysis [17], accumulated AO to the same extent as intact cells. At medium pH of 6.8–7 there is no driving force for AO accumulation, since this is the pH of host and parasite cytosol [2]. However,

appreciable AO accumulation was observed. The above results imply that fluorescence quenching is due mostly to accumulation of AO at high concentrations in acidic compartment(s) of the parasite, primarily the food vacuole. Decrease in fluorescence with AO uptake is due mostly to concentration-dependent self-quenching. AO accumulation driven by a pH gradient is commonly observed in many types of cells and in isolated acidic organelles.

Attempts to localize AO intracellularly by fluorescence microscopy succeeded only partially: the food vacuole became fluorescent shortly after addition of AO to the bathing medium for a brief period (too short for photography). Due to further accumulation AO fluorescence became self-quenched and could not be seen any more.

The suggested accumulation of AO in the parasite's acidic organelles is supported by the fact that fluorescence quenching is reversed either by the protonophore CCCP, or the lysosomotropic agent NH_4Cl (Fig. 1), which are expected to dissipate H^+-gradients. The rate of dissipation is much faster than the rate of accumulation. In the presence of CCCP a new steady-state fluorescent intensity is observed, while with NH_4Cl and nigericin there is a transient reaccumulation of AO. We explain these phenomena according to de Duve *et al.* [18]: AO penetrates across the various membranes of the infected erythrocyte on its way to the food vacuole as a free base, and there it becomes protonated; this causes a rise in the vacuolar pH, which is later lowered by the vacuolar membrane H^+-pump until a steady-state is achieved. Hence, the rate of accumulation reflects pump activity and the concentration of unprotonated AO. In the presence of large concentrations of NH_4Cl the pump cannot overcome the vast excess of this weak base and the transient reuptake of AO is due to the swelling of the food vacuole (see below). With CCCP, the rate of H^+ translocation by the protonophore exceeds the pumping rate and the protons equilibrate throughout the various compartments of the system.

The accumulation of AO in the cells exceeds considerably the value expected for monoprotic base distribution, indicating that some of the dye binds intracellularly, as is commonly observed in different types of cells (see for example Refs 19 and 20). This binding is considered to be proportional to intracellular AO concentration. Although in conjunction with the complex multi-compartmental nature of the malaria-infected erythrocyte, binding poses difficulties in the precise determination of the vacuolar pH, the effect of the H^+ gradient on the uptake of AO is magnified, and thus AO can serve as a highly sensitive H^+-gradient probe.

In order to evaluate this binding and the degree of magnification, infected cells were suspended in RPMI-1640 medium preadjusted to different pHs, the decrease of AO fluorescence was monitored at steady-state, and then CCCP was added to dissipate the pH gradient and the fluorescence intensity was recorded again. AO accumulation increased with increasing medium pH (pH_o), as expected from the increase in the pH gradient (assuming that the vacuolar pH (pH_i) remains constant. As AO has a pK_a of 10.45, it is expected to accumulate inside the

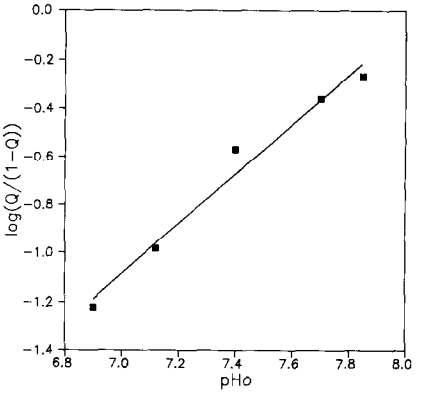

Fig. 2. Accumulation of acridine orange into malaria-infected erythrocytes as a function of medium pH. Erythrocytes infected with trophozoites and schizonts were suspended (0.3 hematocrit, 80% parasitemia) in RPMI-1640 media preadjusted to different pHs with HCl or NaOH, containing 1 μM AO, and allowed to accumulate the dye to steady-state at 37°. The fluorescence signal was recorded and then CCCP (10 μM) was added and the signal recorded again. The values of $Q/(1-Q)$ for each system was calculated and their logarithm plotted against pH_o. The parameters of linear regression were: slope 1.03 ± 0.09; intercept 8.28 ± 0.07; $r = 0.988$.

parasite's food vacuole obeying the equation [21]:

$$\frac{[AO]_i}{[AO]_o} = \frac{[H^+]_i}{[H^+]_o} \quad (1)$$

where the subscripts i and o represent the vacuole and the bathing medium, respectively. To account for binding, the right side of Eqn (1) has to be multiplied by (1 + C), C being the vacuolar binding constant [20]. This relationship assumes that the vacuolar binding sites are far from saturation. Indeed, we found that the AO accumulation ratio (cell to medium) does not change in the 0.07–10.0 μM range (not shown). Since the relative fluorescence quenching (Q) is a direct measure of dye uptake, we can take the expression $Q/(1-Q)$ as equivalent to AO_i/AO_o, where AO_i represents the total amount of AO associated with the food vacuole and AO_o is the total dye in the medium. By taking the log on both sides of Eqn (1) and using $AO_i/V_i = [AO]_i$ and $AO_o/V_o = [AO]_o$, where V_i (=the product of hematocrit, parasitemia and the relative volume of the food vacuole, which is 3.2% of the infected cell [2]) and V_o (essentially equal to 1) represent the respective relative volumes, we get:

$$\log [Q/(1-Q)] = pH_o - pH_i + \log V_i + \log (1 + C) \quad (2)$$

Thus, a plot of $\log [Q/(1-Q)]$ vs pH_o should be a straight line with a slope of 1 and an intercept = $\log V_i - pH_i + \log (1 + C)$. Results of such an analysis, shown in Fig. 2, clearly indicate that the slope (1.03 ± 0.09) is essentially equal to 1. Subtracting $\log V_i$ from the intercept ($= -8.2 \pm 0.07$), we get a value of -4.21 which is equal to $-pH_i + \log (1 + C)$, or $pH_i = 4.21 + \log (1 + C)$. This is as close as one can get to the value of the vacuolar pH in intact

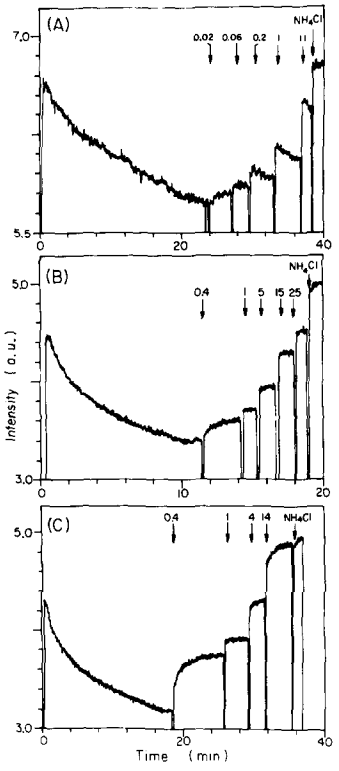

Fig. 3. Effect of antimalarial drugs on acridine orange accumulation in infected cells. Erythrocytes infected with trophozoites and schizonts were suspended (0.4% hematocrit, 90% parasitemia) in RPMI-1640, pH 7.40, 37° and allowed to accumulate AO (1 μM) to steady-state. Aliquots of 3–10 μl of stock drug solutions were then added stepwise, to the indicated concentrations, while continuously monitoring AO fluorescence. NH_4Cl (25 mM) was added at the end to show maximal AO release. (A) Chloroquine. (B) Quinine. (C) Mefloquine.

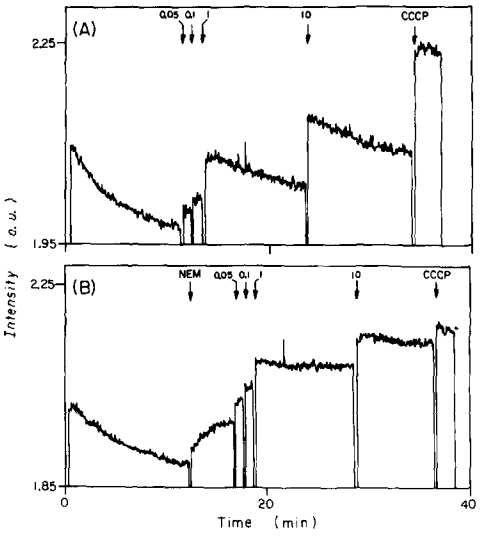

Fig. 4. Effect of CQ on acridine orange accumulation in infected cells as affected by NEM. Experimental conditions are described in the legend to Fig. 3. Fluorescence changes were recorded for longer times to emphasize AO reaccumulation. CCCP (10 μM) was added at the end of the run to show maximal AO release. (A) Control. (B) Titration in presence of 10 μM NEM.

infected cells using this technique. Taking a previously published value of the latter (5.0 [2]) or the value of 4.89 obtained indirectly to intact cells (Geary *et al.* submitted), we get a value for C which is 5.2 or 3.8, respectively. This is obviously the factor by which the pH gradient-dependent accumulation of AO is magnified.

Effects of antimalarial drugs on vacuolar pH

As shown in Fig. 3, titration of AO accumulation with antimalarial drugs resulted in dye release, though distinctive differences were found among the different drugs: CQ caused a rapid AO release (i.e., alkalinization of the food vacuole) followed by a slow gradual reuptake at concentrations above 1 μM; with 7HQ the rate of alkalinization was slower, commensurate with the greater relative polarity of this compound. Q and MQ also alkalinized the vacuoles at high rates but with no further reacidification.

Treatment of infected cells with micromolar concentrations of *N*-ethylmaleimide (NEM), an inhibitor of vacuolar H^+-pumps [22], increased vacuolar pH as expected. (At such low NEM concentrations, this observation can be assigned to H^+-pump inhibition rather than to other non-specific effects on SH-groups of enzymes and/or transport systems, which are usually observed in the millimolar range.) When NEM was added at 10 μM, only partial alkalinization was observed (Fig. 4). Subsequent addition of CQ caused a further rise in vacuolar pH with no transient reuptake of AO, and was more effective in dissipation of the pH gradient than in parallel controls (without NEM), suggesting that the partially inhibited pump cannot overcome the effect of the drug on pH_i.

Although pH_i cannot be calculated accurately from AO accumulation data, the *change* in pH_i can be determined accurately. Following Eqn (2) we get:

$$\log\frac{Q}{1-Q} - \log\frac{Q'}{1-Q'} = -pH_i + pH'_i = dpH_i \tag{3}$$

where Q' is the fluorescence quenching and pH'_i is the vacuolar pH in the presence of the drug. Clearly the product $(1 + C) \cdot V_i$ in Eqn (2) cancels out in the calculation of the change in pH_i and the observed effects of drugs on pH_i or the pH gradient are devoid of the complications inherent to AO binding and are therefore accurate.

The dose–response effect of the diprotic drugs CQ and 7HQ on the alkalinization of the vacuolar pH is shown in Fig. 5. The values for CQ were taken from the immediate effect and not from the steady-state value. Hence, the dose–response of CQ on pH_i as shown in this figure may have to be shifted to the right to some extent. The dose–response effect of the monoprotic compounds MQ, Q and NH_4Cl are shown in Fig. 6. The order of potency of antimalarials in increasing the vacuolar pH, is MQ CQ > Q > 7HQ $\gg$ NH_4Cl. This is also the rank order of their antimalarial effect.

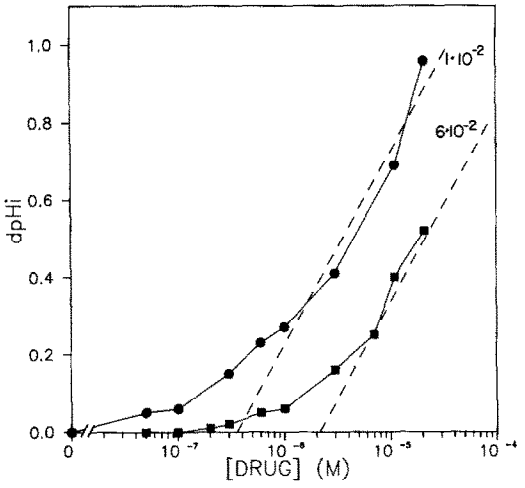

Fig. 5. Dose-dependent alkalinization of the parasite's food vacuole by chloroquine and 7H-quinoleine. Experiments were performed as described in the legend to Fig. 3, and the pH changes in the parasite's acid compartment were calculated according to Eqn (3). The change in pH_i (set arbitrarily at 0 in absence of drug) was plotted against the drug's concentration. (●) Chloroquine; (■) 7H-quinoleine. Also drawn (dashed lines) are the theoretical curves relating dpH_i to drug concentration, calculated using different buffer capacities, as indicated.

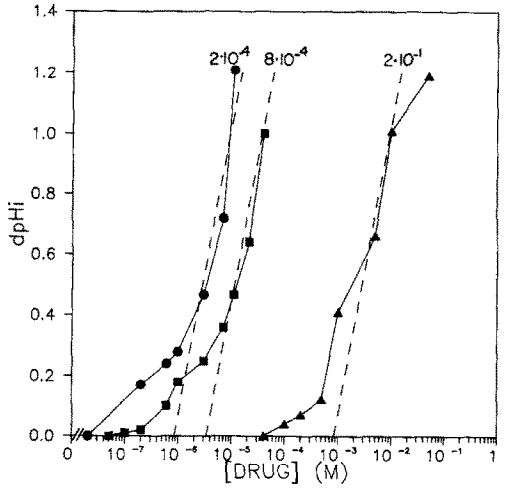

Fig. 6. Dose-dependent alkalinization of the parasite's food vacuole by quinine, mefloquine and NH_4Cl. Experimental and computation details are described in the legend to Fig. 5. (■) Quinine; (●) mefloquine; (▲) NH_4Cl.

Titration of infected cells preloaded with AO and CQ in the presence of NEM resulted in a shift to the left of the dose–response curve. At 15 μM NEM, pH_i changed linearly with $\log[CQ]_o$, unlike the upward convex curve observed at 0 and 8 μM NEM (Fig. 7).

Effects of alkylamines on vacuolar pH

The protonophoric effects of antimalarials on pH_i suggested that the potency of MQ may be related to its greater relative hydrophobicity. In order to test

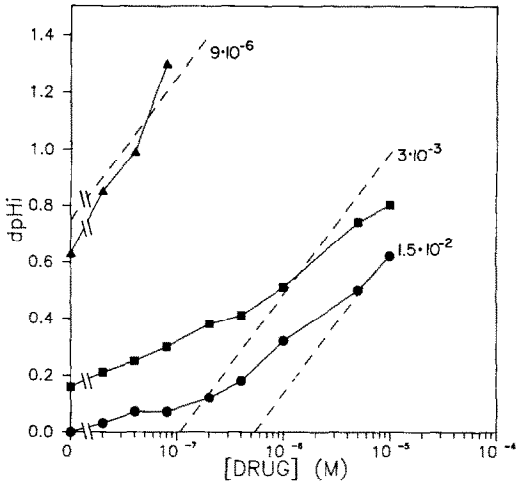

Fig. 7. Dose-dependent alkalinization of the parasite's food vacuole by chloroquine in the presence of NEM. Experimental and computation details are described in the legend to Fig. 5. (●) Control; (■) 8 μm NEM; (▲) 15 μm NEM.

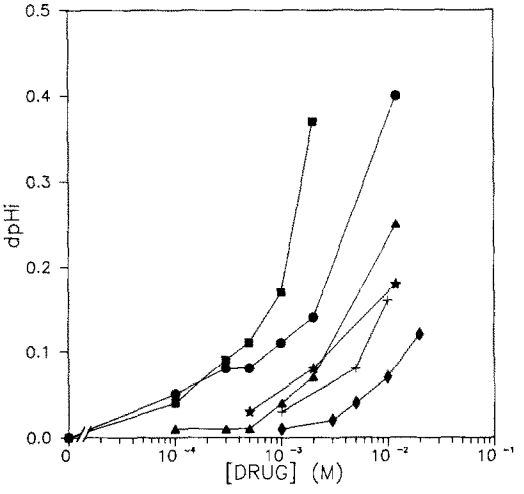

Fig. 8. Dose-dependent alkalinization of the parasite's food vacuole by alkylamines. Experiments and calculations of pH changes were performed as described in the legend to Fig. 5. (■) Octylamine; (●) heptylamine; (★) butylamine; (+) propylamine; (▲) tributylamine; (◆) triethylamine.

this assumption, we assayed the alkalinizing effect of various alkylamines differing in their polarity. The octanol-partitioning of the amines used in this study, given as $\log(P_{octanol})$, are [23]: octyl −2.9; heptyl −2.02; tributyl −1.52; triethyl −1.44; butyl −0.81 and propyl −0.37. Solubility in the membrane has been shown previously to be the major determinant in the diffusion of alkylamines into egg phosphatidyl choline liposomes [24]. As seen from Fig. 8, this is also essentially their rank order in raising the vacuolar pH. All these compounds are monobasic and expected to accumulate to the same extent along the pH gradient. The greater potency of octyl- and heptyl-amines in raising the vacuolar pH could therefore be related to the ability of their protonated

species to translocate across the membrane. The more polar amines are even less effective than ammonia, suggesting that their effect is mostly exerted by proton titration and that their potency is related to their ability to cross membranes as free bases.

Effects of antimalarials, alkylamines and acridine orange on parasite growth

Since different strains of *P. falciparum* differ in their susceptibility to drugs and we sought to correlate this index to their effect on vacuolar pH, the IC_{50} values were determined for the different compounds used in this study. Results, shown in Table 1, indicate that the FCR3 strain is most susceptible to MQ, followed by CQ, Q and 7HQ. The rank order of the antimalarial effect of alkylamines correlates with their hydrophobicity and their potency as pH-dissipators. However, the IC_{50} values of all compounds tested are significantly lower than the concentrations shown above to cause a change in the vacuolar pH. The respective drug concentrations causing a change of 0.3 pH units in the vacuolar pH (a value suggested by Krogstad *et al.* [4] to underlie inhibition of parasite growth) are also shown for the sake of comparison.

AO was also found to inhibit parasite growth. Acridines, including AO, are known to inhibit cell growth as well as DNA synthesis and to cause reduction in RNA and protein synthesis (see Ref. 25 for review). If it were increasing the vacuolar pH, it should have had an additive effect to the action of antimalarials, either by the latter act of vacuolar alkalinization or by putative binding to identical vacuolar sites. In order to test this possibility, the combined effect of AO and CQ has been tested and the results subjected to an isobole analysis [26]. Results shown in Fig. 9 indicate that these two compounds act on different parasite targets inasmuch as their effect is not additive. These results also reconfirm earlier conclusions that CQ, which can also intercalate into DNA (Q is a poor intercalator and MQ does not intercalate at all [27], exerts its antimalarial effect by a different mechanism (see Ref. 1 for a recent review).

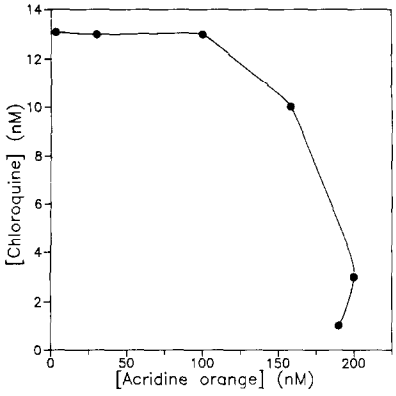

Fig. 9. Combined effect of chloroquine and acridine orange on parasite growth. Growth of parasites in culture in the presence of different combinations of CQ and AO was assayed by [^{3}H]hypoxanthine incorporation and the IC_{50} values were determined as described in Materials and Methods. The IC_{50} values of CQ were plotted against [AO] and those of AO against [CQ].

DISCUSSION

A major controversy exists at the present time concerning the details of the mode of action of quinoline-containing antimalarial drugs. Krogstad and his colleagues contend that these drugs inhibit the digesting activity in the parasite's food vacuole by raising its pH [4, 11]. We claim, on the other hand, that at the high concentrations they reach in the vacuole they inhibit the action of the hydrolases [1–3, 28]. We feel that a clear solution to this problem would itself be an important step forward, and would also provide clues regarding the mechanism of drug

Table 1. Effects of antimalarial drugs and alkylamines on parasite growth and alkalinization of the food vacuole

Compound	IC_{50}	IC_{ALK}	Corrected IC_{ALK}
Mefloquine	8.8×10^{-10}	1.1×10^{-6}	
Quinine	1.0×10^{-7}	4.5×10^{-6}	
Chloroquine	2.1×10^{-8}	1.3×10^{-6}	7.9×10^{-7}
7H-Quinoleine	2.0×10^{-7}	8.1×10^{-6}	4.9×10^{-6}
Octylamine	2.2×10^{-5}	1.1×10^{-3}	
Heptylamine	3.4×10^{-5}	3.0×10^{-3}	
Butylamine	2.2×10^{-4}	1.6×10^{-2}	
Propylamine	3.2×10^{-4}	1.3×10^{-4}	
Triethylamine	5.6×10^{-4}	4.0×10^{-2}	
Tributylamine	6.0×10^{-4}	8.0×10^{-3}	
NH_4Cl	3.1×10^{-3}	1.0×10^{-3}	

The effects of drugs and alkylamines on parasite growth was tested and the IC_{50} values determined as described in Materials and Methods. Also shown are the concentrations causing an increase of 0.3 units (or 0.2 units for alkylamines) in the vacuolar pH (IC_{ALK}) and the corrected concentrations for the diprotic CQ and 7HQ (corrected IC_{ALK}), to account for their depletion from the medium due to cellular uptake (the medium depletion of monoprotic bases is negligible).

resistance and a sound basis for further drug development of antimalarial drugs.

Fluorometric [2, 4] and autoradiographic experiments [29] indicate that the food vacuole of *P. falciparum* is the major acid and CQ-accumulating organelle of the parasite. Ultrastructural [30] and biochemical [8, 9] studies indicated that this is also the site of drug action. The vacuolar pH has been previously determined by measuring the pH-dependent fluorescence of fluorescein which was targetted into the vacuole. However, this procedure involves the disruption of the infected cell in order to discard host cell hemoglobin which quenches the fluorescence. In the present work we have used an indirect method, namely AO accumulation, to evaluate the vacuolar pH in a non-disruptive manner. Although this method is hampered by the extensive intracellular AO-binding and does not allow the precise calculation of the vacuolar pH, it is very sensitive and accurate in reporting changes in this parameter. It should be emphasized that, since this binding is directly proportional to the vacuolar drug concentration (Fig. 2) and preaccumulated AO can be released by so many vastly different agents, it is very likely that binding takes place inside the acidic compartment(s) of the parasite and does not involve substantial binding to cytoplasmic and/or nuclear sites.

Addition of antimalarials to infected erythrocytes resulted in a rapid release of preaccumulated AO (Fig. 3), and in analogy with the effect of CCCP and NH_4Cl (Fig. 1), must be due to an increase in the vacuolar pH. In terms of extracellular concentrations, MQ is more and Q is less effective than CQ in raising the vacuolar pH. However, considering their calculated vacuolar concentrations, the pH-dissipating effect of both is considerably more potent than that of CQ. Compared to the effect of NH_4Cl, which is also monobasic but acting in the millimolar range (Fig. 6) by proton titration [12], Q and MQ are effective in the micromolar range, suggesting that they act mostly by proton shuttling, i.e., entering into the vacuole as a free base, becoming protonated and exiting as such. This suggestion is consistent with the following considerations: both compounds intercalate into phospholipid monolayers [31], implying that they can dissolve into bilayers in their protonated form, a prerequisite for diffusion across the membrane. This effect probably stems from the fact that their quinoline ring is not charged above pH 4–4.5, rendering them more hydrophobic (indeed, the partitioning of Q into octanol is 400 times larger than that of CQ at pH 4.5 [32], e.g., close to that of the food vacuole). The trifluoromethyl moieties of MQ certainly increase its hydrophobicity as compared with Q, and hence its greater potency in pH dissipation. Using everted *E. coli* membrane vesicles where both the membrane potential and the pH gradient can be controlled, we have recently demonstrated that the rate of ΔpH dissipation was directly proportional to the membrane potential, in agreement with the ability of QH^+ and MQH^+ to cross the membrane [33].

CQ is dibasic with pK_as of 8.1 (the ring nitrogen) and 10.2 (the side-chain nitrogen), and is therefore expected to accumulate in the acid interior of the vesicles to the square of the monobasic Q (pK_a = 8.4, on the quinuclidine nitrogen) and MQ (pK_a = 8.6, on the piperidine nitrogen). Under the conditions of the present experiments the pH gradient between the vacuole and the external medium is at least 2.2 [2, 4], meaning that CQ is expected to accumulate 2.5×10^4-fold and Q and MQ should accumulate 160-fold [21]. At a vacuolar pH of 5.2, most of the CQ is in its diprotonated form (CQH_2^{2+}) and the relative concentration of monoprotonated form (CQH^+) is <0.5%, i.e., smaller than those of QH^+ and MQH^+ at equal extracellular concentration and pH_i. Only the unprotonated form of CQ can dissolve into phospholipid membranes; CQH^+ can bind to phosphatidylcholine membranes to a minimal extent [34], but does not intercalate into them [31, 35]. However, CQ binds much more avidly to negatively charged phospholipids [36] suggesting that, given the lipid composition of the parasite membranes [37], CQH^+ (but not CQH_2^{2+}) could in principle translocate across the membrane. In order to act as a protonophore, CQ must therefore penetrate (at least the vacuolar membrane) as a free base. However, if it penetrates as the CQH^+ form [38], it would be much less effective in proton titration. Since CQ acts at relatively low concentrations, we suggest that it penetrates as a free base and acts by proton titration, but inasmuch as it does not reach vacuolar concentrations equivalent to that of ammonia when the pH gradient is fully dissipated, it must also have a protonophoric effect. The relatively poor alkalinizing effect of 7HQ could be explained as follows: substitution of Cl by H on the quinoline ring should considerably decrease the partitioning of this compound into the membrane (in its monoprotonated form), thus diminishing the protonophoric effect. Indeed, 7HQ was not alone in having a smaller overall pH dissipating effect, but the effect was achieved at a slower rate.

The transient effects of CQ (Fig. 3) and NH_4Cl (Fig. 1) on AO accumulation can be readily explained from Eqn (2): these two compounds accumulate at very high concentrations inside the food vacuole. This in turn creates a charge imbalance which drives anions into the vacuole, thereby increasing even further the osmotic concentration in the lumen of this organelle. The resulting water influx causes swelling of the vacuole, as is commonly observed with lysosomes of macrophages treated with lysosomotropic agents [13]. The increase in V_i results in further uptake of AO, even without additional change in pH_i, as predicted from Eqn (2). This interpretation is compatible with the absence of transients in the presence of CCCP, low [CQ], or Q and MQ. The two latter compounds would reach less than millimolar concentrations in the vacuole, with almost no consequence to the osmotic balance. The transients observed with CQ also imply that this drug accumulates as a diprotic base and that the translocation of the CQH^+ species across the vacuolar membrane (at least) is slow relative to that of the free base. If this were not true, CQ should have accumulated as a monoprotic base, with no consequence to the osmotic balance of the vacuole.

Titration of malaria-infected erythrocytes with quinoline-containing antimalarials resulted in a dose-

Table 2. Calculated apparent buffer capacity of the parasite's food vacuole

Compound	Apparent buffer capacity
NH_4Cl	2×10^{-1}
Quinine	8×10^{-4}
Mefloquine	2×10^{-4}
Chloroquine	1×10^{-2}
7H-Quinoleine	6×10^{-2}
CQ + 8 μM NEM	3×10^{-3}
CQ + 15 μM NEM	9×10^{-6}

The theoretical dependence of pH_i on extracellular drug concentration for monoprotic and diprotic weak bases were generated as described by Krogstad and Schlesinger [11]. The resulting curves were fitted to the linear parts of the experimental pH_i titration curves, using the necessary buffer capacity, as shown in Figs 5–7.

dependent release of preaccumulated AO, caused by the alkalinization of the vacuolar lumen. Since Krogstad and Schlesinger [11] have previously suggested that antimalarials act by titration of protons in the food vacuole, we have followed their derivations and calculated the dependence of pH_i on extracellular drug concentration, using different values for the buffer capacity of the vacuole, in order to accommodate the theoretical relationship with experimental results. This analysis provides an independent and quantitative tool to verify the conclusions reached above concerning the protonophoric effect of the drugs. It should be emphasized that the buffer capacity depends not only on the vacuolar buffer concentration, but also on the H^+-pump activity and the basal leak of protons out of the vacuole along their concentration gradient. Reduced pump activity and/or increased leak, as could be mediated by drugs, would obviously result in an apparent lower buffer capacity.

Inspection of Figs 5 and 6 shows that the theoretical curves follow the experimental results of dose-dependent increase in pH_i only at high drug concentrations. This divergence of experiment from theory can be interpreted as follows: at low drug levels, $[H^+]_i$ is reduced with a concomitant diminution of the basal H^+ leak, i.e., apparent increase in buffer capacity. This effect will become progressively less pronounced as pH_i increases, until the experimental and theoretical curves merge. It seems that the horizontal shift from the minimal drug concentrations that cause infintesimal rise in pH_i, to the intersection of the theoretical curve with the abscissa, represents the effect of the basal proton leak on the apparent buffer capacity. The values of buffer capacity taken to make the theoretical curves match the titration curves of each drug vary considerably (Table 2). Thus, the apparent buffer capacity for NH_4Cl is significantly larger than that for the monoprotic drugs Q and MQ. This observation could be due either to the protonophoric effect of the latter compounds, e.g., increasing H^+ leak, or to their inhibition of the pump, or to both. The rank order of the apparent buffer capacities for all drugs is inversely proportional to their relative hydrophobicities, suggesting that they act as protonophores rather than inhibitors of the pump. The buffer capacities for CQ and 7HQ are smaller than that for NH_4Cl and larger than those for Q and MQ, implying that they act mostly by proton titration, but to some extent also by proton shuttling.

Partial inhibition of the pump by NEM results in a decrease in the buffer capacity, and almost complete inhibition (at 15 μM) yields an apparent buffer capacity of 9×10^{-6}, e.g., 22,200-fold less than for NH_4Cl (Fig. 7 and Table 2). This difference probably represents the pump's contribution to the buffer capacity and suggests that the buffer concentration in the vacuole is minimal. For this reason, when NEM-treated cells are titrated with CQ there is no transient reaccumulation of AO, since the minimal amounts of drug needed to raise the vacuolar pH do not provoke an osmotic imbalance with ensuing swelling of the vacuole.

We have recently described similar effects of antimalarial drugs on the ΔpH generated by substrate oxidation across membranes of *E. coli* and interpreted them identically [33]. However, in these membranes CQ was a more efficient pH dissipator than MQ. A similar difference is found among the alkylamines: while in *E. coli* tributylamine is less effective than both butyl- and propylamines, in malaria-infected cells the opposite is true. These results imply that differences in the membrane lipid composition may be instrumental in mediating these protonophoric effects, inasmuch as partitioning of basic amphiphiles into membranes, and hence translocation across them, depends on the lipid composition of the membrane [39].

Overall, the results obtained with alkylamines underscore the importance of the hydrophobicity of weak bases in their alkalinization of acid subcellular organelles [13] either by proton titration or by proton shuttling (the protonophoric effect). However, the similarity of $-\log (P_{octanol})$ for octylamine (2.9), heptylamine (2.02) and Q (2.1 at pH 7.4 or 0.6 at pH 4.5), all monoprotic bases, should have resulted in equipotent effects on the vacuolar pH. However, Q is by far the most effective, indicating that membrane solubility is not the sole governing factor in this effect, and others, such as molecular volume and charge density [40], must be playing some role in determining the rate of translocation of the protonated species of these weak bases.

Although there is no doubt that both antimalarials and the hydrophobic alkylamines are able to raise the vacuolar pH, this effect is observed at concentrations which are substantially higher than their respective IC_{50} values, as was previously demonstrated [2, 4, 6]. Even if one corrects for drug depletion from the medium due to its cellular accumulation at relatively large inoculum size, one could not bring the alkalinizing effect into the antimalarial concentration range. Standing out from this generalization is NH_4Cl, where the concentration causing alkalinization is identical to IC_{50}, and one can conclude that this compound inhibits parasite growth by the classical lysosomotropic effect [18]. We have found recently that lysosomotropic detergents also inhibit parasite growth by vacuolar alkalinization [41], confirming that alkalinization of the food vacuole *per se* could inhibit parasite growth. We must therefore

conclude, as we have suggested previously [1], that the specific antimalarial effect of quinoline-containing drugs is due to their inhibition of vacuolar hydrolases rather than by shifting the vacuolar pH away from the optimal range for enzyme action, an effect caused by many weak bases which have a relatively weak antimalarial activity. The antimalarial effect of alkylamines is presumably the same inasmuch as many cationic amphiphiles inhibit acid phospholipases [42].

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REFERENCES

1. Ginsburg H and Geary TG, Current concepts and new ideas on the mechanism of action of quinoline-containing antimalarials. *Biochem Pharmacol* **36**: 1567–1576, 1987.
2. Yayon A, Cabantchik ZI and Ginsburg H, Identification of the acidic compartment of *Plasmodium falciparum* infected human erythrocytes. *EMBO J* **13**: 2695–2700, 1984.
3. Yayon A, Cabantchik ZI and Ginsburg H, Susceptibility of human malaria parasites to chloroquine is pH dependent. *Proc Natn Acad Sci USA* **82**: 2784–2788, 1985.
4. Krogstad DJ, Schlesinger P and Gluzman IY, Antimalarials increase vesicle pH in *Plasmodium falciparum*. *J Cell Biol* **101**: 2302–2309, 1985.
5. Geary TG, Jensen JB and Ginsburg H, Uptake of [^{3}H]chloroquine by chloroquine-sensitive and chloroquine-resistant strains of *Plasmodium falciparum*: lack of correlation of uptake and sensitivity. *Biochem Pharmacol* **35**: 3805–3812, 1986.
6. Fitch CD, Chloroquine-resistant *Plasmodium falciparum*: difference in handling ^{14}C-amodiaquin and ^{14}C-chloroquine. *Antimicrob Agents Chemother* **3**: 545–548, 1973.
7. Gluzman IY, Schlesinger PH and Krogstad DJ, Inoculum effect with chloroquine and *Plasmodium falciparum*. *Antimicrob Agents Chemother* **31**: 32–36, 1987.
8. Zarchin S, Krugliak M and Ginsburg H, Digestion of host erythrocyte is the primary target for quinoline-containing antimalarials. *Biochem Pharmacol* **35**: 2435–2442, 1986.
9. Zhang Y, Inhibition of hemoglobin degradation in *Plasmodium falciparum* by chloroquine and ammonium chloride. *Exp Parasitol* **64**: 322–327, 1987.
10. Homewood CA, Warhurst DC, Peters W and Baggaley VV, Lysosomes, pH and the antimalarial action of chloroquine. *Nature (Lond)* **235**: 50–52, 1972.
11. Krogstad DJ and Schlesinger PH, A perspective on antimalarial action: effects of weak bases on *Plasmodium falciparum*. *Biochem Pharmacol* **35**: 547–552, 1986.
12. Roos A and Boron WF, Intracellular pH. *Physiol Rev* **61**: 296–434, 1981.
13. Ohkuma S and Poole B, Cytoplasmic vacuolation of mouse peritoneal macrophages and the uptake into lysosomes of weakly basic substances. *J Cell Biol* **90**: 656–664, 1981.
14. Lambros C and Vanderberg JP, Synchronization of *Plasmodium falciparum* erythrocytic stages in culture. *J Parasitol* **65**: 418–420, 1979.
15. Jensen JB, Concentration from continuous cultures of erythrocytes infected with trophozoites and schizonts. *Am J Trop Med Hyg* **27**: 1274–1276, 1978.
16. Desjardins RE, Canfield CJ, Haynes JD and Chulay JD, Quantitative assessment of antimalarial activity *in vitro* by a semiautomated microdilution technique. *Antimicrob Agents Chemother* **16**: 710–718, 1979.
17. Ginsburg H, Handeli S, Friedman S, Gorodetsky R and Krugliak M, Effects of red blood cell potassium and hypertonicity on the growth of *Plasmodium falciparum* in culture. *Z Parasitenk* **72**: 185–199, 1986.
18. de Duve C, de Barsy T, Poole B, Trouet A, Tulkens P and van Hoof F, Lysosomotropic drugs. *Biochem Pharmacol* **23**: 2495–2531, 1974.
19. Lee HC and Forte JG, A study of H^+ transport in gastric microsomal vesicles using fluorescent probes. *Biochim Biophys Acta* **508**: 339–356, 1978.
20. Christen R, Schackmann RW and Shapiro BM, Elevation of the intracellular pH activates respiration and motility of sperm of the sea urchin *Strongylocentrotus purpuratus*. *J Biol Chem* **257**: 14881–14890, 1982.
21. Schuldiner S, Rottenberg H and Avron M, Determination of ΔpH in chloroplasts. 2. Fluorescent amines as a probe for the determination of ΔpH in chloroplasts. *Eur J Biochem* **25**: 64–70, 1972.
22. Harikumar P and Reeves JP, The lysosomal proton pump is electrogenic. *J Biol Chem* **258**: 10403–10410, 1986.
23. Leo A, Hansch C and Elkins D, Partition coefficients and their uses. *Chem Rev* **71**: 525–607, 1971.
24. Bar-On Z and Degani H, Permeability of alkylamines across phosphatidylcholine vesicles as studied by ^{1}H-NMR. *Biochim Biophys Acta* **813**: 207–212, 1985.
25. Nasim A and Brychcy T, Genetic effects of acridine compounds. *Mutat Res* **65**: 261–288, 1979.
26. Hewlett PS and Plackett RL, *An Introduction to the Interpretation of Quantal Response in Biology*. Edward Arnold, London, 1979.
27. Peters W, Howells RE, Portus JH, Robinson BL, Thomas SC and Warhurst DC, The chemotherapy of rodent malaria. XXVII. Studies on mefloquine. *Ann Trop Med Parasitol* **71**: 407–412, 1977.
28. Krugliak M, Waldman Z and Ginsburg H, Gentamicin and amikacin repress the growth of *Plasmodium falciparum* in culture, probably by inhibiting a parasite acid phospholipase. *Life Sci* **40**: 1253–1257, 1987.
29. Aikawa M, High-resolution autoradiography of malaria parasites treated with ^{3}H-chloroquine. *Am J Pathol* **67**: 277–280, 1972.
30. Yayon A, Timberg R, Friedman S and Ginsburg H, Effects of chloroquine on the feeding mechanism of the intraerythrocytic human malarial parasite *Plasmodium falciparum*. *J Protozool* **31**: 367–372, 1984.
31. Ginsburg H and Demel RA, Interaction of hemin, antimalarial drugs and hemin–antimalarial complexes with phospholipid monolayers. *Chem Phys Lipids* **35**: 331–347, 1984.
32. Zamora JM, Pearce HL and Beck WT, Physical–chemical properties shared by compounds that modulate multidrug resistance in human leukemic cells. *Molec Pharmacol* **33**: 454–462, 1988.
33. Nissani E and Ginsburg H, Protonophoric effects of antimalarial drugs and alkylamines in *E. coli* membranes. *Biochim Biophys Acta* **978**: 293–298, 1989.
34. Ferrari V and Cutler DJ, The pH-dependence of chloroquine uptake by phosphatidylcholine vesicles. *J Pharm Pharmacol* **38**: 761–763, 1986.
35. Ginsburg H and Demel RA, The effect of ferriprotoporphyrin IX and chloroquine on phospholipid monolayers: possible implications to antimalarial activity. *Biochim Biophys Acta* **732**: 16–19, 1983.
36. Harder A, Hille K-H and Debuch H, Effect of chloroquine and *O'*,*O*-bis(diethylaminoethyl)hexesterol on acidic phospholipid membranes. *Hoppe-Seyler's Z Physiol Chem* **364**: 997–1001, 1983.
37. Holz GG, Lipids and the malarial parasite. *Bull WHO* **55**: 237–248, 1977.

38. Warhurst DC, Antimalarial schizontocides: why a permease is necessary. *Parasitol Today* **2**: 331–333, 1986.
39. Zachowski A and Durand P, Biphasic nature of the binding of cationic amphipaths with artificial and biological membranes. *Biochim Biophys Acta* **937**: 411–416, 1988.
40. Lieb WR and Stein WD, Non-Stokesian nature of transverse diffusion within human red cell membranes. *J Membrane Biol* **92**: 111–119, 1986.
41. Cabantchik ZI, Silfen J, Firestone RA, Krugliak M, Nissani E and Ginsburg H, Effects of lysosomotropic detergents on the human malarial parasite *Plasmodium falciparum* in *in vitro* culture. *Biochem Pharmacol* **38**: 1271–1277, 1989.
42. Lüllmann-Rauch R, Drug-induced lysosomal storage disorders. In: *Lysosomes in Applied Biology and Therapeutics* (Eds. Dingle JT, Jacques PJ and Shaw IH), Vol. 6, North-Holland, Amsterdam, 49–130, 1979.