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COMMENTARY

A PERSPECTIVE ON ANTIMALARIAL ACTION: EFFECTS OF WEAK BASES ON *PLASMODIUM FALCIPARUM*

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The most important antimalarials (including chloroquine, quinine, and mefloquine) are weak bases [1] which are positively charged at physiologic pH and bind to many macromolecules, including polyanions such as nucleic acids [2, 3]. Chloroquine has been studied extensively in macrophages and fibroblasts [4, 5], and it has been shown to inhibit processes which depend upon the acidic environment of the endosome/lysosome system such as receptor-mediated endocytosis [6], the intracellular targeting of lysosomal enzymes [7], and lysosomal proteolysis [8]. Although recent studies of the effects of chloroquine on mammalian cells have focused almost exclusively on the weak base effect [4, 5], other mechanisms have also been considered as potential modes of chloroquine action against plasmodia. These include the inhibition of nucleic acid synthesis [2, 3, 9], and binding to ferriprotoporphyrin IX [10].

In this paper we examine the weak base effect, the effects of chloroquine and other weak bases on mammalian cells, potential mechanisms of chloroquine action against plasmodia, potential explanations for the discrepancy between the chloroquine concentrations that increase vesicle pH in mammalian cells versus susceptible plasmodia (10–60 μM vs 5–20 nM), and a model we have developed to quantitate the effects of mono- and diprotic weak bases on vesicle pH based on our recent studies of vesicle pH in fibroblasts and *Plasmodium falciparum* [11].

THE WEAK BASE EFFECT

Weak bases are typically present in both their charged (protonated) and uncharged (neutral) forms, although the charged forms predominate at physiologic pH. At alkaline pHs near their p*K*s (typically 8–10), the amounts of the two forms are roughly equal (Fig. 1). In contrast, under the more acidic conditions of acid intracellular vesicles (pH 5), almost all molecules are in the charged (protonated) form (Fig. 1).

As formulated by de Duve *et al.* [12], the explanation for the weak base effect is based on three assumptions: (1) the neutral (uncharged) forms of weak bases readily cross both plasma and vesicle membranes, (2) these membranes are impermeable (or much less permeable) to the charged (protonated) forms of weak bases, and (3) lysosomes and other membrane-bound compartments (vesicles) with an acid pH exist in normal eucaryotic cells. The logical consequences of these assumptions are consistent with the known effects of weak bases on both mammalian cells and plasmodia [12, 13]: (1) weak bases are concentrated (by protonation) in their non-diffusible form within acid intracellular vesicles (Fig. 2), (2) the concentration of weak bases in acid intracellular vesicles increases both the pH and the osmolality of those vesicles, and (3) swelling and/or membrane leakiness occurs in these vesicles because of the water flow (cytosol to vesicle) caused by the osmotic gradient across the membrane.

This theory is consistent with the antimalarial activity of chloroquine and other weak bases, with the concentration of chloroquine in the parasite vesicle reported by Aikawa [14], and with the swelling of the parasite vesicle described by a number of investigators [14, 15]. However, it has not been possible to measure the pH of the parasite vesicle to test this hypothesis directly, and this hypothesis does not explain why plasmodia are much more susceptible to chloroquine than mammalian cells.

EFFECTS OF CHLOROQUINE AND OTHER WEAK BASES ON MAMMALIAN CELLS

Studies of mammalian cells in the last 10–15 years have defined several acid intracellular compartments and established their importance for endocytosis and the targeting of lysosomal enzymes [16, 17]. Chloroquine (and other weak bases) inhibits a large number of processes in eucaryotic cells, presumably by alkalinizing their acid vesicles [7, 18–28]. The wide variety of these effects suggests that the acid pH of intracellular vesicles is essential for the normal function of most eucaryotic cells.

POTENTIAL MECHANISMS OF CHLOROQUINE ACTION AGAINST PLASMODIA

In addition to the possibility that chloroquine might act against the malaria parasite by increasing vesicle pH, other potential mechanisms that have been proposed include the inhibition of nucleic acid

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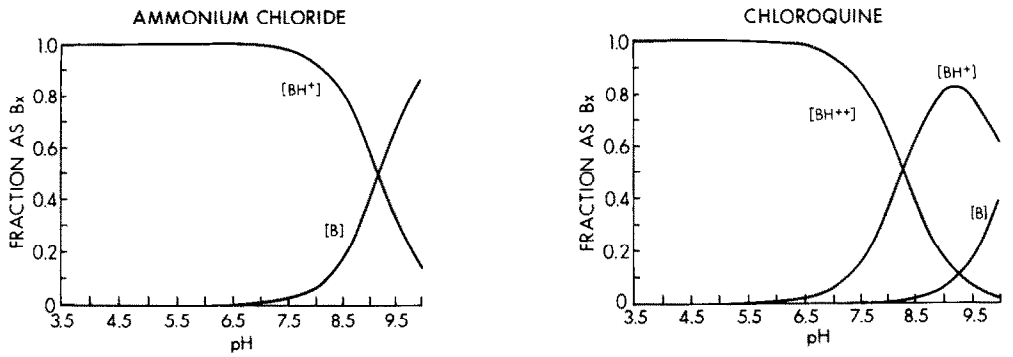

Fig. 1. Effect of pH on the protonation of weak bases. The concentrations of the free (unprotonated) form of a weak base (B) such as NH_4Cl (left panel) decrease markedly as pH decreases from 10 to 4, and those of the charged (protonated) form (BH^+) increase. With a diprotic weak base such as chloroquine (right panel), the concentrations of the diprotonated form also increase at lower pH. Data plotted are derived from calculations based on the p*K*s of these compounds.

synthesis [2, 3, 9] and binding to ferriprotoporphyrin IX [10].

Inhibition of nucleic acid synthesis

At physiologic pH, chloroquine is a positively charged cation that readily binds to DNA, RNA, and other polyanions. Studies by a number of investigators have shown that chloroquine inhibits DNA and RNA synthesis in bacterial and mammalian cells [2, 3], and that it can also intercalate into the DNA helix (with the chloroquinoline ring between base pairs and the cationic side chain protruding from the helix) [9].

However, the chloroquine concentrations which inhibit nucleic acid synthesis are much greater than those that inhibit the growth of chloroquine-susceptible strains of *P. falciparum* (1–2 mM vs 5–20 nM) [2, 3]. Although parasitized erythrocytes concentrate chloroquine several hundred-fold [29], even those concentrations are several orders of magnitude less than the chloroquine concentrations that inhibit nucleic acid synthesis (1–2 μM 1–2 mM). Thus, for this mechanism to be important, the parasite would need to concentrate chloroquine an additional 100- to 1000-fold in the compartment(s) where nucleic acid synthesis was occurring.

Binding to ferriprotoporphyrin IX (FP)

As the intraerythrocytic malaria parasite matures, it accumulates a dark pigmented material in its vacuole [30]. Most malariologists view this pigmented material as the non-degradable residuum from the ingested hemoglobin, and several have suggested that it may be similar or identical to FP. In particular, Fitch and his colleagues have shown that FP lyses red cells as well as murine and human malaria parasites [10, 31, 32]. They have demonstrated that chloroquine binds to FP with a stoichiometry of 0.5 : 1 [33],

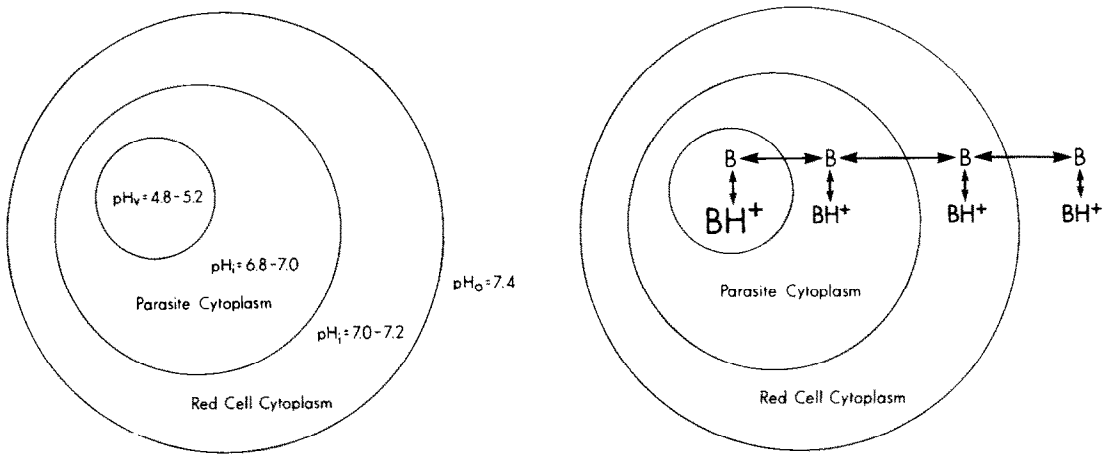

Fig. 2. Effect of an acid intracellular compartment on the distribution of weak bases. Left panel: Studies by several investigators have shown that intracellular vesicles have an acid pH (pH_v) approximately 4.8 to 5.2 in medium at physiologic pH, i.e. an outside pH (pH_o) of 7.4. Estimates of red cell cytoplasmic pH (pH_i) and parasite cytoplasmic pH have been 7.0 to 7.2 and 6.8 to 7.0 respectively. Right panel: According to the assumptions of de Duve *et al.* [12] (see text for details), the neutral uncharged forms of weak bases (B) readily cross both plasma and vesicle membranes (i.e. their concentrations are equal in all three compartments). In contrast, the protonated (charged) forms of weak bases (BH^+) do not cross membranes readily. Thus, their concentration is greatest in acid vesicles, and slightly greater in the cytosol than in the external medium.

and that the chloroquine : FP complex also lyses both red cells and plasmodia [10, 32]. They have hypothesized that malaria parasites normally produce an endogenous FP-binding protein which protects them from the potentially lethal effects of free (unbound) FP [34], and have recently found FP-binding activity in the cytosol of *P. berghei* parasites [35].

There is no question that FP is a membrane-lytic agent. However, at least two unresolved issues remain: (1) despite the apparent absence of pigment in chloroquine-resistant *P. berghei* [36], the available biochemical data do not demonstrate differences in the amount or type of pigment produced by chloroquine-susceptible and chloroquine-resistant strains of *P. falciparum*, and (2) studies comparing FP-binding proteins in chloroquine-susceptible and -resistant strains are not yet available to determine whether differences in these proteins could explain chloroquine resistance.

Antimalarials as weak bases

In addition to these hypotheses, Homewood and others have suggested that chloroquine may act as a weak base [37]. To test this hypothesis, it is necessary to estimate the pH of parasite vesicles and the effect of chloroquine and other antimalarials on that pH. Recent studies by ourselves [11] and by Yayon *et al.* [38] have shown that the parasite contains acid vesicles. The techniques for estimating the pH of intracellular compartments have been carefully reviewed [39] and will be discussed here briefly. Micro pH electrodes have been used to measure intracellular pH in large cells and in some cultured cells. However, it is not practical to place such an electrode into a small (0.5 μm diameter) intracellular vesicle such as an endosome, lysosome or parasite vesicle. Nuclear magnetic resonance (NMR) has been used to estimate intracellular pH in mammalian cells [39] and in malaria parasites*. However, the small volume of the parasite vesicle relative to the volume of the surrounding cytoplasm precludes the detection of a signal from a reporter group within the parasite vesicle. Although the distribution of weak bases such as [^{14}C]methylamine can be used to determine the pH of an acid compartment in a two-compartment system, this approach is not useful in the parasitized red cell system where there are four compartments (medium, red cell cytoplasm, parasite cytoplasm, and the parasite vesicle) and the relative volumes of the compartments are unknown and unequal. Spectroscopic and fluorescent techniques suffer from the same defects unless it is possible to confine the pH-sensitive fluorescent molecule to the compartment of interest. Then fluorescent probes have the necessary sensitivity to determine the pH of small intracellular compartments.

Using the fluorescein–dextran (FD) technique originally described by Ohkuma and Poole [4], it is possible to measure vesicle pH in *P. falciparum* [11, 38] by incorporating FD into the cytoplasm of uninfected red cells and subsequently infecting those red cells with parasites which take up FD (with hemoglobin) from the red cell cytoplasm by endocytosis as they mature. Once the host red cell has been lysed, FD is then present only in the parasite vesicle [11]. These studies have shown that the parasite vesicle is normally acid (baseline pH 5.2 to 5.4) [11, 38], and that the concentrations of weak bases which inhibit parasite growth *in vitro* are virtually identical to those that increase vesicle pH (e.g. an EC_{50} of 194 nM for chloroquine vs 100–1000 nM chloroquine concentrations to increase vesicle pH by 0.3 to 0.4 pH units) [11]. Similar observations were also made with quinine (an EC_{50} of 415 nM vs 100–1000 nM concentrations to increase vesicle pH) and mefloquine (an EC_{50} of 10 nM vs 10 nM concentrations to increase vesicle pH) [11]. In addition, both the EC_{50} and the chloroquine concentration necessary to increase vesicle pH were significantly lower in a chloroquine-susceptible strain of *P. falciparum* (an EC_{50} of 6 vs 194 nM, and a chloroquine concentration of 1–10 vs 100–1000 nM to increase vesicle pH) [11]. These results suggest that the action of the most important antimalarials is closely associated with their ability to increase parasite vesicle pH.

Potential explanations for the susceptibility of plasmodia to chloroquine

The effects of chloroquine and other similar antimalarials on vesicle pH in mammalian cells can be explained by their pKs (10.2 and 8.3 for chloroquine) and the ΔpH between the initial pH of the acid vesicle and the external medium (considering a difference of 2 pH units as producing a driving force for the 100-fold concentration of a monoprotic weak base). However, chloroquine increases vesicle pH at much lower concentrations in both resistant and susceptible *P. falciparum* (100–1000 and 1–10 nM) [11] than in fibroblasts or macrophages (10–60 μM) [4, 5]. These results cannot be explained by the pKs of the drug and/or the ΔpH across the vesicle membrane despite the fact that chloroquine is a diprotic weak base (see below). They suggest that *P. falciparum* parasites are intrinsically more susceptible to chloroquine than mammalian cells. To quantitate the effects of monoprotic and diprotic weak bases on vesicle pH, we have developed a model based on the pKs of the weak bases being studied and on the ΔpH between the medium and vesicle.

DEVELOPMENT OF A MODEL TO EXAMINE THE EFFECTS OF WEAK BASES ON THE pH OF ACID VESICLES

Assuming that the concentration of the neutral (unprotonated) weak base and the pKs are the same in the vesicle and the medium, it is possible to calculate the total concentration of weak base in the acidic compartment $[TB]_v$,† if pH_v and pH_o are

* W. Vine, A. H. Fairlamb and D. Couburn, Abstract, Society of Magnetic Resonance in Medicine, New York (1984).

† Abbreviations/nomenclature: The subscripts o and v indicate medium (outside) and intravesicular concentrations respectively. TB indicates all forms (total) of the weak base, and B, BH^+ and BH^{2+} are the free, mono- and diprotonated species of the weak base respectively. The dissociation constants for weak bases are indicated as K_1 (the more alkaline dissociation constant) and K_2 (the more acidic dissociation constant for diprotic bases). The negative logarithms of the dissociation constants are indicated as pK_1 and pK_2, in the same way that hydrogen ion concentration is represented as pH.

known, and the protonated form of the weak base is essentially impermeant:

$$\frac{TB_v}{TB_o} = \frac{1 + 10^{(pK - pH_v)}}{1 + 10^{(pK - pH_o)}} \quad (1)$$

Although the protonated form of a weak base is not absolutely impermeable to biological membranes, it is typically 100- to 1000-fold less permeable than the neutral (unprotonated) form of the weak base [39]. As a result, this assumption yields a good approximation of the total concentration of the weak base in the acid vesicle (equation 1). In the case of chloroquine, one may formulate a similar equation that describes the distribution of a diprotic weak base:

$$\frac{TB_v}{TB_o} = \frac{1 + 10^{(pK_1 - pH_v)} + 10^{(pK_1 + pK_2 - 2pH_v)}}{1 + 10^{(pK_1 - pH_o)} + 10^{(pK_1 + pK_2 - 2pH_o)}} \quad (2)$$

Both of these equations (equations 1 and 2) indicate that the total concentration of weak base in the vesicle is greater than the total concentration of weak base in the medium ($TB_v > TB_o$) when vesicle pH is less than medium (outside) pH ($pH_v < pH_o$) (i.e. weak bases are concentrated in vesicles with a pH more acidic than the surrounding medium). Based on de Duve's first assumption [12], the excess concentration of weak base in the vesicle ($[TB]_v - [TB]_o$) must arise from protonation of the initially neutral form of the weak base, which enters the vesicle in the unprotonated form and then consumes protons (thus raising vesicle pH).

In the case of mammalian lysosomes, a number of studies have functionally characterized the Mg^{2+}-ATP-dependent proton pump responsible for vesicle acidification [40–42]. To raise lysosomal pH, the weak base must overcome the ability of this pump to acidify the lysosome and any buffering by the contents of the vesicle. If vesicle pH (pH_v) can be measured, it is possible to estimate the total buffering capacity of the vesicle by delivering an acid or base load to the vesicle interior and determining the change in vesicle pH that follows. If one assumes that the weak base distributes across the vesicle membrane according to the relationships given above, the free protons removed from the interior of the vesicle by the entering weak base can be calculated from vesicle pH (pH_v) and the pK(s) of the bases involved. For a monoprotic weak base, the concentration of protons consumed is defined by:

$$[BH^+]_v = \frac{[TB]_v}{\frac{K_1}{[H^+]_v} + 1} \quad (3)$$

For a diprotic weak base, the appropriate equation is:

$$([BH^+]_v + 2[BH^{2+}]_v) = \frac{[TB]_v}{\frac{(K_1)(K_2)}{[H^+]_v^2} + \frac{(1)}{[H^+]_v} + 1} + \frac{[TB]_v}{\frac{(K_1)}{[H^+]_v} + 1 + \frac{[H^+]_v}{K_2}} \quad (4)$$

The ratio of this calculated value to the measured pH change provides an estimate of the total buffering capacity of the vesicle.

Using this approach, we have defined a three-dimensional surface relating these three variables (pH_v, $[TB]_o$, and total buffering capacity). The results of these calculations for NH_4Cl and chloroquine (shown in two dimensions in Figs. 3 and 4) predict changes in pH_v as a function of TB_o, at several values of the total vesicle buffering capacity. The ability of chloroquine to raise pH_v at lower $[TB]_o$ than NH_4Cl, and the different shape of the chloroquine contour line are both a result of its being a diprotic weak base (equation 4). We have also plotted experimental measurements of vesicle pH in cultured fibroblasts and malaria parasites at several $[TB]_o$

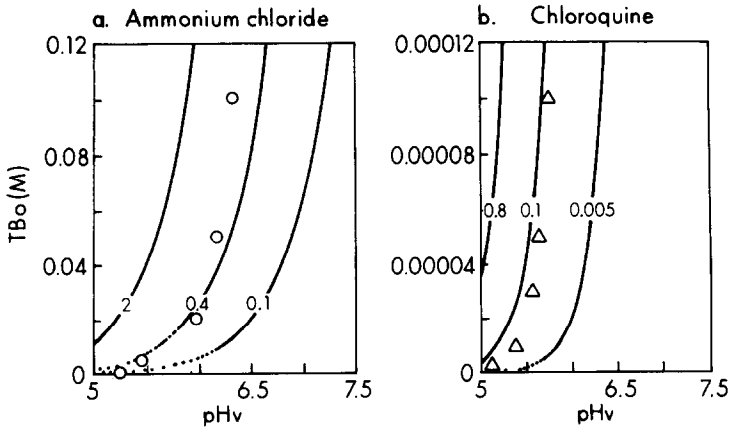

Fig. 3. Effect of weak bases on vesicle pH in fibroblasts. Based on the relationship of vesicle pH (pH_v) to extracellular weak base concentration (TB_o), one can plot contour lines which represent the total buffering capacity of the vesicle (see text for details). The experimental results observed with fibroblasts using both ammonium chloride (panel a, open circles) and chloroquine (panel b, open triangles) closed parallel the calculated contour lines (based on data from Ref. 11).

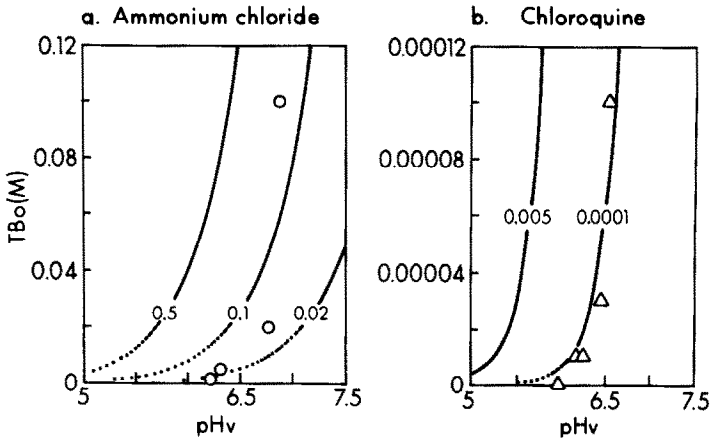

Fig. 4. Effect of weak bases on vesicle pH in chloroquine-resistant *P. falciparum*. The experimental results observed with chloroquine-resistant *P. falciparum* using ammonium chloride (panel a, Fig. 4) are similar to those obtained with fibroblasts (panel a, Fig. 3). In contrast, the buffering capacity of the parasite vesicle observed with chloroquine (panel b, Fig. 4) is approximately 700-fold less than that observed with fibroblasts (panel b, Fig. 3) (based on experimental data from Ref. 11).

concentrations on these surfaces (Fig. 4). Within the experimental accuracy of these measurements, the proposed mechanism of weak base concentration in acid vesicles provides an explanation for our results with fibroblasts, but not for those with *P. falciparum*. It is also clear that a number of factors may affect pH_v. For example, as the concentration of the weak base is increased, vesicle osmolality must also be increasing. Lysosomes have been observed to swell when the cell is exposed to a weak base. The effect of osmotic forces increases in vesicle water, and membrane tension in these vesicles are unknown, but may account for some of the decreasing change in pH_v observed at higher NH_4Cl concentrations.

Using these values for total buffering capacity, the apparent buffering capacity of the parasite vesicle is approximately 700-fold less in the presence of chloroquine than it is with NH_4Cl (Fig. 4). In contrast, in fibroblast lysosomes, there is only a 4-fold difference (Fig. 3). Recent data from our laboratory suggest that there is an additional 10 to 100-fold difference in apparent buffering capacity between NH_4Cl and chloroquine in chloroquine-susceptible parasites. These results indicate that chloroquine raises the pH of acid vesicles in *P. falciparum* at external concentrations that cannot account for its ability to overcome the total buffering capacity of the vesicle (as defined by the effect of NH_4Cl on vesicle pH) by the weak base effect alone. They suggest that either (a) the parasite has an additional means of concentrating chloroquine in its vesicles, or (b) that chloroquine reduces the total buffering capacity of the parasite vesicle (e.g. by inhibiting the proton pump or by itself transporting protons across the vesicle membrane).

DISCUSSION

Historically, the treatment of *P. falciparum* infection in humans has relied upon quinine and chloroquine. The recent appearance of significant chloroquine resistance has emphasized the fact that the mechanism of action of these drugs against the parasite is unclear. Of the three proposed mechanisms of chloroquine action in *P. falciparum*: (a) inhibition of nucleic acid synthesis, (b) binding to ferriprotoporphyrin IX, and (c) increase of the pH in the parasite's acid vesicle, the first two have an uncertain correlation between *in vitro* studies and *in vivo* conditions (see "Potential Mechanisms of Chloroquine Action against Plasmodia" above), and the third has not previously been accessible to experimental study. We have reviewed each of the proposed mechanisms, and discussed our recent observations on the effect of chloroquine on the acid intracellular vesicles of *P. falciparum*. We observed both a temporal and a quantitative relationship between the biological activity of chloroquine (and other antimalarials) against the parasite, and the response of parasite vesicle pH. Based on this information, we believe that increasing vesicle pH is the most likely explanation for the antiplasmodial effects of chloroquine and other similar antimalarials.

The model we have developed for the concentration of weak bases in acid vesicles is consistent with the known effects of mono- and diprotic weak bases (such as NH_4Cl and chloroquine respectively) on mammalian cells and with the effects of NH_4Cl on *P. falciparum* (Figs. 3 and 4). However, it does not explain the effects of chloroquine and other antimalarials against *P. falciparum*, which occur at concentrations 2–3 orders of magnitude less than those which can be explained by their p*K*s and the pH difference alone.

From the ammonium chloride data (Figs. 3a and 4a), we infer that the intrinsic buffering capacity of *P. falciparum* acid vesicles is similar to that of mammalian fibroblasts. From the chloroquine data, we conclude that chloroquine has a disproportionate effect on vesicle pH in *P. falciparum*, in comparison with mammalian cells. Chloroquine-resistant *P. falciparum* parasites have a response more similar to fibroblasts, which is more consistent with a simple weak base mechanism of action. Although our most

recent studies demonstrate that chloroquine-susceptible parasites take up approximately 2.5 times as much drug as chloroquine-resistant parasites,* it is not yet clear whether this effect alone is sufficient to explain their greater susceptibility to the drug. In our opinion, fundamental study of this question (why plasmodia are *hypersusceptible* to chloroquine) provides an important opportunity to define mechanisms of antimalarial action and resistance.

In summary, the most important antimalarials (chloroquine, quine, and mefloquine) are weak bases which increase parasite vesicle pH at their biologically active concentrations. Their effects on the parasite (rapid increase of vesicle pH and vesicle swelling) are similar to their effects on mammalian cells. Based on the p*K*s of the weak bases tested and on measurements of vesicle pH, we have developed a model that relates vesicle pH and the extracellular weak base concentration to vesicle-buffering capacity. This model predicts the effects of ammonium chloride on vesicle pH in fibroblasts and *P. falciparum*, and the effects of chloroquine on vesicle pH in fibroblasts. However, chloroquine increases vesicle pH in *P. falciparum* at lower concentrations than predicted by the model. This result cannot be explained by the p*K*s of chloroquine and the pH difference between the parasite vesicle and the external medium alone. It is not yet clear whether the ability of chloroquine to increase vesicle pH in parasites at nanomolar concentrations is due entirely to an additional concentrating mechanism not present in mammalian cells, or whether additional mechanisms such as inhibition of the parasite vesicle proton pump or rapid equilibration of protons across the vesicle membrane are also involved.

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A Mathematical Appendix, which describes the stepwise derivation of equations 1–4, is available from the authors on request.

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