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COMMENTARY

CURRENT CONCEPTS AND NEW IDEAS ON THE MECHANISM OF ACTION OF QUINOLINE-CONTAINING ANTIMALARIALS*

HAGAI GINSBURG†‡ and TIMOTHY G. GEARY§‖

†Department of Biological Chemistry, The Hebrew University of Jerusalem, Jerusalem 91904, Israel; and §Department of Microbiology and Public Health, Michigan State University, East Lansing, MI 48824-1101, U.S.A.

In spite of ages of successful use of the natural quinoline-containing antimalarial drug quinine, and decades of the synthetic analog chloroquine, very little is known about their mode of action. The resurgence of malaria in the late 1950s due to emerging drug resistance induced the search for new medications. Most of the experimental effort in the chemotherapy of malaria has been devoted to empirical screening of new formulations using mostly murine and avian models. Over 250,000 compounds have been thus screened for antimalarial activity at huge expense, but very few new drugs have emerged. This frustrating result underscores the necessity for a rational approach based on a more comprehensive understanding of the physiology and biochemistry of the parasite and its interaction with its host. Too few workers have studied the mode of action of drugs in conjuction with ongoing biochemical and physiological processes in the maturing intraerythrocytic parasite. Important areas, such as electron transport and lipid metabolism, have been almost totally neglected and others, such as the mechanism of hemoglobin digestion, have only recently received attention and remain essentially unknown.

In this brief essay, we shall consider current concepts on the mode of action of aminoquinoline drugs, with a major emphasis on chloroquine (CQ), and their limitations in view of new experimental evidence. We shall review briefly the effects of CQ and some of its congeners on eukaryotic cells and on model biophysical systems and attempt to extrapolate from these findings to the possible modes of action of the drugs in malaria parasites, and the mechanism(s) of drug resistance.

Current concepts on the antimalarial mode of action of animoquinolines

Initially, interactions with DNA were thought to underlie drug toxicity for malaria parasites [1–4]. However, this idea has been rejected [5, 6] based on several lines of evidence, namely, that some quinoline-containing antimarlarials, e.g. mefloquine, do not interact with DNA [7, 8], that the concentration of CQ necessary to achieve this effect is not reached in the nucleus of the parasite (see below), that there is no selectivity in the interaction of CQ with DNA from parasite or host cells [9], and that physicochemical analyses are inconsistent with this proposal [10, 11].

Two major hypotheses are currently available: that these drugs act as lysosomotropic agents [12–16] or, alternatively, that they form a lytic or membrane-toxic complex with ferriprotoporphyrin IX (FP), a proposed product of the parasite digestion of hemoglobin (Hb) [17]. According to the first mechanism, the drugs accumulate in the parasite by virtue of their weak base properties [18] into an acidic compartment(s), with consequent disruption of function due to the resulting alkalinization. The second assumes that the compounds accumulate by interacting with free FP, resulting in damage to critical parasite organelle membranes.

Membrane toxicity mechanism

The best developed theory has been advanced by Fitch and co-workers, proposing that CQ accumulation by parasitized erythrocytes is due to the specific binding of CQ to FP [17]. A high molecular weight complex can form between FP (or other porphyrins) and CQ [19–25] and other such drugs [26–28], with an affinity comparable to that of the proposed high-affinity CQ binding site suggested for malaria-infected erythrocytes [20], although the affinity and the stoichiometry of FP–CQ complexation have been contested recently [23, 24, 28].

In vitro, FP is highly lytic when applied externally to a variety of cells, including free parasites and uninfected erythrocytes [29–33]. Recently, it has been shown that FP produced inside erythrocytes also causes lysis [34]. CQ has a bimodal effect on FP-induced lysis; low concentrations enhance it but higher concentrations (0.5 mM) have no effect. If, as has been proposed, FP is produced during Hb digestion in the food vacuole [17, 35], then it is reasonable to assume that malaria pigment (hemozoin) forms in the parasite as a mechanism of sequestering and thus detoxifying FP [17, 36]. Since FP complexed with CQ remains lytic to normal mouse [30] and human [37] erythrocytes as well as malaria parasites [33], it has been proposed that the mechanism of action of CQ involves the complexing of the drug with transiently free FP, before the latter is sequestered into pigment, resulting in damage by the FP–CQ complex to parasite structures [17].

Although this theory has many attractive aspects, particularly in the critical area of differential

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‡ To whom correspondence should be sent.

‖ Current address: The Upjohn Co., Kalamazoo, MI 49001, U.S.A.

parasite/host toxicity, evidence has been accumulating which is difficult to reconcile with its major points. This evidence may be conveniently discussed under four headings which represent the major components of the theory.

First, it must be assumed that FP exists in the parasite in an accessible state for a period of time sufficient to allow its interaction with CQ. Biochemically, FP has never been demonstrated to exist even transiently in malaria parasites [35, 38, 39]. That it is present at all is assumed from studies on the composition of hemozoin granules [38]. However, it is clear that the methods used to isolate and characterize hemozoin greatly affect interpretation of its composition [40], and its exact chemistry is still unknown. It has been claimed that application of photoacoustic spectroscopy to rodent malaria parasites in the presence of CQ reveals the presence of in site CQ–FP complexes [41, 42]. Unfortunately, experimental details were lacking. Neither the concentration of CQ nor the time of exposure was stated, and the data were apparently obtained from saponin-treated parasites, a step which compromises the viability of these cells [36]. These data are also contradicted by observations made with another technique. We recently have used Mössbauer Effect Spectroscopy as a tool to analyze iron metabolism in *Plasmodium berghei* [43]. This physical method nondestructively characterizes the molecular environment of iron in iron-containing molecules [44]. The Mössbauer spectrum of the pigment iron was found to be trivalent, high spin, with parameters significantly different from those of any known iron porphyrin compound, instead resembling that of the iron-binding protein ferritin. These findings are difficult to reconcile with what is currently known about hemozoin composition [38], but the use of the sensitive spectroscopic method avoids the difficulties of hemozoin purification which have plagued this area of research. Using Mössbauer Effect Spectroscopy, we could also demonstrate the absence of free FP in *P. berghei*-infected rat erythrocytes and that the spectroscopic changes induced by CQ in aqueous solutions of FP could not be detected in infected erythrocytes. This is compelling evidence that CQ–FP complexes do not occur in *P. berghei*. Iron may be sequestered in hemozoin without the porphyrin, so that we cannot exclude the complexation of free protoporphyrin or other metabolic products thereof with CQ [19, 23, 24], still resulting in lysis. However, free protoporphyrin could not be detected in *Plasmodium falciparum*-infected erythrocytes (H. Ginsburg, unpublished observations).

Second, it has been asserted that the accumulation of CQ into malaria parasites resulted from the formation of FP–CQ complexes [17, 20]. This possibility is at odds with the observation that NH_4Cl can almost completely displace intracellular CQ from *P. falciparum*-infected erythrocytes [45], but it does not alter the ability of CQ to complex with FP [45]. Furthermore, if transiently-free FP accounts for the accumulation of the drug, then steady-state free FP concentrations in the food vacuole must be high enough to account for the rapid and extensive uptake. The high-affinity site proposed to account for drug accumulation, to the total intracellular concentration of 5 μM [46], is presumed to be free FP. Since the drug accumulates selectively in the food vacuole [15, 47], which occupies at most 5% of the volume of the infected cell, the local concentration must approach 100 μM, far higher than that necessary to cause total cell lysis [29, 33]. We have found, recently, using a very high specific activity preparation of [^{3}H]CQ, that CQ reaches concentrations in infected cells of at least 50 μM, or 3 mM in the food vacuole, within less than 30 sec (initial external [CQ] = 10^{-6} M) [48]. Steady-state levels of FP required to achieve such rapid accumulation are far higher than physiologically or metabolically feasible. We have calculated recently that, at the maximal rate of Hb digestion, FP concentration in the food vacuole would rise at a rate of 280 μM/min [49]. To avoid lysis, the rate of FP sequestration must be considerably larger, thus reducing the concentrations of the putative CQ receptor much below the required level.

Third, this theory assumes that parasite killing is due to the effect of CQ–FP complexes [17]. However, numerous lines of evidence do not support this contention. First, it should be possible to correlate the rank order of quinoline-containing antimalarials in accumulating and/or binding to FP and in killing malaria parasites. This is not the case, especially considering amodiaquine, quinine, and mefloquine [50, 51]. Drug–FP interactions are in fact marked by low specificity, with no similarity to structure–activity relationships found for antimalarial activity [52]. Furthermore, the earliest detectable effect of CQ should be disruption of the food vacuole. Ultrastructural studies [53] clearly show no damage to the vacuolar membrane after a 40-min exposure to CQ, at which time extremely high intracellular concentrations are present [45, 48, 54], and no intermixing of macromolecules between the food vacuoles and the cytoplasm can be seen. Permeabilization of the food vacuole membrane to ions is unlikely, since this should dissipate the pH gradient, which is the driving force for drug accumulation, and consequently reduce intracellular drug levels. Most importantly, the enhancing effect of CQ on FP-induced lysis is biomodal, i.e. it is conspicuous in the micromolar range but totally absent above 0.5 mM [29]. Thus, at the vacuolar drug levels achieved with <30 sec, CQ should have no effect on lysis. The time course of CQ uptake is also clearly incompatible with the lytic action of intracellular FP–CQ complexes. At 10^{-6} M external CQ, the most sensitive stages of *P. falciparum* (trophzoites and schizonts) begin to show toxicity only after about 2 hr [55]. Intracellular CQ concentrations reach levels far higher than those required for lysis [33] much sooner than this [13, 45, 48, 56]. Indeed, after a 1-hr exposure to 10^{-6} M CQ, vacuolar levels of over 3 mM are reached [48] but, if these parasites are washed after a short while (<1 hr) and placed in drug-free medium, very little decrease in viability is observed [55]. These data cannot be explained by the CQ–FP hypothesis.

Finally, it has been proposed that CQ resistance is achieved by an absolute reduction in the amount of FP available for CQ complex formation [17, 33].

In fact, this theory was originally advanced after it was found that some CQ-resistant strains of *P. berghei* demonstrate a dramatic decrease in the number of pigment granules compared to CQ-sensitive strains [57–61]. These observations were thought to localize the site of CQ action to Hb digestion and pigment formation [62]. However, it was quickly found that this observation did not extend to CQ-sensitive and -resistant strains of *P. falciparum* [63] nor to all CQ-resistant *P. berghei* strains [64], making the relevance of the initial observations uncertain. Most importantly, using Mössbauer Effect Spectroscopy, we recently showed that, although pigment granules cannot be visually detected in CQ-resistant *P. berghei*, pigment was in fact present in amounts equal to that found in the CQ-sensitive strain [43, 65]; the two strains did not differ in extent of Hb degradation but rather in the macro-aggregation of pigment molecules to form visually detectable granules.

Since pigment could not be observed in CQ-resistant *P. berghei*, it was assumed that the reduction in CQ accumulation observed in these strains [62] is due to the reduction or absence of pigment formation. Since there is no difference in pigment formation between CQ-sensitive and -resistant strains of *P. falciparum*, reduction of CQ accumulation observed in CQ-resistant parasites of this species [56] was assumed to be due to an unspecified difference in FP sequestration, which did not extend to amodiaquine, a close structural analog of CQ [66, 67]. However, this theory predicts that the receptor for CQ is decreased effectively in amount in CQ-resistant *P. falciparum*, which implies that some degree of cross-resistance to other 4-aminoquinolines exists. This is not the case *in vivo* [68, 69] or *in vitro* [70], even for the close structural analog 3-methylchloroquine. It is difficult to accept the assertion that the receptor would be selectively unavailable only to CQ [71], and it would be more prudent to assume that the actual receptor, if it indeed exists at all, has not been identified yet.

Perhaps most at odds with the possibility that CQ resistance is due to diminished availability of FP is the observation that, at least in *P. falciparum*, the differences in CQ accumulation between sensitive and resistant strains are not nearly as great as the absolute differences in CQ sensitivity [48]. At external CQ concentrations of 10^{-7} M, which are ineffective in killing CQ-resistant strains, intracellular levels in these strains exceed those achieved with external concentrations of 3.2×10^{-8} m, lethal to sensitive strains [48]. Thus, there is no absolute relationship between intracellular drug levels and toxicity, as must be true for the FP–CQ complex theory. In this regard, a series of strains of *P. falciparum* which differ by >20-fold in sensitivity to the drug show only 2- to 4-fold differences in drug accumulation [48]. Furthermore, calculations of drug vacuolar concentrations, when those strains were exposed to drug levels equal to their respective IC_{50} (drug concentrations needed for 50% inhibition of parasite growth assayed in the 48-hr *in vitro* test), showed an increase in vacuolar [CQ] in correlation with IC_{50}. This result indicates that the capacities for drug accumulation are similar among the different strains and suggests that the intracellular target for CQ must display a differential susceptibility in drug-resistant strains. Recent data to the contrary [72] can be explained by the fact that these workers measured CQ accumulation between 28 and 47 hr after exposure, by which time the parasites almost certainly were dead [55] and thus had lost any accumulative capability.

Finally, it has been demonstrated recently that mefloquine and quinine totally abolish the CQ enhancement of FP-induced hemolysis [37]. If the membrane toxicity mechanism was indeed operative, mefloquine and quinine should protect the parasite against chloroquine. This is not the case *in vitro* [73].

Consideration of CQ-induced autophagy and pigment clumping

Chloroquine induces autophagy in mammalian cells [74–79] and in malaria parasites [38, 80]. Autophagous vesicles containing clumped pigment particles, Hb, and ribosomes form 35–80 min after drug administration. This process is energy dependent and is competitively inhibited by most other antimalarials (which alone do not cause autophagy) and non-competitively by inhibitors of protein and RNA synthesis, which alone are exceedingly toxic to the parasite [81], so that the specificity of the effect is questionable. The presence of a clumping receptor site for CQ has been proposed (clearly different than the high-affinity site suggested in Ref. 18) and some structural requirements of the putative receptor have been proposed [82]. However, affinities of antimalarials for the clumping site or the high-affinity site correlate poorly with potency in killing parasites, indicating that the identification of CQ receptor sites is premature. Further difficulties in the use of pigment clumping as a model for CQ action are found in the observations that some inhibitors of CQ-induced clumping have additive or even potentiating effects when combined with CQ in parasite killing assays [73, 83, 84]. Although the details of CQ-induced autophagy at the molecular level remain unknown, this process may have an explanation, discussed below, which is compatible with data available from malaria parasites and from observations on phagosome-lysosome fusion in macrophages [85].

CQ as a lysomotropic agent

The presence of compartments inside malaria parasites which accumulate CQ is well documented by autoradiographic experiments with [^{3}H]CQ [47]. The exclusive presence of acid phosphatase in the same organelles [86] has led to their identification as the acidic food vacuoles [87], where the degradation of host cell cytosol occurs. Data on the distribution of CQ in *P. berghei*-infected mouse erythrocytes could be interpreted in support of this compartmentation [88]. By analogy to phagolysosomes of mammalian cells, this compartment is expected to have an acidic pH [89]. Recently, we showed that this is indeed the case; the food vacuole pH of *P. falciparum* is 5.0 to 5.2 [90], whereas the pH values of the parasite cytoplasm and the host cell cytosol are approximately equal and about 2 pH units more alkaline. This result has subsequently been confirmed [15, 48]. The mechanism(s) by which this pH

is maintained is unknown, but, from data obtained with lysosomes, two mechanisms should be considered.

First, from studies on the permeability properties of lysosomal membranes, it is possible that a Donnan-type equilibrium exists [cf. Ref. 91]. Second, there exists a Mg^{2+}-ATP-driven proton pump which translocates protons into the lysosomal interior at the expense of metabolic energy [92, 93]. It is possible that these mechanisms operate in parallel and both contribute to the acidification. The validity of the analogy drawn between mammalian phagolysosomes and the parasite food vacuoles is enhanced by indirect evidence for a proton pump which operates in the parasite lysosomes and/or food vacuoles. Thus, CQ uptake into infected erythrocytes is strongly inhibited by metabolic poisons [13, 16], suggesting the functional presence of an ATP-driven proton pump. The inhibition of CQ accumulation by uncouplers [13, 66], protonophores [94] and lysosomotropic agents [45, 66] indicates that the driving force for drug accumulation is indeed a pH gradient. However, lysosomotropic agents do not prevent CQ accumulation altogether, indicating the contribution of a Donnan-type maintenance of a pH gradient.

The absolute dependence of CQ accumulation on the existence of a pH gradient between the accumulating acidic compartment and the extracellular medium can be seen from data in *P. berghei*-infected mouse red cells [66] and in *P. falciparum*-infected human erythrocytes [90]. In both cases, it was demonstrated that decreasing the medium pH to ≤ 6.2 totally abolished CQ accumulation. At this pH, the pH gradient approaches negligible levels, which, combined with the effect of an acidic pH on cellular metabolic pathways such as glycolysis, could reduce ΔpH to essentially zero. Similar findings have been reported for the accumulation of CQ and other weak bases by mouse macrophages [95].

These data indicate that CQ accumulates by pH gradients into acidic organelles in malaria parasites. Since the drug can be doubly protonated, the accumulation ratio corresponds to the square of the ratio in proton concentration between the organelle and the medium [96]. If CQ accumulates selectively into acidic compartments, then at pharmacological concentrations of the drug (10^{-7} to 10^{-6} M) CQ concentrations inside these organelles at an extracellular pH of 7.4 can reach millimolar levels [48, 90]. The fact that such high local concentrations of CQ can be achieved must be considered in the analysis of mechanisms of action.

In addition to the food vacuole, malaria parasites also contain organelles which are morphologically typical lysosomes [53, 97]. Since most attention has been paid to drug effects on the food vacuoles, it is convenient to discuss these organelles separately in evaluating the lysosomotropic hypothesis. The possibility that CQ acts on the food vacuoles has the inherent attraction of providing an explanation for the specificity of the drug for malaria parasites, since most mammalian cells lack organelles identical to them in function. The most obvious possibility is that the accumulation of the drug inside the vacuole raises the pH above the optimum required for the function of parasite enzymes involved in Hb digestion, which have acidic pH optima [12–16, 98–100]. As mentioned, evidence is now available to show that the pH of the *P. falciparum* food vacuole is 5.0 to 5.2 [90]. It appears, however, that alkalinization *per se* is an insufficient explanation of drug toxicity. Calculations of the vacuolar pH from the distribution of the weak base methylamine into infected erythrocytes in the presence of CQ indicate that no substantial alkalinization occurs within the therapeutic drug concentration range [90]. This is certainly due to the ability of vacuolar H^+-pumps to maintain an acidic pH even in the presence of CQ [48]. Also, vacuolar pH attained in the presence of lethal [CQ] is proportional to the IC_{50} value in different strains. Furthermore, although NH_4Cl increased intravacuolar pH to a greater extent than CQ, this compound is considerably less toxic than CQ [45, 101]. If alkalinization were the mechanism of action, the rank order of quinoline-containing antimalarials on the basis of simple pK_a values should be strongly correlated with their rank order of potency. If this were true, drugs such as mefloquine and quinine, which can also be doubly protonated but have lower pK_a values than CQ and thus can accumulate to a lesser extent, should be less potent than CQ. This is not the case [70]. Considering available structure–activity data [70, 102, 103], it is clear that modifications which would not be expected to change pK_a values have profound effects on activity; if alkalinization alone caused parasite death, it should be possible to demonstrate toxicity for *all* basic and dibasic compounds solely on the basis of their pK_a. This also is not the case. Thus, alternative mechanisms of inhibition must be sought.

It must be noted that some experimental evidence suggesting that CQ kills malaria parasites via a lysosomotropic mechanism has been presented recently [15]. However, these studies employed saponin-treated parasites, which, by virtue of the induced leakiness to ions [36], may not be fully functional with respect to the maintenance of the food vacuole pH. Furthermore, these data show that pH alterations induced by CQ, quinine and mefloquine are not equally antimalarial. Both CQ and quinine kill malaria parasites at concentrations which have a very small effect on vacuolar pH (as we have found with CQ [48]), whereas much larger increases in vacuolar pH due to mefloquine are tolerated without apparent ill effect. Despite eloquent arguments in support of this mechanism [16], we believe other effects are involved in the antimalarial action of CQ.

Recently it is has been demonstrated that CQ, amodiaquinine, primaquine and mefloquine inhibit a *P. falciparum* acid protease which digests Hb [98, 104]. The observation that the inhibition by CQ was more effective as pH increased [98] suggests a mechanism by which the toxicity of the drug could be achieved by the concerted action of high concentrations and slight alkaninization. Acid proteases and aminopeptidases which are inhibited by antimarlarials have been identified in other *Plasmodium* spp. as well [98, 100, 105–107]. However, since some of these enzymatic activities were maximal at neutral pH, great prudence must be exercised in implicating them in drug action. Neutral proteases cannot function in the acidic cellular compartments where and

only where inhibitory drug concentrations can be reached. This is an important topological aspect which must not be disregarded and which simultaneously raises some basic questions concerning the mechanism and location(s) of Hb digestion by the parasite. Inhibition of Hb digestion caused by CQ could kill the parasite. Although at the trophozoite and schizont stages, which are the most sensitive to CQ [55], the parasites are freely permeable to amino acids in serum or RPMI-1640 [36, 108–110], some amino acids such as arginine and lysine are unable to enter through the permeability pathways induced by the parasite in the host cell membrane; thus, the parasite could be starved for some amino acids in this situation. That host cytosol digestion is essential for parasite growth and development is evident from the fact that covalent cross-linking of Hb inhibits parasite growth and prevents Hb digestion by cathepsin D and parasite proteases [111]. In recent experiments it was found that therapeutically relevant CQ concentrations inhibit Hb digestion and efflux of amino acids from infected erythrocytes [49]. Interestingly, the relative abundance of amino acids appearing in the medium closely matched their relative abundance in hemoglobin. Furthermore, lysosomal effects of CQ exposure, which could be separated from the vacuolar effects, where not observed at lethal [CQ], in line with the selective antimalarial activity of this drug. These observations, especially when coupled with ultrastructural observations [54], call into question the relevance of the inhibition of the Hb digestion enzyme from *P. falciparum* by CQ–FP complexes but not by CQ alone [104]. While sufficiently high concentrations of CQ–FP complexes to cause this effect are unlikely to occur (see above), the accumulation of undigested vesicles containing host cell cytoplasm in the food vacuole [54] also calls into question the relevance of direct protease inhibition to the lethal action, since in this situation Hb is not exposed to the protease anyway.

CQ causes swelling of lysosomes in *P. falciparum* [53], similar to the effect seen in mammalian lysosomes [112]. The accumulation of CQ in these organelles is expected to shift the pH away from the optimum of a variety of lysosomal hydrolases [18] but, for the reasons cited above for the food vacuole, it is clear that simple alkalinization of lysosomes is not a sufficient explanation for CQ toxicity.

An alternate explanation is that these drugs directly inhibit the function of lysosomal enzymes. CQ inhibits the breakdown of endogenous proteins [113] and inhibits proteases in fibroblasts, mouse kidney subcellular suspensions, and rat liver lysosomes [114]. The lysosomal acid protease cathepsin B is strongly inhibited by CQ [114]. However, inhibition of lysosome function is not observed in *P. falciparum* at therapeutic [CQ] [49].

Another group of target enzymes for CQ is lysosomal phospholipases [115, 116]. We have demonstrated recently that ingestion of host cell cytosol by *P. falciparum* results in the release of the contents of membrane-bound endocytic vesicles into the food vacuole [53, 54]. The breakdown of the membrane structure of these vesicles, which precedes cytosol digestion, is inhibited by CQ; this process may reasonably be assumed to involve phospholipases [53]. Lysosomal phospholipases are indeed inhibited by CQ [117–120]; this has been proposed as the mechanism of CQ-induced lipidosis in mammalian liver cells [121, 122]. Quinacrine, another potent antimalarial drug, is widely used as a phospholipase inhibitor [119, 123]; the drug forms a relatively stable complex with phospholipids which limits the access of phospholipase to substrate [124].

A need for an alternative mode of action

There can be little doubt that CQ and other quinoline-containing antimalarials accumulate in malaria parasites, by virtue of their weak base properties, specifically into acidic organelles and that this is necessary for the drug toxicity. It is also clear that drug accumulation is necessary but insufficient to generate the killing effect, and more effort must be directed toward understanding the effects of high concentrations of these drugs on biochemical processes in these organelles. Suggestions that CQ may act through inhibition of ornithine decarboxylase [125] may be questioned simply on the basis of differential compartmentation of the drug and the enzyme. The observation that CQ alters glutathione metabolism in *P. berghei* [126] must first be extended to *P. falciparum* before its relevance can be assessed.

In light of the numerous inconsistencies which have arisen between current hypotheses and experimental observations, it is clear that a new approach to the problem of the mechanism of action of CQ is required. This is not to suggest that the formation of FP–CQ complexes and/or the alkalinization of acidic cellular compartments do not contribute to the drug effect; in all likelihood, they do. Nonetheless, neither hypothesis is sufficient to explain the available data.

Basic observations concerning CQ action

In considering the mechanism of CQ action, especially in *P. falciparum*, several pertinent experimental facts are inescapable, including (1) trophozoites and schizonts are considerably more sensitive to CQ than ring stages, and require a 1- to 2-hr exposure to pharmacologically relevant CQ concentrations for eliciting irreversible toxicity [55]; (2) the rate of uptake of CQ is extremely rapid, reaching very high intracellular levels long before the time noted above [45, 48, 90]. Thus, high concentrations must be maintained for a considerable time to generate irreversible effects; (3) since the drug is concentrated in acidic parasite organelles, the concentration in these compartments (at relevant external concentrations) reaches millimolar levels; alkalinization with NH_4Cl causes a rapid and essentially complete efflux of CQ [45]; (4) resistance to CQ does not predictably result in cross-resistance to other quinoline-containing antimalarials [68–70]; (5) although CQ-resistant parasites accumulate somewhat less drug than sensitive strains, the intracellular drug concentration necessary for killing increases proportionately to drug resistance [48]; and (6) the drug is selectively toxic to malaria parasites.

Alternate directions in theories of CQ action

Considering the available data on CQ effects in malaria parasites and in other cells, some new directions in research become evident. We suggest that

the major toxic effect of high (e.g. >1 μM) concentrations of CQ involves the disruption of food vacuole and lysosomal function, with consequent release of hydrolases and degradation of parasite components. Lower but therapeutically relevant concentrations interrupt digestion of host cell cytoplasm, resulting in nutrient deprivation. It is likely that the relative importance of these effects varies with CQ concentration, time of exposure, and parasite strain, as well as with different quinoline-containing antimalarials; these effects are discussed in detail below.

Chloroquine has a demonstrable effect on membrane stability ([127]; H. Ginsburg, M. Krugliak and A. Yayon, unpublished observations); at low concentrations ($\leq 10^{-4}$ M) it stabilizes erythrocyte membranes, but at higher concentrations ($\geq 10^{-3}$ M) it labilizes them. Our recent findings [48] show that vacuolar concentrations of CQ exceed 10^{-3} M when infected erythrocytes are exposed to 10^{-7} to 10^{-6} M solutions. However, the fact that major ultrastructural alterations in the food vacuole membrane are not seen, whereas extensive vacuolization of lysosomes is evident, suggests a differential drug effect on these two acidic compartments. The vacuolar pH is likely to be slightly more alkaline than that of the primary lysosomes (derived from endoplasmic reticulum) due in part to their smaller surface/volume ratio relative to that of lysosomes and in part to the buffering capacity of the large amount of amino acids derived from Hb degradation (approximately 0.5 mmole of hemoglobin monomers per liter cells are degraded per hour resulting in the production of 13 mM amino acids per minute inside the food vacuole at the trophozoite stage). As mentioned earlier, the theory of weak base accumulation [96] predicts that dibasic CQ will concentrate in acidic compartments proportional to the square of the proton concentration gradient across the separating membrane. Thus, if the lysosomal compartment was only 0.5 pH units more acidic than the food vacuole, CQ should concentrate to a 10-fold greater extent in the former. This simple calculation illustrates the possibility of a differential effect on the lysosomes, although the functional consequences are not necessarily greater. Furthermore, CQ may behave somewhat differently than other weak bases. In mouse peritoneal macrophages, CQ causes extensive vacuolization at relatively low concentrations but the effect is much less pronounced at slightly higher concentrations [94]. The current dogma holds that such vacuolization results from the high accumulation of weak bases into lysosomes, resulting in osmotic swelling. Higher CQ concentrations could disrupt lysosomal membrane permselectively, dissipating the proton and/or osmotic gradient and decreasing vacuolization. Consequently, lysosomal enzymes would be released, as demonstrated in liver cells [128] and in rat retina [129], with resultant damage to cytoplasmic elements. Such an event has not yet been demonstrated in *P. falciparum* [49].

Other effects of CQ must also be considered, particularly the interactions of the drug with phospholipids (PL). The basis for this idea relies on studies recently summarized by Lüllmann-Rauch [130], which indicate that protonated forms of amphiphilic drugs interact with various PL by both hydrophobic and electrostatic forces. Binding of such drugs alters the physico-chemical properties of PL and reduces their susceptibility to hydrolysis by phospholipases. Both CQ [117] and quinacrine [119–123] are potent inhibitors of phospholipase action, and this effect is observed at concentrations even below those achieved in parasite acidic organelles. The binding interaction results in the accumulation of both drug and acidic PL in hepatic lysosomes.

Some points of this model have been substantiated experimentally in lipid membrane models. Thus, drug–lipid interactions have been demonstrated by NMR techniques [131]; these results indicated a requirement for a protonated amino group in the drug side chain and a charged group in the PL, but the affinity of binding is primarily dependent upon the hydrophobicity of the drug [132]. It was also found that cholesterol inhibits the PL–drug interaction. The PL–drug interaction reduces the electrostatic charge of membrane-aqueous interfaces and results in a reduction of phospholipase-mediated hydrolysis of PL [133].

Chloroquine binds preferentially to acidic PL [134, 135] and is displaced, as are other drugs, from the PL–membrane interface by Ca^{2+} [132]; binding to zwitterionic phosphatidylcholine also occurs [136, 137]. It was concluded that CQ was bound to an extent less than expected from its hydrophobicity, due to its relatively high electrostatic charge. This conclusion is supported by the observation that CQ is unable to intercalate into PL monolayers [138], whereas substantial intercalation is observed with quinine and mefloquine [51].

CQ–PL interactions offer an intriguing explanation of the phenomenon of reversible CQ-resistance seen in *P. berghei* [5]. In this parasite, some drug-resistant phenotypes require constant CQ exposure; when the drug is withdrawn from experimental animals, CQ sensitivity returns. It is well established that chronic exposure to CQ causes the accumulation of acidic phospholipids and an increased cholesterol:PL ratio in rat liver [139]. Since the liver is the main source of plasma lipids, which are known to be in dynamic equilibrium with erythrocyte membranes [140], and since the parasite is unable to synthesize fatty acids and cholesterol *de novo* [141], it is highly likely that the lipid composition of the parasite depends to some extent on host lipid metabolism. Since, as noted above, increases in membrane cholesterol:PL ratios decrease the interaction of CQ with model membranes, alterations in cholesterol availablity may change parasite cholesterol levels and thus drug sensitivity. Thus, it is possible that unstable CQ resistance may originate from the response of the host to the drug, rather than from a true genetic change in the parasite. Preliminary analyses of the lipid composition of *P. falciparum*-infected erythrocytes show that CQ-resistant strains have higher (~40%) cholesterol: PL ratios than do sensitive strains (H. Ginsburg, unpublished observations). This difference alone could account for drug resistance. Parenthetically, we have found recently that

cholesterol considerably increases the effect of CQ on FP intercalation into PL monolayers [138], a process which underlies the lytic effect of CQ–FP complexes. Thus, the CQ-resistant strain of *P. falciparum*, which contains a higher cholesterol:PL ratio than a CQ-sensitive strain, should have been more sensitive (rather than more resistant) to the drug if the membrane toxicity mechanism was indeed operative. This result offers additional indirect evidence against the CQ–FP complex theory.

Drugs such as CQ can also affect the transport and processing of lysosomal enzymes [142], events which require an acidic pH. Lysosomotropic agents block the delivery of newly synthesized hydrolases to lysosomes and also the pH-dependent dissociation of some receptor–ligand complexes during receptor-mediated endocytosis [143, 144]. These observations have been made in mammalian cells, but similar processes may function in malaria parasites; this could explain the CQ inhibition of feeding and/or intracellular protein turnover which is essential for rapidly developing and differentiating cells [cf. Ref. 145], particularly malaria parasites [37]. It is interesting to note that, at subtoxic CQ levels, ring stage parasites do not mature although their viability is maintained [55].

The observation that CQ inhibits parasite feeding by preventing the breakdown of single-membrane vesicles in the food vacuole [53] is perhaps best explained by the inhibition of phospholipase activity, but inhibition of phospholipases may have additional effects. Parasite growth and development are accompanied by a large increase in the membrane surface area of the organism. Augmentation of all the membrane systems of the infected cells causes the total lipid content to increase, but the lipid composition of the parasite differs from that of the erythrocyte in many respects [141]. Exchange of lipids between the erythrocyte and parasite membranes is probably mediated by lipid-exchange proteins [140] of host cell origin, but the synthesis of the parasite-specific PL requires an extensive phospholipase activity, another potential target for antimalarial drugs. It is intriguing to note that the stage of the parasite life cycle which is most sensitive to CQ [55] is also characterized by a high rate of PL synthesis [146].

The interplay between drug concentration and the length of exposure needed to elicit an inhibitory effect could also apply to the possible effect of CQ on lysosomal structure and function. Exposure of trophozoites and schizonts of *P. falciparum* to 10^{-6} M CQ requires about 2 hr for a 50% decrease in viability, whereas exposure to 10^{-7} M does not reduce viability even after 8 hr of exposure, instead requiring 48 hr for a similar effect [55]. These results could be interpreted as evidence of concentration-dependent lysosomal toxicity. Exposure to relatively high (e.g. 10^{-6} M) CQ concentrations could result in destabilization of lysosomes, release of hydrolases, and the consequent irreversible and progressive destruction of parasite enzymes, membranes, and macromolecules. The effect of 10^{-6} M CQ is highly stage-dependent; young ring stages, which are relatively inert metabolically, and mature schizonts, which have completed differentiation, are relatively less sensitive to the drug [55]. These observations suggest that such stages could express little lysosomal activity. Interestingly, it was found that young ring stages (12–16 hr after reinvasion) could be arrested morphologically at this stage by CQ and fail to mature or degenerate even after 48 hr of drug exposure [55]. Maturation undoubtedly requires extensive intracellular processing of lipids and proteins, mediated at least in part by lysosomes [145]. The delayed effects of lower CQ concentrations (10^{-7} M) could be due to inhibition of the function or the sorting and processing of food vacuolar and lysosomal enzymes, thus interfering with nutrition and cellular differentiation. One could speculate that, when lysosomal function is inhibited by CQ, delivery of hydrolases through fusion with the food vacuole in primate malaria species [53, 97] or with endocytic vesicles in murine malaria species [87] is preferred over autophagy which mediates intracellular protein and phospholipid turnover [147, 148]. Under these conditions, the parasite could be killed by the inability to differentiate and mature. One could also speculate that the effects of high CQ concentrations cause the rapid disappearance of parasites from the blood during treatment [5], whereas chemoprophylaxis, which involves lower CQ concentrations, could be effective by the second mechanism.

A proposed hypothesis of CQ action: PL interactions

Undoubtedly, CQ action requires its accumulation in malaria-infected erythrocytes to extremely high levels. Such accumulation into acidic subcellular organelles, e.g. food vacuoles and lysosomes, is driven by a proton gradient. The latter is established by simultaneous inwards energy-driven proton pumping and the backflux of H^+ along a concentration gradient. Hydrophobic, unprotonated weak bases diffuse rapidly across cellular membranes along their concentration gradient and become trapped in acidic compartments upon protonation, since membranes are much less permeable to the protonated species. Such accumulation demonstrably leads to a rapid alkalinization of these compartments [48, 89, 95]. However, further proton pumping could lead to reacification since the reduced H^+ gradient results in a decrease in H^+ backflux and hence a larger net H^+-uptake. Drug uptake and concomitantly that of charge neutralizing anions result in osmotic swelling and the appearance of translucent vacuoles. At low drug concentrations pump activity could be sufficient for reacidification to the original pH, but at higher concentrations (e.g. $\geq 1\ \mu$M) it fails to do so due to efflux of protonated drug along its huge concentration gradient, and probably also due to drug-induced membrane permeabilization [48]. Such backflux obviously shuttles protons across the membrane of the acidic compartment, thereby leading to further alkalinization [149]. Some weak bases, such as tributylamine, raise organelle pH but cause no vacuolization; in fact, such compounds inhibit the uptake of other bases and subsequent vacuolization [113]. These observations can be explained by the relatively high permeability of the protonated form of tributylamine. It could also account for the fact that the IC_{50} of methylamine for

malaria parasites is 4×10^{-4}M, whereas that of NH_4Cl was 10-fold higher (T. Geary, unpublished observations). A similar mechanism could account for the inhibition of CQ-induced autophagy in malaria parasites by other quinoline-containing antimalarials as well as by β-adrenergic agonists and antagonists [82] which are also weak bases [103]. Similarly, it could explain the inhibition of phagosome–lysosome fusion in macrophages by ammonia and primary amines [150]. If a lysosomotropic drug is added prior to CQ exposure, as was the case in many pigment clumping experiments, the food vacuole pH would have been increased, thus reducing the driving force for CQ accumulation and preventing it from reaching a sufficient concentration for clumping. It should be expected that different bases have different efficiencies in eliciting autophagy and phagosome–lysosome fusion. Many of the experiments examining the interaction of CQ and other drugs at the proposed high-affinity site could be similarly re-evaluated; this holds true also for the experiments indicating a relationship between drug uptake and autophagy and energy metabolism.

It is very possible that drug accumulation could depend on membrane lipid composition inasmuch as both H^+ and protonated drug leaks as well as pump activity are expected to be affected. Although it is premature at the present time to speculate how these independent processes are affected by membrane lipids, the observation that the vacuolar pH of drug-sensitive *P. falciparum* strains is more acidic and is more rapidly restored upon alkalinization [48] strongly suggests a major role for PL composition in drug resistance. Once inside acidic compartments, the high concentrations of CQ could inhibit cellular processes such as host cytosol digestion, PL metabolism, and lysosomal function and enzyme targeting and processing, as discussed above. Particularly important in this regard may be phospholipase inhibition, resulting in the cessation of digestion of host cell cytoplasm [49, 54]. Possible explanations for CQ resistance and for the lack of cross-resistance to similar drugs are also evident. Drug potency in this model is a function of both the ability of the drug to be concentrated in acidic organelles and the potency of drug interaction with membranes and phospholipases. Mefloquine and quinine, for instance, are somewhat more and less potent, respectively, than CQ *in vitro* against *P. falciparum* ([70], T. Geary, unpublished observations), but should reach significantly lower intracellular concentrations than CQ (see above). Thus, one would predict that these drugs would be somewhat more potent at interacting with membranes and hydrolases than CQ. Mefloquine is indeed a more effective inhibitor of a parasite acid protease than CQ [99]. Quinacrine, which is also a dibasic amine, was somewhat more potent than chloroquine *in vitro* (T. G. Geary, unpublished observations). It is a more potent inhibitor of acidic phospholipases than CQ [121]. It is also possible that the alkalinization due to CQ may be relatively less important than that achieved by mefloquine and quinine, which are able to intercalate into the lipid phase and hence be more effective in proton shuttling. However, recent data [15] suggest that mefloquine may be differentiated from both CQ and quinine in this regard. Resistance could be acquired by PL alterations which influence the membrane interactions with these drugs and/or which change the membrane permeability to protonated bases. The permeability of amphipathic charged molecules across membranes depends on intrinsic drug properties, including charge distribution, lipophilicity, and on the ability to interact with membrane components, e.g. PL. If one assumes that drug translocation occurs through the lipid phase of the membrane, then alterations in the proportion of acidic PL will affect the charge-dependent component of permeability, while changes in the saturation of PL alkyl chains and/or cholesterol content will affect the hydrophobicity-dependent factor of partitioning as well as the diffusion coefficient of the specific drug. Since little is known about the accumulation fo quinoline-containing antimalarials in parasites or their interactions with membranes, in conjunction with PL composition, it is not yet possible to make definite predictions. Our preliminary results concerning the lipid composition of CQ-sensitive and -resistant strains of *P. falciparum* point favorably in this direction.

In summary, it is apparent that CQ accumulates in *P. falciparum* as a weak base and becomes highly concentrated in the acidic food vacuole. The ability of this compartment to accomplish this may be greater than that of host cell lysosomes, conceivably due to stronger buffering capacity, more active H^+ pumps, and perhaps to its much reduced surface : volume ratio. Once inside, sufficiently high CQ concentrations exist to block the digestion of host cell cytoplasm. This may be due to inhibition of parasite phospholipases needed to rupture the vesicles containing erythrocyte stroma. This may deprive the parasite of needed amino acids derived from Hb, and/or ribose, derived from host cell ATP, which is apparently utilized in parasite nucleic acid synthesis (T. Geary, unpublished observations). Variations in potency among the quinoline-containing antimalarials and variable cross-resistance among them may be determined by the exact composition of PL in the relevant membranes, which control the ability of these drugs to inhibit phospholipases. In addition, certain antimalarial effects of CQ may be mediated by disruption of normal lysosomal function. This inhibition may be far more critical to the parasite, with its extremely rapid developmental plan, then to the host cell.

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