A Common Mechanism for Blockade of Heme Polymerization by Antimalarial Quinolines*

(Received for publication, August 10, 1998, and in revised form, September 10, 1998)

David J. Sullivan, Jr.‡‡, Hugues Matile‡, Robert G. Ridley¶ and Daniel E. Goldberg**

From the ‡Department of Molecular Microbiology and Immunology, Johns Hopkins School of Hygiene and Public Health, Baltimore, Maryland 21205, §Hoffmann-La Roche, Pharmaceuticals Division, Pharma Research Preclinical, Basel, Switzerland, CH-4070 and ¶Howard Hughes Medical Institute, Departments of Medicine and Molecular Microbiology, Washington University School of Medicine, St. Louis, Missouri 63110

The antimalarial quinolines are believed to work by blocking the polymerization of toxic heme released during hemoglobin proteolysis in intraerythrocytic Plasmodium falciparum. In the presence of free heme, chloroquine and quinidine associate with the heme polymer. We have proposed that this association of the quinoline-heme complex with polymer caps the growing heme polymer, preventing further sequestration of additional heme that then accumulates to levels that kill the parasite. In this work results of binding assays demonstrate that the association of quinoline-heme complex with heme polymer is specific, saturable, and of high affinity and that diverse quinoline analogs can compete for binding. The relative quinoline binding affinity for heme polymer rather than free heme correlates with disruption of heme polymerization. Mefloquine, another important antimalarial quinoline, associated with polymer in a similar fashion, both in cultured parasites and in the test tube. In parasite culture, blocking heme release with protease inhibitor was antagonistic to mefloquine action, as it is to chloroquine action. These data suggest a common mechanism for quinoline antimalarial action dependent on drug interaction with both heme and heme polymer.

Plasmodium falciparum, the etiologic parasite of severe malaria, ingests up to 75% of its host erythrocyte hemoglobin into an acidic food vacuole. There, hemoglobin degradation occurs, providing amino acids for parasite maturation. Proteolysis of hemoglobin releases toxic heme, which accumulates in the food vacuole to concentrations estimated at 400 μM (1). Lacking heme oxygenase activity to catabolize the porphyrin moiety, parasites polymerize the highly reactive free heme into a metabolically inert crystalline material called hemozoin. This substance is a polymer of undefined length, in which the iron of one heme is coordinated to the side chain propionate carboxylate of the next heme (2).

Heme polymerization can be achieved in the test tube using parasite extracts (3, 4), preformed hemozoin nucleation (5), chemical synthesis (β-hematin) (6), synthetic lipids (7), or P. falciparum histidine-rich proteins (PfHRPs)1 found in the food vacuole for initiation of polymerization (8). The widely used antimalarial quinolines such as chloroquine and quinidine inhibit the polymerization of heme generated by each of these methods. Other characteristics of quinoline action that bear on a role in interrupting heme polymerization are stage specificity for killing of parasites actively degrading hemoglobin and releasing heme, hyperconcentration to millimolar levels in the food vacuole from nanomolar levels in plasma, and food vacuolar swelling as an early morphologic effect of quinoline treatment (9). The ability of quinolines to inhibit in vitro heme polymerization correlated well with P. falciparum culture efficiency in one study (10), although a separate study failed to find a strong correlation, and it was suggested that variable accumulation of different compounds may have accounted for the lack of correlation (11).

A previous theory postulated that the chloroquine-induced disruption of heme polymerization results from sequestration of polymerization substrate by binding of drug to free heme, which exists in equilibrium between the monomer and μ-oxo dimer (12). Avidity of binding to free heme, probably in the μ-oxo dimer, by a number of quinolines shows correlation with inhibition of in vitro heme polymerization (10). There are some exceptions. Chloroquine binds free heme with 20–100-fold greater affinity than quinidine yet is equipotent at blocking heme polymerization (9, 13). Artemisinin also binds free heme but does not inhibit heme polymerization (14, 15). Another postulate is that chloroquine may interact directly with a polymerization protein (4). However, the characterized polymerization proteins, the PfHRPs, have no significant affinity for quinolines, and the quinolines inhibit the hemozoin elongation reaction in the absence of protein (5, 6, 16).

Our previous studies found that [3H]chloroquine and [3H]quinidine incubated with cultured intraerythrocytic parasites becomes associated with hemozoin by electron microscopic autoradiography and by subcellular fractionation (16). In vitro the quinoline incorporation into heme polymers depended on the addition of free heme. The rate of quinoline incorporation paralleled heme extension of the polymer. These data suggested that a quinoline-heme complex incorporates at the extension site of the polymer to cap further chain extension, which stops the sequestration of toxic heme. The current study probes further the binding of the quinoline-heme complex to polymer. The data implicate the affinity of the quinoline-heme complex for polymer as an important determinant of the ability of diverse quinolines to block heme polymerization.

1 The abbreviation used is: Pf HRP, Plasmodium falciparum histidine-rich protein.
EXPERIMENTAL PROCEDURES

Parasite Culture—P. falciparum clones HB3 (chloroquine-sensitive) and Dd2 (chloroquine-resistant) were grown at 2% hematocrit and 10% parasitemia by the method of Trager and Jensen (17) with the substitution of AlbuMax II (Life Technologies, Inc.) for serum (5, 18). Synchrony was maintained by n-sorbitol treatment (19). Early trophozoite cultures in 15-ml plates were incubated for 20–24 h with 0.25 of [3H]mefloquine with a specific activity of 14 Ci/mmol (a gift from Hoffmann-La Roche).

Materials—Parasite-derived hemoglobin was prepared by washing lysates of trophozoites 2–3 times in 2% SDS, followed by proteinase K digestion overnight, three additional washes in 2% SDS, incubation in 6 M urea for 3 h, washing three more times in 2% SDS, and finally five washes with distilled water. After each wash, the material was centrifuged at 13,000 g for 15 min, and the resulting pellet was resuspended by brief sonication in the next wash solution. β-Hematin was prepared by the method of Egan et al. (6). The product was washed to remove unreacted free heme with 2% SDS and 100 mM sodium bicarbonate, pH 9.1, until clear, washed with 2% SDS (three times), and finally washed with distilled water (five times). P. f. HRP-initiated hemoglobin was purified as described previously (16). [3H]chloroquine (Du-Pont NEN) had a specific activity of 27 Ci/mmol (1 Ci = 37 Gbq). [3H]Quinidine (American Radiolabeled Chemicals, St. Louis, MO) had a specific activity of 15 Ci/mmol. Unlabeled mefloquine was a gift of The Walter Reed Army Institute of Research (Washington, DC). All other chemicals were purchased from Sigma.

Heme Binding Assay—202.5 nCi of [3H]chloroquine or 225 nCi of [3H]quinidine were incubated overnight at 37 °C with 5 μM hemin in 1.5 ml of 100 mM sodium acetate, pH 5.0. 2 μM parasite-derived hemoglobin (total heme content) was added for an additional 24-h incubation. The suspension was pelleted (13,000 g for 10 min) and resuspended in 50 mM Tris-HCl, pH 8.0, by gentle sonication. The sonicate was layered on a 1.7 M sucrose cushion in 50 mM Tris and ultracentrifuged as described previously (14). Polymer extension was minimal using 5 μM heme as opposed to the 50 μM heme used previously. In other experiments, varied concentrations of unlabeled drug were incubated with 1H-labeled drug and hemin for the first 24 h of incubation. The incorporation into heme polymer of 5 or 10 μM [3H]chloroquine or 1Hquinidine, respectively, was assessed with or without addition of unlabeled drug.

Depolymerization Assay—Synchronized trophozoite lysates of parasite clones HB3 and Dd2 were sonicated in 50 mM Tris-HCl, pH 8.0, and total heme content was quantitated. 5 nmol (heme content) of parasite lysate, parasite-derived hemoglobin, chemically synthesized heme polymer, or P. f. HRP II-initiated heme polymer were incubated in 1 ml of 500 mM sodium acetate buffer, pH 5.0, with or without addition of 5 mM chloroquine or quinidine at 37 °C for 72 h. Incubations were pelleted and washed on pH 9.1, 100 mM sodium bicarbonate, pH 9.1, and 2% SDS. An additional wash with 2% SDS was done before quantification.

Mefloquine Binding Assays—Assessment of mefloquine binding in cultured intraerythrocytic parasites and to hemoglobin in vitro was performed as described previously (16).

Quinoline/Protease Inhibitor Culture Experiments—Drug combinations were assessed in culture as described previously (20).

RESULTS

Binding of Heme-Quinoline Complex to Heme Polymer—Heme polymer was incubated with different concentrations of chloroquine or quinidine in the presence of hemin. Specific, saturable binding that could be competed by excess cold quinoline was observed. Fig. 1A shows a Scatchard analysis, which allowed estimation of apparent K_d values in the 250–400 nM range. Both chloroquine and quinidine have similar high affinity binding in the submicromolar range of drug concentrations, although quinidine displays a more complex low affinity binding component at ~1 μM concentration. Because the target for binding, heme polymer, is insoluble, these estimates must be considered apparent and not true K_d values. In Fig. 1B the slope remained constant when the heme or heme polymer (data not shown) concentration was varied. Binding curves

Fig. 1. Chloroquine and quinidine binding to hemozoin. A, saturation binding assays using 5 μM hemin, [3H]chloroquine (filled squares), [3H]quinidine (open circles), and 2 μM hemozoin (heme content) were performed in triplicate. B, binding assay using [3H]quinidine in which hemin was increased to 10 μM (filled triangles) and also decreased to 2.5 μM (open triangles). C, [3H]Chloroquine binding assay using synchronized trophozoite parasites harvested by saponin lysis followed by sonication. Crude lysates were equalized to 2 μM hemozoin (heme content) and incubated with 5 μM hemin and [3H]chloroquine. B/F, bound/free; CQ sens., chloroquine-sensitive clone; CQ resist., chloroquine-resistant clone.
using cell lysates from HB3, a chloroquine-sensitive clone, or Dd2, a chloroquine-resistant parasite clone, had similar slopes (Fig. 1C).

The ability of disparate unlabeled drugs to compete with \(^{3}H\)chloroquine or in the drug-heme complex polymer binding assay was assessed (Fig. 2). Drugs that do not inhibit heme polymerization, such as ampicillin, verapamil, artemesinin, primaquine, and 8-OH-quinoline, did not compete for binding at a concentration of 5 \(\mu M\), which is >10 times the concentration of chloroquine needed for half-maximal binding. Drugs that are potent inhibitors of heme polymerization, such as amodiaquine, mefloquine, quinidine, quinacrine, and chloroquine, competed well, although quinine competed to a lesser extent.

Heme Polymer Derived from Different Sources Is Not Depolymerized—A previous report had shown that heme polymer derived from the mouse parasite Plasmodium yoelii depolymerized when incubated in millimolar concentrations of quinolines (21). Using similar assay conditions but a longer period of 72 h, no depolymerization was seen for heme polymer in crude P. falciparum parasite lysates from both chloroquine-sensitive clone HB3 and chloroquine-resistant clone Dd2, for hemozoin extensively purified from parasites, for chemically synthesized heme polymer, or for polymer initiated by recombinant PfHRP II (data not shown). In each case, 20–30% of polymer was lost with continued washes, but the amount lost was within 2% for 5 \(\mu M\) chloroquine or 5% for 5 \(\mu M\) quinidine of that for heme polymer with no added drug.

Mefloquine Associates with Hemozoin in Cultured Parasites and Incorporates into Heme Polymer in Vitro—Mefloquine is a clinically used antimalarial quinoline that displays activity disparate from chloroquine in different P. falciparum strains. In the competition assay (Fig. 2), mefloquine was able to block \(^{3}H\)quinoline incorporation. To examine its activity further, \(^{3}H\)mefloquine was incubated with intraerythrocytic P. falciparum in culture for 20 h. Parasites were lysed, and mefloquine was isolated using a sucrose cushion. Half of parasite-associated mefloquine co-purified with hemozoin (Fig. 3A). Nearly all of the radiolabel that was sedimentable in controls without a sucrose cushion sedimented through sucrose with hemozoin. These results are similar to those reported for chloroquine and quinidine (16). Controls in which \(^{3}H\)mefloquine was added to unlabeled parasites at lysis showed no copurification with heme polymer on the sucrose cushions. In the in vitro quinoline incorporation assay (Fig. 3B), labeled mefloquine required the presence of free heme for incorporation into heme polymer, as is also the case with chloroquine and quinidine.

The Protease Inhibitor RO-4388 Antagonizes Mefloquine Action on Cultured Parasites—Plasmepsin I is an aspartic protease implicated in the initial steps of hemoglobin degradation.

---

**FIG. 2.** Competition for binding with antimalarial quinolines. 5 \(\mu M\) \(^{3}H\)chloroquine was incubated with 5 \(\mu M\) hemin in the presence of various compounds added at 5 \(\mu M\). Error bars indicate S.E. for quadruplicate counts.

**FIG. 3.** Binding of \(^{3}H\)mefloquine to intraerythrocytic and isolated hemozoin. A, sonicated HB3 trophozoite lysates were processed on sucrose cushions to yield hemozoin pellets (16). Duplicate cultures were incubated with 0.25 \(\mu Ci\) of \(^{3}H\)mefloquine processed with a sucrose cushion (filled bars) and with a sucrose cushion (open bars) and with 0.125 \(\mu Ci\) of \(^{3}H\)mefloquine and a sucrose cushion (horizontal striped bars), and control cultures were incubated without \(^{3}H\)mefloquine but with radiolabeled drug added at parasite lysis (hatched bars). B, overnight incubations of \(^{3}H\)mefloquine with 10 nmol of hemozoin (heme content) and 50 \(\mu M\) hemin (MFQ+HZ+HEME), \(^{3}H\)mefloquine with hemozoin (MFQ+HZ), \(^{3}H\)mefloquine with heme (MFQ+HEME), or \(^{3}H\)mefloquine alone (MFQ) in 100 \(\mu M\) sodium acetate, pH 5.0, were processed through sucrose cushions. Triplicate determinations of radioactivity associated with pellets are shown.
and heme release that occurs in the parasite food vacuole. Inhibitors of this enzyme have been shown to be antagonistic to chloroquine action in cultured parasites, presumably because less heme is released, preventing chloroquine from effecting accumulation of toxic monomer (20). Isobologram analysis of the combination of mefloquine with RO-4388 (Fig. 4), suggests that these agents are antagonistic in culture, much as chloroquine and this protease inhibitor are antagonistic.

**DISCUSSION**

The quinoline antimalariais have been used as specific therapy for hundreds of years, yet debate has continued on the precise mechanism of action. Inhibition of the unique process of heme polymerization in the acidic food vacuole of *P. falciparum* has been postulated to result in toxic accumulation of the highly reactive heme moiety, which kills the parasite, perhaps through oxidative damage of membranes or other cellular targets (12). Our previous work showed that chloroquine accumulates bound to hemozoin in intraerythrocytic parasites and that this binding is dependent on the presence of free heme. These observations led to the proposal of the capping mechanism of the inhibition of heme polymerization (16). It was suggested that chloroquine binds to heme as it is released during hemozoin degradation, forming a complex that can then add on to the growing polymer, terminating extension and blocking further heme incorporation. We have now explored further the interaction of quinoline-heme complexes with hemozoin. Binding to polymer is saturable and specific. Apparent affinity is similar for chloroquine and quinidine, which fits well with their comparable abilities to inhibit heme polymerization. This binding of the heme-drug complex to hemozoin can be competed by other quinoline antimalariais, although not by δ-hydroxyquinoline, which binds heme less well and does not inhibit polymerization. Artemisinin and, to some extent, primaquine bind heme but do not compete in the polymer binding assays, consistent with its lack of activity in heme polymerization inhibition assays. Unrelated compounds (ampicillin and verapamil) do not compete. These data suggest that both the ability to form a heme-drug complex and the ability of that complex to bind to heme polymer are important determinants of heme polymerization blockade. The results may explain why quinidine, which is 20–100-fold less potent than chloroquine at binding heme, is as potent at inhibiting polymer formation.

Hemozoin from chloroquine-sensitive or -resistant strains had similar affinities for the chloroquine-heme complex. This adds to the body of evidence suggesting that chloroquine resistance is a result of failure of drug to reach the target site, rather than alteration of the target itself.

A previous study had shown that *P. yoelii* hemozoin could be depolymerized by quinolines. Synthetic β-hematin was not depolymerized in these assays (21). In our experiments, under similar conditions, no significant depolymerization of heme polymer from *P. falciparum* or from other sources was observed.

Mefloquine is a lipophilic quinoline alcohol in wide clinical use. Like chloroquine and quinidine, it also binds to heme polymer in a heme-dependent fashion and diminishes binding of the other labeled quinolines in a competition assay. In culture, it associates with hemozoin. The activities of mefloquine and a protease inhibitor that blocks heme release in the food vacuole are antagonistic. In all of these behaviors, then, mefloquine is like the other quinolines studied. Despite the distinct lack of correlation of parasite resistance patterns to the different antimalarial quinolines, the action of these drugs on binding to and inhibiting extension of heme polymer is strikingly similar. We would suggest that it is likely that these diverse agents have a similar mechanism of action. Understanding the basis of antimalarial quinoline affinity for heme polymer and its relation to drug action will promote development of new drugs that can elude resistance mechanisms by stronger interactions with the heme polymer as well as by overcoming transporter-mediated resistance mechanisms.

**REFERENCES**

1. Francis, S. E., Sullivan, D. J., Jr., and Goldberg, D. E. (1997) *Annu. Rev. Microbiol.* 51, 97–123
2. Slater, A. F., Swiggard, W. J., Orton, B. R., Flitter, W. D., Goldberg, D. E., Cerami, A., and Henderson, G. B. (1991) *Proc. Natl. Acad. Sci. U. S. A.* 88, 325–329
3. Dorn, A., Vippagunta, S. R., Matile, H., Bubendorf, A., Vennerstrom, J. L., and Ridley, R. G. (1998) *Biochem. Pharmacol.* 55, 737–747
4. Slater, A. F. G., and Cerami, A. (1992) *Nature* 355, 167–169
5. Dorn, A., Stoffel, R., Matile, H., Bubendorf, A., and Ridley, R. (1995) *Nature* 374, 269–271
6. Egan, T., Ross, D., and Adams, P. (1994) *FEBS Lett.* 352, 54–57
7. Bendrat, K., Berger, B. J., and Cerami, A. (1995) *Nature* 378, 138
8. Sullivan, D. J., Jr., Gluzman, I. Y., and Goldberg, D. E. (1996) *Science* 271, 219–222
9. Slater, A. F. G. (1993) *Pharmacol. & Ther.* 57, 203–235
10. Dorn, A., Vippagunta, S. R., Matile, H., Jaquet, C., Vennerstrom, J. L., and Ridley, R. G. (1998) *Biochem. Pharmacol.* 55, 727–736
11. Hawley, S. R., Bray, P. G., Munthin, M., Atkinson, J. D., O'Neill, P. M., and Ward, S. A. (1995) *Antimicrob. Agents Chemother.* 42, 682–686
12. Ridley, R. G., Dorn, A., Vippagunta, S. R., and Vennerstrom, J. L. (1997) *Ann. Trop. Med. Parasitol.* 91, 559–566
13. Chou, A. C., Chevi, R., and Fitch, C. D. (1980) *Biochemistry* 19, 1543–1549
14. Hong, Y.-L., Yang, Y.-Z., and Meshnick, S. R. (1994) *Mol. Biochem. Parasitol.* 63, 121–128
15. Asawamahasakda, W., Ittarat, I., Chang, C.-C., McElroy, P., and Meshnick, S. R. (1994) *Mol. Biochem. Parasitol.* **67**, 183–191
16. Sullivan, D. J., Jr., Gluzman, I. Y., Russell, D. G., and Goldberg, D. E. (1996) *Proc. Natl. Acad. Sci. U. S. A.* **93**, 11865–11870
17. Trager, W., and Jensen, J. B. (1976) *Science* **193**, 673–675
18. Flores, M. V., Berger-Eiszele, S. M., and Stewart, T. S. (1997) *Parasitol. Res.* **83**, 734–736
19. Lambros, C., and Vanderberg, J. P. (1979) *J. Parasitol.* **65**, 418–420
20. Moon, R. P., Tyas, L., Cena, U., Rupp, K., Bur, D., Jaquel, C., Matile, H., Lotachar, H., Gruninger-Leitch, F., Kay, J., Dunn, B. M., Berry, C., and Ridley, R. G. (1997) *Eur. J. Biochem.* **244**, 552–560
21. Pandey, A. V., and Tekwani, B. L. (1997) *FEBS Lett.* **402**, 236–240