Vol. 195, No. 1, 1993
August 31, 1993

CONTROL OF HEME POLYMERASE BY CHLOROQUINE AND OTHER QUINOLINE DERIVATIVES

Albert C. Chou[1] and Coy D. Fitch

Department of Internal Medicine, Saint Louis University
School of Medicine, Saint Louis, Missouri 63104

Received July 13, 1993

Summary: To evaluate the response of heme polymerase to treatment of malaria with chloroquine, we used mice infected with *Plasmodium berghei*. Six hours after treatment with 3 μmoles of chloroquine intraperitoneally per mouse, heme polymerase activity in parasitized erythrocytes decreased from 238 to 37 nanomoles of ferriprotoporphyrin IX polymerized per hour per μmole of ferriprotoporphyrin IX in preformed hemozoin, and nonhemozoin ferriprotoporphyrin IX increased *in vivo* from 40 to 123 nanomoles per ml of packed, parasitized erythrocytes. Other 4-aminoquinoline derivatives were similar in effect to chloroquine. Treatment with quinine, mefloquine, primaquine, or naphthalene derivatives caused no reduction in heme polymerase activity. In contrast to 4-aminoquinoline derivatives, quinine and mefloquine, which are quinolinemethanol derivatives, antagonized the effect of chloroquine.

Because malaria parasites are adapted to feed on hemoglobin, they produce and must detoxify ferriprotoporphyrin IX. Detoxification involves a newly discovered enzyme, heme polymerase (1, 2), which catalyzes the formation of β-hematin polymers. β-Hematin is comprised of FP molecules linked together through ferric iron in the porphyrin ring and carboxylate ions of propionate sidechains (3). It is nontoxic because it is insoluble in water at physiologic or acidic pH values (4), and it is stored in acidic vacuoles (5) where it accounts for the birefringent, dark brown color of malaria pigment (hemozoin) (4).

Chloroquine treatment of malarious mice reduces the activity of heme polymerase and causes the accumulation of nonhemozoin FP during incubation of hemolysates (1). Since this response to chloroquine may be the key to understanding the antimalarial action of quinoline drugs, we have evaluated the accumulation of nonhemozoin FP *in vivo* in response to treatment with chloroquine and other quinoline derivatives. These studies are the first to evaluate the specificity of the control of heme polymerase by quinoline derivatives.

[1]To whom correspondence should be addressed. Fax: (314)268-5108.

Abbreviation: FP, ferriprotoporphyrin IX.

0006-291X/93 $4.00

Methods

Male Swiss (CF-1) mice weighing approximately 25 g were purchased from Sasco, Inc. (Omaha, Nebraska) and infected by intraperitoneal passage of the chloroquine-susceptible NYU-2 strain of *Plasmodium berghei*. Care for the mice was provided in accord with Saint Louis University guidelines. The characteristics of this line of parasites have been described (6). On the sixth day of infection, when parasitemias were high, the mice were treated with the various drugs. To obtain parasitized erythrocytes, the mice were anesthetized with ether, an axillary incision was made, and blood was collected in ice-cold standard medium (68 mM sodium chloride, 4.8 mM potassium chloride, 1.2 mM magnesium sulfate, and 50 mM sodium phosphate, pH 7.4) containing 5 mM glucose and heparin. The blood from several mice was pooled for each experiment, after which the erythrocytes were collected by centrifugation and washed thrice by resuspension in ice-cold standard medium with 5 mM glucose followed by centrifugation to remove plasma and buffy coat.

To prepare a lysate, parasitized erythrocytes were suspended to a hematocrit of 25% in ice-cold standard medium with 5 mM glucose and frozen immediately by dropping the suspension one drop at a time into liquid nitrogen. These frozen droplets were stored at -132° C for a few days (usually 3 or less) before thawing by raising the temperature to 4° C to produce the lysate. Storage at -132° C had no effect on the content of hemozoin or nonhemozoin FP or on heme polymerase activity.

Nonhemozoin FP and preformed hemozoin FP (hemozoin FP content of the lysate prior to incubation) were measured in an aliquot of 0.2 ml of ice-cold lysate, which was added to 1.8 ml of ice-cold 0.1 M sodium acetate (pH 5.0) and immediately centrifuged at 27,000 xg for 15 minutes at 4° C. The resulting pellet was washed by resuspending it in 2.5 ml of ice-cold 0.1 M sodium acetate (pH 5.0), with the aid of sonication, and centrifugation at 27,000 xg and 4° C to recover the insoluble material. This pellet in turn was similarly washed with 2.5 ml of ice-cold standard medium. Finally the pellet was suspended in 2.5% SDS at room temperature, and preformed hemozoin FP was separated from nonhemozoin FP by centrifugation to permit measurement of the FP content of each fraction (1).

For routine measurement of heme polymerase activity, 0.2 ml of the lysate was added to 1.8 ml of an incubation mixture to achieve a final concentration of 300 μM FP and 75 μM sodium acetate (pH 5.0). For the experiments presented in Fig. 3, the incubations also included protease inhibitors, which are identified in the legend, to inhibit the release of FP from hemoglobin. The incubations were conducted at 37° C in capped tubes which were attached to a slowly rotating wheel for 4 hours. Previous studies showed that the reaction is linear for more than 6 hours under these conditions (1). After incubation, the pellet was obtained by centrifugation, washed as described above, and suspended in 2.5% SDS to permit isolation and measurement of postincubation hemozoin FP (1). The difference between postincubation hemozoin FP and preformed hemozoin FP is the amount of FP polymerized to β-hematin during the incubation. In these experiments, the amount of preformed hemozoin FP correlated well with parasitemia (correlation coefficient >0.8).

Parasitemia was determined by microscopic examination of Giemsa-stained blood films. The infections were asynchronous, the parasitemias were approximately 1000 parasites per 1000 erythrocytes on the average, and numerous erythrocytes were infected with several parasites. Consequently, the total number of parasite nuclei were counted for calculation of parasitemia.

Treatment consisted of intraperitoneal injection of one of the drugs shown in Fig. 1 and Table 1 dissolved in 0.1 ml of 0.9 percent aqueous sodium chloride or absolute alcohol 6 hours before the mice were anesthetized to obtain blood. After demonstrating that injections of the sodium chloride solution or alcohol had no effect on heme polymerase activity or on the amounts of hemozoin or nonhemozoin FP, control mice received no injections. The salt forms of the drugs were quinine hydrochloride, primaquine diphosphate, mefloquine hydrochloride, WR 88685 (base), WR 54470 hydrochloride, chloroquine diphosphate, quinacrine dihydrochloride, amodiaquine dihydrochloride, amopyroquine dihydrochloride, SN 10274 dihydrochloride, SN 11438 diphosphate, and WR 29623 (base). Drugs with the SN prefix are

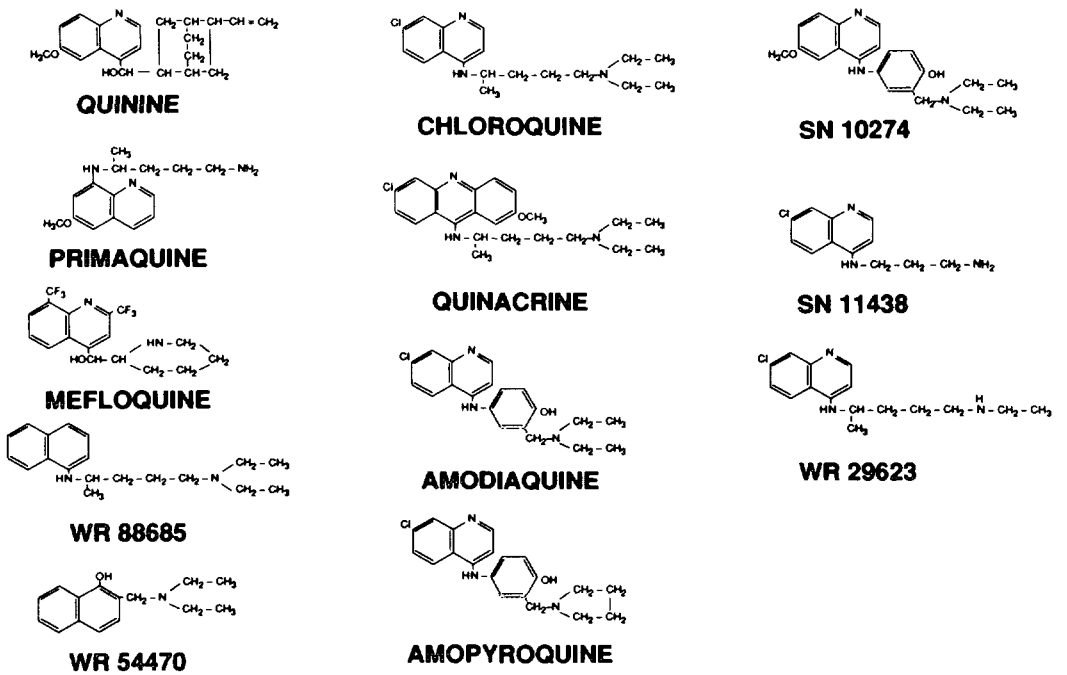

Fig. 1. Drugs and their structural formulas.

described in the survey by Wiseogle (7) and were provided by L.H. Schmidt. Drugs with the WR prefix and mefloquine were obtained from the Walter Reed Army Institute of Research, Washington, D.C.; amodiaquine and amopyroquine were obtained from Parke-Davis, a Division of Warner-Lambert Company, Morris Plains, New Jersey; and the remaining drugs were purchased from Sigma Chemical Company, Saint Louis, Missouri. Alcohol was used as the vehicle for administration of mefloquine, WR 88685, and WR 29623.

Results

The structural formulas of the drugs tested are shown in Fig. 1. Tables 1 and 2 present the principal results. Treatment of malarious mice with chloroquine caused an 85 percent reduction in heme polymerase activity, in agreement with our earlier report (1). Similar reductions were caused by quinacrine and each of the 4-aminoquinoline derivatives but not by primaquine, quinine, mefloquine, or the naphthalene derivatives. In fact, the results presented in Table 2 revealed that treatment with quinine or mefloquine antagonizes the control of heme polymerase by chloroquine. This difference between 4-aminoquinoline and quinolinemethanol derivatives *in vivo* is remarkable since both have been shown to inhibit heme polymerase activity *in vitro* (2), a finding which we confirmed for *P. berghei* in the present work (Fig. 2).

Four compounds were tested for ability to inhibit heme polymerase *in vitro* (Fig. 2), and each caused a decrease in β-hematin synthesis. Chloroquine and amodiaquine, were the most potent, but their 50 percent inhibitory concentrations still were high, approximately 40 μM. The

Table 1. SPECIFICITY OF THE CONTROL OF HEME POLYMERASE BY ANTIMALARIAL DRUGS

Drug	Dose[a]	FP[b]	Hemozoin[c]	Heme Polymerase[d]
None	None	40 ± 16 (16)[e]	1,055 ± 247 (16)	238 ± 40.5 (9)
Quinine	6	69 ± 17 (5)	1,510 ± 416 (5)	273 ± 38.6 (5)
Mefloquine	3 - 5	70 ± 24 (4)	913 ± 149 (4)	286 ± 65.3 (4)
Primaquine	3	58 ± 28 (4)	1,088 ± 479 (4)	263 ± 34.6 (4)
WR 88685	4 - 6	57 ± 27 (3)	1,490 ± 127 (3)	207 ± 37.4 (3)
WR 54470	5 - 6	41 (2)	1,425 (2)	239 (2)
Chloroquine	3	123 ± 39 (11)	798 ± 111 (11)	36.6 ± 12.9 (9)
Quinacrine	3	129 ± 22 (3)	697 ± 107 (3)	33.2 ± 24.5 (3)
Amodiaquine	4	186 ± 29 (3)	930 ± 121 (3)	33.1 ± 5.8 (3)
Amopyroquine	5	236 ± 23 (3)	1,151 ± 134 (3)	61.7 ± 19.2 (3)
SN 10274	3 - 4	141 ± 39 (3)	1,018 ± 215 (3)	78.1 ± 22.6 (3)
SN 11438	4	150 ± 38 (3)	1,068 ± 51 (3)	47.0 ± 9.7 (3)
WR 29623	6	157 ± 58 (3)	1,125 ± 105 (3)	50.8 ± 19.6 (3)

[a] The dose is given in μmoles of drug injected per mouse.
[b] Nanomoles of nonhemozoin FP per ml of packed, parasitized erythrocytes, corrected for differences in parasitemia as follows: the amount of FP per ml of packed erythrocytes was divided by the number of parasites per 1000 erythrocytes and multiplied by 1000.
[c] Nanomoles of preformed hemozoin FP per ml of packed, parasitized erythrocytes corrected for parasitemia as described for nonhemozoin FP.
[d] Nanomoles of FP incorporated into hemozoin per hour per μmole of FP in preformed hemozoin.
[e] Means ± S.D. and number of experiments are shown.

Table 2. ANTAGONISM OF THE EFFECT OF CHLOROQUINE ON HEME POLYMERASE

Time (Hours)	Chloroquine[a]	Chloroquine Quinine[b]	Chloroquine Quinine (twice)[c]	Chloroquine Mefloquine[d]
		(Heme Polymerase Activity)[e]		
0	272 ± 20[f]	267 ± 17	254 ± 27	254 ± 11
2	106 ± 2	340 ± 30	324 ± 46	312 ± 35
4	71 ± 6	175 ± 14	376 ± 67	----
6	58 ± 6	129 ± 36	370 ± 41	348 ± 29

[a] 3 μmoles per mouse were injected intraperitoneally at 0 hour.
[b] 3 μmoles of chloroquine and 6 μmoles of quinine per mouse were injected simultaneously intraperitoneally at 0 hour.
[c] 3 μmoles of chloroquine and 6 μmoles of quinine per mouse were injected simultaneously intraperitoneally at 0 hour plus another 6 μmoles of quinine were injected intraperitoneally 2 hours later.
[d] 3 μmoles of chloroquine and 4.8 μmoles of mefloquine per mouse were injected simultaneously intraperitoneally at 0 hour.
[e] Nanomoles of FP incorporated into hemozoin per hour per μmole of FP in preformed hemozoin.
[f] Means ± S.D. for three experiments are shown for each treatment.

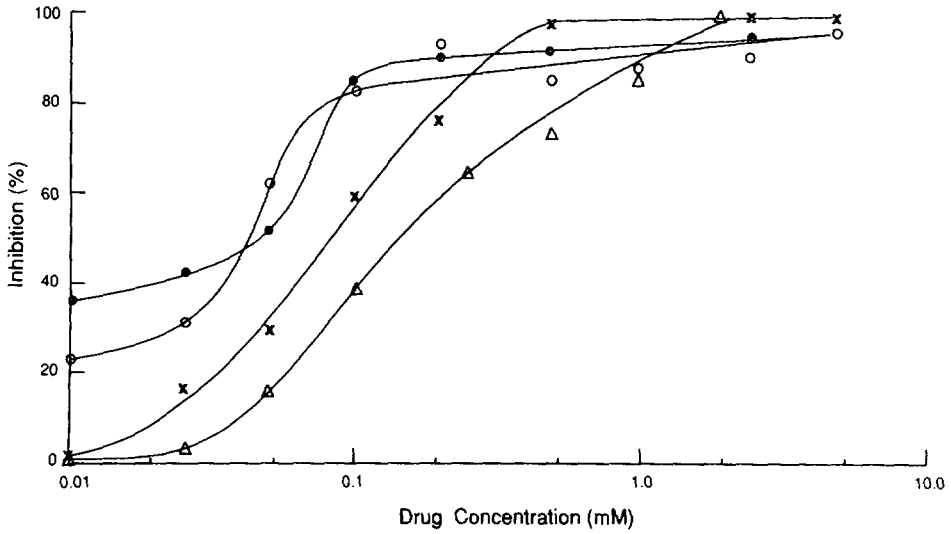

Fig. 2. Inhibition of heme polymerase by antimalarial drugs *in vitro*. The incubation mixture consisted of 0.2 ml of lysate prepared from a 25 percent suspension of washed, parasitized erythrocytes, 50 μM pepstatin A, 100 μM trans-epoxysuccinyl-L-leucylamido(4-guanidino)-butane, 150 μM FP, 75 μM sodium acetate (pH 5.0), and varying concentrations of chloroquine (•), amodiaquine (○), quinine (×), and mefloquine (△) in a total volume of 2 ml. The incubation was conducted at 37° C for 4 hours.

50 percent inhibitory concentrations were 80 μM for quinine and 150 μM for mefloquine. Since amodiaquine, quinine, and mefloquine all compete with chloroquine for binding to FP (8, 9) and since high concentrations of each of them are required to inhibit heme polymerase, it is possible that they simply compete with heme polymerase for FP *in vitro*, rather than interacting directly with the enzyme.

Returning to the data in Table 1, chloroquine caused a significant reduction ($P < 0.001$, Student's t-test) in preformed hemozoin FP and a significant increase ($P < 0.001$) in nonhemozoin FP. These values may be taken as approximations of the values *in vivo* since the temperature during preparation was not allowed to rise above 4° C, and protease and heme polymerase activities are minimal at 4° C (1). In previous experiments, we found that nonhemozoin FP produced *in vitro* includes toxic FP, i.e. FP available to bind chloroquine with high affinity (1). The other 4-aminoquinoline derivatives and quinacrine, which incorporates the 4-aminoquinoline structure, also caused nonhemozoin FP to accumulate.

Discussion

The β-hematin of hemozoin is derived from hemoglobin through a series of steps, which include ingestion of hemoglobin (10), digestion of the globin moiety with release of FP (11), and finally FP polymerization (1, 2). Consequently, reduction in the activity of heme polymerase, the enzyme responsible for the last step in the process, would be expected to cause a pile-up of precursors, such as FP, partially degraded hemoglobin, and ingested hemoglobin, all of which would be detected as nonhemozoin FP.

The precursor of most interest with regard to the antimalarial effect of chloroquine is FP itself. Both it and its high-affinity complex with chloroquine are cellular toxins, which lyse membranes (12, 13), inhibit parasite proteases (14), and probably have a variety of other toxic effects. Thus, the current demonstration that nonhemozoin FP accumulates *in vivo* as a consequence of the control of heme polymerase by chloroquine supports our hypothesis that FP toxicity underlies the antimalarial activity of chloroquine and related drugs (15).

The specificity of the control of heme polymerase by chloroquine is of particular interest because it distinguishes between 4-aminoquinoline derivatives and quinolinemethanol derivatives. The former reduce heme polymerase activity whereas the later do not. In fact, the quinolinemethanol derivatives antagonize the effect of 4-aminoquinoline derivatives *in vivo*. These results could not have been predicted from studies *in vitro* since both groups of quinoline derivatives inhibit heme polymerase in the test tube. Clearly, more is involved *in vivo* than reversible inhibition of the enzyme. Consistent with this conclusion, repeated washing to remove chloroquine from the insoluble fraction of hemolysates prepared from the blood of treated mice has no effect on the activity of heme polymerase (1). It appears, therefore, that chloroquine and other 4-aminoquinoline derivatives control heme polymerase activity *in vivo* by reducing the quantity of the active enzyme, either by inhibiting biosynthesis or by causing inactivation.

Acknowledgment

We thank Yi-Feng Chen for technical assistance.

References

1. Chou, A. C., and Fitch, C. D. (1992) Life Sci. 51, 2073-2078.
2. Slater, A. F. G., and Cerami, A. (1992) Nature (London) 355, 167-169.
3. Slater, A. F. G., Swiggard, W. J., Orton, B. R., Flitter, W. D., Goldberg, D. E., Cerami, A., and Henderson, G. B. (1991) Proc. Natl. Acad. Sci. (USA) 88, 325-329.
4. Fitch, C.D., and Kanjananggulpan, P. (1987) J. Biol. Chem. 262, 15552-15555.
5. Yayon A., Cabantchik Z. I., and Ginsburg H. (1984) EMBO J. 3, 2695-2700.
6. Fitch C. D. (1969) Proc. Natl. Acad. Sci. 64, 1181-1187.
7. Wiseogle, F. Y. (1946) A Survey of Antimalarial Drugs 1941-45, pp. 1863-1921, Vol. 2. J.W. Edwards, Inc., Ann Arbor.
8. Chou A. C., Chevli R., and Fitch C. D. (1980) Biochemistry 19, 1543-1549.
9. Chevli, R., and Fitch, C. D. (1982) Antimicrob. Agents Chemother. 21, 581-586.
10. Rudzinska, M. A., and Trager W. (1957) J. Protozool. 4, 190-199.
11. Goldberg, D. E., Slater, A. F. G., Cerami, A., and Henderson G. B. (1990) Proc. Natl. Acad Sci. USA 87, 2931-2935.
12. Chou, A. C., and Fitch, C. D. (1981) J. Clin. Invest. 68, 672-677.
13. Orjih, A. U., Banyal, H. S., Chevli, C., and Fitch, C. D. (1981) Science 214, 667-669.
14. Vander Jagt, D. L., Hunsaker, L. A., and Campos, N. M. (1986) Mol. Biochem. Parasitol. 18, 389-400.
15. Fitch, C. D. (1986) Parasitol. Today 2, 330-331.