

IN VITRO AND IN VIVO REVERSAL OF CHLOROQUINE RESISTANCE IN *PLASMODIUM FALCIPARUM* WITH PROMETHAZINE

A. M. J. ODUOLA, A. SOWUNMI, W. K. MILHOUS, T. G. BREWER, D. E. KYLE, L. GERENA, R. N. ROSSAN,
L. A. SALAKO, AND B. G. SCHUSTER

Department of Pharmacology and Therapeutics, Postgraduate Institute for Medical Research and Training, College of Medicine,
University of Ibadan, Ibadan, Nigeria; Division of Experimental Therapeutics, Walter Reed Army Institute of Research,
Washington, District of Columbia; Gorgas Memorial Laboratory, Panama City, Panama

Abstract. The effect of combining promethazine with chloroquine was examined against *Plasmodium falciparum* *in vitro* in the *Aotus-P. falciparum* model and in bioassays from volunteers given promethazine. The combination of chloroquine plus promethazine (1×10^{-6} M) reversed chloroquine resistance in standard *P. falciparum* clones and patient parasite isolates from Nigeria. The combination reduced the 50% inhibitory concentrations (IC₅₀s) for chloroquine against resistant parasites by 32–92%. Coadministration of promethazine with chloroquine also demonstrated a dose-dependent effect in *Aotus* monkeys infected with chloroquine-resistant *P. falciparum*. Monkeys were given a chloroquine dose (20 mg/kg of body weight for seven days), which normally has no effect on parasitemia, plus 10, 20, 40, or 80 mg of promethazine/kg of body weight. In one monkey, parasitemia was suppressed at the lowest promethazine dose, but re-treatment with 20 mg/kg resulted in clearance of parasitemia. Initial treatment with chloroquine and 20 or 40 mg/kg of promethazine cleared parasitemia in some animals followed by recrudescence. Re-treatment at higher doses cured one monkey and resulted in initial clearance and delayed recrudescence 28 or 63 days after treatment in two monkeys. Recrudescence parasitemia in the two monkeys was low (10 parasites/ μ l of blood) and subsequently cleared without re-treatment. An *in vitro* bioassay model was developed to examine the effects of clinically achievable doses of promethazine on parasites susceptibilities *in vitro*. Plasma samples taken at hourly intervals from patients given a single oral dose of 25 mg of promethazine decreased the IC₅₀ values for chloroquine by 20–58% with the most significant reductions occurring in plasma obtained from volunteers 3–4 hr after ingestion. Plasma obtained from two volunteers 6 hr after ingestion of the drug demonstrated no effect on chloroquine susceptibility, suggesting that study of the pharmacokinetic disposition and potential interaction is warranted to optimize the dose regimen in patients for antimalarial efficacy. Historic use of this drug combination for treatment or prevention of chloroquine-associated pruritus or as an antiemetic suggest that the combination is safe and effective when used at standard dosages. The results from this study demonstrate that promethazine is a potent modulator of chloroquine resistance. Clinical evaluation of therapeutic regimens is required to validate clinical efficacy of this promising combination for treatment of uncomplicated chloroquine-resistant malaria.

The reversal of chloroquine resistance by compounds with little intrinsic antimalarial activity is a well established phenotype of drug-resistant *Plasmodium falciparum*.¹ In the past decade, numerous compounds have been shown to reverse resistance *in vitro* to chloroquine in parasite isolates from various geographic areas;^{2–9} some of these compounds also reverse chloroquine resistance in animal models.^{3, 10–12} Although limited clinical studies have failed to demonstrate a reversal of chloroquine resistance in human infections,^{7, 13} potential clinical application of the phenomenon remains valid and provides a potentially innovative strategy to treat chloroquine-resistant malaria.

Promethazine is an antihistamine that acts by competing with histamine for H-1 receptor sites on effector cells. This H-1 antagonist is also used as adjunct therapy in the treatment of malaria in English-speaking west African countries. The drug is given as an antiemetic with chloroquine to prevent or alleviate chloroquine-associated pruritus.¹⁴ Commonly, a dose of 5.0–10 mg is given simultaneously, or just prior to administration of chloroquine in children with falciparum malaria in Nigeria; in adults, daily doses of 25 mg are well tolerated. In this report, the *in vitro* and *in vivo* effects of promethazine on chloroquine-resistant *P. falciparum* were evaluated. The data from both *in vitro* and *in vivo* experiments demonstrate that promethazine is a potent modulator of chloroquine resistance in falciparum malaria.

MATERIALS AND METHODS

Parasites and drug susceptibility testing. Ten isolates of *P. falciparum* obtained from patients in Nigeria were tested *in vitro* for susceptibilities to chloroquine, desethylchloroquine, quinine, mefloquine, and halofantrine alone and in combination with promethazine or verapamil. The chloroquine-susceptible west African clone D6 and the multidrug-resistant Indochina clone W2¹⁵ were used as reference parasites. Parasites obtained from patients at the University College Hospital in Ibadan, Nigeria were adapted to continuous culture using standard techniques at the Walter Reed Army Institute of Research where these tests were conducted.¹⁵ The parasites were cultured in human erythrocytes (type A⁺, 6.0% hematocrit) *in vitro* in RPMI 1640 culture medium supplemented with 10% human plasma.^{16, 17} Each culture was maintained in 50-ml sealed culture flasks (Corning Glass Works, Corning, NY) at 37°C in an atmosphere of 3–5% O₂, 2.5–4.0% CO₂, and 90% N₂ (premixed bottled gas; Potomac Airgas, Hyattsville, MD). A modification of the semiautomated microdilution technique in which the hematocrit was 1.5% and the initial parasitemia was 0.5–0.8% was used to test the parasites' susceptibilities to drugs. Suspensions of parasites, drug(s), and ³H-hypoxanthine were incubated in microtitration plates for 42–46 hr at 37°C as described previously.^{4, 5, 18} Inhibition of ³H-hypoxanthine incorporation by 50% (IC₅₀) was determined using a nonlinear regression analysis of the concentration-response curve.

Evaluation of *in vitro* enhancing activities of promethazine. Quantitative analysis of the increased activity of the quinoline-containing antimalarial drug when combined with promethazine was done by comparing concentration-response curves for chloroquine alone and in the presence of several fixed, subinhibitory concentrations of promethazine. Effects of each fixed concentration of promethazine on the response of the parasites (IC_{50}) to the antimalarial drugs were expressed as the response modification index (RMI).⁵ The RMI was calculated by the following formula: $RMI = IC_{50(A,B)}/IC_{50(A)}$, where drug A is a quinoline-containing antimalarial and B is promethazine. An RMI of 1.0 represents no change in the IC_{50} for the quinoline antimalarial drug combined with promethazine. The RMI values < 1.0 represent the degree of potentiation or synergism.

Determination of biologic activity. A bioassay was used at the University of Ibadan to measure the enhancing effect of promethazine in plasma from volunteers that had received promethazine. Three volunteers 19–25 years old were given 25 mg of promethazine each as a single oral dose (equal to a total daily dose for an adult). Similar blood samples were obtained from two additional volunteers who did not take any drug and used as controls. Blood samples (5 ml) were obtained from each volunteer prior to ingestion of the drug (if any) and every hour for 6 hr after ingestion of the drug. The volunteers were observed for tolerance and any reaction to the drug. Plasma from each volunteer was collected and used in the bioassay to determine the ability of promethazine in the volunteer plasma to reverse chloroquine resistance in clones of *P. falciparum in vitro*. The effect of promethazine in plasma from volunteers was evaluated by using a modification of the semiautomated microdilution technique.¹⁸ The IC_{50} for chloroquine versus chloroquine-resistant clone W2 was measured in the presence of plasma routinely used for continuous culture of *P. falciparum* in the laboratory. Similar data were obtained for plasma obtained from volunteers before taking promethazine. Reduction of the IC_{50} value was used as an index of promethazine concentration in the plasma and its biological activity for reversing resistance to chloroquine *in vitro*. The protocol for the studies in the volunteers and patients was reviewed and approved by the Joint University College Hospital/University of Ibadan Ethical Committee. Potential volunteers were given both oral and written explanations of the study and their informed consent was obtained prior to enrollment in the study.

Evaluation of *in vitro* activities in a monkey model. The effect of promethazine plus chloroquine on infection with the chloroquine-resistant Vietnam Smith/RE strain of *P. falciparum* in owl monkeys (*Aotus lemurinus lemurinus*) was evaluated at the Gorgas Memorial Laboratory as described previously.^{3,11} Six monkeys were each inoculated intravenously with 5.0×10^6 trophozoites of the parasite. The monkeys were maintained following procedures and husbandry practices outlined in the *Guide for the Care and Use of Laboratory Animals* as described previously.¹⁹ Infection in each monkey was monitored by microscopic examination of Giemsa-stained blood smears prepared daily for 15 consecutive days after inoculation; enumeration of parasitemia was done using the Earle-Perez method.²⁰ Response of infection to treatment regimen was followed for 100 days in each monkey. Primary treatment was initiated five days after par-

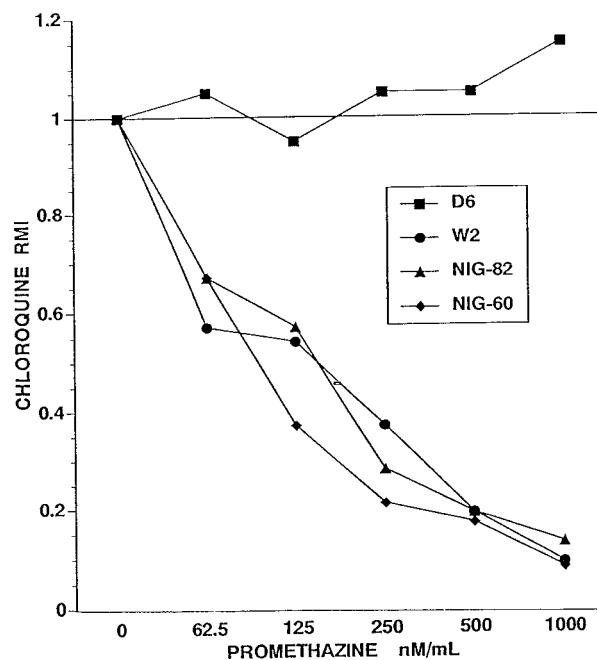


FIGURE 1. Effects of serial concentrations of promethazine on the susceptibility of chloroquine-sensitive (clone D6 -■-), resistant (clone W2 -●-), and Nigerian isolates (NIG-82 -▲- and NIG-60 -◆-) of *Plasmodium falciparum* to chloroquine *in vitro*. The response modification index (RMI) is the ratio of the 50% inhibitory concentrations (IC_{50} s) for chloroquine plus the concentration of promethazine, and chloroquine. An RMI of 1.0 represents no change in the IC_{50} for chloroquine when combined with promethazine. The RMI values < 1.0 represent the degree of potentiation or synergism.

asite inoculation in each monkey when parasitemia ranged from 0.1 to 5.0×10^3 parasites/ml. Additional treatment was initiated if there was recrudescence of infection during the 100 days of follow-up. Each treatment consisted of daily oral administration of a combination of chloroquine and promethazine. Doses of promethazine (10 mg/kg, 20 mg/kg, 40 mg/kg, or 80 mg/kg) were combined with the standard dose of chloroquine (20 mg/kg) daily for seven days. Two of the monkeys treated with chloroquine (20 mg/kg) alone were used as controls.

RESULTS

Potentiation of chloroquine *in vitro*. Simultaneous exposure of isolates and cloned strains of *P. falciparum* to chloroquine plus subinhibitory concentrations of promethazine increased the intrinsic schizontocidal activities of chloroquine against the resistant parasites. This enhanced effect on chloroquine occurred in the presence of 17.8–284 ng/ml of promethazine and increased the intrinsic schizontocidal activity of chloroquine against the resistant isolates by 32–92% (Figure 1 and Table 1). Identical concentrations of promethazine increased intrinsic activities of desethylchloroquine and quinine against the same parasites by 49–89%, and 29–79%, respectively. The IC_{50} s for desethylchloroquine against the isolates ranged from 7.08 ng/ml to 56.89 ng/ml and were reduced to 6.3–35 ng/ml in the presence of 284 ng/ml of promethazine. Similarly, IC_{50} values for quinine against the parasites ranged from 20.38 ng/ml to 57.73

TABLE 1

Reversal of chloroquine resistance with promethazine or verapamil in Nigerian isolates and reference cloned strains of *Plasmodium falciparum* in vitro*

Parasites	IC ₅₀ (ng/ml)		
	Chloroquine	Chloroquine plus promethazine†	Chloroquine plus verapamil‡
Clone D6	2.36	1.51	2.10
Nig-9204	3.01	3.60	2.15
Nig-9245	3.27	3.58	3.52
Nig-9171	5.79	3.89 (32)	1.67 (71)
Nig-919	6.76	6.73 (1)	5.40 (20)
Nig-9163	14.42	1.74 (88)	4.22 (70)
Nig-23	16.05	3.53 (78)	5.61 (65)
Nig-9274	21.02	6.59 (69)	5.85 (72)
Nig-82	45.89	3.47 (92)	6.18 (86)
Nig-9273	35.66	5.67 (92)	7.07 (80)
Clone W2	47.1	3.43 (92)	7.30 (85)
Nig-60	57.5	9.01 (84)	5.13 (91)

* IC₅₀ = 50% inhibitory concentration. Values in parentheses are the percent reductions in the IC₅₀.

† 1 × 10⁻⁶ M promethazine.

‡ 1 × 10⁻⁶ M verapamil.

ng/ml, and were reduced to 5.9–26.5 ng/ml in the presence of promethazine (284 ng/ml). Similar combinations did not have significant effect on susceptibilities of sensitive parasites to chloroquine, desethylchloroquine, or quinine. Incubation of parasites with identical concentrations of promethazine alone reduced growth of both chloroquine-resistant and -sensitive parasites by 10% or less. Combination of promethazine with mefloquine or halofantrine did not have any significant effects on the parasites susceptibilities to the antimalarial drugs. The IC₅₀ values for promethazine against either chloroquine-sensitive or -resistant parasites ranged from 443 ng/ml to 2,500 ng/ml (Table 2), confirming that the drug is not a potent antimalarial drug when used alone.

In vivo/in vitro biologic activities. The effects of plasma obtained from human volunteers who took promethazine on susceptibilities of the chloroquine-resistant W2 clone in a bioassay for determining reversal of chloroquine resistance are shown in Table 3. The IC₅₀ for chloroquine against the resistant clone was reduced by 20–58% when the antimalarial drug was combined with plasma samples obtained at specific time intervals after the volunteers ingested promethazine. The most significant reduction in the IC₅₀ value occurred 3–4 hr after ingestion of the drug. The IC₅₀ values for chloroquine when combined with plasma obtained from the three volunteers 3 or 4 hr after ingestion of promethazine

TABLE 2

Comparative susceptibilities of Nigerian isolates and reference cloned strains of *Plasmodium falciparum* to chloroquine, mefloquine and promethazine in vitro*

Parasites	IC ₅₀ (ng/ml)		
	Chloroquine	Mefloquine	Promethazine
Clone D6	2.36	11.02	1,550
Nig-9204	3.01	9.72	1,822.45
Nig-9171	5.79	4.52	1,193.1
Nig-919	6.76	6.25	1,180.26
Nig-9163	14.28	1.26	445.62
Nig-82	50.79	0.87	443.3
Clone W2	51.79	0.84	1,615.0

* IC₅₀ = 50% inhibitory concentration.

TABLE 3

Susceptibility of chloroquine-resistant reference clone W2 of *Plasmodium falciparum* to chloroquine in the presence of plasma from human volunteers given 25 mg of promethazine and a control volunteer that did not take any drug*

Time†	IC ₅₀ (ng/ml)			
	Control	Volunteer	Volunteer	Volunteer
0	20.08	55.73	23.2	22.82
1	26.46	71.34	20.55	23.96
2	25.34	68.49	17.16	26.28
3	23.12	25.23 (54.7)	9.73 (58)	23.83
4	20.87	NA‡	18.26	12.55 (45)
6		71.76	NA‡	23.46

* IC₅₀ = 50% inhibitory concentration. Values in parentheses are the percent reductions in the IC₅₀.

† Plasma obtained from volunteer at designated time interval. The plasma was not heat inactivated to maintain drug integrity. Variation in IC₅₀ value at 0 hr from the volunteers is probably due to the lack of heat inactivation and antiparasitic activities in the semi-immune plasma.

‡ NA = not available (sample was contaminated with bacteria).

were reduced by 45%, 54.7%, and 58%, respectively (Table 3). Combination of plasma samples obtained from the control volunteers at identical times did not reduce the IC₅₀ values for chloroquine against the parasites.

Activities in a monkey model. Response of infection in *Aotus* monkeys treated with chloroquine alone or a combination of chloroquine and promethazine showed that primary treatment with the combination ameliorated infection while chloroquine alone had no effect on parasitemia (Table 4). Additional treatment with higher doses of promethazine resulted in cure of two monkeys with an infection that is normally chloroquine resistant. Parasitemia was cleared in three of the four monkeys treated initially with the standard dose of chloroquine combined with either 10 mg/kg (one monkey) or 20 mg/kg (two monkeys) of promethazine. Infection in one monkey receiving chloroquine plus 10 mg/kg of promethazine was only suppressed. This monkey was cured following treatment with chloroquine and 20 mg/kg of promethazine. Infections in the other three monkeys recurred between seven and 17 days after completing the initial treatment (Table 4). Subsequent treatment in these monkeys resulted in cure of the infection in one monkey given 20 mg/kg of chloroquine and 80 mg/kg of promethazine. Infection in the other two monkeys was again cleared but recrudescence occurred 28 and 63 days after completing

TABLE 4

Response of chloroquine-resistant infection of the *Plasmodium falciparum* Smith/RE strain in *Aotus* monkeys (*Aotus lemurinus lemurinus*) to treatment with chloroquine and promethazine

	Treatment			
	CQ* + 10.0†	CQ* + 20.0†	CQ* + 40.0‡	CQ* + 80.0‡
Monkey #84055	S§	CD¶		
Monkey #89022	CL (7)#	CL (9)	CL (28)###	
Monkey #86040		CL (17)	CL (63)###	
Monkey #89014		CL (8)	CL (8)	CD¶

* Chloroquine, 20 mg/kg/day for seven days.

† Promethazine (mg/kg) given twice a day for seven days.

‡ Promethazine (mg/kg) given daily for seven days.

§ S = parasitemia was suppressed.

¶ CD = animal was cured of drug-resistant infection with the combination.

CL () = parasitemia was cleared but infection recrudesced in (8) days.

Parasitemia at recrudescence was low (10 parasites/μl of blood) and cleared without additional treatment (after 60 and 40 days in #89022 and #86040, respectively).

treatment. Parasitemia in the two monkeys at recrudescence was low, less than 10 parasites/ μ l of blood. The recrudescence infection cleared without re-treatment in the two animals (Table 4).

DISCUSSION

The results of this study demonstrate that promethazine, an H-1 antagonist, is a potent modulator of chloroquine resistance in *P. falciparum* isolates and clones. Promethazine enhanced the activity of chloroquine against resistant parasites *in vitro* and *in vivo*. Perhaps most importantly, potent chloroquine reversal activity was demonstrated in a bioassay of plasma from volunteers that had taken promethazine.

The costs of a complete dosage of mefloquine, halofantrine, or the derivatives of qinghaosu (artemether and artesunate) are prohibitively high for most residents of the poor malaria-endemic countries. This economic reality makes it imperative that cheaper alternatives be available for treatment of malaria. The phenomenon of reversal of chloroquine resistance could play an important role in efforts to control drug-resistant malaria at economically feasible costs, especially in Africa. A combination of chloroquine with promethazine, or some other resistance modulator drugs, would be substantially cheaper than any of the currently available antimalarial drugs. Unfortunately, a decade has elapsed since the description of the reversal phenomenon¹ without a clear choice among the potential resistance reversal agents or significant preclinical studies leading to development of a novel resistance modulator drug. Results of the present studies suggest that promethazine has potential to fill this need in the interim.

The limited success among compounds tested in a monkey model^{3, 11} and the failure in human volunteers^{7, 13, 21} has been attributed in part to protein binding and pharmacokinetic interactions of the potential combinations. Both cyproheptadine and desipramine reversed resistance in *P. falciparum* *in vitro* and in animal models,^{2, 3, 7, 11} but failed to enhance antimalarial activity of chloroquine in patients infected with *P. falciparum*.^{7, 13} Evaluation of blood obtained from a volunteer who took cyproheptadine also failed to show reversal of resistance *in vitro*.²¹ The discrepancies between these *in vitro* and *in vivo* observations with potential combination drugs are a major concern for a successful clinical application of the reversal phenomenon. Obviously, the selection of a successful combination must be based on several factors: these include pharmacokinetics, pharmacodynamics, toxicity, and the ability to reverse chloroquine resistance.

Binding of drugs to plasma proteins, especially albumin and acute phase proteins is known to decrease antimalarial drug efficacy^{22, 23} and has been suggested to account for failure of desipramine in clinical studies.²⁴ The ability of promethazine in volunteers plasma to enhance chloroquine activity (after taking drug orally) suggests the H-1 antagonist might retain its resistance reversal activity *in vivo*. In addition, coadministration of promethazine has been shown to increase blood levels of chloroquine.²⁵

There is an urgent need to capitalize on the potential chemotherapeutic advantages of the chloroquine resistance reversal phenomenon for the treatment of drug-resistant malaria. The present observations suggest that promethazine is

a good candidate for further studies. Unlike cyproheptadine,²¹ promethazine retained detectable reversing activities in human plasma after oral administration of drug to human volunteers. In addition, both chloroquine and promethazine are commonly used in combination in anglophone countries of west Africa (for other reasons) and appear to be safe and well tolerated. Data on pharmacokinetic interactions of the two drugs are warranted to optimize dosage regimens of promethazine plus chloroquine for a combination therapy in chloroquine-resistant malaria.

Acknowledgments: We thank Dr. James Peggins and Dr. R. Keith Martin for critical review of the manuscript. We are grateful to Drs. O. A. T. Ogundahunsi and G. O. Omitowoju for contributions to this study, and to Margaret Bell for secretarial assistance.

Financial support: This investigation received financial support from the UNDP/World Bank/WHO Special Program for Research and Training in Tropical Diseases and the Rockefeller Foundation Biotechnology Career Fellowship Program.

Authors' addresses: A. M. J. Oduola, A. Sowunmi, and L. A. Salako, Department of Pharmacology and Therapeutics, Postgraduate Institute for Medical Research and Training, College of Medicine, University of Ibadan, Ibadan, Nigeria. W. K. Milhous, T. G. Brewer, D. E. Kyle, L. Gerena, and B. G. Schuster, Division of Experimental Therapeutics, Walter Reed Army Institute of Research, Washington, DC 20307-5100. R. N. Rossan, 9122 W. Viking Road, Las Vegas, NV 89117-6844.

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