

## In Vitro and In Vivo Potentiation of Chloroquine against Malaria Parasites by an Enantiomer of Amlodipine

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**A combination of chloroquine and amlodipine, a derivative of 1,4-dihydropyridine calcium channel blocker, was tested against *Plasmodium falciparum* in vitro and *P. yoelii* in mice. The dextrorotary enantiomer of amlodipine, practically devoid of calcium channel blocking action, increased chloroquine accumulation inside the infected mouse erythrocytes and potentiated chloroquine action against the resistant strains of *P. falciparum* in vitro and *P. yoelii* in mice. Unlike the racemate, the dextrorotary amlodipine was not toxic to the host animal, even at the highest dose of 250 mg/kg. No potentiating effect was noted in the chloroquine-susceptible strains of *P. falciparum*. The results of this study indicate that chloroquine potentiation of amlodipine is probably independent of calcium channels and that a combination therapy of the dextrorotary enantiomer of amlodipine and chloroquine might be a potentially useful therapeutic strategy against chloroquine-resistant falciparum malaria.**

Chloroquine has been the drug of choice for treating *Plasmodium falciparum* malaria for more than 40 years. However, the prophylactic and therapeutic utility of chloroquine is largely limited today because of the spread of the chloroquine-resistant strains of *P. falciparum* in most malaria-endemic regions (5). Therefore, new therapeutic approaches are urgently needed.

The mechanism of chloroquine resistance is thought to involve an enhanced drug efflux from the drug-resistant malaria parasites (17). This efflux process is supposed to be responsible for the failure of chloroquine to accumulate inside erythrocytes parasitized by resistant parasites to the extent that has been observed with susceptible parasites (7, 21, 32). This efflux process can be inhibited by several calcium channel blockers (22, 29). Similar observations were reported with tricyclic antidepressants (4) and tricyclic anti-histaminics (25). The latter agents possess an indirect calcium antagonist action on either calmodulin, a calcium-binding protein (27), or other calcium-dependent enzymes (20).

The use of combination therapy with chloroquine and calcium antagonists is probably unsuitable in cases of human malaria because of the high doses of calcium antagonists necessary to reverse chloroquine resistance (4). One of the possible means to circumvent this problem of host toxicity may be the use of stereospecific drugs (2, 16), since the dextrorotary (+)-enantiomer of verapamil has been shown to reverse chloroquine resistance in vitro (37). Amlodipine, a 1,4-dihydropyridine derivative of nifedipine, has a highly stereospecific property on the calcium channels. The affinity of its levorotary (–)-enantiomer to the calcium channels is 1,000 times superior to that of its dextrorotary (+)-enantiomer (1). On the basis of this stereospecificity, the chloroquine potentiating actions of the racemic mixture and the (+)-enantiomer of amlodipine were compared in this study. We report that (+)-amlodipine potentiated chloroquine in vitro and in vivo, without lethal effects, and led to a

substantial increase in chloroquine concentration in the infected mouse erythrocytes.

### MATERIALS AND METHODS

**In vitro drug activity measurement.** Three culture-adapted strains of *P. falciparum* were used in the study. Strain FCM 29/Cameroon (chloroquine resistant) was isolated and cloned by the dilution method. Strains FCM 6/Thailand (chloroquine resistant) and L-1/Gabon (chloroquine susceptible) were isolated in our laboratory. These strains were maintained in type A human erythrocytes by the in vitro culture method of Trager and Jensen (30). The culture medium consisted of RPMI 1640 medium with HEPES (*N*-2-hydroxyethylpiperazine-*N'*-ethanesulfonic acid; 25 mM), NaHCO<sub>3</sub> (25 mM), and 10% pooled human serum. Chloroquine sulfate (Specia-Rhone Poulenc, Vitry, France) was dissolved in sterile, distilled water. Twofold dilutions were distributed in 24 flat-bottomed well plates to obtain final concentrations ranging from 12.5 to 1,600 nM in triplicate. Stock solutions of the racemate and the (+)-enantiomer of amlodipine maleate (Pfizer Central Research, Sandwich, United Kingdom) were prepared in sterile, distilled water. For the determination of the intrinsic antimalarial activity of amlodipine, twofold dilutions were distributed, in triplicate, in the tissue culture plates to give final concentrations ranging from 0.3 to 40 μM. The drug interaction between chloroquine and amlodipine was studied by comparing the activities of chloroquine alone with that of chloroquine in the presence of a fixed subinhibitory concentration (2.5 μM) of amlodipine.

The semi-microtest was used to determine the in vitro susceptibility of the falciparum malaria parasites (19). Briefly, the inoculum (700 μl per well) consisted of a suspension of parasitized erythrocytes (2.5% hematocrit; starting parasitemia, 0.1 to 1.0%) in complete culture medium. The plates were incubated at 37°C in 5% O<sub>2</sub>–5% CO<sub>2</sub>–90% N<sub>2</sub> for 42 h. [<sup>3</sup>H]hypoxanthine (1 μCi per well; Amersham, Les Ullis, France) was added 24 h before the end of the incubation period to assess parasite maturation. The suspension was collected and washed on glass fiber filter

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TABLE 1. IC<sub>50</sub> of chloroquine and amlodipine (racemate or dextrorotatory enantiomer) for each drug alone or in combination against *P. falciparum*<sup>a</sup>

Strain	IC <sub>50</sub> (10 <sup>-9</sup> M) ± SEM				
	Amlodipine alone		Chloroquine alone	Chloroquine + 2.5 × 10 <sup>-6</sup> M amlodipine	
	Racemate	Dextrorotatory enantiomer		Racemate	Dextrorotatory enantiomer
FCM 29-C1 (Cameroon)	6,740 ± 220	7,040 ± 210	823 ± 14	158 ± 7	298 ± 10
FCM6 (Thailand)	4,660 ± 120	5,080 ± 90	569 ± 17	73 ± 5	101 ± 7
L-1 (Gabon)	11,380 ± 190	12,310 ± 310	20 ± 0.4	22 ± 1.0	21 ± 1.3

<sup>a</sup> Combination experiments were conducted in the presence of a constant subinhibitory concentration of amlodipine that had been determined to have no significant effect on parasite growth. All tests were run in triplicate.

papers, and the amount of radioactivity incorporated into the parasites was measured by use of a liquid scintillation counter (Wallac 1410; Pharmacia, Uppsala, Sweden). Concentration-response curves were analyzed by log dose-probit analysis to obtain the 50 and 90% inhibitory concentrations (IC<sub>50</sub> and IC<sub>90</sub>) (12).

**In vivo drug activity measurement.** The line N67 of *P. yoelii* subsp. *nigeriensis* was kindly supplied by O. Godard, Centre Nicolas Grillet, Rhone Poulenc Research. This parasite strain is resistant to chloroquine and susceptible to pyrimethamine and mepacrine (24). Female CD1 mice (between 6 and 8 weeks of age) were used in the study. Their mean weight was 20 ± 2 g. The mice were kept at a temperature of 22 ± 3°C and provided with a standard diet and water. Chloroquine diphosphate salt (Sigma Chemical Co., St. Louis, Mo.) was dissolved in 0.9% NaCl so that each 0.2-ml aliquot contained a dose of 1.5 mg/kg. Amlodipine was dissolved in 0.9% NaCl so that each 0.3-ml aliquot contained doses ranging from 0.4 to 5 mg (corresponding to 20 to 250 mg/kg [body weight]).

The 4-day test was carried out as described by Peters (24). Parasitized erythrocytes were obtained from a donor mouse with rising parasitemia (>20% infected erythrocytes) and diluted with 0.9% NaCl to obtain 0.2-ml aliquots containing 10<sup>7</sup> infected erythrocytes. Mice were inoculated intravenously in the tail vein with 10<sup>7</sup> infected erythrocytes on day zero. For each experiment, the mice were randomly assigned to a group of 5 or 10 infected mice. The drugs were administered orally via nasogastric tube (amlodipine) or subcutaneously (chloroquine) once daily from day zero to day 3. On day 4, thin blood films were made from the tail blood and stained with Giemsa stain and the parasitemia was determined. All experiments included a drug-free control group, a chloroquine-treated group, and groups treated with different doses of the racemic mixture or the (+)-enantiomer of amlodipine administered alone or in combination with chloroquine.

**Chloroquine accumulation in mouse erythrocytes.** After inoculation with 10<sup>7</sup> infected erythrocytes, the mice were given 1.5 mg of chloroquine per kg alone or in combination with 30 or 40 mg of amlodipine [racemate or (+)-enantiomer] per kg. Six hours later, blood samples were collected from the retroorbital sinus, and the cells were separated from plasma by rapid centrifugation. The concentrations of chloroquine and its main metabolite, monodesethylchloroquine, were determined in both the cell pellet and plasma by a specific assay based on high-performance liquid chromatography (26) with fluorescence detection (3). The method is sensitive and reproducible, with limits of detection of 0.3 ng/ml for both chloroquine and its metabolite.

## RESULTS

**Chloroquine potentiation in vitro.** Both the racemate and the (+)-enantiomer of amlodipine showed weak intrinsic antimalarial activities in vitro, with the IC<sub>50</sub> values ranging from 4.7 to 12.3 μM (Table 1 and Fig. 1). Both the racemate and (+)-amlodipine, at the concentration of 2.5 μM, reduced the IC<sub>50</sub> for chloroquine significantly (*P* < 0.05) in the chloroquine-resistant strains FCM 29 and FCM 6. The racemic mixture of amlodipine was slightly more effective than its (+)-enantiomer. No potentiating effect was produced by these compounds in the chloroquine-susceptible strain L-1 at the same drug concentration.

**Chloroquine potentiation in vivo.** To test chloroquine potentiation in vivo, a total of five separate experiments were conducted. The mean parasitemia of nontreated control mice varied from 55 to 72% (mean, 68%) on day 4. All but one (98%) of the nontreated control mice died between days 5 and 7. The daily dose of 1.5 mg of chloroquine per kg alone

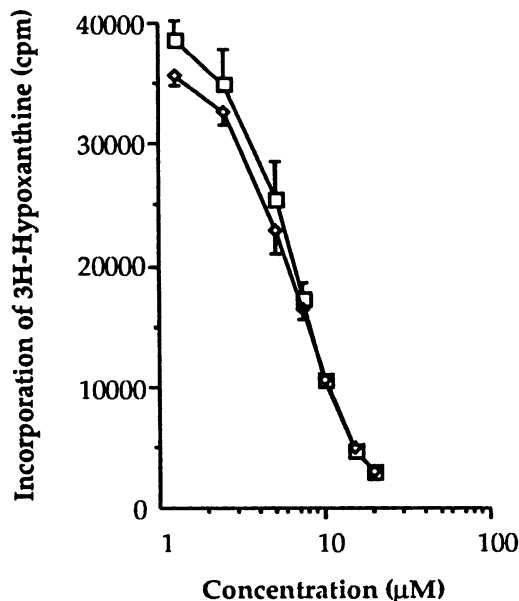


FIG. 1. In vitro intrinsic antimalarial activity of racemate (◇) and dextrorotatory enantiomer (□) of amlodipine against the chloroquine-resistant clone FCM 29. Linear regression analysis showed no statistical difference between the concentration-response curves of the enantiomers. At a concentration of 2.5 μM, parasite growth inhibition was less than 10%. Bars denote standard deviations.

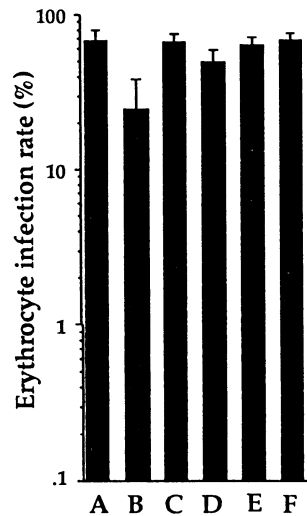


FIG. 2. Mean parasitemia on day 4 in untreated control mice (A) and in mice treated with 1.5 mg of chloroquine per kg (B), 30 mg of racemate amlodipine per kg (C), 40 mg of racemate amlodipine per kg (D), 30 mg of (+)-amlodipine per kg (E), or 40 mg of (+)-amlodipine alone per kg (F). Bars denote standard deviations.

marginally affected the parasite growth, with a mean parasitemia of 24% on day 4 (Fig. 2). Most (71%) of the mice treated with chloroquine alone died before day 10. Given alone, both racemate and (+)-amlodipine, were totally inactive at doses of 30 or 40 mg/kg daily. The racemic mixture was toxic at 40 mg/kg, since one of five mice died by day 4 at that dose.

With the combination therapy of the racemate of amlodipine and chloroquine (1.5 mg/kg), parasite growth was significantly inhibited in a dose-dependent manner. The parasitemia decreased from 0.8 to 0.3% in mice receiving 20- to 40-mg/kg doses of the racemate of amlodipine in combination with chloroquine (Fig. 3). Thirty- and 40-mg/kg doses of the racemate of amlodipine combined with chloroquine were toxic, with the death of 2 of 25 and 2 of 15 mice, respectively, before termination of treatment. Higher doses of the racemate (50 to 250 mg/kg) combined with chloroquine showed 60 to 100% mortality rates on day 2.

When increasing doses of the (+)-enantiomer of amlodipine were given with chloroquine (1.5 mg/kg), a similar pattern of enhanced schizontocidal activity was observed without lethal effects, even at the highest dose of 250 mg of (+)-amlodipine per kg plus 1.5 mg of chloroquine per kg. At the highest dose, all mice were still alive on day 10.

**Chloroquine accumulation in infected erythrocytes.** Treatment with the racemate (30 or 40 mg/kg) and (+)-amlodipine (30 or 40 mg/kg) increased chloroquine accumulation, as represented by the cell-to-plasma concentration ratio, by approximately sixfold in the erythrocytes obtained from *P. yoelii*-infected mice, as compared to the erythrocytes obtained from the mice treated by chloroquine alone (Table 2). Monodesethylchloroquine was seen to accumulate similarly inside the infected erythrocytes.

## DISCUSSION

Commercially available amlodipine is a racemic mixture of the (+)- and (–)-enantiomers which is used for the treatment of hypertension and ischemic heart disease. Essentially all of

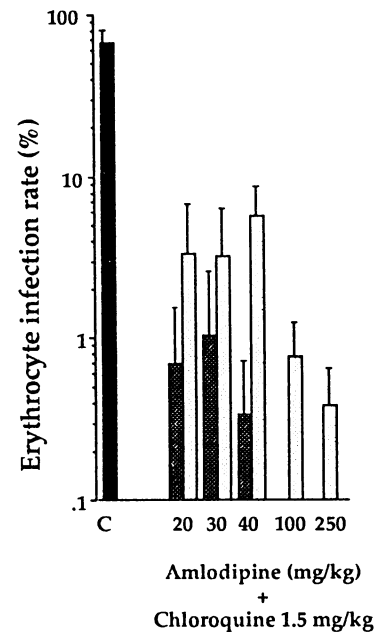


FIG. 3. Mean parasitemia on day 4 in untreated control mice (black bar) and in mice receiving chloroquine (1.5 mg/kg) plus racemate (dark gray bars) or (+)-enantiomer (light gray bars) of amlodipine (20 to 250 mg/kg). Bars denote standard deviations. The highest dose of racemate amlodipine shown is 40 mg/kg because of its toxicity.

the calcium channel blocker activity of amlodipine resides in the (–)-amlodipine (1). Although several calcium channel blockers share this feature, the stereospecificity is particularly high in the case of amlodipine. The enantiomeric ratio of verapamil is only 10:1 (6), implying that, when given at the large doses required for potentiating chloroquine action, the less active enantiomer can still act on the calcium channel and produce cardiovascular toxic effects. This is not the case with amlodipine, whose enantiomeric ratio is 1,000:1. The present study confirms our initial hypothesis that acute toxic effects to the host can be minimized by using an enantiomer practically devoid of calcium antagonist action.

Our results demonstrate that the racemate and the (+)-enantiomer of amlodipine potentiate chloroquine action against the resistant strains of malaria parasites both in vitro and in vivo. Although the (+)-enantiomer of amlodipine required slightly higher doses than the racemate to potentiate chloroquine (confirming the in vitro data), the apparent lack of acute toxicity in the host animal allowed a 10-fold increase in the dose regimens. In the in vivo experiments, both compounds increased chloroquine accumulation in the infected erythrocytes. An intraerythrocytic accumulation of monodesethylchloroquine, a major metabolite of chloroquine which possesses an antimalarial activity (31), was also noted. The latter observation confirms the in vitro potentiation of monodesethylchloroquine by calcium antagonists, reported by other authors (18), and may have important implications in further augmenting the schizontocidal action of chloroquine in vivo.

Despite the fact that (+)-amlodipine is practically devoid of calcium channel blocking activity, chloroquine potentiation was observed in our experiments with this compound. Other studies have shown that (+)-enantiomers of phenylalanylamine groups of calcium channel blockers, such as vera-

TABLE 2. Chloroquine accumulation in erythrocytes from mice infected with chloroquine-resistant *P. yoelii* N67<sup>a</sup>

Drug measured in cells and plasma	Mean cell-to-plasma ratio ( $\pm$ SD) of drug concn in mice treated with:				
	Chloroquine alone at 1.5 mg/kg	Chloroquine at 1.5 mg/kg + racemate at:		Chloroquine at 1.5 mg/kg + dextrorotatory enantiomer at:	
		30 mg/kg	40 mg/kg	30 mg/kg	40 mg/kg
Chloroquine	5.0 $\pm$ 0.8	29.4 $\pm$ 16.2	34.9 $\pm$ 6.2	36.2 $\pm$ 13.8	28.4 $\pm$ 13.9
Monodesethyl-chloroquine	1.1 $\pm$ 0.1	6.0 $\pm$ 0.8	8.0 $\pm$ 0.8	12.6 $\pm$ 3.7	6.9 $\pm$ 0.2

<sup>a</sup> After inoculation with 10<sup>7</sup> trophozoites, mice were given 1.5 mg of chloroquine alone per kg or in combination with 30 or 40 mg of amlodipine (racemate or dextrorotatory enantiomer) per kg. Six hours later, chloroquine and its main metabolite, monodesethylchloroquine, were assayed in both cells and plasma by high-performance liquid chromatography (3, 26).

pamil, gallopamil, and devapamil, reverse drug resistance in vitro against *P. falciparum* (37) as well as in cancer cells (13). In addition, the drug concentration of amlodipine required to potentiate chloroquine action in vitro was close to 1,000 times higher than the concentration required to inhibit calcium channels (1). These observations confirm that the reversal of chloroquine resistance is independent of calcium channels.

Either an interaction with a putative malarial P glycoprotein or an interference in the mitochondrial or lysosomal functions might be involved in chloroquine potentiation. It is unknown whether the mechanisms of action of calcium channel blocker are similar in the parasitized cell and in the mammalian system. However, analogies between chloroquine resistance in malaria parasites and multidrug resistance in neoplastic cells are numerous (15, 17, 22, 23, 29). At the genetic level, an amplification of a malarial gene (*Pfmdr1* or *Pfmdr2*) homologous with the mammalian multidrug-resistant (*mdr*) gene has been implicated in chloroquine resistance (8, 9, 36). However, resistance to chloroquine and rapid chloroquine efflux were not shown to be linked to the presence or amplification of either *Pfmdr1* or *Pfmdr2* (35). At the protein level, contradictory observations on the presence or absence of a P glycoprotein-like structure in malaria parasites were reported (14, 38). Both calcium channel blockers and calmodulin inhibitors inhibit, in voltage-independent and calmodulin-independent manners, numerous enzymes present in malaria parasites (10, 16, 28). Some of these enzymes may be involved in the transport of chloroquine into malaria parasites (11, 33). Although the calcium channels are most probably not involved, the mechanism of reversal of chloroquine resistance at the molecular level is not clear at present.

Our data suggest that the combination of chloroquine and (+)-amlodipine may be effective in treating chloroquine-resistant *falciparum* malaria in man. Before considering its use, additional studies are needed to investigate the potential interactions of (+)-amlodipine with the pharmacodynamic and pharmacokinetic parameters of chloroquine (34). In mice, the metabolism of chloroquine did not appear to be modified by (+)-amlodipine, since the ratios of the concentrations of chloroquine and monodesethylchloroquine in blood were similar in mice that received chloroquine with or without amlodipine (data not shown). Our data demonstrate that (+)-amlodipine can potentiate in vivo chloroquine action and may represent a potential therapeutic strategy against chloroquine-resistant *P. falciparum*.

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